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13. ABSTRACT *(Maximum 200* Saxitoxin (STX), a potent neurotoxin, is the causative agent of paralytic shellfish poisoning in humans. The North American bullfrog, Rana catesbeiana, contains a plasma protein called saxiphilin that binds STX with high affinity and specificity. Since saxiphilin may be useful as a detection reagent or an antidote for STX, we have investigated the biochemical properties of saxiphilin and cloned cDNA encoding this protein. Native saxiphilin is a polypeptide of 825 amino acid residues (Mr = 90,901) that contains one binding site for [^{3}H]STX per molecule with an equilibrium dissociation constant of K_D = 0.2 nM. The amino acid sequence of saxiphilin, deduced from cDNA isolated from bullfrog liver, exhibits substantial homology to members of the transferrin family of Fe^{3+}-binding proteins. However, biochemical and immunochemical analyses confirm that saxiphilin is a unique protein that is distinct from bullfrog serum transferrin. The mechanism of [^{3}H]STX binding to saxiphilin, including the pH-dependence and temperature-dependence, was characterized in detail. Recombinant saxiphilin was expressed in insect cells using a baculovirus vector and the STX-binding site was localized to the C-lobe domain of the protein. A phylogenetic survey found saxiphilin-like activity in arthropods, fish, amphibians and reptiles.

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FOREWORD

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Edward G Moylan 10/11/96

PI - Signature Date

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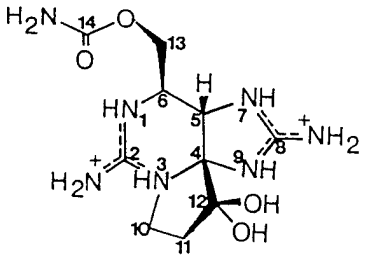
FINAL REPORT [DAMD17-93-C-3069]

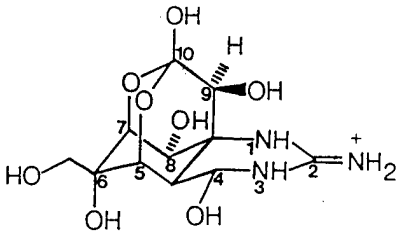
Principal Investigator and Author: Edward G. Moczydlowski, Ph. D

Title of Project: Cloning and Functional Analysis of Saxiphilin, a Saxitoxin-Binding Protein from the Bullfrog

Introduction: Saxitoxin (STX, Fig. 1) is a natural product that is chemically defined as a tricyclic perhydropurine derivative (Shimizu, 1986). It is a divalent cation in aqueous solution by virtue of two cyclized guanidinium groups with pKa's of 8.2 and 11.5 (Shimizu et al., 1981). STX and various STX derivatives are naturally produced by certain species of marine dinoflagellates and freshwater cyanobacteria (blue-green algae) (Hall et al, 1990; Carmichael, 1990). These microbes produce saxitoxin leading to the contamination of edible shellfish and fresh water sources. Saxitoxin poisoning is an important problem of environmental concern that has a significant economic impact in the fishing industry. "Red tide" blooms of dinoflagellates in the ocean and cyanobacterial blooms of inland waters have occurred with increased frequency in recent years due to organic waste pollution that favors microbial growth (Anderson, 1994; Carmichael, 1994). Unwitting human consumption of shellfish contaminated with STX is the cause of the food poisoning syndrome known as "paralytic shellfish poisoning" that can result in respiratory paralysis and death in severe cases (Anderson, 1994; Gessner et al, 1996). STX is a potent blocker of voltage sensitive Na^+ channels that mediate the fast depolarizing phase of action potentials in electrically excitable cells (Ritchie & Rogart, 1977). This neurotoxin binds with nanomolar affinity to an extracellular site on voltage-sensitive Na^+ channels in nerve and muscle. [^{3}H]STX is a commercially available radiochemical that is used by neurochemists to quantitate the density of Na^+ channels, an integral membrane protein of excitable tissues.



SAXITOXIN



TETRODOTOXIN

Figure 1. Chemical structures of the neurotoxins, saxitoxin (STX) and tetrodotoxin (TTX). Saxiphilin, a soluble protein from the bullfrog homologous to transferrin, specifically binds STX with a K_D of ~200 pM but does not bind TTX (Llewellyn et al., 1994). Voltage-sensitive Na^+ channels of electrically excitable cells bind both STX and TTX in a competitive fashion at an extracellular site that has been localized to the pore entrance (Terlau et al., 1991).

Over the past several years, our laboratory has identified and characterized a specific site of STX binding that is different from the Na^+ channel interaction described above. An anomalous STX-binding component originally observed in studies of bullfrog tissues led to the discovery of saxiphilin, a soluble protein that specifically binds [^{3}H]STX with a high affinity K_D of 0.2 nM (Moczydlowski et al., 1988; Mahar et al., 1991). Purification of the native protein from frog plasma showed that saxiphilin is a 91 kDa polypeptide (Li & Moczydlowski, 1991). Subsequent cloning of saxiphilin cDNA revealed that it is a structural homolog of the transferrin family of proteins (Morabito & Moczydlowski, 1994).

Transferrins are an important family of Fe^{3+}-binding proteins that include serum transferrin in plasma, lactoferrin in milk and other secretions, melanotransferrin which is bound to the plasma membrane of melanoma cells and other cell types, and ovotransferrin found in egg white of birds (Crichton, 1991; Welch, 1992). Transferrins bind Fe^{3+} with very high affinity ($K_D \simeq 10^{-20}$ M) in a bicarbonate-dependent fashion and thus provide a soluble, biologically available form of inorganic iron. Most transferrins consist of a single glycosylated polypeptide of approximately 680-700 residues that binds two Fe^{3+} ions, one in each of two homologous domains called the N-lobe and C-lobe, that arose from an internal duplication. Serum transferrin (Tf) is responsible for the intracellular delivery of Fe^{3+} to vertebrate cells via the cycle of transferrin-receptor mediated endocytosis (Thorstensen & Romslo, 1990). Other important functions of transferrin and lactoferrin are related to infectious diseases and iron toxicity. Strong chelation of iron by transferrins inhibits bacterial growth in biological fluids since iron is a limiting requirement for growth and cell division (Griffiths & Bullen, 1987). Low levels of free iron maintained by Tf and lactoferrin also minimizes the potential toxicity of Fe^{3+}/Fe^{2+} caused by iron-dependent generation of hydroxyl free radical (Griffiths, 1987).

In regard to these findings, the U. S. Army Medical Research and Materiel Command funded a three-year contact grant on "Cloning and Functional Analysis of Saxiphilin, a Saxitoxin-Binding Protein from the Bullfrog." The primary goal of this project as outlined in the statement of work (USAMRMC Log. No. 90296008) is as follows: "The contractor shall furnish all equipment, personnel, facilities and supplies required to clone and express bullfrog saxiphilin in a recombinant system and to develop large-scale preparation procedures for research quantities of the recombinant saxiphilin for possible use as a research tool or countermeasure against saxitoxin."

This report represents a comprehensive summary of all work conducted on this project. Our efforts have yielded new basic information on the structure and biochemical properties of saxiphilin. In particular, a cDNA encoding saxiphilin has been cloned from bullfrog liver, and the exact structural relationship of saxiphilin to transferrin proteins has been established. Recombinant saxiphilin has been successfully expressed in cultured insect cells using a baculovirus vector. Most of the results reported here in abbreviated form are described in detail in five recent publications that have resulted primarily from this contract (Li et al., 1993; Morabito and Moczydlowski, 1994; Llewellyn and Moczydlowski, 1994; Morabito et al., 1995; Llewellyn et al., 1996). These latter

publications include a complete description of the methodology and data analysis. Interested readers are referred to these primary sources for additional details.

Body of Report:

1. Saxiphilin is biochemically and functionally distinct from bullfrog serum transferrin. The discovery of saxiphilin occurred in series of experiments designed to investigate the possible heterogeneity of Na^+ channel isoforms in frog skeletal muscle (Mahar et al., 1991). Our laboratory was using [^{3}H]STX to characterize the recovery of Na^+ channels in various membrane fractions isolated after homogenization and sucrose gradient fractionation. These studies yielded an unusual observation. A significant fraction of the [^{3}H]STX binding sites in the frog muscle preparation was insensitive to competitive displacement by tetrodotoxin (TTX). TTX is a Na^+ channel toxin with only one cyclic guanidinium group (Fig. 1) that is found in certain puffer fish, newts and many other toxic animal species (Fuhrman, 1986; Yasumoto et al., 1986). Its exact biochemical origin in vertebrates is unknown but some researchers believe that it is produced by bacteria (Yasumoto et al., 1986; Yotsu et al., 1987). Voltage-sensitive Na^+ channels are universally known to be blocked by STX and TTX in a competitive fashion (with K_i's in the range of 2-20 nM), although there are Na^+ channel isoforms that have lower affinity for these toxins (K_i's = 100 - 1000 nM) such as those of mammalian heart muscle (Ritchie & Rogart, 1977; Guo et al., 1987). The unusual STX binding site that we observed in frog muscle exhibited subnanomolar affinity for [^{3}H]STX but was completely insensitive to 100 μM TTX.

Upon searching the literature, we found that a similar binding site in frog heart had been previously observed by Dolye and coworkers, but this site was present in a soluble form (Doyle et al., 1982; Tanaka et al., 1984). They hypothesized that the soluble, TTX-insensitive, STX-binding site was an unusual soluble form of a Na^+ channel protein. Since this was a provocative observation, we pursued it and found that virtually all tissues of the bullfrog contain soluble [^{3}H]STX binding activity that is released upon homogenization. This activity is also present in frog plasma at a concentration of about ~300 nM [^{3}H]STX sites. Since it soon became clear that this soluble binding activity was a biochemically distinct entity from Na^+ channels, we coined the name *saxiphilin* to describe the new protein (Mahar et al, 1991).

The first direct information on the molecular nature of saxiphilin was obtained by purification of the soluble [^{3}H]STX-binding activity from bullfrog plasma. Li et al. (1993) devised a two-step purification procedure which showed that the purified saxiphilin protein is a 90 kDa polypeptide as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purification was accomplished by chromatography of bullfrog plasma on a heparin-Sepharose column followed by chromatofocusing. Partial sequencing of the purified saxiphilin protein revealed that tryptic fragments of this 90 kDa protein exhibit considerable sequence homology (40-70% identity) to the transferrin family of Fe^{3+}-binding proteins.

The unexpected homology to transferrin led to the question of whether saxiphilin is actually a form of frog transferrin. This question was addressed by the study of Li et al. (1993). In this work, transferrin was purified from bullfrog plasma and shown to be distinct from saxiphilin on the basis of its size (~78 kDa for Tf vs. 90 kDa for saxiphilin), visible absorption spectrum, and ligand-binding properties. High affinity binding of [^{3}H]STX was found to be a distinctive property of saxiphilin that was not exhibited by transferrins from various animal species. All commercially available Tf proteins that have been tested, as well as bullfrog apo- and holo-transferrin, do not bind [^{3}H]STX (Li et al., 1993). Thus it appears that saxiphilin is an evolutionary relative of Tf that has diverged to bind a different ligand and perform a different function.

Conversely, under conditions appropriate for transferrins, purified saxiphilin does not bind $^{55}Fe^{3+}$, implying that it is not involved in iron metabolism. The study of Li et al. (1993) also found that polyclonal antibodies raised against native saxiphilin precipitated [^{3}H]STX binding activity from whole bullfrog transferrin. In an enzyme-linked immunosorbent assay using native proteins, antisaxiphilin antibodies weakly cross-reacted with transferrin from bullfrog and a number of other species. Likewise antibodies against human transferrin cross-reacted with saxiphilin in a similar immunosorbent assay. These results conclusively demonstrated that saxiphilin is not identical to bullfrog transferrin but it is structurally related to the transferrin family.

2. Determination of the primary sequence of saxiphilin by molecular cloning. Partial sequence information was used to design degenerate PCR primers that could be used to amplify saxiphilin cDNA. The template cDNA was synthesized by reverse transcription using oligo-dT primed RNA extracted from bullfrog liver. A nested PCR strategy yielded a 450 bp band that appeared to be a good candidate for saxiphilin cDNA. This fragment was used as a probe to screen a bullfrog liver library resulting in the isolation of a full-length clone of saxiphilin cDNA (Morabito & Moczydlowski, 1994; 1995). The primary sequence of saxiphilin is shown in Fig. 2 (Genbank accession No. U05246). The saxiphilin protein is a single polypeptide of 844 residues. The N-terminal 19 residues correspond to a secretory signal sequence resulting in an 825 residue mature protein with a calculated molecular weight of 90,901. The saxiphilin sequence can be readily aligned by similarity to transferrins as illustrated in Fig. 2 which identifies residues identical to those of human lactoferrin.

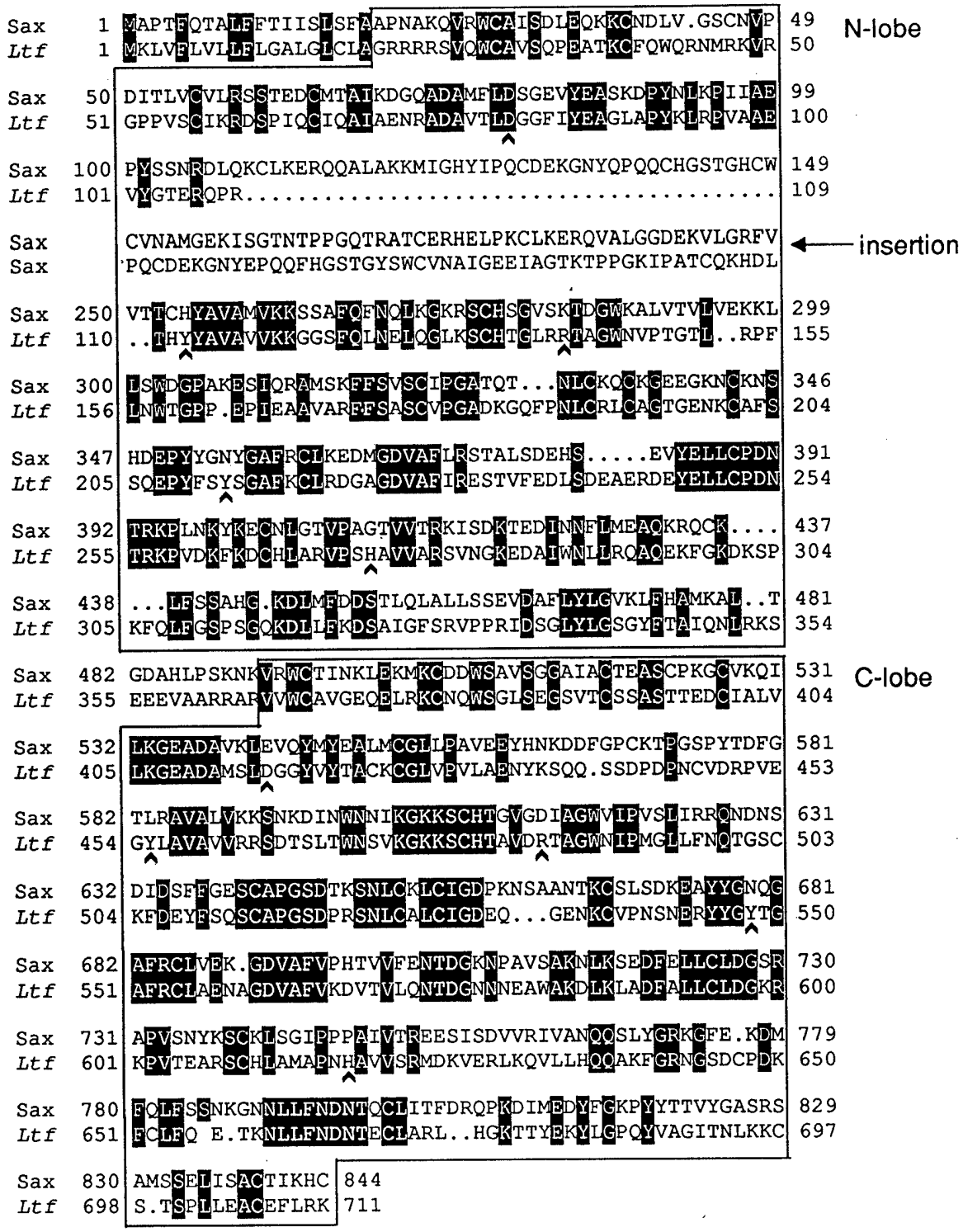


Figure 2. Structural comparison of saxiphilin and lactoferrin. The figure shows a sequence alignment of saxiphilin (Sax) and human lactoferrin (Ltf). Identical residues are highlighted in black boxes. Regions corresponding to the homologous N- and C-lobes of lactoferrin are outlined in large boxes. In each lobe, five conserved residues that coordinate Fe^{3+}/HCO_3^- in transferrins (D, Y, R, Y, H) are identified by the symbol "^" in the lactoferrin sequence.

Fig. 3 shows the domain structure and location of 14 disulfide bonds in saxiphilin as predicted on the basis of homology to transferrins and the crystal structure of lactoferrin (Anderson et al., 1989). A unique 143-residue insertion sequence occurs in saxiphilin at a location corresponding to the N1-N2 hinge region of lactoferrin. This insertion is a tandem duplication containing two copies of the Thyr-1 modular domain that is shared by a number of proteins in the protein database (Fig. 4).

Lack of Fe^{3+} binding by saxiphilin is readily explained by amino acid substitutions of nine out of ten highly conserved ligand-binding residues for Fe^{3+}/HCO_3^{1-} (1 Asp, 2 Tyr, 1 His and 1 Arg) located in the two homologous domains of Tf proteins (Baker, 1994; Morabito & Moczydlowski, 1994). The following amino acid substitutions at these sites in saxiphilin are as follows (using residue numbers based on human Tf): Y95H, R124K, Y188N and H249G in the N-lobe; D392E, Y426L, R456D, Y517N, H585P in the C-lobe (Fig. 2).

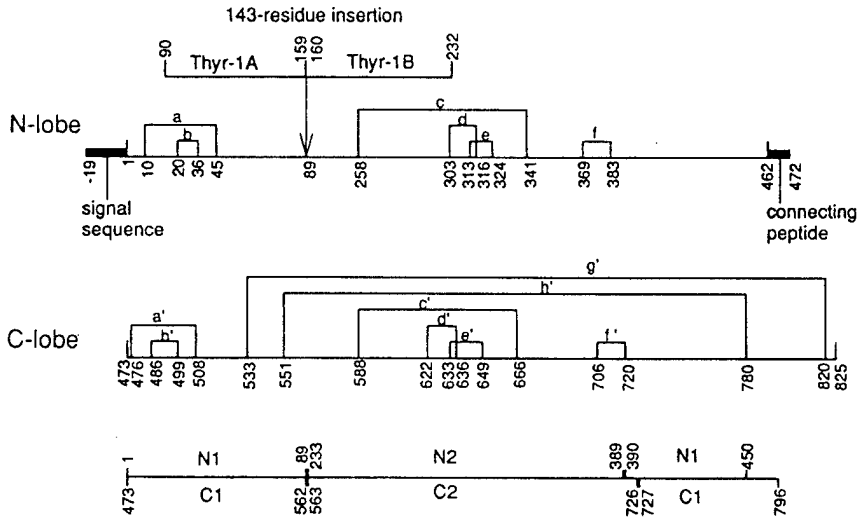


Figure 3. Diagram of the linear sequence of saxiphilin. This diagram shows major structural features of saxiphilin as predicted by homology to transferrins. The first 19 residues (-19 to -1) are a secretory signal peptide. Residues 1-462 and 473-796 comprise the N-lobe and C-lobe, respectively. Connected pairs of residues labeled a-f and a'-h' correspond to fourteen predicted disulfide bonds. Residues 90-232 are a unique insertion in saxiphilin that consists of two tandem thyroglobulin Type-1 domains (Thyr-1A and Thyr-1B). The bottom diagram shows the location of residues that comprise predicted subdomains N1, N2, C1, and C2, and also shows that the unique insertion at residue 89 occurs at a hinge region between N1 and N2. Figure taken from Morabito et al., 1995.

As noted above, saxiphilin also differs from Tf proteins by the presence of a unique insertion sequence of 143 residues in the N-terminal domain. This insertion is found at a location of the saxiphilin molecule that would be predicted to form a hinge region observed in the N-lobe of lactoferrin between subdomains N1 and N2 (Figs. 2 & 3, Anderson et al., 1989). Sequence analysis and a search of the protein database reveals that this insertion sequence consists of a tandem duplication (67% identity) containing a

recognized protein module known as a type-1 repetitive element of thyroglobulin (Thyr-1) (Malthiery & Lissitzky, 1987). Such ~50-residue Thyr-1 domains (Fig. 4) have been identified in diverse proteins that include: thyroglobulin, epithelial glycoprotein cell surface antigen (Simon et al., 1990), invariant chain Ii of major histocompatibility class II complex (MHC-II) (Koch et al., 1987), the cell matrix protein nidogen (Mann et al., 1989), insulin-like growth factor binding proteins (Shimasaki et al., 1991), and a testis-specific multidomain proteoglycan called testican (Alliel et al., 1993). The function of the Thyr-1 module is unknown but work on the invariant chain Ii of the MHC-II complex suggests that it may serve as an intracellular transport signal from an early to a late endosomal compartment (Peterson & Miller, 1992).

```
Sax (90-159)    KCLKERQQALA--KKMIGHYIPQCDEKGNYQPQQCHGSTGHCWCVNAMGEKISGTNTPPGQTRATCERHELP-
                ||||||| ||:   |::|:::|||||||||||:||| ||||| :|||||:|| | || ||||   |||::|:|
Sax (160-232)   KCLKERQVALGGDEKVLGRFVPQCDEKGNYEPQQFHGSTGYSWCVNAIGEEIAGTKTPPGKIPATCQKHDLVT

nidogen (842-889)              GMFVPQCDEYGHYVPTQCHHSTGYCWCVDRDGRELEGSRTPPGMRPP-C
invariant chain (210-248)      GAFRPKCDENGNYMPLQCHGSTGYCWCVFPNGTEVPHTK
EGP (93-123)                   GLYDPDCDESGLFKAKQCNG-TSMCWCVNTAG
thyroglobulin 1.1 (29-73)        YVPQCAEDGSFQTVQCQNDGRSCWCVGANGSEVLGSRQP-GR-PVAC
thyroglobulin 1.2 (97-141)       YLPQCQDSGDYAPVQCDVQHVQCWCVDAEGMEVYGTRQL-GR-PKRC
thyroglobulin 1.5 (597-639)      FVPSCTTEGSYEDVQCFSGE--CWCVNSWGKELPGSRVRDGQ-PR-C
thyroglobulin 1.6 (664-707)      FVPACTSEGHFLPVQCFNSE--CYCVDAEGQAIPGTRSAIGK-PKKC
```

Figure 4. Homology relationships of the 143-residue insertion sequence unique to saxiphilin. The upper two sequences are a pair-wise alignment of saxiphilin residues 90-159 and 160-232 showing two-fold homology within the 143-residue insertion. A vertical line marks an identity and a colon indicates a conservative substitution. The lower seven sequences illustrate homology to a modular domain observed in many other proteins that is known as a thyroglobulin Type-1 motif. The comparison sequences are mouse nidogen (Mann et al., 1989), rat invariant chain Ii of MHC-II complex (McKnight et al. 1989), human epithelial glycoprotein (EGP) (Simon et al., 1990) and human thyroglobulin (Malthiery & Lissitzky, 1987). Figure taken from Morabito & Moczydlowski, 1995.

The tissue distribution of saxiphilin mRNA was investigated by comparing hybridization signals using probes corresponding to nearly full-length saxiphilin DNA and a region coding for the unique insertion domain of saxiphilin. This survey showed that saxiphilin mRNA was abundant in frog liver and lung with minor amounts detected in brain and pancreas. No hybridization was observed in spleen, kidney, stomach, skeletal muscle, heart, or unfertilized eggs (Morabito & Moczydlowski, 1994). These studies of saxiphilin mRNA distribution identified the liver as an active locus of saxiphilin gene expression and synthesis. This finding is consistent with the notion that saxiphilin, like transferrin, is secreted from the liver into blood plasma where it is available for distribution to other tissues.

3. Characterization of the mechanism of saxitoxin binding to purified native saxiphilin. Saxiphilin specifically binds STX (K_D = 0.2 nM) and various STX derivatives (K_D range = 0.2 to 170 nM) as assayed by binding competition with [^{3}H]STX (Mahar et al., 1991). To date, we have not found any other organic compound that significantly competes with STX binding to saxiphilin, even at a concentration of 10 mM (Llewellyn et al., 1994).

The stoichiometry of [^{3}H]STX binding to pure saxiphilin indicates that there is only one binding site for STX per 91 kDa polypeptide. STX binding to saxiphilin is inhibited by low pH with half-inhibition at pH 5.7. H^+ inhibits [^{3}H]STX binding in an allosteric manner by slowing the toxin association rate and enhancing the dissociation rate. The kinetics of H^+ inhibition can be quantitatively described by a model in which the protonation of a single residue modulates toxin binding with a pKa of 7.2 in the STX-free form of saxiphilin and a pKa of 4.3 in the STX bound form of saxiphilin. Various divalent transition metal cations and trivalent lanthanide cations inhibit [^{3}H]STX binding to saxiphilin in the range of 1-100 mM. Some of these effects appear to be due a competitive binding interaction since 10 mM Pr^{3+} inhibits [^{3}H]STX binding by slowing the toxin association rate without affecting the dissociation rate, as expected for simple competition. The carboxylate modifying reagent trimethyloxonium inhibits toxin binding in an STX-protectable fashion, implying that acidic Asp and Glu residues form part of the STX binding site (Llewellyn & Moczydlowski, 1994).

The pH dependence of Fe^{3+} binding to serum Tf is an important aspect of the delivery of Fe^{3+} to cells by the process of receptor-mediated endocytosis. The dissociation rate of Fe^{3+} from Tf is a very slow process at an extracellular pH of 7.4, but it is strongly enhanced at an endosomal pH value in the range of 5.5 (Lestas, 1976; Chasteen & Williams, 1981; Dautry-Varsat et al. 1983). The drop in intra-endosomal pH brought about by an H^+-pump ATPase on the endosomal membrane thus results in Fe^{3+}-release from Tf and subsequent movement of iron out of the endosome for storage and utilization. Despite the structural differences of saxiphilin from Tf, [^{3}H]STX binding to saxiphilin is inhibited by low pH, via an enhancement of the [^{3}H]STX dissociation rate, over the same pH range as that of Fe^{3+}-binding to serum Tf. Although it may be a coincidence, the similar pH dependence of ligand binding to Tf and saxiphilin suggests that saxiphilin may also function in the cellular internalization of a small molecule by pH-regulated, receptor-mediated endocytosis.

4. Functional expression of recombinant saxiphilin and a C-lobe fragment of saxiphilin. In beginning experiments to express and produce recombinant saxiphilin, we first looked to the transferrin field for an appropriate method. Expression of functionally active recombinant human serum Tf and human lactoferrin has been successfully achieved in the mammalian BHK cell line (baby hamster kidney cells) using the pNUT expression vector (Mason et al., 1991, 1993; Day et al., 1992). Since high expression levels in the range of 10-125 mg protein/liter have been reported for this latter system, we first tried

this approach for saxiphilin. Despite several attempts, we have not yet been able to subclone saxiphilin cDNA into the pNUT vector. It seems that saxiphilin cDNA is not very well behaved with respect to the blunt-end ligation necessary for insertion into the SmaI restriction site of the pNUT vector, which lacks a convenient multiple cloning site. However, we have successfully expressed saxiphilin in various mammalian cell lines (e. g., HEK293 human embryonic kidney; HEPG3 human hepatoma) using the pcDNA3 vector (Invitrogen) and selection for stable transfectants by neomycin resistance. Unfortunately, these cell lines exhibited rather low levels of secreted saxiphilin (~2 pmol/ml) and did not seem to be very promising for large scale expression (unpublished data).

The best expression system for saxiphilin that we have found thus far is the use of baculovirus-mediated expression in the Sf9 or HighFive insect cell lines as described in Morabito et al. (1995). With this system we have obtained expression levels of 2.2-6.1 mg saxiphilin secreted / liter culture medium as quantitated by [^{3}H]STX binding. Figure 5 summarizes the basic functional properties of recombinant saxiphilin produced by baculovirus-mediated expression. The molecular mass, affinity for STX (K_D = 0.22 nM), binding kinetics and pH-dependence of the recombinant saxiphilin protein are essentially indistinguishable from native saxiphilin.

We also constructed a deletion mutant of saxiphilin lacking the N-lobe by splicing the coding region for the C-terminal 361-residue fragment to that of the first 20 N-terminal residues containing the native secretory signal sequence. A baculovirus vector coding for this construct directs the secretion of a ~38 kDa C-lobe derivative of saxiphilin (called C-sax) that is recognized by anti-saxiphilin polyclonal antibody. C-sax exhibits a somewhat a lower affinity K_D of ~0.9 nM for [^{3}H]STX due to a 4-fold faster dissociation rate. These results nevertheless demonstrate that the STX binding site and residues that determine the pH dependence of toxin binding are located within the C-lobe domain of saxiphilin (Morabito et al., 1995).

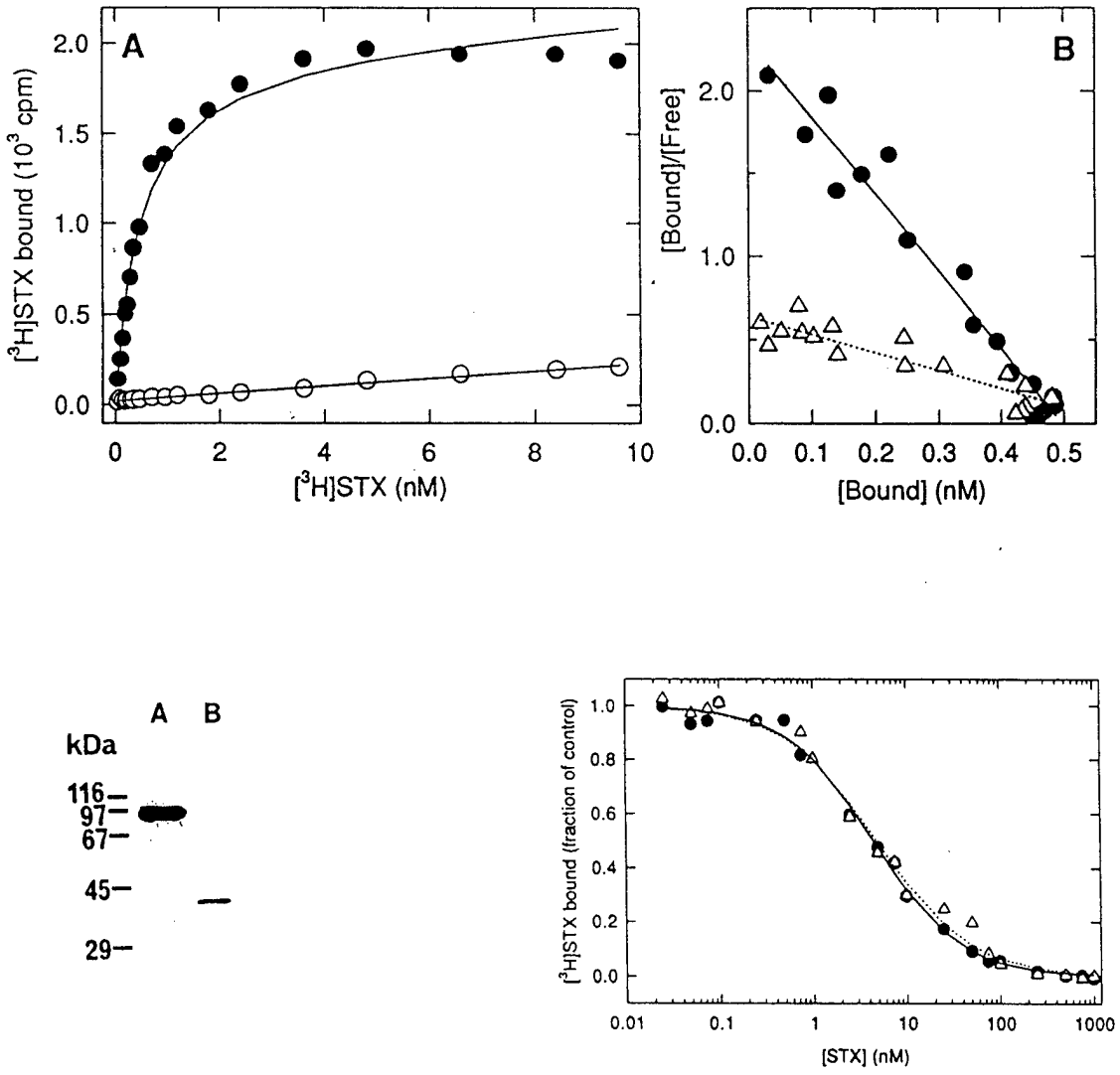


Figure 5. Characterization of recombinant saxiphilin expressed in insect cells. The top panel (A, B) shows results of an assay of [^{3}H]STX binding to culture medium from insect cells infected with a baculovirus vector encoding saxiphilin (R-sax) or the C-lobe of saxiphilin (C-sax). Panel A shows raw binding data for R-sax in the absence (●) or presence (○) of 10 μM STX to assess non-specific binding. Panel B shows Scatchard plots of [^{3}H]STX binding to R-sax (●, K_D = 0.22 nM) or C-sax (Δ, K_D = 0.93 nM). The panel in the lower left shows an immunoblot of culture medium from insect cells expressing either R-sax (lane A) or C-sax (Lane B). Samples were subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane and probed with anti-saxiphilin antibody. The positions of the immunoreactive bands agree well with the theoretical molecular mass of R-sax (91 kDa) and C-sax (40 kDa). The panel in the lower left shows a titration of [^{3}H]STX binding by unlabeled STX for R-sax (●) or C-sax (Δ), indicating competitive inhibition at a single-class of sites. Figures take from Morabito et al., 1995.

Using conventional methods similar to those previously used to purify native saxiphilin from frog plasma (Li and Moczydlowski, 1991), we have successfully purified recombinant saxiphilin to homogeneity. We adapted this purification scheme for 5 liter batches of medium harvested from insect cells grown in suspension. The routine practicality of saxiphilin production and purification are facilitated by the ability to use commercially available serum-free medium for insect cell culture (e.g., HyQ CCM3 medium from Hyclone).

5. Phylogenetic Survey of Saxiphilin Activity. Since saxiphilin has been purified, characterized from the bullfrog, *Rana catesbeiana*, it is important to know whether this unusual Tf homolog is peculiar to frogs or whether it is broadly expressed in the animal kingdom. To address this question, we recently completed an extensive phylogenetic survey of saxiphilin activity (Llewellyn et al., 1996). The premise of the survey is that saxiphilin activity as characterized in the bullfrog (Mahar et al., 1991; Llewellyn and Moczydlowski, 1994) has a unique and well defined biochemical pharmacology of [^{3}H]STX binding. Thus, this assay can be used to identify the presence of a saxiphilin-like protein in plasma, hemolymph and crude tissue extracts from diverse animal species.

Using this approach we found saxiphilin-like activity in certain arthropods (e.g., spiders, a centipede, and crabs) and in three classes of vertebrates (fish, amphibians and reptiles). Figure 6 shows saturation binding isotherms of high-affinity [^{3}H]STX binding measured in five diverse species: *Bufo marinus* (cane toad), *Naja naja kaouthia* (Thailand cobra), *Thamnophis sirtalis* (garter snake), *Gambusia affinus* (mosquito fish) and *Ethmostigmus rubripes* (an Australian centipede). Analysis of the kinetics, pharmacology, and pH-dependence of the binding activity from all of these species confirmed that it was characteristically "saxiphilin-like." For example, the competition-binding experiments of Fig. 7 show that soluble [^{3}H]STX-binding activity in the cobra, mosquito fish, and the centipede display similar structure-activity relationships for the binding of several naturally occurring STX derivatives.

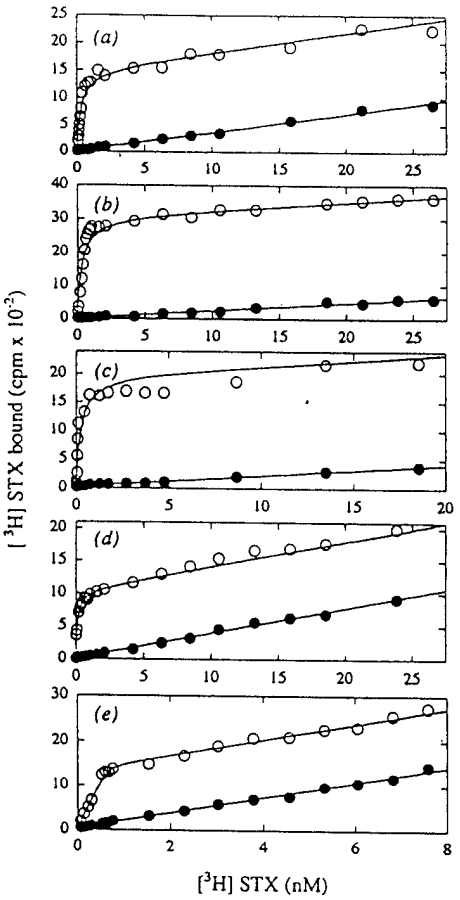


Figure 6. Saxiphilin-like activity in diverse animal species. The five panels above demonstrate the presence of saxiphilin-like activity in an amphibian, two reptiles, a fish and an arthropod. A constant amount of soluble protein extracted from various animal species was assayed for binding at the indicated concentrations of total [^{3}H]STX, where filled circles indicate the non-specific component in the presence of excess unlabeled STX. (a) plasma from *Bufo marinus*, cane toad; (b) plasma from *Naja naja kaouthia*, Thailand cobra; (c) plasma from *Thamnophis sirtalis*, garter snake; (d) crude soluble extract from *Gambusia affinis*, mosquito fish; (e) hemolymph from *Ethmostigmus rubripes*, an Australian centipede. Figure taken from Llewellyn et al., 1996.

A striking finding that emerged from this work was the exceedingly high STX-binding affinity of saxiphilin proteins from some animal species. For example, the calculated equilibrium dissociation binding constant (K_D) for [^{3}H]STX was approximately 10^{-12} M for the cane toad (*Bufo marinus*), the mosquito fish (*Gambusia affinis*), and the centipede (*Ethmostigmus rubripes*). Such evolutionary conservation of high affinity for a particular ligand argues that the STX-saxiphilin interaction reflects a conserved biological function.

The survey results point to the conclusion that the saxiphilin gene probably arose during the origin of invertebrates and has been conserved in many vertebrate species. The phylogenetic pattern of saxiphilin expression that we have observed is very similar to that of transferrin, which has been identified in some insects and all vertebrates that have been

studied (Bartfeld & Law, 1990; Jamroz et al., 1993; Kurama et al., 1995, Welch, 1990). Our findings suggest that saxiphilin may have arisen directly from an ancestral bi-lobed transferrin molecule that lost its ability to bind Fe^{3+} but gained an ability to bind a different ligand such as STX. The results of the species survey suggest that the saxiphilin gene is likely to have a general biological function since it is present in the genome of phylogenetically diverse classes of animals.

However, we have not yet found evidence of a saxiphilin protein in the plasma of birds or mammals, including humans. This may mean that the gene is limited to ectothermic (cold-blooded) animals. It may also mean that we have not yet identified the tissue of expression or conditions that induce expression of the saxiphilin gene in birds or mammals.

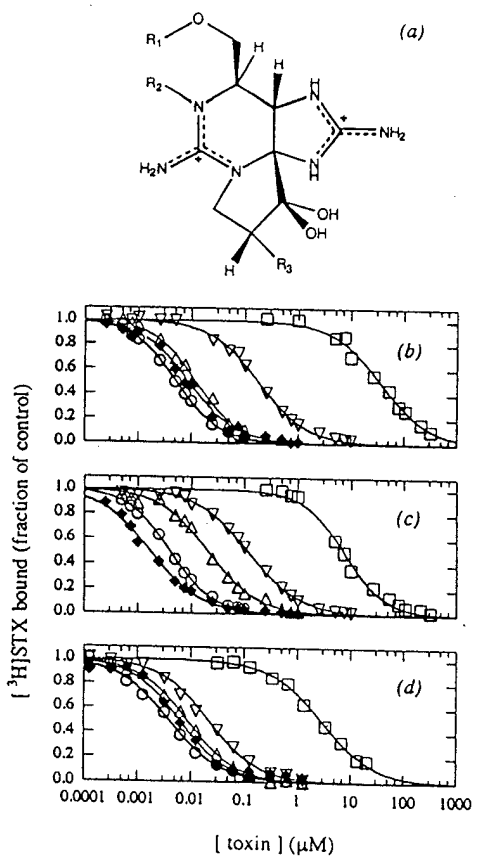


Figure 7. Structure-activity relationships for STX and four STX derivatives assayed by competitive inhibition of [^{3}H]STX binding (a) Chemical structure of five tested STX derivatives: STX (O) , R_1 = $CONH_2$, R_2 = H, R_3 = H; decarbamoyl saxitoxin (filled diamond), R_1 = H, R_2 = H, R_3 = H; neosaxitoxin (Δ) R_1 = $CONH_2$, R_2 = OH, R_3 = H; natural derivative B1 (upside down triangle), R_1 = $CONHSO_3^-$, R_2 = H, R_3 = H; natural derivative C1 (open square), R_1 = $CONHSO_3^-$, R_2 = H, R_3 = OSO_3^-. (b) plasma of *Naja naja kaouthia*, Thailand cobra; (c) extract of *Gambusia affinis*, mosquito fish; (d) hemolymph of *Ethmostigmus rubripes*, centipede. Data taken from manuscript of Llewellyn et al., (1996).

Conclusions: This project has resulted in the following accomplishments and original findings:

1. Saxiphilin is a unique member of the transferrin superfamily of proteins. It does not appear to function directly in iron transport as judged by a lack of Fe^{3+}-binding activity. The inability of saxiphilin to bind Fe^{3+} is explained by substitutions in the primary amino acid sequence of nearly all of the conserved residues of transferrins that are known to coordinate directly with Fe^{3+} and HCO_3^- in the two homologous binding sites for Fe^{3+}/HCO_3^-.

2. The complete primary amino acid sequence of bullfrog saxiphilin has been deduced by molecular cloning of saxiphilin cDNA. The sequence indicates that saxiphilin is secreted from frog liver as an 825-residue polypeptide after cleavage of a 19-residue signal sequence. Sequence alignment with transferrins whose structures have been solved by X-ray diffraction indicates that saxiphilin contains at least 14 conserved disulfide bonds.

3. Binding titration of pure native saxiphilin with [^{3}H]STX shows that there is one high affinity STX-binding site per molecule with a KD of ~0.2 nM at 0° C and pH 7.4.

4. Binding of STX to saxiphilin is inhibited by decreasing pH with half-maximal inhibition occurring at pH 5.7. The inhibition of STX binding by H^+ is the combined result of a slower association rate and a faster dissociation rate. The pH-dependent kinetics of STX binding can be explained by an allosteric model in which protonation of a single titratable residue results in a low affinity conformation of saxiphilin with respect to STX.

5. The cloned cDNA encoding saxiphilin has been inserted into a baculovirus expression vector that directs the synthesis of functionally active protein in cultured insect cells. A similar baculovirus constructed to express a 40 kDa fragment of saxiphilin corresponding to the C-lobe domain also exhibits [^{3}H]STX-binding activity, showing that the STX-binding site is located in this portion of the molecule.

6. Saxiphilin-like activity has been detected in a number of arthropods, fish, amphibians and reptiles. This indicates that the saxiphilin gene has an ancient origin in animal evolution and may have arisen from an ancestral transferrin-like protein. The widespread occurrence of saxiphilin throughout the arthropod and vertebrate lineages suggests that this protein may have a general biological function.

The structural similarity of saxiphilin to Tf raises the question of how this particular structure could be adapted to bind different ligands. Possible insight to this question comes from the observation of Baker et al. (1987) that the fold of the N-lobe and C-lobe

of lactoferrin bears a striking similarity to the structures of the bacterial periplasmic binding proteins. These latter molecules reside in the periplasmic space between the inner and outer membrane of many species of bacteria and are involved in the active transport of many kinds of inorganic ions and small molecules such as phosphate, sulfate, several amino acids and numerous sugars (Ames, 1986). Although these binding proteins have a low level of sequence identity to each other or to the N-lobe and C-lobe of transferrins, they all have a similar size (~330 residues) and tertiary fold based on two subdomains connected by a flexible hinge region, with each subdomain formed by a twisted β-sheet surround by α-helices. The crystal structures of many of these proteins has been solved with their substrate bound (Quiocho et al., 1987;, Quiocho, 1990). These structures indicate that the interdomain cleft of this structure has been adapted to form a binding site for many different small molecules by the formation of different bonding interactions. In particular, Baker et al. (1987) noted that the topology of the secondary structural elements of the sulfate binding protein of *Salmonella typhimurium* is very similar to that of the N-lobe of lactoferrin. This observation has led to the suggestion that the transferrin/lactoferrin structure might be used as a scaffold to engineer the binding of various small molecules (Baker and Lindley, 1992).

The identification of saxiphilin as a non-Fe^{3+}-binding member of the Tf family suggests that other such proteins may exist. Our work on saxiphilin has led us to the notion that transferrins may comprise a superfamily of structurally related proteins with diverse biological functions besides those known to be linked to iron metabolism. Recent developments in the fields of Tf and lactoferrin biochemistry support this contention.

A non-Fe-binding homolog of transferrin in pig plasma has recently been characterized and described as a potent inhibitor of certain isoforms of carbonic anhydrase. This protein named pICA by C. A. Fierke and coworkers is a 79 kDa glycoprotein that binds to carbonic anhydrase II in a 1:1 complex and inhibits this enzyme with a K_i of 0.5 nM (Roush and Fierke, 1992). Cloning and sequence analysis (Genbank Accession No. U36916) has shown that pICA is 65% identical to porcine transferrin. Equilibrium dialysis studies indicate that pICA does not bind iron (Wuebbens et al., 1994). The lack of iron binding by this Tf-like protein is presumably due to at least three substitutions of Fe^{3+}/HCO_3^- ligand binding residues: R124W in the N-lobe; R456T, Y517F in the C-lobe (human Tf numbering). Together with our studies of saxiphilin, the discovery of pICA indicates that vertebrate genomes contains at least two genes coding for proteins that are closely related to Tf, but which do not appear to be directly involved in Fe^{3+} transport. Molecular biological and biochemical studies are clearly needed to identify such genes and investigate their functions. It is also intriguing that two other relatives of the Tf family, human melanotransferrin (Baker et al., 1992) and a moth transferrin (Bartfeld and Law, 1990), have lost the ability to bind Fe^{3+} in the C-lobe (Baker, 1994), the same lobe that binds STX in saxiphilin (Morabito et al., 1995).

Emerging developments in the lactoferrin field also point to multiple functions and isoforms of this protein. Human lactoferrin itself has recently been proposed to bind specific DNA sequences and function as an activator of gene transcription (He and

Furmanski, 1989). It has been suggested that lactoterrin secreted by neutrophils is taken up by lymphocytes and transported to the nucleus where it functions as a transcription factor that is involved in immunomodulation (Baeuerle, 1995). Lactoferrin also has been found to have a bactericidal peptide domain within its sequence that is capable of killing numerous species of bacteria (Bellamy et al., 1992; Tomita et al. 1994). In addition, a non Fe-binding isoform of lactoferrin has been isolated from human milk and characterized as an RNAase that is hypothesized to provide protection against transmission of retroviruses (Furmanski et al., 1989; Ramaswamy et al., 1993). While still tentative, such emerging findings on new and diverse functions of transferrin-related proteins may lead to a revision of the conventional idea that the function of the transferrin protein family is strictly related to iron binding.

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Li, Y., Llewellyn , L., & Moczydlowski, E. 1993. Biochemical and immunochemical comparison of saxiphilin and transferrin, two structurally related plasma proteins from *Rana catesbeiana*. *Mol. Pharmacol.* 44: 742-748.

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Biochemical and Immunochemical Comparison of Saxiphilin and Transferrin, Two Structurally Related Plasma Proteins from *Rana catesbeiana*

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SUMMARY

Saxiphilin is a ~90-kDa protein in bullfrog plasma that binds the neurotoxin saxitoxin (STX) with high affinity (K_d, ~0.2 nM). The relationship between saxiphilin and transferrin was examined because partial sequencing of saxiphilin previously revealed an unexpected homology to members of the transferrin family of Fe^{3+}-binding proteins. Transferrin was purified from bullfrog plasma and shown to be distinct from saxiphilin on the basis of its size (~78 kDa), chromatographic behavior, visible absorption spectrum, and ligand-binding properties. High affinity binding of [^{3}H]STX was found to be a distinctive property of saxiphilin that was not exhibited by transferrins from various species of animals. Conversely, under conditions appropriate for transferrins, purified saxiphilin did not bind $^{55}Fe^{3+}$, implying that it is not involved in iron metabolism. Polyclonal antibodies raised against native saxiphilin precipitated [^{3}H]STX-binding activity from whole bullfrog plasma. On immunoblots such antibodies recognized the denatured saxiphilin protein but only weakly labeled bullfrog transferrin. In an enzyme-linked immunosorbent assay using native proteins, antisaxiphilin antibodies weakly cross-reacted with transferrin from bullfrog and a number of other species. Likewise, antibodies against human transferrin cross-reacted with saxiphilin in a similar immunosorbent assay. These results lead to the conclusion that saxiphilin is not bullfrog transferrin but is structurally related to the transferrin family. As a novel member of the transferrin superfamily, saxiphilin may help to uncover new functions mediated by this class of proteins.

STX, a small heterocyclic guanidinium compound, is a potent neurotoxin that is produced by certain dinoflagellates and cyanobacteria (1). A well known aspect of the biology of STX is the widespread distribution of this toxin in various marine animals. In toxicology, STX is associated with the problem of "paralytic shellfish poisoning" that sporadically occurs in conjunction with plankton blooms (2). Paralysis induced by STX poisoning is due to blockage of voltage-dependent Na^+ channels of electrically excitable cells at an external site associated with the conducting pore (3). In addition to STX-sensitive Na^+ channels, tissues from various amphibians and reptiles have been found to contain a different soluble protein, named saxiphilin, that specifically binds STX with high affinity (4, 5). At present, it is unknown whether STX binding to saxiphilin has any physiological significance, but such a protein might have useful pharmacological applications in reversal of STX block of excitable cells and/or in antidote therapy.

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Partial sequencing of saxiphilin purified from plasma of the North American bullfrog (*Rana catesbeiana*) revealed that fragments of this 90-kDa protein exhibit considerable sequence homology (40–70% identity) to vertebrate transferrins (6). Transferrins are a family of monomeric Fe^{3+}-binding glycoproteins of M_r ~80,000, including serum transferrin, lactoferrin, melanotransferrin, and ovotransferrin (7, 8). Some of these proteins exhibit antimicrobial activity due to their high affinity for Fe^{3+} (K_d, ~10^{-20} M); however, the essential role of serum transferrin is to supply eukaryotic cells with Fe^{3+}, which is necessary for growth as a requisite cofactor of numerous metalloproteins. Transferrins contain two internally homologous domains that each bind one Fe^{3+} ion and one bicarbonate anion, except for human melanotransferrin (9, 10) and a transferrin isolated from the tobacco hornworm *Manduca sexta* (11), which both appear to have only one functional Fe^{3+}-binding domain.

Because bullfrog transferrin has not yet been sequenced or cloned, the unexpected homology between saxiphilin and members of the transferrin family raised the possibility that saxiphilin is an unusual derivative or isoform of transferrin itself.

ABBREVIATIONS: STX, saxitoxin; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

To address this question, we have separately purified saxiphilin and transferrin from bullfrog plasma and compared the size, ligand-binding properties, and immunochemical cross-reactivity of these two proteins. The results indicate that saxiphilin is a distinct protein that does not bind Fe^{3+} but nevertheless contains related antigenic determinants that reflect underlying structural similarity to transferrins.

Experimental Procedures

Materials. Various materials and animals used in this study were obtained from the following commercial sources: [^{3}H]STX (20–40 Ci/mmol) and $^{55}FeCl_3$ (70 Ci/mol) from Amersham; STX and Pansorbin (*Staphylococcus aureus* cells) from Calbiochem; Biogel P6 from Bio-Rad; CNBr-activated Sepharose 4B, S-Sepharose, Sephadex G-200, and DEAE-Sephadex A-50 from Pharmacia; NTA, apotransferrin from human, horse, cow, and rabbit, transferrin from mouse and guinea pig, human lactoferrin, chicken ovotransferrin, and goat antiserum against human transferrin from Sigma; peroxidase-conjugated goat IgG against rabbit immunoglobulins and peroxidase-conjugated rabbit IgG against goat IgG from Cappel (Organon Teknika Corp.); and adult bullfrogs (*Rana catesbeiana*) from Connecticut Valley Biological Supply.

Purification of saxiphilin and preparation of antisaxiphilin antibodies. Saxiphilin, assayed by [^{3}H]STX binding, was purified to homogeneity from bullfrog plasma by column chromatography on heparin-Sepharose and chromatofocusing as described previously (6). Polyclonal antiserum to native saxiphilin was raised in rabbits by Pocono Rabbit Farm and Laboratory (Canadensis, PA). Antigen injections of pure saxiphilin followed a schedule of 200 μg of protein in Freund's complete adjuvant injected intradermally on the first day, 100 μg in Freund's incomplete adjuvant injected intradermally on day 14, and 25-μg intramuscular boost injections in incomplete adjuvant on day 28 and every 4 weeks thereafter. A high titer of antisaxiphilin antibodies was observed after 5 weeks with the enzyme-linked immunosorbent assay described below. Antiserum (~15 ml) was collected from weekly bleeds. Antisera obtained from two different rabbits exhibited similar levels of reactivity.

Antisaxiphilin antibodies used in this work were affinity-purified using saxiphilin as the ligand. Pure saxiphilin (0.5 mg) was covalently coupled to 0.5 ml of swelled CNBr-activated Sepharose 4B according to the manufacturer's instructions. The prepared column was equilibrated with bicarbonate buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3), and 0.1 ml of rabbit antisaxiphilin antiserum diluted to 1 ml in bicarbonate buffer plus 0.5% Tween 20 detergent was applied and recycled three times through the column. The column was then washed with 10 ml of bicarbonate buffer and eluted with 5 ml of 100 mM glycine, pH 2.8. A small peak of specific antibodies that eluted after the pH step was neutralized to pH 7.5 with Tris base, stored frozen, and used in various immunoassays.

Purification of bullfrog transferrin. A sample (10 ml) of previously collected bullfrog plasma was thawed and supplemented with 10 mM $NaHCO_3$ and 15 μM $Fe(NTA)_2$ premixed with 45 μCi of $^{55}FeCl_3$. This ^{55}Fe-labeled plasma sample was first subjected to gel filtration chromatography on a 2.5- × 50-cm column of Sephadex G-200 equilibrated with 100 mM Tris·HCl, 1 M NaCl, pH 7.8, and was eluted at 12 ml/hr with the same buffer. A peak of soluble, protein-bound ^{55}Fe eluting after the void volume was pooled and dialyzed against 3 liters of 20 mM Tris·HCl, pH 7.8. This sample was applied to a 2.5- × 25-cm column of DEAE-Sephadex A-50 equilibrated with 20 mM Tris·HCl, pH 7.8, was eluted at 12 ml/hr with a linear gradient of 400 ml of 20–500 mM Tris·HCl, pH 7.8, and was collected in 12.5-ml fractions. Aliquots (10 μl) of various fractions were assayed for ^{55}Fe by liquid scintillation counting. Specific binding of [^{3}H]STX was assayed on 10-μl fraction aliquots as described (6). Protein was monitored by measuring absorbance at 280 nm, and the salt gradient was monitored by measuring conductivity of 1/100 dilutions of various fractions. A single major peak (Fig. 1) of ^{55}Fe was identified as bullfrog transferrin by its characteristic orange-pink color and visible absorption spectrum (Fig. 2A) with a maximum at 465 nm.

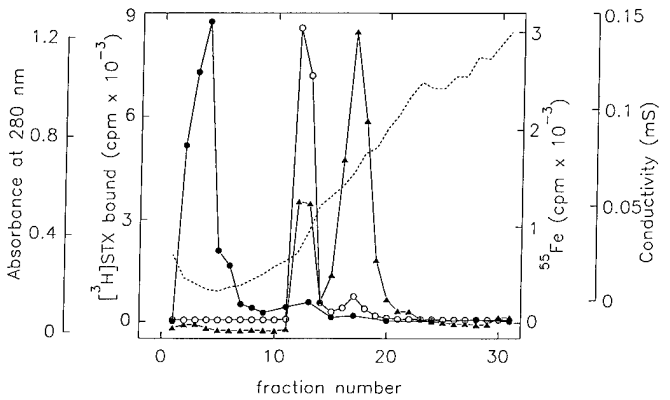

Fig. 1. Separation of [^{3}H]STX- and ^{55}Fe-binding activities in bullfrog plasma. A 10-ml sample of bullfrog plasma was labeled with $^{55}Fe(NTA)_2$, partially purified by gel filtration on a column of Sephadex G-200, and subjected to anion exchange chromatography on a column of DEAE-Sephadex A-50, as described in Experimental Procedures. Various fractions from the DEAE-Sephadex A-50 column were assayed for protein (▲), specific binding of [^{3}H]STX (●), ^{55}Fe (○), and conductivity (– – –). Similar results were obtained in three different experiments.

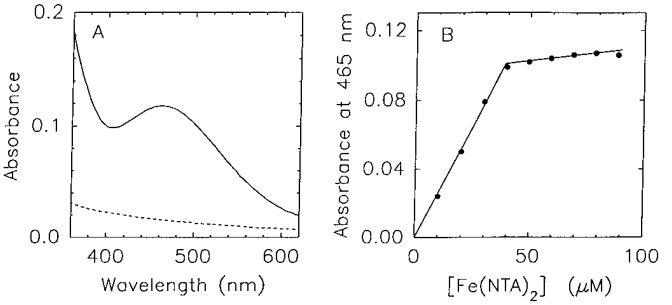

Fig. 2. Visible absorption spectrum and Fe^{3+} titration of bullfrog apotransferrin. A, Spectrum of 1.6 mg/ml apotransferrin before (– – –) and after (——) addition of 50 μM $Fe(NTA)_2$. B, Absorbance change at 465 nm measured after addition of the indicated concentrations of $Fe(NTA)_2$ to 1.6 mg/ml apotransferrin.

Iron was removed from purified transferrin by addition of 1 mM NTA and 2 mM EDTA, adjustment to pH 4.2 with acetic acid, and incubation overnight at 0°. The sample was then dialyzed against 1 mM MOPS-NaOH, 100 mM NaCl, pH 7.4, and lyophilized to 1 ml. Residual chelating agent was removed by gel filtration on a 15-ml column of Biogel P6 eluted with 100 mM NaCl, 1 mM MOPS-NaOH, pH 7.4. The yield of apotransferrin was ~8 mg.

Because [^{3}H]STX binding experiments indicated that bullfrog transferrin purified by the method described above contained a trace amount of contaminating saxiphilin, an additional purification step was undertaken for use in immunochemical assays. S-Sepharose was used because saxiphilin is a basic protein and was previously found to adsorb to this medium (5). A column (4 ml) of S-Sepharose was equilibrated with 25 mM sodium acetate, 10 mM MES-NaOH, pH 6.0. Bullfrog apotransferrin (320 μg) diluted to 1 ml with equilibration buffer was applied to the column at 15 ml/hr. The column was eluted with 24 ml of 100 mM sodium acetate and 12 ml of 150 mM sodium acetate in 10 mM MES-NaOH, pH 6.0. The peak of protein eluting after the final step of 150 mM sodium acetate was pooled, dialyzed against 10 mM NaCl, 1 mM HEPES-NaOH, pH 7.4, and concentrated by lyophilization.

Fe^{3+} titration of apotransferrin and absorption spectrum. Stock solutions of 10 mM $Fe(NTA)_2$ were prepared fresh by dissolving $FeCl_3 \cdot 6H_2O$ in an acidic solution of NTA at a ratio of 2.2 NTA/Fe and adjusting the pH to 4.0 with NaOH. Fe^{3+} binding to apotransferrin was monitored by measuring absorbance at 465 nm with a Perkin-Elmer

λ9 UV-visible scanning spectrophotometer. Absorbance was recorded 10 min after addition, with mixing, of consecutive 2-μl aliquots of 5 mM $Fe(NTA)_2$ to a quartz cuvette (1-cm path length) containing 1.0 ml of 1.6 mg/ml apotransferrin in 20 mM HEPES-NaOH, 20 mM $NaHCO_3$, 100 mM NaCl, pH 7.4. At the equivalence point, the spectrum of Fe_2-transferrin was recorded against a reference cuvette containing titration buffer minus protein. To determine the stoichiometry of Fe^{3+} binding, the transferrin protein concentration was based on quantitative amino acid analysis performed by the Yale Protein Chemistry Facility. The amino acid composition of bullfrog transferrin was similar to that reported previously (12). Protein determined by this method was corrected for proline, cysteine, and tryptophan, which were not measured directly but were assumed to be equal to 10.7 weight percent, based on the known composition of transferrin cloned from *Xenopus laevis* (13).

Immunoprecipitation of [^{3}H]STX-binding activity. A sample of 35 μg of bullfrog plasma was incubated with 11 nM [^{3}H]STX, 10 mM MOPS-NaOH, 200 mM choline chloride, pH 7.4, and various amounts (0.2–25 μg) of affinity-purified antisaxiphilin antibodies were added in a final volume of 0.5 ml. Control reactions included 40 μM STX or preimmune rabbit serum instead of specific antibodies. Samples were incubated for 1 hr on ice, and 50 μl of Pansorbin (10%, w/v, *S. aureus* cells) were added for an additional 1-hr incubation. The samples were centrifuged at 12,000 × *g* for 3 min. The pellets were washed three times in incubation buffer and counted in a scintillation counter.

Immunoblots. Samples of 1–10 μg of bullfrog plasma, purified saxiphilin, and transferrin were subjected to SDS-PAGE (14) using 7.5% polyacrylamide gels and were electroblotted onto nitrocellulose membranes (Gelman Biotrace NT) using a Bio-Rad Trans-Blot apparatus, as described (6). The membrane blots were probed with affinity-purified antisaxiphilin antibody (1/1500 dilution of 0.25 mg/ml antibody) and developed according to instructions for the Western blot analysis system (Amersham), which uses a peroxidase-conjugated anti-rabbit antibody and a chemiluminescence reaction to expose film.

Enzyme-linked immunosorbent assay. Purified saxiphilin or transferrin from various sources was diluted to 5 μg/ml in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2), and 50 μl were added to individual wells of a polystyrene microtiter plate (Corning 25860). The plates were incubated at 4° overnight for adsorption of antigens. After the plate was drained, each well was blocked with 150 μl of 4% nonfat dry milk in PBS and incubated for 1 hr at room temperature. The plates were drained and 50 μl of various serial dilutions (in 4% milk) of rabbit antisaxiphilin antibody or goat anti-human transferrin antiserum were added to each well. After a 1-hr incubation, the drained plate was washed three times with 150 μl/well of 4% milk. Each well then received 150 μl of a 1/500 dilution of peroxidase-conjugated second antibody. After a 1-hr incubation, wells were washed three times with 150 μl of 4% milk and then two times with PBS. This was followed by addition of 75 μl/well of substrate solution (0.4% *o*-phenylenediamine, 0.0126% H_2O_2, in PBS). After a 30-min incubation, 50 μl of 5 N H_2SO_4 were added to stop the reaction and absorbance at 490 nm was read with a microtiter plate spectrophotometer. The same protocol was followed for experiments with increasing antigen concentrations at fixed antibody dilutions of 1/2000.

Results

Chromatographic separation of [^{3}H]STX-binding activity and $^{55}Fe^{3+}$-binding activity. $^{55}Fe^{3+}$ and [^{3}H]STX were used to monitor binding activity of these ligands during chromatographic separation of transferrin from saxiphilin in bullfrog plasma. Plasma transferrin was prelabeled with a complex of $^{55}Fe^{3+}$ and NTA, in the presence of 10 mM bicarbonate. NTA serves to provide a soluble form of chelated Fe^{3+} that can readily bind to apotransferrin (7). Transferrin was purified from ^{55}Fe-labeled plasma by gel filtration on Sephadex G-200, followed by anion exchange chromatography on DEAE-Sephadex. Assays of fractions from the Sephadex G-200 column revealed co-migration of [^{3}H]STX-binding activity and protein-bound ^{55}Fe after the void volume (data not shown). This initial co-migration of the two binding activities on a gel filtration column is consistent with the similar elution volumes of saxiphilin activity and human transferrin previously observed using high performance size exclusion chromatography (5). Further fractionation on DEAE-Sephadex of the Sephadex G-200 pool containing protein-bound ^{55}Fe resolved saxiphilin and transferrin activity into two distinct peaks (Fig. 1). Consistent with the high isoelectric point (pI ~10.9) of saxiphilin, as determined by isoelectric focusing (5), [^{3}H]STX-binding activity readily passed through the DEAE-Sephadex column at pH 7.8 and low ionic strength. In contrast, a single major peak corresponding to bullfrog transferrin eluted from this column at higher ionic strength, as identified by a characteristic orange-pink color of the fractions containing ^{55}Fe.

After removal of Fe^{3+} from purified bullfrog transferrin by prolonged incubation at pH 4.2 in the presence of 1 mM NTA and 2 mM EDTA, the protein was further characterized by spectrophotometric titration of absorbance at 465 nm with $Fe(NTA)_2$. This titration (Fig. 2B) exhibited a sharp equivalence point characteristic of other transferrins and a slope of 2590 M^{-1} cm^{-1}/Fe^{3+} site, which is similar to reported values for the extinction coefficient of human transferrin (ϵ_{465} = 2500–2600 M^{-1} cm^{-1}) (15, 16). An Fe^{3+}-binding capacity of 1.9 mol of Fe^{3+}/mol of bullfrog transferrin was calculated by dividing the observed $Fe(NTA)_2$ equivalence point by the protein concentration, as determined by quantitative amino acid analysis, and assuming a protein molecular weight (M_r = 77,640) equal to that of transferrin from the African clawed frog *Xenopus laevis* (13). This Fe^{3+}-binding capacity is consistent with two functional Fe^{3+} sites/transferrin molecule, as found for all known serum transferrins from other vertebrates (7). The absorption spectrum of bullfrog transferrin (Fig. 2A) exhibited an Fe^{3+}-dependent maximum in the visible region at 465 nm and a minimum near 405 nm, which is typical of transferrins from various sources (7). The spectrum of Fe^{3+}-saturated bullfrog transferrin was also characterized by absorbance ratios of A_{465}/A_{280} = 0.046 and A_{465}/A_{410} = 1.18. The former ratio is similar to that of native human Fe_2-transferrin (A_{465}/A_{280} = 0.046) (17) and the recombinant form of the amino-terminal half-molecule of human Fe-transferrin (A_{465}/A_{280} = 0.048) (18). However, the A_{465}/A_{410} ratio of bullfrog transferrin is somewhat lower than that of the native and recombinant forms of human transferrin (A_{465}/A_{410} = 1.34–1.41) (16, 18, 19). The absence of an absorbance peak at 410 nm implies that the preparation is not contaminated by heme, as also noted in other preparations of bullfrog transferrin (20, 21).

Demonstration of different molecular weights of purified saxiphilin and transferrin by SDS-PAGE. A comparative analysis of purified saxiphilin and transferrin preparations by SDS-PAGE is shown in Fig. 3. Purified transferrin exhibited a single predominant band migrating with an apparent molecular weight of 78,000 ± 1,000. The transferrin band was at the same position as one of the major protein bands of whole plasma, consistent with a typical serum protein content of ~10% transferrin (12). Saxiphilin was separately purified from bullfrog plasma by a procedure involving chromatofocusing, as described previously (6). Saxiphilin migrated on SDS-PAGE with a distinctly higher apparent molecular weight of

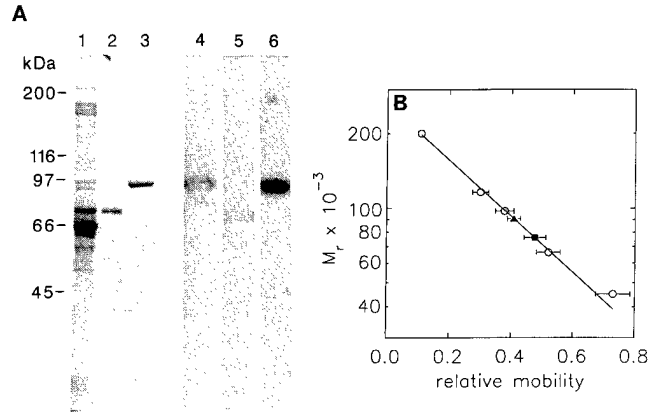

Fig. 3. SDS-PAGE and immunoblots of whole plasma, transferrin, and saxiphilin. A, *Lanes 1*, *2*, and *3*, SDS-PAGE of 10 μg of bullfrog plasma, 1 μg of bullfrog transferrin, and 1 μg of bullfrog saxiphilin, respectively, stained with Coomassie blue. *Lanes 4*, *5*, and *6*, immunoblots of a duplicate of *lanes 1*, *2*, and *3*, respectively, probed with rabbit antisaxiphilin antibodies detected by peroxidase-conjugated anti-rabbit antibody and Amersham Enhanced Chemiluminescence reagents. B, Compilation of data from five SDS-PAGE experiments showing the relative mobility of bullfrog transferrin (■) and saxiphilin (▲) with respect to five molecular weight markers (○), i.e., myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 97,400), bovine serum albumin (M_r 66,200), and hen ovalbumin (M_r 45,000).

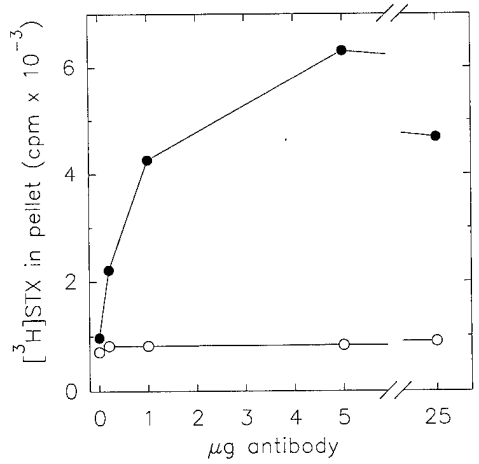

Fig. 4. Immunoprecipitation of [^{3}H]STX-binding activity from bullfrog plasma by antisaxiphilin antibodies. Bullfrog plasma (35 μg) was incubated with 11 nM [^{3}H]STX in the absence (●) or presence (○) of unlabeled STX and was subjected to precipitation with increasing amounts of antisaxiphilin antibody, as described in Experimental Procedures. *Data points* are the means of duplicate determinations. Similar results were obtained in three experiments.

90,000 ± 3,000 (Fig. 3B). The saxiphilin band exhibited a positive reaction when stained for carbohydrate by the periodic acid Schiff method (22) (data not shown). This indicates that, like transferrin, saxiphilin is a glycoprotein.

Discrimination of saxiphilin and transferrin by antisaxiphilin antibodies on immunoblots. Antiserum to native saxiphilin was raised in rabbits and polyclonal antibodies were affinity-purified on a column of saxiphilin coupled covalently to Sepharose 4B. Such antibodies were capable of immunoprecipitating [^{3}H]STX-binding activity from samples of crude bullfrog plasma (Fig. 4). This precipitation was dependent on the amount of antibody added, was abolished by an excess of unlabeled STX, and did not occur with preimmune rabbit serum. Samples of bullfrog plasma, transferrin, and saxiphilin subjected to SDS-PAGE were electroblotted onto nitrocellulose membranes. The resulting protein blots were probed with antisaxiphilin antibodies and developed by a chemiluminescence-based detection technique. Typical results in Fig. 3A show a strong reaction with pure saxiphilin and specific detection of an equivalent band in the sample corresponding to crude plasma. In contrast, the band corresponding to pure bullfrog transferrin exhibited weak reactivity in this assay. Control experiments using rabbit serum collected before immunization with saxiphilin showed no reaction (data not shown). The results of Figs. 3 and 4 demonstrate that the 90-kDa [^{3}H]STX-binding protein previously characterized as saxiphilin (6) is distinct from bullfrog serum transferrin, on the basis of size and reactivity to antisaxiphilin antibodies.

Evidence that saxiphilin and transferrin have different ligand-binding properties. Purified saxiphilin and bullfrog apotransferrin were compared in sensitive binding assays for $^{55}Fe^{3+}$ and [^{3}H]STX. In case the ability of saxiphilin to bind iron was masked under the conditions of the experiment with whole plasma in Fig. 1, we separately incubated pure saxiphilin and transferrin (as a control) with excess $^{55}Fe(NTA)_2$ and 10 mM $NaHCO_3$ at pH 5. After 12 hr of incubation, this mixture was adjusted to pH 7 and protein-bound $^{55}Fe^{3+}$ was separated from free $^{55}Fe(NTA)_2$ on a size exclusion column (Biogel P6). This technique allowed us to readily measure binding of $^{55}Fe^{3+}$ to bullfrog apotransferrin but showed no detectable binding of $^{55}Fe^{3+}$ to saxiphilin (data not shown). Correspondingly, when transferrins from various species were tested in comparison with saxiphilin for specific binding of 5 nM [^{3}H]STX, none of the nonamphibian transferrin proteins displayed significant binding of this neurotoxin (Table 1). In this experiment, 2 μg of various transferrins were tested versus 0.0074 μg of saxiphilin, to enhance detection of possible low affinity binding of [^{3}H]STX by transferrins. Bullfrog transferrin purified by chromatography on DEAE-Sephadex did exhibit a small amount of [^{3}H]STX binding, but the low specific activity of this binding (~12 pmol/mg) (Table 1) suggested that it could be due to trace contamination (~0.1%) by saxiphilin. This residual [^{3}H]STX binding was greatly reduced by subjecting bullfrog transferrin

TABLE 1

Lack of [^{3}H]STX binding by various transferrins

Samples of various transferrins (2 μg) or saxiphilin (0.0074 μg) were incubated in 100 μl of 100 mM MOPS-NaOH, pH 7.4, 100 mM NaCl, 10 mM $NaHCO_3$, 5 nM [^{3}H]STX, in the absence or presence of 10 μM unlabeled STX. After 1 hr at 0°, bound [^{3}H]STX was separated from free ligand as described (6). Values of cpm were converted to pmol bound using the measured specific activity of [^{3}H]STX (13,980 cpm/pmol). Specific binding is reported as the difference between assays in the absence and presence of 10 μM STX. Results are expressed as specific activity (pmol bound per mg of the tested protein), and reported values are the mean ± standard deviation of four measurements.

Sample	[^{3}H]STX bound
	pmol/mg
Human apotransferrin	0.2 ± 0.1
Human lactoferrin	0.0 ± 0.4
Bovine apotransferrin	−0.1 ± 0.6
Horse apotransferrin	−0.1 ± 0.3
Rabbit apotransferrin	0.3 ± 0.3
Guinea pig transferrin	−0.1 ± 0.3
Mouse transferrin	0.0 ± 0.1
Chicken ovotransferrin	0.1 ± 0.2
Bullfrog apotransferrin (before S-Sepharose)	12 ± 1.1
Bullfrog apotransferrin (after S-Sepharose)	1.7 ± 0.1
Bullfrog saxiphilin	12,000 ± 1,200

to an additional step of chromatography on S-Sepharose (Table 1), a medium that was previously found to effectively adsorb saxiphilin (5). The measured specific activity of [^{3}H]STX binding to pure saxiphilin (12,000 pmol/mg) is approximately equivalent to a 1:1 stoichiometry for a 90-kDa protein. A more rigorous Scatchard analysis of [^{3}H]STX binding to pure saxiphilin has confirmed this 1:1 binding stoichiometry.[1] Thus, our results indicate that saxiphilin binds 1 mol of [^{3}H]STX/mol of protein, whereas bullfrog transferrin binds 2 mol of Fe^{3+}/mol of protein.

Evidence of immunological cross-reactivity between saxiphilin and various transferrins. The antigenic relationship between saxiphilin and bullfrog transferrin was explored further by examining the reactivity of affinity-purified antisaxiphilin antibodies in an enzyme-linked immunosorbent assay. Fig. 5 shows the results of an experiment in which fixed amounts (250 ng) of pure saxiphilin and bullfrog transferrin were incubated with serial dilutions of antibody. As expected from the results of immunoblot analysis (Fig. 3A), the antisaxiphilin antibody was strongly reactive with saxiphilin, but a weak reaction with bullfrog transferrin was also detected at high antibody concentration. As noted above, [^{3}H]STX binding measurements suggested that nominally pure preparations of bullfrog transferrin may contain trace amounts of saxiphilin. Because such contamination could affect the interpretation of the immunosorbent assay results, we compared bullfrog transferrin before and after additional purification by S-Sepharose chromatography. This latter procedure reduced but did not completely eliminate the reactivity of bullfrog transferrin with antisaxiphilin antibodies (Fig. 5). The reduction in reactivity produced by further purification is consistent with the suggestion, stated above, that trace contamination by saxiphilin, on the order of ~0.1%, is the likely source of low level [^{3}H]STX binding observed for the transferrin preparation before the S-Sepharose step (Table 1). Because the two proteins were purified from the same source, trace contamination is an inherent problem that makes it difficult to establish whether the residual cross-reactivity observed in the experiment of Fig. 5 is a genuine reflection of antigenic similarity.

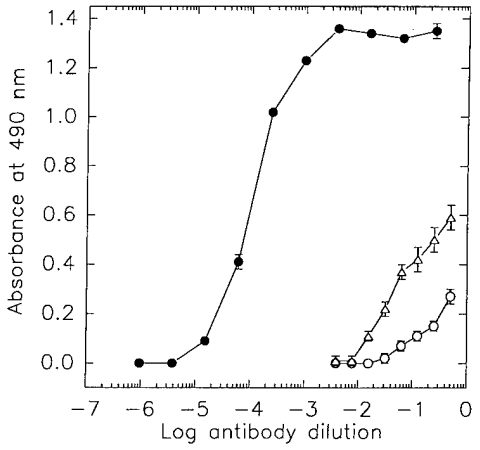

Fig. 5. Enzyme-linked immunoassay of saxiphilin and bullfrog transferrin with antisaxiphilin antibodies. Microtiter wells were coated with 250 ng of pure saxiphilin (●), bullfrog transferrin purified by chromatography on DEAE-Sephadex (△), or bullfrog transferrin subjected to an additional purification step of chromatography on S-Sepharose (○). The wells were then incubated with increasing dilutions of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody, as described in Experimental Procedures. *Data points* for transferrin are the mean and standard deviation of six determinations. *Data points* for saxiphilin are the mean of duplicates.

To pursue this relationship indirectly, several other transferrins from various species were also studied. We found that antisaxiphilin antibodies cross-reacted with different affinities with transferrins from species such as cow, human, and horse (Fig. 6A). Rabbit transferrin was essentially unreactive in this assay. Because these other transferrin samples contained no detectable [^{3}H]STX-binding activity and we have not observed saxiphilin-like activity in a mammalian species, it seems unlikely that this interspecies cross-reactivity is due to contamination by a saxiphilin-like protein. A similar pattern of species-specific cross-reactivity was observed when the test antigen concentration was increased at a fixed antibody concentration (Fig. 6B).

The antigenic relationship between saxiphilin and various

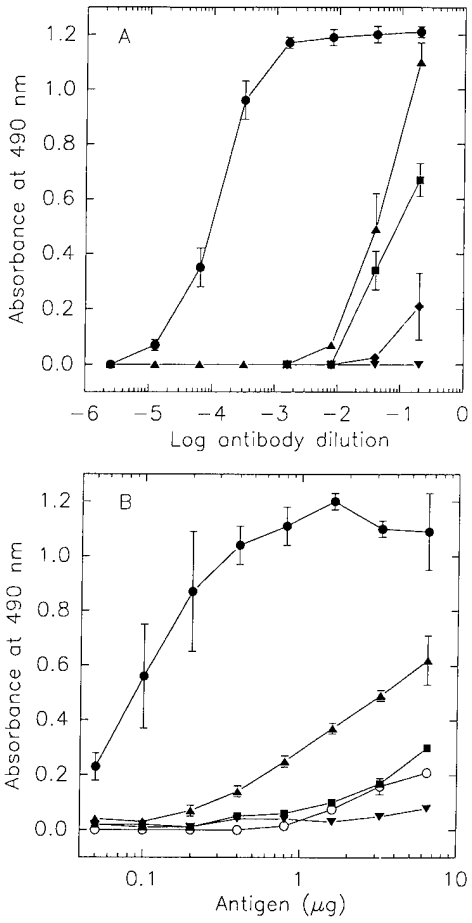

Fig. 6. Cross-reactivity of various transferrins with antisaxiphilin antibodies in an enzyme-linked immunosorbent assay. A, Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody, as described in Experimental Procedures. B, Microtiter wells coated with increasing amounts of various antigens were incubated with a 1/2000 dilution of antisaxiphilin antibody and assayed using peroxidase-coupled second antibody. ●, Bullfrog saxiphilin; ▲, bovine transferrin; ■, human transferrin; ◆, horse transferrin; ▼, rabbit transferrin; ○, bullfrog transferrin after S-Sepharose chromatography. *Data points* are the mean and standard deviation of four determinations.

[1] L. Llewellyn and E. Moczydlowski, unpublished observations.

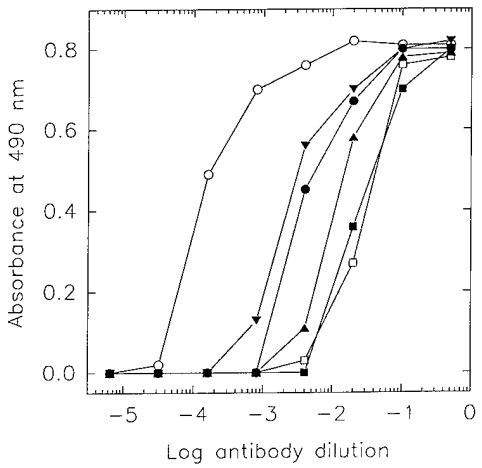

Fig. 7. Cross-reactivity of saxiphilin and various transferrins with anti-human transferrin antibodies in an enzyme-linked immunosorbent assay. Microtiter wells coated with 250 ng of various antigens were incubated with increasing dilutions of anti-human transferrin antibody and assayed using peroxidase-coupled second antibody as described in Experimental Procedures. ○, Human transferrin; ▼, rabbit transferrin; □, chicken ovotransferrin; ▲, bovine transferrin; ■, bullfrog transferrin; ●, bullfrog saxiphilin. *Data points* are the average of duplicate determinations.

transferrin proteins was also examined by testing, in a similar enzyme-linked immunosorbent assay, the reactivity of commercially obtained goat antibodies raised against human serum transferrin. In this experiment bullfrog saxiphilin was recognized with efficiency similar to that of various nonhuman transferrins, including those of rabbit, cow, and chicken (Fig. 7). Saxiphilin was actually somewhat more reactive than bullfrog transferrin in this assay. As judged by cross-recognition of frog saxiphilin and transferrins of several different species by polyclonal antibodies, saxiphilin behaves immunologically as a relative of the transferrin family.

Discussion

Results presented here, together with information obtained by partial sequencing of native saxiphilin (6) and recent cloning of saxiphilin cDNA,[2] establish that saxiphilin is a functionally and structurally unique member of the transferrin superfamily of proteins. High affinity binding of [^{3}H]STX is the property that originally led to the discovery of saxiphilin (4, 5, 23), but the functional significance of this interaction, if any, is presently unknown. Curiously, the phylogenetic distribution of saxiphilin appears to be limited to certain amphibians and reptiles that presently include *Rana catesbeiana* (bullfrog), *Rana sylvatica* (wood frog), *Bufo marinus* (marine or cane toad), *Ambystoma tigrinum* (tiger salamander), *Notophthalamus viridescens* (red spotted newt), *Taricha granulosa* (rough skinned newt), and *Thamnophis sirtalis* (garter snake).[3] Although the existence of STX and STX derivatives in the marine food chain is well documented (1, 24, 25), reports of STX in the terrestrial freshwater environment are limited to its production by a cyanobacterium, *Aphanizomenon flos-aquae* (26, 27). In contrast to the marine ecosystem, there is little information to suggest that STX plays a significant role in freshwater chemical ecology (28). Thus, the phenomenon of constitutive production by certain amphibians and reptiles of a plasma protein with high affinity for STX poses an interesting biological mystery.

Because partial sequencing of purified bullfrog saxiphilin previously revealed homology to the transferrin family of proteins (6), the purpose of the present work was to determine whether saxiphilin is derived directly from transferrin. Our results eliminate this possibility, because we have shown that saxiphilin is biochemically distinct from transferrin by a number of criteria. The saxiphilin protein is significantly larger than most transferrin proteins, migrating with an M_r of ~90,000 on SDS-PAGE, compared with ~78,000 for bullfrog transferrin (Fig. 3). Saxiphilin exhibits basic charge characteristics, as judged by an apparent pI of 10.7 (5), adsorption to an S-Sepharose cation exchange column (5), lack of adsorption to a DEAE-Sephadex anion exchange column (Fig. 1), and elution behavior on a chromatofocusing column (6). In contrast, bullfrog transferrin exhibits a pI in the range of 6.3–6.6 (12), which is consistent with adsorption to DEAE-Sephadex at pH 7.8 and low ionic strength (Fig. 1). Also, the two proteins clearly bind different ligands. Saxiphilin does not appear to bind $^{55}Fe^{3+}$ under conditions appropriate for transferrins, and purified samples of saxiphilin are colorless, implying the lack of spectroscopically active bound metal ions. Conversely, [^{3}H]STX does not bind to bullfrog transferrin or any of a large number of commercially available apotransferrins in our standard assay. The small amount of [^{3}H]STX binding detected for nominally pure transferrin from bullfrog (Table 1) can be attributed to trace contamination by saxiphilin (Fig. 5).

The conclusion that saxiphilin is not directly derived from bullfrog transferrin is also supported by the results of immunochemical experiments using polyclonal antibodies against saxiphilin and human transferrin (Figs. 5 and 6). Both of these antibodies clearly discriminate the two frog proteins. The demonstrated specificity of the antisaxiphilin antibodies suggests that they will be useful tools in immunohistochemical studies.

Apart from showing that saxiphilin and transferrin are different proteins, our immunochemical studies also confirm that they are structurally related. The extent of this structural relationship has recently been revealed by cloning of a cDNA from bullfrog liver that appears to correspond to an mRNA transcript of the coding sequence for saxiphilin.[2] This sequence information is helpful in interpreting the present immunological results. The saxiphilin cDNA clone predicts a secreted protein molecular weight of 90,818, which is consistent with the value reported in this paper (90,000 ± 3,000) for native saxiphilin determined by SDS-PAGE (Fig. 3). Except for one large gap due to a unique insertion of 144 residues in saxiphilin, pairwise sequence alignments of the deduced saxiphilin sequence with the sequences of various members of the transferrin family reveal amino acid sequence homology on the order of 51% identity with transferrin from *X. laevis* (African clawed frog) and 39–44% identity with various human transferrins (serum transferrin, lactoferrin, and melanotransferrin). Such sequence alignments show many short regions of almost complete identity and other regions of practically no homology between saxiphilin and various members of the transferrin family.[2] The immunological cross-reactivity observed here is consistent with such sequence homology and is typical of that found when polyclonal antibodies raised against one member of a protein family are assayed for reactivity with closely related

[2] M. Morabito and E. Moczydlowski. Cloning of bullfrog saxiphilin reveals a unique relative of the transferrin family that binds saxitoxin. Submitted for publication.

[3] L. Llewellyn, P. Bell, and E. Moczydlowski, unpublished observations.

members of the same protein family from other species. Such cross-reactivity may be expected to depend on the number of shared epitopes between related antigen proteins and the relative concentrations and affinities of various immunoglobulins in the polyclonal serum.

The cloned sequence of saxiphilin also explains the lack of Fe^{3+} binding reported in this paper. X-ray crystallography (10, 29) has shown that the two Fe^{3+}/HCO_3^- binding sites of transferrin are each formed by five highly conserved ligand residues, i.e., one aspartate, two tyrosines, one histidine, and one arginine. Alignment of the saxiphilin clone with the sequences of known transferrins reveals that only one of 10 of these critical residues is conserved in saxiphilin.[2] This finding leads to the prediction that both of the analogous binding domains of saxiphilin are nonfunctional with respect to Fe^{3+}, as confirmed here by the lack of detectable $^{55}Fe^{3+}$ binding.

In summary, saxiphilin may be recognized as a structural relative of the transferrin family that does not bind Fe^{3+}. Current information on this protein and its structural similarity to transferrin lead us to propose that saxiphilin may serve as a transport protein for an unidentified endogenous ligand or as an element of a detoxification system for a toxin acquired from the environment. The availability of antisaxiphilin antibodies described in this work and a cDNA clone encoding saxiphilin[2] will facilitate analysis of this protein and should help to elucidate its actual physiological role.

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Pharmacology

Molecular cloning of bullfrog saxiphilin: A unique relative of the transferrin family that binds saxitoxin

(neurotoxin/sodium channel/thyroglobulin domain/amphibian/molecular evolution)

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ABSTRACT **Plasma and tissues of certain vertebrates contain a protein called saxiphilin that specifically binds the neurotoxin saxitoxin with nanomolar affinity. We describe the isolation of a cDNA clone of saxiphilin from liver of the North American bullfrog (*Rana catesbeiana*). The cDNA sequence encodes a protein that is evolutionarily related to members of the transferrin family of Fe^{3+}-binding proteins. Pairwise sequence alignment of saxiphilin with various transferrins reveals amino acid identity as high as 51% and predicts 14 disulfide bonds that are highly conserved. The larger size of saxiphilin (91 kDa) versus serum transferrin (≈78 kDa) is primarily due to a unique insertion of 144 residues. This insertion contains a 49-residue domain classified as a type 1 repetitive element of thyroglobulin, which is shared by a variety of membrane, secreted, and extracellular matrix proteins. Saxiphilin also differs from transferrins in 9 of 10 highly conserved amino acids in the two homologous Fe^{3+}/HCO_3-binding sites of transferrin. Identification of saxiphilin implies that transferrin-like proteins comprise a diverse superfamily with functions other than iron binding.**

Saxitoxin (STX) is tricyclic organic molecule that is produced by various dinoflagellates and cyanobacteria (1). STX ranks among the most potent paralytic neurotoxins by virtue of its nanomolar blocking effect on voltage-sensitive Na^+ channels of neurons and skeletal muscle. In previous studies using [^{3}H]STX to measure STX-binding sites of Na^+ channels, an unusual high-affinity binding site ($K_d \approx 0.2$ nM) was found in soluble extracts of frog heart (2) and skeletal muscle (3). Further work showed that this soluble STX-binding site is associated with an ≈90-kDa monomeric protein (named saxiphilin) that is present in bullfrog plasma at a concentration of ≈300 nM (4, 5). Partial amino acid sequences of purified saxiphilin were found to exhibit similarity to members of the transferrin family (5). However, saxiphilin is biochemically and functionally distinct from bullfrog serum transferrin (6). Transferrins are a family of ≈80-kDa proteins noted for their exceptionally high affinity for Fe^{3+} with a K_d in the range of 10^{-20} M (7). By transporting Fe^{3+} into eukaryotic cells through binding to the transferrin receptor and subsequent endocytosis, serum transferrin functions as an important growth factor required for synthesis of Fe^{3+}-containing proteins.

This paper describes the isolation of a cDNA clone encoding saxiphilin from bullfrog liver.‡ Sequence analysis indicates that saxiphilin is an evolutionary relative of the transferrin family but differs in two major respects. Saxiphilin has substitutions of 9 of the 10 highly conserved residues that form the two Fe^{3+}/HCO_3-binding sites of transferrin. It also has a unique insertion of 144 residues that contains a type 1 thyroglobulin domain (Thyr-1). These findings lead to the conclusion that saxiphilin originated from an ancestor of the transferrin family but diverged to perform a different function. The unique ability of saxiphilin to bind STX and the similarity of its tissue distribution with that of transferrin suggest that its physiological role may be to transport or sequester an endogenous organic molecule rather than Fe^{3+}

MATERIALS AND METHODS

Isolation of a cDNA Corresponding to Saxiphilin. Adult bullfrogs (*Rana catesbeiana*) were purchased from Connecticut Valley Biological Supply (Southampton, MA). Total RNA from bullfrog liver was prepared (8) and further purified on a CsCl step gradient (9). First strand cDNA was synthesized using $(dT)_{15}$ primer and murine leukemia virus reverse transcriptase according to recommendations of the manufacturer (GIBCO/BRL). The following degenerate oligonucleotides, X, Y, and Z, were designed from tryptic peptides of saxiphilin (5), Sax-133 (X and Y) and Sax-101 (Z), and they were synthesized by the Yale Medical School Protein and Nucleic Acid Facility: X sense (CAA/GTAT/CATGTAT/CGAA/GGCIC/TTIATGTGT/CGG), Y sense (GAA/GTAT/CCAT/CAAT/CAAA/GGAT/CGAT/CTTT/CGGICC), and Z antisense (CCA/GTCIGTA/GTTT/CTCA/GAAIACIACIGTG/ATCIGG). X and Z were first used as primers for PCR using the oligo(dT)-tailed cDNA as a template. The reaction was run for 30 cycles with 5 μM each of X and Z. The cycle was 1 min of denaturation at 94°C, 1 min of annealing at 47°C, and 2 min of extension at 72°C. The final cycle included an extension of 7 min at 72°C. A PCR product of ≈450 bp was obtained by reamplification of the latter reaction mixture using Y and Z by nested PCR run under the same conditions. All PCR reactions used Amplitaq DNA polymerase (Perkin–Elmer/Cetus). The ≈450-bp product was purified on a 2% agarose gel and cloned in the plasmid vector pCR1000 (Invitrogen). A number of clones were isolated and sequenced (10) using Sequenase (United States Biochemical).

One of the PCR-derived clones containing saxiphilin sequence was used as a hybridization probe to screen a bullfrog liver cDNA library. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography of total RNA extracted from liver of adult bullfrog and used to synthesize double-stranded cDNA using the ZAP-cDNA kit from Stratagene. The cDNA was cloned in the Lambda ZAPII vector following the Stratagene protocol. Five micrograms of poly(A)$^+$ RNA yielded about 2.4×10^6 recombinant phages prior to amplification. Five hundred thousand recombinant phages were screened using a *Bam*HI–*Eco*RI fragment isolated from the PCR-derived cDNA clone according to the map in Fig. 1. The screening of the library was performed by standard proce-

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Abbreviations: STX, saxitoxin; Thyr-1, type 1 thyroglobulin domain.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U05246).

dures (9). One of the positive clones isolated from the screen contained the DNA sequence shown in Fig. 2.

Analysis of Saxiphilin mRNA. Five micrograms of poly(A)$^+$ RNA from bullfrog liver was fractionated by electrophoresis on a 1% agarose gel containing formaldehyde (8) and transferred to a Hybond-N$^+$ nylon membrane (Amersham). The filter was first hybridized to probe 1 (Fig. 5*a*, lane 1) and then stripped and hybridized to probe 2 (Fig. 5*a*, lane 2). Probe 1 was a 2.25-kb *Sma* I fragment derived from the saxiphilin cDNA clone (Fig. 1). Probe 2 was a 0.43-kb fragment specific to the unique insertion sequence in saxiphilin (Fig. 1) that was prepared by PCR amplification of nucleotides 303–738 in Fig. 2. The probes were labeled with [^{32}P]dCTP using a randomly primed labeling kit (Boehringer Mannheim). Alkali fixation, prehybridization, and removal of the first probe were performed according to Amersham. Autoradiography of the filters was performed by exposure to film for 1 hr with an intensifying screen. Probes 1 and 2 were similarly used to detect saxiphilin message in total RNA extracted from various bullfrog tissues in a slot blot hybridization assay. Hybridization was performed at 65°C and the final wash was in 1× SSC/0.1% SDS for 1 hr at 50°C (Fig. 5*a*) or 0.2× SSC (Fig. 5*b*).

RESULTS AND DISCUSSION

Identification of Saxiphilin cDNA. Cloning of saxiphilin was accomplished by combining PCR amplification of an 0.45-kb fragment of cDNA with screening of a bullfrog liver cDNA library. One of the positive clones from the library contained a 2681-bp cDNA with an open reading frame of 845 amino acids. Fig. 1 shows a restriction map of the cDNA clone and the location of the PCR-amplified probe used to screen the library. The nucleotide sequence of the cDNA clone and the deduced amino acid sequence of saxiphilin are shown in Fig. 2. The clone contains a 5′ untranslated region of 23 bp followed by an ATG codon for Met-1 and a TAA termination codon following Cys-845. The N terminus of the mature protein begins at Ala-20 as recognized by a 25-residue sequence previously obtained by Edman degradation of the intact native protein (5). The 19-residue sequence preceding Ala-20 corresponds to a secretory signal sequence as found for all known transferrins (7). The 3′ end of the clone contains a consensus sequence (AATAAA) for polyadenylylation that

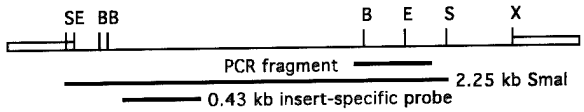

FIG. 1. Restriction map of saxiphilin cDNA. Solid bars indicate a PCR fragment (0.45 kb) used to isolate a full-length cDNA clone and probes corresponding to a *Sma* I restriction fragment and a saxiphilin-specific insertion used in mRNA hybridization experiments. Restriction sites are *Bam*HI (B), *Eco*RI (E), *Sma* I (S), and *Xho* I (X).

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                                                                -23 GGCACGAGGAGGCACTGAGAAGG
    ATGGCTCCGACTTTCCAAACAGCTCTGTTTTTCACCATCATTAGCTTGAGCTTTGCGGCACCAAATGCAAAACAAGTCCGGTGGTGTGCGATATCAGATCTTGAGCAGAAG 111
  1 M  A  P  T  F  Q  T  A  L  F  F  T  I  I  S  L  S  F  A  A  P  N  A  K  Q  V  R  W  C  A  I  S  D  L  E  Q  K
    AAGTGCAATGATCTAGTGGGATCCTGCAATGTCCCGGACATTACCCTTGTGTGTGTGCTTAGATCCTCCACTGAGGACTGTATGACAGCTATAAAGGATGGACAAGCAGAT 222
 38 K  C  N  D  L  V  G  S  C  N  V  P  D  I  T  L  V  C  V  L  R  S  S  T  E  D  C  M  T  A  I  K  D  G  Q  A  D
    GCCATGTTCTTAGATAGTGGAGAGGTTTATGAAGCTTCAAAGGATCCTTATAACCTAAAGCCAATTATTGCAGAACCATACAGTTCCAATAGAGACTTGCAAAAATGCTTA 333
 75 A  M  F  L  D  S  G  E  V  Y  E  A  S  K  D  P  Y  N  L  K  P  I  I  A  E  P  Y  S  S  N  R  D  L  Q  K  C  L
    AAGGAGCGTCAACAAGCTCTGGCAAAGAAGATGCATCTGGTCATTATATTCCACAATGTGATGAAAAGGGCAATTACCAACCACAGCAGTGCCATGGCAGCACTGGGCACT 444
112 K  E  R  Q  Q  A  L  A  K  K  M  H  L  V  I  I  F  H  N  V  M  K  R  A  I  T  N  H  S  S  A  M  A  A  L  G  T
    GCTGGTGTGTTAACGTCAATGGGTGAAAAGATTTCTGGAACAAATACTCCACCTGGACAAACACGAGCTACATGTGAAAGGCATGAATTGCCAAAATGCTTGAAGGAACGC 555
149 A  G  V  L  T  S  M  G  E  K  I  S  G  T  N  T  P  P  G  Q  T  R  A  T  C  E  R  H  E  L  P  K  C  L  K  E  R
    CAGGTAGCTCTGGGAGGAGACGAGAAAGTGTTAGGTCGTTTTGTTCCTCAGTGTGATGAAAAGGGCAATTATGAACCACAACAGTTCCATGGCAGCACTGGGTACAGCTGG 666
186 Q  V  A  L  G  G  D  E  K  V  L  G  R  F  V  P  Q  C  D  E  K  G  N  Y  E  P  Q  Q  F  H  G  S  T  G  Y  S  W
    TGTGTTAATGCAATCGGTGAAGAGATTGCTGGAACAAAAACTCCACCTGGAAAAATACCAGCTACATGTCAAAAACACGATTTGGTTACTACATGTCATTACACAGTTGCT 777
223 C  V  N  A  I  G  E  E  I  A  G  T  K  T  P  P  G  K  I  P  A  T  C  Q  K  H  D  L  V  T  T  C  H  Y  T  V  A
    ATGGTGAAAAAGTCCAGCGCGTTCCAGTTTAATCAGCTGAAAGGCAAGAGGTCCTGTCACTCAGGTGTATCAAAGACAGATGGCTGGAAAGCTCTTGTGACTGTTCTTGTT 888
260 M  V  K  K  S  S  A  F  Q  F  N  Q  L  K  G  K  R  S  C  H  S  G  V  S  K  T  D  G  W  K  A  L  V  T  V  L  V
    GAAAAGAAGTTGTTGTCCTGGGATGGACCTGCCAAAGAATCCATTCAACGAGCCATGTCAAAGTTCTTCTCAGTCAGCTGTATCCCTGGAGCAACCCAAACCAACCTCTGC 999
297 E  K  K  L  L  S  W  D  G  P  A  K  E  S  I  Q  R  A  M  S  K  F  P  S  V  S  C  I  P  G  A  T  Q  T  N  L  C
    AAGCAATGTAAGGGAGAGGAAGGGAAGAACTGTAAGAACAGCCATGATGAGCCATACTATGGCAACTATGGAGCATTCAGATGTTTGAAAGAAGATATGGGAGATGTAGCT 1110
334 K  Q  C  K  G  E  E  G  K  N  C  K  N  S  H  D  E  P  Y  Y  G  N  Y  G  A  F  R  C  L  K  E  D  M  G  D  V  A
    TTTCTGAGAAGCACAGCTTTGTCAGATGAACATTCAGAGGTGTATGAGCTTCTGTGTCCAGACAATACTAGGAAGCCCCTTAACAAATACAAGGAGTGTAACCTAGGAACA 1221
371 F  L  R  S  T  A  L  S  D  E  H  S  E  V  Y  E  L  L  C  P  D  N  T  R  K  P  L  N  K  Y  K  E  C  N  L  G  T
    GTTCCAGCAGGTACAGTGGTGACCAGAAAAATCAGTGACAAGACTGAGGACATCAATAACTTTCTCATGGAGGCTCAGAAAAGGCAATGCAAATTGTTCAGTTCTGCACAT 1332
408 V  P  A  G  T  V  V  T  R  K  I  S  D  K  T  E  D  I  N  N  F  L  M  E  A  Q  K  R  Q  C  K  L  F  S  S  A  H
    GGGAAAGATTTGATGTTTGATGATTCTACCTTACAATTAGCTCTTCTCTCATCTGAAGTGGATGCGTTCCTCTATCTCGGAGTAAAATTGTTCCATGCCATGAAAGCACTG 1443
445 G  K  D  L  M  F  D  D  S  T  L  Q  L  A  L  L  S  S  E  V  D  A  F  L  Y  L  G  V  K  L  F  H  A  M  K  A  L
    ACTGGAGATGCACATCTTCCATCCAAAAATAAAGTGCGGTGGTGTACAATAAATAAACTGGAAAAGATGAAGTGTGATGATTGGTCAGCTGTAAGCGGTGGGGCCATTGCA 1554
482 T  G  D  A  H  L  P  S  K  N  K  V  R  W  C  T  I  N  K  L  E  K  M  K  C  D  D  W  S  A  V  S  G  G  A  I  A
    TGCACAGAGGCATCCTGTCCAAAGGGTTGTGTTAAACAGATTCTGAAAGGTGAAGCTGATGCAGTGAAACTTGAGGTACAATACATGTATGAGGCTTTGATGTGCGGACTG 1665
519 C  T  E  A  S  C  P  K  G  C  V  K  Q  I  L  K  G  E  A  D  A  V  K  L  E  V  Q  Y  M  Y  E  A  L  M  C  G  L
    CTGCCAGCAGTAGAAGAATACCACAATAAAGATGATTTTGGGCCCTGTAAAACCCCTGGATCCCCATACACAGATTTTGGGACACTGCGTGCTGTAGCCTTGGTAAAAAAA 1776
556 L  P  A  V  E  E  Y  H  N  K  D  D  F  G  P  C  K  T  P  G  S  P  Y  T  D  F  G  T  L  R  A  V  A  L  V  K  K
    AGCAACAAAGACATCAACTGGAACAACATTAAAGGCAAGAAGTCCTGCCATACTGGTGTTGGTGATATTGCTGGCTGGGTTATCCCTGTTAGTCTTATTAGAAGGCAGAAT 1887
593 S  N  K  D  I  N  W  N  N  I  K  G  K  K  S  C  H  T  G  V  G  D  I  A  G  W  V  I  P  V  S  L  I  R  R  Q  N
    GACAATTCTGATATTGATTCCTTCTTCGGTGAGAGCTGTGCTCCTGGATCAGATACTAAATCCAACCTCTGTAAACTGTGCATTGGTGACCCCAAGAATTCCGCGGCCAAT 1998
630 D  N  S  D  I  D  S  F  F  G  E  S  C  A  P  G  S  D  T  K  S  N  L  C  K  L  C  I  G  D  P  K  N  S  A  A  N
    ACAAAATGTTCTCTCAGTGATAAGGAGGCCTATTATGGAAACCAAGGTGCCTTTAGATGTCTGGTGGAAAAAGGAGATGTGGCATTTGTGCCTCACACTGTTGTATTTGAA 2109
667 T  K  C  S  L  S  D  K  E  A  Y  Y  G  N  Q  G  A  F  R  C  L  V  E  K  G  D  V  A  F  V  P  H  T  V  V  F  E
    AACACAGATGGTAAAAATCCAGCAGTGTGGGCAAAAAATTTGAAATCAGAAGATTTTGAACTATTATGTTTGGATGGCTCACGTGCCCCTGTCAGTAATTACAAGAGCTGC 2220
704 N  T  D  G  K  N  P  A  V  W  A  K  N  L  K  S  E  D  F  E  L  L  C  L  D  G  S  R  A  P  V  S  N  Y  K  S  C
    AAACTTTCAGGCATCCCACCTCCCGCCATTGTCACCCGGGAAGAGAGCATCAGTGATGTTGTAAGAATTGTTGCTAATCAACAGTCACTGTATGGACGGAAGGGATTTGAA 2331
741 K  L  S  G  I  P  P  P  A  I  V  T  R  E  E  S  I  S  D  V  V  R  I  V  A  N  Q  Q  S  L  Y  G  R  K  G  F  E
    AAAGATATGTTCCAGTTGTTTTCTTCAAATAAAGGCAACAACCTCCTCTTCAATGACAACACTCAGTGCCTGATTACATTTGATAGACAACCAAAAGACATTATGGAGGAT 2442
778 K  D  M  F  Q  L  F  S  S  N  K  G  N  N  L  L  F  N  D  N  T  Q  C  L  I  T  F  D  R  Q  P  K  D  I  M  E  D
    TACTTTGGGAAACCCTACTACACTACAGTGTATGGTGCGAGCAGATCTGCCATGTCATCAGAACTGATATCTGCCTGCACAATTAAACACTGTTAAGAAGATCTAGCAAAC 2553
815 Y  F  G  K  P  Y  Y  T  T  V  Y  G  A  S  R  S  A  M  S  S  E  L  I  S  A  C  T  I  K  H  C  *
    ATTTGTGCTATAACTTTCTGCCTGGTTGGCACGTTGATCTTCTTTTTAATCCATATATCACTTCTGTTTTCTTTTTGTTTTTAAATAAATATTTAGCCTACCATG(A)18 2676
```

FIG. 2. Nucleotide sequence and deduced amino acid sequence of a cDNA clone of saxiphilin from bullfrog liver. Solid underlines mark the amino acid sequences of six fragments of native saxiphilin identified previously (5).

is 22 bp upstream of the poly(A) tail. Identification of the cloned sequence as saxiphilin was confirmed by finding the sequences of all five tryptic fragments (underlined in Fig. 2) previously reported (5) as well as the N-terminal sequence of the native protein. The predicted molecular weight of the 826-residue mature protein is 90,818, which is in good agreement with that of the native protein (90,000 ± 3000) as estimated by polyacrylamide gel electrophoresis (5, 6).

Homology between Saxiphilin and Transferrins. A sequence comparison search (11, 12) (SwissProt and GenBank data base) using the deduced amino acid sequence of saxiphilin established an evolutionary relationship to the transferrin family of Fe^{3+}-binding proteins. This is illustrated in Fig. 3 by an alignment of saxiphilin, serum transferrin from the African clawed frog (*Xenopus laevis*) (13), and human serum transferrin (14). Considerable sequence similarity is found throughout the whole alignment except for a 144-residue insertion that occurs after Gln-89 of saxiphilin. If this large insertion is considered as a gap, pairwise sequence alignments (17) between saxiphilin and various transferrins yield values of 28%, 44%, and 51% identity with transferrin from tobacco hornworm (*Manduca sexta*) (18), human serum (14), and *X. laevis* (13), respectively.

Another key feature identifying saxiphilin as an evolutionary relative of transferrin is the presence of internal duplication between residues 20–487 and 488–845 (Met-1 numbering) as detected by dot plot analysis (19) (not shown). The transferrin protein family is characterized by similarity between the first ≈350 N-terminal residues and the last ≈350 C-terminal residues, which indicates that the protein arose from an intragenic duplication (7). This internal duplication is the basis of the bilobal tertiary structure and similar secondary structure folding pattern of the N-terminal half (N lobe) and C-terminal half (C lobe) of human lactoferrin (15) and rabbit serum transferrin (16) as deduced by x-ray crystallography. Excluding the 144-residue insertion, saxiphilin contains the same percentage of internal similarity as *Xenopus* transferrin (38% identity), but somewhat less than that exhibited by various human transferrins (44–48% identity). The transferrin family can also be recognized by a large number of conserved disulfide bonds (7, 16, 20). In human lactoferrin six disulfide bonds appear at homologous positions in the N lobe and C lobe. All 12 of these disulfide bonds appear to be conserved in saxiphilin as identified by sequence alignment. In Fig. 3 these are labeled *a–f* and *a′–f′* in the putative N and C lobe regions of the sequence, respectively. Human lactoferrin and several other transferrins also contain four additional disulfides in the C lobe that are not present in the N lobe. Two of these can be identified in saxiphilin, labeled as *g′* and *h′* in Fig. 3.

In most known transferrins, both the N lobe and C lobe domains contain a high-affinity ($K_d \approx 10^{-20}$ M) binding site for Fe^{3+}. X-ray crystallography (15, 16) and sequence analysis have previously shown that ligand residues in these two Fe^{3+}-binding sites are highly conserved (7, 20). In each lobe, Fe^{3+} is coordinated by the same four residues: Asp-63 (Asp-392), Tyr-95 (Tyr-426), Tyr-188 (Tyr-517), and His-249 (His-585), with sequence numbers corresponding to human serum transferrin in the N lobe (C lobe), respectively. Physiological binding of Fe^{3+} to these two sites in transferrin is also known to require bicarbonate anion (HCO_3^-), which appears to bridge (15) between Fe^{3+} and the highly conserved

```
       -19                    1       a          b                     b         a                *
Sax     MAPTFQTALF FTIISLSFAA PNAKQVRWCA ISDLEQKKC- ---NDLVGSC NVPDITLVCV LRSSTEDCMT AIKDGQADAM FLDSGEVYEA SKDPYNLKPI   77
Xltf    MDFSLRVALC LSMLALCLAI QKEKQVRWCV KSNSELKKC- ---KDLVDTC KNKEIKLSCV EKSNTDECSL LFRKTMQMQF VWTGGDVYKG SLQPYNLKPI   77
Hstf    MRLAVGALLV CAVLGLCLAV P-DKTVRWCA VSEHEATKCQ SFRDHMKSVI PSDGPSVACV KKASYLDCIR AIAANEADAV TLDAGLVYDA YLAPNNLKPV   80

                                                    !
Sax     IAEPYSSNRD LQKCLKERQQ ALAKKMHLVI IFHNVMKRAI TNHSSAMAAL GTAGVLTSMG EKISGTNTPP GQTRATCERH ELPKCLKERQ VALGGDEKVL  177
Xltf    MAENYGSHTE TD-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   89
Hstf    VAEFYGSKED PQ-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   92

                                                                      *                          c     *
Sax     GRFVPQCDEK GNYEPQQFHG STGYSWCVNA IGEEIAGTKT PPGKIPATCQ KHDLVTTCHY TVAMVKKSSA FQFNQLKGKR SCHSGVSKTD GWKALVTVLV  277
Xltf    ---------- ---------- ---------- ---------- ---------- ------TCYY AVAVVKKSSK FTFDELKDKK SCHTGIGKTA GWNIIIGLLL  133
Hstf    ---------- ---------- ---------- ---------- ---------- ------TFYY AVAVVKKDSG FQMNQLRGKK SCHTGLGRSA GWNIPIGLL-  135

                                    d             e   d        e           *     c
Sax     EKKLLSWDGP AKESIQRAMS KFFSVSCIPG ATQ---TNLC KQCKGEEGKN CKNSHDEPYY GNYGAFRCLK EDMGDVAFLR STALSDEHS- ----EVYELL  369
Xltf    ERKLLKWAGP DSETWRNAVS KFFKASCVPG AKE---PKLS QLCAGIKEHK CSRSNNEPYY NYAGAFKCLQ DDQGDVAFVK QSTVPEEFH- ----KDYELL  225
Hstf    ---YCDLPEP -RKPLEKAVA NFFSGSCAPC ADGTDFPQLC QLCPG----- CGCSTLNQYF GYSGAFKCLK DGAGDVAFVK HSTIFENLAN KADRDQYELL  226

        f              f      *
Sax     CPDNTRKPLN KYKECNLGTV PAGTVVTRKI SDKTEDINNF LMEAQ----- -KRQ-CKLFS SAHG-KDLMF DDSTLQLALL SSEVDAFLY- -LGV---KLF  456
Xltf    CPDNTRKSIK EYKNCNLAKV PAHAVLTRGR DDKSKDIIEF LQEAQ----- -KTQECKLFR LPG-----MG KGSNFQGQRS ESYSPPIFYG QFSVPRSRLF  314
Hstf    CLDNTRKPVD EYKDCHLAQV PSHTVVARSM GGKEDLIWEL LNQAQEHFGK DKSKEFQLFS SPHG-KDLLF KDSAHGFLKV PPRMDAKMYL GYE-----YV  320

                                  a'         b'            b'        a'               *          g'                 h'
Sax     HAMKALTG-- -DAHLPSKNK -VRWCTINKL EKMKCDDWSA VSGGAIACTE ASCPKGCVKQ ILKGEADAVK LEVQYMYEAL MCGLLPAVEE YHNKDDFGPC  552
Xltf    QCIQALKEGV KEDDSAAQVK -VRWCTQSKA EKTKCDDWTT ISGGAIECTE ASTAEECIVQ ILKGDADAVT LDGGYMYTAG LCGLVPVMGE YYDQDDLTPC  413
Hstf    TAIRNLREGT CPEAPTDECK PVKWCALSHH ERLKCDEWSV NSVGKIECVS AETTEDCIAK IMNGEADAMS LDGGFVYIAG KCGLVPVLAE NYNKSD--NC  418

                     *                         c'     *                              d'          e' d'             e'
Sax     KTPGSPYTDF GTLRAVALVK KSNKDINWNN IKGKKSCHTG VGDIAGWVIP VSLIRRQNDN SDIDSFFGES CAPGSDTKSN LCKLCIGDP- KNSAANTKCS  651
Xltf    QRSCSQAK-- GVYYAVAIVK KG-TQVSWSN LRGVKTCHTA VGRTAGWNIP VGLITSETAN CDFASYVGES CAPGSDVKSN LCALCIGDPE KLSEREKKCS  510
Hstf    ED-----TPE AGYFAVAVVK KSASDLTWDN LKGKKSCHTA VGRTAGWNIP MGLLYNKINH CRFDEFFSEG CAPGSKKDSS LCKLCMGS-- ----GLNLCE  507

                *       c'                                           f'              f'     *
Sax     LSDKEAYYGN QGAFRCLVEK -GDVAFVPHT VVFENTDGKN PAVWAKNLKS EDFELLCLDG SRAPVSNYKS CKLSGIPPPA IVTREESISD VVRIVANQQS  750
Xltf    PSASEAYYGY SGAFRCLVEK -GQVGFAKHT TVFENTDGKN PAGWAKDLKS EDFELLCPDG SRAPVTDYKR CNLAEVPAHA VVTLPDKREQ VAKIVVNQQS  609
Hstf    PNNKEGYYGY TGAFRCLVEK -GDVAFVKHQ TVPQNTGGKN PDPWAKNLNE KDYELLCLDG TRKPVEEYAN CHLARAPNHA VVTRKDKEAC VHKILRQQQH  606

                                          h'                                                     g'
Sax     LYGRKGFE-K DMFQLFSSNK GNNLLFNDNT QCLITFDRQP KDIMEDYFGK PYYTTVYGAS RSAMSSELIS ACTIKHC                         826
Xltf    LYGRKGFQ-K DIFQMFQSTG GKDLLFKDST QCL--LEIPS KTTMQEFLGD KYHTAVTSLN KCSTSNEASW LPAQFHSCMK IYMIVDCPLS II       698
Hstf    LFGSNVTDCS GNFCLFRS-E TKDLLFRDDT VCLAKL--HD RNTYEKYLGE EYVKAVGNLR KCSTSS-LLE ACTFRRP                         679
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FIG. 3. Homology relationships of saxiphilin. The amino acid sequence of saxiphilin (Sax) is aligned with *X. laevis* transferrin (Xltf) (13) and human serum transferrin (Hstf) (14). The alignment was produced by using the PILEUP program of the Genetics Computer Group analysis package (12). Gaps in the alignment are shown as a hyphen (–). Residues that are identical in two of three proteins at any position are shown in boldface type. Position 1 is the N terminus and residues −19 to −1 correspond to the signal sequence. A single consensus site for N-linked glycosylation in saxiphilin is noted by an exclamation point (!). Asterisks (*) denote the positions of 10 highly conserved residues in the two Fe^{3+}/HCO_3^- sites of transferrins (15, 16). The locations of 14 probable disulfide bonds in saxiphilin were identified by homology to human lactoferrin (15) and are labeled as *a–f* in the N lobe and *a′–h′* in the C lobe above underlined pairs of Cys (C) residues.

```
Sax (90-160)    KCLKERQQAL-AKKMHLVIIFHNVMKRAITNHSSAMAALGTAGVLTSMGEKISGTNTPPGQTRATCERHELP-
                ||||||| || :    |  :      ::  :     :  |    :  :|| | || ||||   |||::|:|
Sax (161-233)   KCLKERQVALGGDEKVLGRFVPQCDEKGNYEPQQFHGSTGYSWCVNAIGEEIAGTKTPPGKIPATCQKHDLVT

Sax (178-226)                    GRFVPQCDEKGNYEPQQFHGSTGYSWCVNAIGEEIAGTKTPPGKIPATC
nidogen (842-889)                GMFVPQCDEYGHYVPTQCHHSTGYCWCVDRDGRELEGSRTPPGMRPP-C
invariant chain (210-248)        GAFRPKCDENGNYMPLQCHGSTGYCWCVFPNGTEVPHTK
EGP (93-123)                     GLYDPDCDESGLFKAKQCNG-TSMCWCVNTAG
thyroglogulin 1.1 (29-73)          YVPQCAEDGSFQTVQCQNDGRSCWCVGANGSEVLGSRQP-GR-PVAC
thyroglobulin 1.2 (97-141)         YLPQCQDSGDYAPVQCDVQHVQCWCVDAEGMEVYGTRQL-GR-PKRC
thyroglobulin 1.5 (597-639)        FVPSCTTEGSYEDVQCFSGE--CWCVNSWGKELPGSRVRDGQ-PR-C
thyroglobulin 1.6 (664-707)        FVPACTSEGHFLPVQCFNSE--CYCVDAEGQAIPGTRSAIGK-PKKC
```

FIG. 4. Homology relationships of the 144-residue insertion unique to saxiphilin. The upper two sequences are a pairwise alignment of saxiphilin residues 90–160 and 161–233 showing significant two-fold internal homology within the 144-residue insertion. A vertical line marks an identity and a colon marks a conservative substitution. The lower eight sequences illustrate homology between residues 178–226 within the saxiphilin insertion and a domain observed in other proteins that is known as a type 1 repeat of thyroglobulin (22). The comparison sequences are mouse nidogen (23), rat invariant chain (24), human epithelial glycoprotein (EPG) (25), and human thyroglobulin (22). Residues in boldface type are identical to those in saxiphilin and are present in at least three of the eight sequences.

residue, Arg-124 (Arg-456). In Fig. 3 the positions of these 10 critical residues are identified with asterisks; only 1 of these residues is found to be absolutely conserved in saxiphilin at Asp-60. Two of these residues in the N lobe have also apparently diverged in *X. laevis* transferrin (Asp-63 → Thr, Arg-124 → Lys); however, direct measurement of the Fe^{3+}-binding capacity of transferrin from *R. catesbeiana* indicates the presence of two functional Fe^{3+}-binding sites (6). Two other members of the transferrin family are known to contain only one functional Fe^{3+}-binding site [i.e., *M. sexta* transferrin (18) and human melanotransferrin (21)], but saxiphilin is the only example of a related protein in which nearly all of the Fe^{3+}-binding site residues have diverged.

Homology Relationships of the Insertion Sequence. The 144-residue insertion found in saxiphilin has several interesting features. Dot plot analysis (19) of the insertion sequence reveals that this domain also appears to have arisen from a duplication event. Fig. 4 shows an alignment of the two halves of the insertion sequence (saxiphilin residues 90–160 and 161–233) indicating 35% identity that is mainly clustered at the ends of the alignment. Assuming that saxiphilin/transferrin homology corresponds to a similar tertiary structure, then the insertion would be predicted to occur at a location between the previously identified d and e strands of β-sheet in the crystal structure of lactoferrin (15). This location is at the hinge region separating domains 1 and 2 that form the Fe^{3+}-binding pocket in the N lobe of lactoferrin and suggests that the saxiphilin insertion could form an autonomous globular domain that would not disrupt the basic folding pattern of lactoferrin. The 144-residue insertion also contains the only consensus site (N-X-T/S) for N-linked glycosylation in saxiphilin at Asn-119 (Fig. 3), suggesting that part of this region forms a surface domain.

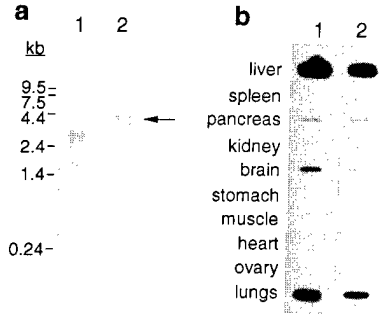

FIG. 5. Characterization and tissue distribution of saxiphilin mRNA. (*a*) Northern blot analysis of poly(A)$^+$ RNA from bullfrog liver. Five micrograms of poly(A)$^+$ RNA was fractionated on agarose, transferred to nylon, and consecutively hybridized with a 2.25-kb *Sma* I probe (lane 1) and a 0.43-kb insert-specific probe (lane 2). The arrow at the right points to the band identified as saxiphilin mRNA. (*b*) The same probes were hybridized to 5 μg of total RNA from various bullfrog tissues using a slot blot apparatus: 2.25-kb *Sma* I probe (lane 1); 0.43-kb insert-specific probe (lane 2).

A sequence comparison search (11, 12) against the 144-residue insertion identified a portion of the C-terminal half of the insertion that is 59% identical to a 48-residue fragment (Fig. 4) of nidogen (23), a ubiquitous ≈150-kDa cell matrix protein. This particular region of nidogen is itself a repetitive domain that has been previously shown to be related to a particular class of 10 repetitive domains (Thyr-1) found in thyroglobulin (22). As indicated in Fig. 4, such Thyr-1 repetitive elements have also been identified in several small integral membrane proteins: invariant chain subunit I of class II major histocompatibility complex (24, 26) and two related cell surface antigens called EPG (epithelial glycoprotein) (25) and GA733 (27). A class of insulin-like growth factor-binding proteins (28) and the B1 chain of laminin (29) also contain Thyr-1 domains. Along with saxiphilin, versions of this domain thus occur in a wide variety of membrane, secreted, and cell matrix proteins. The function of such repetitive Thyr-1 elements is unknown, but they have been suggested to play a role in intracellular protein transport and secretion (26).

Expression of Saxiphilin Message in Bullfrog Tissues. Northern blot analysis (Fig. 5*a*) was performed using the 2.25-kb *Sma* I fragment (Fig. 1) derived from the saxiphilin cDNA clone as a probe of poly(A)$^+$ RNA from bullfrog liver. Two bands were detected, an intense band at ≈3.1 kb and a fainter band at ≈4.4 kb (Fig. 5*a*, lane 1). The same filter was then stripped and hybridized to a 0.43-kb probe (Fig. 1) corresponding to the saxiphilin-specific insertion extending from nucleotide position 303 to position 738 in Fig. 2. This probe hybridized strongly to the upper ≈4.4-kb band (Fig. 5*a*, lane 2), indicating that this band corresponds to saxiphilin mRNA. The intense lower band at ≈3.1 kb in lane 1 is presumably the result of cross-hybridization with transferrin mRNA, which is known to be highly abundant in liver (30). This interpretation is consistent with the high degree of similarity between saxiphilin and transferrin mRNA and the relative abundance of the two proteins in bullfrog plasma (i.e., the concentration of transferrin is ≈100-fold greater than saxiphilin) (4–6).

The relative level of saxiphilin mRNA in various bullfrog tissues was investigated by hybridizing total RNA with the same probes used for the Northern blot analysis (Fig. 5*b*). The highest amount of saxiphilin mRNA was detected in liver, followed by lung, pancreas, and brain. In a previous study (4), we found that bullfrog kidney, heart, and ovaries have high levels of soluble [^{3}H]STX binding activity but this does not appear to correspond to a high level of mRNA. Such differences in the tissue distribution of saxiphilin mRNA versus protein activity as detected by [^{3}H]STX binding may reflect cellular uptake of plasma saxiphilin through a cell surface receptor or differences in protein turnover rates. Rat transferrin is synthesized principally in liver but also at lower

levels in brain and testis (30). Transferrin secreted from liver appears to be the source of transferrin found in plasma and other tissues such as intestine, which do not express transferrin mRNA (30). The possibility exists that saxiphilin and serum transferrin utilize similar mechanisms of gene expression, secretion, and internalization through the process of receptor-mediated endocytosis (7). Further work will be necessary to determine whether there is a receptor for saxiphilin analogous to the transferrin receptor.

Saxiphilin was originally discovered by its binding affinity for STX ($K_d \simeq 0.2$ nM), an interaction that exhibits a high degree of chemical specificity (4). Although our understanding of the functional significance of this binding interaction is incomplete, it has allowed us to identify a relative of the transferrin family that does not appear to be involved in iron metabolism. By analogy to other protein superfamilies that can be recognized on structural grounds, the case of saxiphilin and transferrin implies that transferrin-like proteins comprise a superfamily with functions more diverse than those associated with Fe^{3+} binding. Based on the high degree of sequence similarity, it appears that saxiphilin arose from a two-lobed transferrin ancestor by a process that eventually led to substitution of most of the Fe^{3+}-coordinating residues. The insertion sequence in the N lobe probably arose through a duplication event and exon shuffling of a Thyr-1 domain (27). The wide occurrence and repetition of this domain within proteins suggest that it may serve as a recognition site for a protein–protein interaction.

Although saxiphilin and transferrins bind different ligands (6), the pH dependence of STX dissociation from saxiphilin (L. Llewellyn and E.M., unpublished data) is similar to that of Fe^{3+} dissociation from transferrin, which is important in the delivery of iron to cells (7). This suggests that the mechanism of ligand binding and release in saxiphilin and transferrin are functionally analogous. The crystal structure of lactoferrin indicates that the Fe^{3+}-binding cavity (diameter = 10 Å) is potentially large enough to accommodate an organic molecule (15). On this basis, we hypothesize that saxiphilin may function in delivering or removing an endogenous ligand. Although it is known that STX is widely distributed in various marine invertebrates in association with plankton blooms (1), there is scant information on the chemical ecology of STX in freshwater ecosystems. However, at least one species of freshwater cyanobacteria has been found to synthesize STX (31). Thus, in frogs it is possible that saxiphilin may participate in a detoxification mechanism for neutralizing a microbial toxin. In broader terms, the recognition of a transferrin-like protein that binds an organic molecule is suggestive of a physiological system for transport and sequestration of small molecules that might ultimately be exploited for antidote therapy or drug delivery.

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Pharmacology. In the article "Molecular cloning of bullfrog saxiphilin: A unique relative of the transferrin family that binds saxitoxin" by Maria A. Morabito and Edward Moczydlowski, which appeared in number 7, March 29, 1994, of *Proc. Natl. Acad. Sci. USA* (**91,** 2478–2482), the authors request that the following corrections be noted. In the course of studies that involved resequencing saxiphilin cDNA, a few errors in the published sequence were discovered. The most significant error was an inadvertent insertion of three noncontiguous nucleotide bases. Correction of this error results in a revised translation of the coding sequence within the previously identified 144-residue insertion domain. This latter region is actually a sequence of 143 residues that is absent in other members of the transferrin protein family as previously noted. Furthermore, 31 consecutive amino acids within the insertion domain have been revised as shown below in a corrected Fig. 4. Analysis of the revised sequence indicates that the insertion domain is a tandem duplication with 67% identity (instead of the previously reported 35% identity) for an alignment of residues 90–159 with 160–232. As shown in Fig. 4, this insertion domain of saxiphilin contains two type 1 thyroglobulin module domains (Thyr-1) instead of one, as reported previously. The 143-residue revised insertion does not contain a consensus site for N-linked glycosylation suggested previously. Two additional nucleotide bases in the sequence were corrected. One of these changes did not affect the translation and the other one resulted in a change of the previous saxiphilin residue Thr-238 to corrected Ala-237. In summary, the reported saxiphilin cDNA contains an open reading frame of 844 residues. Removal of the 19-residue secretory signal sequence gives a predicted molecular weight of 90,901 for this 825-residue secreted protein. The saxiphilin sequence has been corrected in the Genbank data base (accession no. U05246).

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Sax (90-159)    KCLKERQQALA--KKMIGHYIPQCDEKGNYQPQQCHGSTGHCWCVNAMGEKISGTNTPPGQTRATCERHELP-
                ||||||| ||:   |::|:::||||||||||:||| ||||| :|||||:|| | || ||||   |||::|:|
Sax (160-232)   KCLKERQVALGGDEKVLGRFVPQCDEKGNYEPQQFHGSTGYSWCVNAIGEEIAGTKTPPGKIPATCQKHDLVT

nidogen (842-889)                GMFVPQCDEYGHYVPTQCHHSTGYCWCVDRDGRELEGSRTPPGMRPP-C
invariant chain (210-248)        GAFRPKCDENGNYMPLQCHGSTGYCWCVFPNGTEVPHTK
EGP (93-123)                     GLYDPDCDESGLFKAKQCNG-TSMCWCVNTAG
thyroglobulin 1.1 (29-73)          YVPQCAEDGSFQTVQCQNDGRSCWCVGANGSEVLGSRQP-GR-PVAC
thyroglobulin 1.2 (97-141)         YLPQCQDSGDYAPVQCDVQHVQCWCVDAEGMEVYGTRQL-GR-PKRC
thyroglobulin 1.5 (597-639)        FVPSCTTEGSYEDVQCFSGE--CWCVNSWGKELPGSRVRDGQ-PR-C
thyroglobulin 1.6 (664-707)        FVPACTSEGHFLPVQCFNSE--CYCVDAEGQAIPGTRSAIGK-PKKC
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FIG. 4. Homology relationships of the 143-residue insertion unique to saxiphilin. The upper two sequences are a pairwise alignment of saxiphilin residues 90–159 and 160–232 showing significant twofold internal homology within the 143-residue insertion. A vertical line marks an identity and a colon marks a conservative substitution. The lower seven sequences illustrate homology between residues 105–153 and 177–225 within the saxiphilin insertion and a domain observed in other proteins that is known as a type 1 repetitive module of thyroglobulin (22). The comparison sequences are mouse nidogen (23), rat invariant chain (24), human epithelial glycoprotein (EGP) (25), and human thyroglobulin (22). Residues in boldface type are identical to those in saxiphilin and are present in at least four of the nine sequences.

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Characterization of Saxitoxin Binding to Saxiphilin, a Relative of the Transferrin Family That Displays pH-Dependent Ligand Binding†

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ABSTRACT: Saxiphilin is a 91 kDa saxitoxin-binding protein that is homologous to members of the transferrin family of Fe^{3+}-binding proteins noted for pH-dependent release of Fe^{3+}. The mechanism of toxin binding to purified native saxiphilin from the bullfrog (*Rana catesbeiana*) was studied using [^{3}H]saxitoxin. At pH 7.4 and 0 °C, [^{3}H]saxitoxin binds to a single site on saxiphilin with a K_D of ~0.2 nM. The pH dependence of [^{3}H]saxitoxin binding follows a one-site titration curve in the range of pH 9–4 with maximal binding from pH 9 to 7 and half-inhibition at pH 5.7. Inhibition of toxin binding at low pH is the combined result of a decrease in the rate of toxin association and an increase in the rate of toxin dissociation. The dependence of the apparent rate constants for [^{3}H]saxitoxin association and dissociation on [H^+] can be accounted for by a four-state model of allosteric interaction between the toxin-binding site and a single titratable residue of saxiphilin with a pK_a of 7.2 in the toxin-free form and 4.3 in the toxin-bound form. From 0 to 25 °C, the temperature dependence of [^{3}H]saxitoxin binding to saxiphilin is characterized by $\Delta H°$ = −8.3 kcal mol^{-1}, $\Delta S°$ = 13.8 cal mol^{-1} K^{-1}, and activation energies of 22.5 kcal mol^{-1} for dissociation and 11.1 kcal mol^{-1} for association. Binding of [^{3}H]saxitoxin to saxiphilin is competitively inhibited with low affinity by a variety of divalent metal and lanthanide cations. Inhibition of toxin binding by the carboxyl-methylating reagent trimethyloxonium is prevented by pre-equilibration with [^{3}H]saxitoxin, implicating the presence of one or more carboxyl groups in the binding site. Functional similarities suggest that the saxitoxin-binding site of saxiphilin is located in an interdomain cleft analogous to the location of one of the two homologous Fe^{3+}-binding sites of transferrins. On the basis of residue substitutions between saxiphilin and transferrins, it is proposed that the saxitoxin-binding site is located in the carboxy terminal lobe of saxiphilin and that binding is modulated by protonation of a conserved histidine residue.

Saxiphilin is a soluble protein that binds saxitoxin (STX)[1] with high affinity and specificity. Saxiphilin from the North American bullfrog (*Rana catesbeiana*) has been studied most extensively (Mahar et al., 1991; Li & Moczydlowski, 1991; Li et al., 1993); however, similar activity is present in a variety of ectothermic vertebrates (L. Llewellyn, J. Lynch, P. Bell, and E. Moczydlowski, unpublished results). Using [^{3}H]STX binding as an assay, saxiphilin was purified from bullfrog plasma and identified as a 91 kDa protein related to transferrin (Li & Moczydlowski, 1991). The ligand, STX, is a potent neurotoxin with a structure distinguished by two cyclized guanidinium groups (Figure 2). STX or "paralytic shellfish poison" is produced by certain cyanobacteria and dinoflagellates and accumulated by numerous invertebrate and vertebrate species in marine environments (Hall et al., 1990). Neurotoxicity of STX is a result of the blockage of voltage-dependent sodium channels that mediate the depolarizing phase of action potentials. The large (~210 kDa) α-subunit of sodium channels contains one extracellular binding site for STX that is located in an external vestibule close to the entrance of the ion-selective pore (Catterall, 1992).

In contrast to sodium channels, the physiological function of saxiphilin is presently unknown. Partial sequencing of the native protein (Li & Moczydlowski, 1991) provided information that led to the isolation from bullfrog liver of a full-length cDNA clone encoding saxiphilin (Morabito & Moczydlowski, 1994). The primary sequence confirmed that saxiphilin is homologous to members of the transferrin family of Fe^{3+}-binding proteins, which are also known as siderophilins. For example, pairwise alignment of saxiphilin with human lactoferrin shows 44% sequence identity at the amino acid level, similar 2-fold internal homology, and apparent conservation of 14 disulfide bonds. However, the presence of a unique insertion of 144 residues, the substitution of most of the conserved Fe^{3+}-coordination residues, and a lack of $^{55}Fe^{3+}$ binding discriminate saxiphilin from other members of the transferrin family (Li et al., 1993; Morabito & Moczydlowski, 1994).

The transferrin family includes serum transferrin, lactoferrin, ovotransferrin, and melanotransferrin. These latter proteins exhibit bicarbonate-dependent binding of Fe^{3+}, with a high affinity, K_D, for Fe^{3+} estimated to be ~10^{-20} M for human transferrin (Aisen et al., 1978). Serum transferrin sequesters, transports, and ultimately delivers Fe^{3+} to eukaryotic cells by the process of receptor-mediated endocytosis

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[1] Abbreviations: BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STX, saxitoxin; TMO, trimethyloxonium tetrafluoroborate; Tris, tris(hydroxymethyl)aminomethane.

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(Dautry-Varsat, 1986). The related protein lactoferrin serves as a bacteriostatic agent in milk and other secretions by limiting free Fe^{3+} required for growth of microorganisms (Griffiths & Bullen, 1987). Ovotransferrin (conalbumin) performs a similar function in egg white of birds. Melanotransferrin is an Fe^{3+}-binding protein associated with the plasma membrane of melanoma cells; however, its exact function is unknown (Rose et al., 1986; Baker et al., 1992). The crystal structures of transferrin and lactoferrin are characterized by two homologous lobes that each contain a ferric ion coordinated by two tyrosines, one histidine, one aspartic acid, and one bicarbonate anion cofactor that is also hydrogen-bonded to a conserved arginine residue (Bailey et al., 1988; Anderson et al., 1989). An important aspect of the function of serum transferrin is the pH-dependent release of Fe^{3+} in the range of pH 6–4 (Lestas, 1976; Van Renswoude et al., 1982). Physiologically, after endocytosis of serum transferrin bound to the transferrin receptor, acidification of the internal endosome compartment by an H^+-ATPase triggers the release of Fe^{3+} from transferrin for biosynthesis of other iron-containing proteins.

The identification of saxiphilin as a structural relative of the transferrin family suggests that such proteins may have other biochemical functions besides their known role in iron metabolism and homeostasis. To pursue the function of saxiphilin, this study is an initial investigation of the mechanism of STX binding to saxiphilin as compared to the known mechanism of Fe^{3+} binding to transferrin. We find that native saxiphilin has a binding stoichiometry of one [^{3}H]STX-binding site per molecule, in contrast to the two Fe^{3+}-binding sites of most transferrins. The pH dependence of [^{3}H]STX binding to saxiphilin is similar to the pH dependence of Fe^{3+} binding to serum transferrin and is consistent with an equilibrium between high- and low-affinity conformations of the binding site controlled by protonation of a histidine residue. We also find that certain divalent and lanthanide metal cations competititvely inhibit [^{3}H]STX binding to saxiphilin with low affinity, which implies that the STX-binding site contains residues that form a weak metal ion-binding site. These similarities suggest that STX binds to one lobe of saxiphilin in a manner analogous to that of transferrin and lactoferrin, in which Fe^{3+} is bound in a clawlike fashion between two flexible domains connected by a hinge region (Bailey et al., 1988; Anderson et al., 1989). Particular amino acid residues that may be involved in STX binding and the pH dependence of saxiphilin are suggested on the basis of structural homology to transferrin.

MATERIALS AND METHODS

Materials. [^{3}H]STX labeled by ^3H_2O exchange (Ritchie et al., 1976) was purchased from Amersham and standardized as described (Moczydlowski et al., 1988). The working specific activity was 25 400 cpm/pmol using Ecoscint scintillation fluid (National Diagnostics). Stock solutions of STX (Calbiochem) were diluted in 1 mM citrate buffer (pH 5.0). Mops, Mes, and Hepes were obtained from Sigma, and Tris base was from American Bioanalytical. Heparin-Sepharose CL-6B, PBE 118, Polybuffer 96, and Pharmalyte 8–10.5 ampholytes were from Pharmacia LKB. AG50W-X2 cation exchange resin (100–200 mesh, H^+ form) was purchased from Bio-Rad. Trimethyloxonium tetrafluoroborate (TMO) was from Aldrich. Chloride salts of metal cations and lanthanides were from Alfa. Other chemicals were reagent grade from J. T. Baker. Water was deionized with a Milli-Q system from Millipore.

Purification and Stability of Saxiphilin. Saxiphilin was purified from bullfrog (*R. catesbeiana*) plasma by modification of the procedure of Li and Moczydlowski (1991). The chromatofocusing resin was PBE 118 rather than PBE 94. After chromatofocusing, ampholytes were removed by gel permeation chromatography on a column of Sephacryl S-300 (1.4 × 30 cm) equilibrated with 1 mM Hepes-NaOH, 100 mM NaCl (pH 7.4). To avoid inactivation by freeze–thawing, aliquots of purified saxiphilin were diluted 1:1 with 40% glycerol in 1 mM Hepes-NaOH, 100 mM NaCl (pH 7.4) and quick frozen in liquid N_2. Thawed saxiphilin was also stabilized by addition of 0.1 mg/mL BSA in all assays and dilutions of the protein. In all experiments, protein which had been frozen only once was used to ensure maximal activity. Protein concentration of pure saxiphilin was based on quantitative amino acid analysis performed by the Yale Protein Chemistry Facility and corrected for proline, cysteine, and tryptophan which were assumed to be 10.7% by weight according to the known amino acid composition (Morabito & Moczydlowski, 1994). Saxiphilin concentrations were calculated from the protein molecular weight of 90 818 deduced from saxiphilin cDNA (Morabito & Moczydlowski, 1994).

Measurement of [^{3}H]STX Binding. [^{3}H]STX bound to saxiphilin was measured in 100 μL aliquots applied to 1 mL minicolumns of AG50W-X2 resin (100–200 mesh, converted to $Tris^+$ form and pre-equilibrated with 20 mM Tris-HCl, pH 7.4, 10 mg/mL BSA). Rapid elution (10 s) of bound [^{3}H]-STX into scintillation vials was achieved after addition of sample and wash (0.5 mL of 20 mM Tris-HCl, pH 7.4) by applying pressure to the column with a syringe. At neutral pH, this cation exchange resin selectively binds free [^{3}H]-STX and not the [^{3}H]STX/saxiphilin complex which is larger than the exclusion limit (<2700 MW). Binding isotherms for Scatchard analysis were obtained by varying total [^{3}H]-STX concentration from 0.05 to 10 nM. Samples for equilibrium binding assays were allowed to equilibrate for at least 1 h. Nonspecific binding was determined with 10 μM unlabeled STX. All binding experiments were performed at 0 °C except for studies of the temperature dependence of [^{3}H]STX binding (Figure 5) where sample temperature was maintained with a circulating water bath. Binding assays are based on means of duplicate determinations that did not differ by more than 10%.

A stock buffer mixture of Tris (0.5 M)/Mes (0.25 M)/acetic acid (0.25 M), adjusted with tetramethylammonium hydroxide or HCl, was used in studies of the pH dependence of [^{3}H]STX binding. This buffer mixture is designed to maintain a relatively constant ionic strength throughout the pH range of 4–9 (Ellis & Morrison, 1982). The final buffer concentration in the assay was either 20 or 100 mM in Tris, as noted in figure legends. For binding experiments where pH was varied to pH < 6.5, the minicolumns were pre-equilibrated with 100 mM Tris-HCl (pH 7.4) to maintain constant recovery of saxiphilin. Reported pH values for the buffers were measured at assay concentrations and temperatures with a Corning Model 150 ion analyzer.

Dissociation and Association Time Course. For dissociation, saxiphilin was first equilibrated with 4.8 nM [^{3}H]STX at the desired assay conditions. After removing two aliquots (100 μL) to determine the initial value of bound [^{3}H]STX, 10 μM STX was added to begin exchange with the radioligand. Aliquots (100 μL) were removed at various time intervals and applied to cation exchange columns to determine the time course of dissociation. The association time course was similarly determined by initiating the reaction with 19.2 nM

[^{3}H]STX after pre-equilibration of ~0.5 nM saxiphilin at assay conditions for 30 min. The equilibrium level of binding was established by following the reaction for periods up to 1 h.

Effect of Divalent Metals and Lanthanide Cations on [^{3}H]-STX Binding. Binding inhibition titrations were performed with chloride salts of various divalent metal and lanthanide cations in the presence and absence of 100 mM $NaHCO_3$, using 20 ng/mL saxiphilin (~0.2 nM), 100 mM NaCl, 4.8 nM [^{3}H]STX, and 100 mM Mops-NaOH at a final pH of 7.45. Samples were incubated for 1 h before assay of bound [^{3}H]STX. For experiments with Pr^{3+}, sample incubation was also extended to 6 h. Association and dissociation kinetics of [^{3}H]STX binding in the absence or presence of 10 mM $PrCl_3$ and 100 mM $NaHCO_3$ were determined as described above with 34 ng/mL saxiphilin (~0.4 mM) in 100 mM NaCl, 100 mM Mops-NaOH (pH 7.4). In all experiments with varying concentrations of metal chlorides in the absence or presence of $NaHCO_3$, the assay mixture was maintained at pH 7–7.4 throughout the titration. No precipitation was observed in the assay samples.

Effect of TMO Modification on [^{3}H]STX Binding. Since TMO is hydrolyzed in water (MacKinnon & Miller, 1989), saxiphilin was exposed to this reagent as rapidly as possible after it was dissolved. Various amounts of TMO (2–14 mg) were weighed into tubes that were flushed with N_2 and sealed. Aliquots (200 μL) of a buffered solution containing saxiphilin, BSA, and NaCl were added to each tube and vortexed. After 10 min, [^{3}H]STX plus water was added to a final volume of 250 μL. Final conditions for the assay were 63 ng/mL (~0.7 nM) saxiphilin, 19.2 nM [^{3}H]STX, 100 mM NaCl, 100 mM Tris-HCl, pH 8.6. After equilibration for 30 min, bound [^{3}H]-STX was measured in duplicate 115 μL aliquots. Control binding was defined with an identical sample without TMO. Nonspecific binding was measured in a sample with 10 μM STX. For ligand protection experiments, saxiphilin was first pre-equilibrated with 19.2 nM [^{3}H]STX for 30 min in the assay buffer before exposure to TMO for 10 min. The TMO experiment of Figure 9 was performed four times with similar results.

Miscellaneous. SDS–PAGE (7% T, 2.7% C) was performed according to Laemmli (1970) with a water-cooled slab gel (0.75 mm × 12 cm × 16 cm) apparatus. Samples for electrophoresis were heated at 90 °C in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue R-250 according to Diezel et al. (1972). B_{max} and K_D values for a one-site binding model were obtained by Scatchard analysis using the LIGAND computer program (Munson & Rodbard, 1980) obtained from Biosoft. Parameter fits to binding competition curves, exponential kinetics, and a four-state model of the pH dependence of [^{3}H]STX binding were obtained with the nonlinear fitting utility of Sigmaplot 4.1 (Jandel Scientific).

RESULTS

Stability and Stoichiometry of [^{3}H]STX Binding to Saxiphilin. Preparations of saxiphilin used for this study were virtually homogeneous as judged by a single major band on SDS–PAGE (Figure 1A). As noted previously (Li et al., 1993), the apparent molecular weight of saxiphilin estimated by SDS–PAGE is in good agreement with the protein molecular weight of 90 818 deduced from the cDNA sequence (Morabito & Moczydlowski, 1994). To ensure maximal activity for determination of toxin-binding stoichiometry, the stability of purified saxiphilin was investigated. Figure 1B shows that saxiphilin is subject to denaturation by freeze–thawing as noted by complete loss of [^{3}H]STX-binding activity after four repetitive cycles of freezing in liquid N_2. This loss of activity was attenuated by addition of 20% glycerol or ethylene glycol to the freezing buffer, but these cryoprotectants did not completely eliminate this form of denaturation (Figure 1B). To minimize such loss of activity, saxiphilin was aliquoted in small volumes in buffer containing 20% glycerol and thawed only once before use.

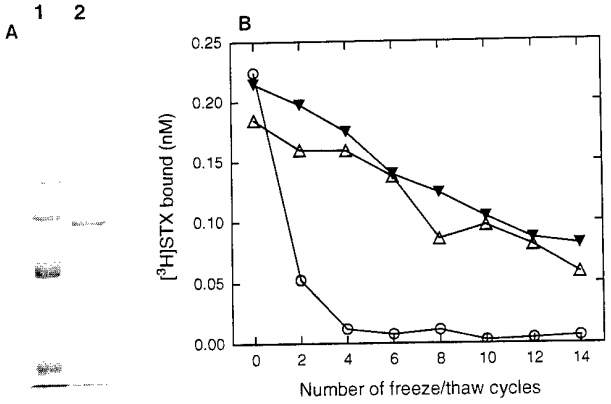


FIGURE 1: Sensitivity of purified saxiphilin to freeze–thawing. (A) SDS–PAGE (7% total acrylamide) of saxiphilin used for determination of [^{3}H]STX-binding stoichiometry. Lane 1, 1.7 μg each of M_r standards (top to bottom): β-galactosidase (116 000), phosphorylase b (97 400), bovine serum albumin (67 000), bovine carbonic anhydrase (29 000); lane 2, 0.8 μg of saxiphilin. (B) Effect of freeze–thawing on [^{3}H]STX-binding activity of saxiphilin and partial protection by glycerol and ethylene glycol. A sample of saxiphilin (27 ng/mL) (~0.3 nM) without cryoprotectant (○) or with 20% glycerol (▼) or 20% ethylene glycol (△) was subjected to repeated cycles of freeze–thawing and assayed for [^{3}H]STX-binding activity.

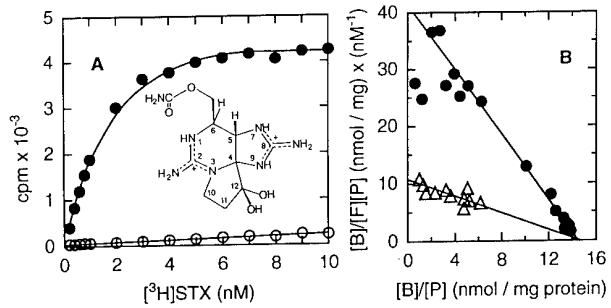


FIGURE 2: Stoichiometry and K_D of [^{3}H]STX binding to pure saxiphilin. (A) Binding isotherm obtained with increasing concentrations of total [^{3}H]STX in the presence of 200 mM NaCl, 10 mM Mops-NaOH (pH 7.45), and 120 ng/mL saxiphilin. Samples were assayed in the absence (●) or presence (○) of 10 μM STX to assess nonspecific binding. The insert shows the structure of STX. (B) Scatchard transformation of the data in panel A (●) is fit with a K_D of 0.35 ± 0.02 nM and a B_{max} of 14.5 ± 0.2 nmol/mg of protein. Results are also shown for a similar [^{3}H]STX titration at pH 5.55 (△) in the presence of 100 mM Tris/50 mM Mes/50 mM acetic acid, 100 mM NaCl and fit with a K_D of 13.6 ± 3.6 nM and a B_{max} of 14.7 ± 2.9 nmol/mg of protein.

Figure 2A shows raw data from a binding titration of a sample of pure saxiphilin with increasing concentrations of [^{3}H]STX. The low level of "nonspecific" binding measured in the presence of 10 μM unlabeled STX is characteristically observed with highly purified saxiphilin. The corresponding Scatchard plot of Figure 2B shows that [^{3}H]STX-binding data obtained at pH 7.45 and 0 °C are consistent with a homogeneous class of sites with K_D = 0.35 ± 0.02 nM and B_{max} = 14.5 ± 0.2 nmol/mg of protein. A similar experiment with a different preparation of saxiphilin gave values of K_D

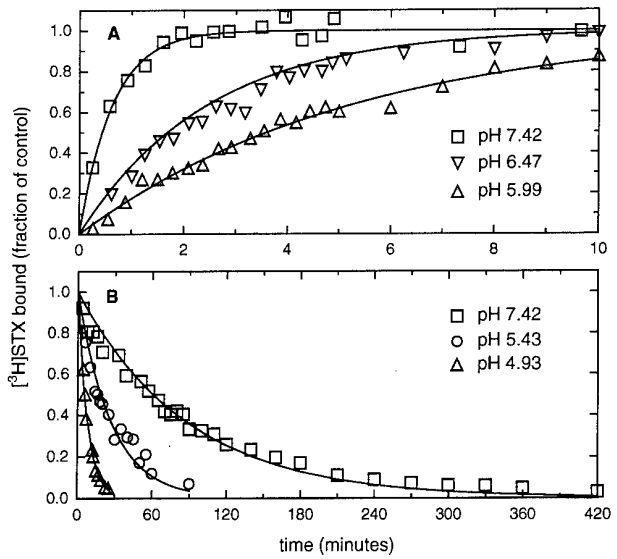


FIGURE 3: Effect of pH on association and dissociation kinetics of [^{3}H]STX binding to saxiphilin. (A) Time course of [^{3}H]STX association at three different pH values was determined with 19.2 nM [^{3}H]STX and 46 ng/mL (~0.5 nM) saxiphilin in the presence of 100 mM NaCl, 100 mM Tris/50 mM Mes/50 mM acetic acid buffer adjusted to pH 5.99 (△), 6.47 (▽), and 7.42 (□). (B) Time course of [^{3}H]STX dissociation at three different pH values was determined after equilibrating 4.8 nM [^{3}H]STX and 27 ng/mL saxiphilin (~0.3 nM) for 30 min in the same buffer adjusted to pH 4.93 (△), 5.43 (○), and 7.42 (□). Binding data are normalized to the equilibrium value at long times (A) or the initial value (B). Data points at each pH value are fit to a single exponential with rate constants: (A) pH 5.99, 3.2×10^{-3} s^{-1}; pH 6.47, 6.85×10^{-3} s^{-1}; pH 7.42, 15.5×10^{-3} s^{-1}; (B) pH 4.93, 21.3×10^{-4} s^{-1}; pH 5.43, 4.9×10^{-4} s^{-1}; pH 7.42, 1.44×10^{-4} s^{-1}. Some overlapping data points have been omitted to relieve crowding.

= 0.16 ± 0.02 nM and B_{max} = 13.9 ± 0.4 nmol/mg of protein. The mean value of B_{max} based on 10 determinations is 14.1 ± 1.4 nmol of [^{3}H]STX bound/mg of protein. The theoretical value of B_{max} for a protein with a molecular mass of 90.8 kDa is 11.0 nmol/mg for one STX-binding site and 22.0 nmol/mg for two STX-binding sites. Since the linear Scatchard plot indicates one class of binding sites in the protein preparation, we conclude that the B_{max} data are consistent with one [^{3}H]-STX-binding site per saxiphilin molecule. The discrepancy between the measured B_{max} value of 14.1 nmol/mg and the theoretical value of 11.0 nmol/mg is probably due to calibration errors in the specific activity of [^{3}H]STX and/or the measurement of protein concentration. On the basis of the structural homology of saxiphilin to members of the transferrin family (Morabito & Moczydlowski, 1994) which bind two Fe^{3+} ions, this stoichiometry suggests that only one of the two homologous lobes of saxiphilin contains a functional STX-binding site.

Effect of pH on the Kinetics of [^{3}H]STX Binding. Binding of [^{3}H]STX to saxiphilin is inhibited by a decrease in pH. Scatchard analysis (Figure 2B) indicates that the K_D of [^{3}H]-STX is 13.6 ± 3.6 nM at pH 5.55 (0 °C) which is ~40-fold lower affinity than the K_D of 0.35 nM at pH 7.45. However, the extrapolated B_{max} value of [^{3}H]STX binding does not appear to be altered at low pH (Figure 2B). This indicates that the number of available binding sites does not depend on [H^+] and that the decrease in affinity must be due to altered kinetics of [^{3}H]STX binding.

The reduction in affinity for [^{3}H]STX at low pH is the combined result of a decrease of the association rate (Figure 3A) and an increase of the dissociation rate (Figure 3B). The time course of association of [^{3}H]STX to saxiphilin was measured under pseudo-first-order conditions with the concentration of ligand (19.2 nM [^{3}H]STX) approximately 20-fold greater than the concentration of binding sites. Under these conditions, the association time course was closely approximated by a single exponential, giving an apparent rate constant of $k_p = 15.5 \times 10^{-3}$ s^{-1} at pH 7.42 (Figure 3A). Reduction of the pH to 6.47 and 5.99 resulted in a 2.3- and 4.8-fold decrease in the apparent association rate, respectively. When the dissociation time course of [^{3}H]STX was measured by the rate of exchange with excess unlabeled STX, the kinetics were well-described by a single-exponential process under a wide range of conditions (e.g., Figure 3B). At pH 7.42 and 0 °C, the dissociation of [^{3}H]STX from saxiphilin occurs with a halftime of ~80 min corresponding to a rate constant of $k_d = 1.44 \pm 0.03 \times 10^{-4}$ s^{-1}. This rate is enhanced by a factor of 3.4- and 14.8-fold at pH 5.43 and 4.93, respectively. The observed pseudo-first-order association rate constant (k_p) for [^{3}H]STX at pH 7.42 may be converted to a bimolecular association rate constant (k_a), using the familiar expression derived from the rate expression for a reversible bimolecular reaction: $k_p = [STX]k_a + k_d$ (Fersht, 1985). This gives a value of $k_a = 8.0 \times 10^5$ s^{-1} M^{-1}. Using the ratio of k_d/k_a to calculate the equilibrium dissociation constant at pH 7.42 gives K_D = 0.18 nM, which is close to the value (0.16–0.35 nM) derived from Scatchard analysis at this pH. This agreement between kinetic and equilibrium measurements further supports the assumptions of a single binding site for [^{3}H]STX and first-order kinetics.

Mechanism of Inhibition of [^{3}H]STX Binding to Saxiphilin by H^+. Figure 4A is a plot of the equilibrium concentration of bound [^{3}H]STX measured at various pH values from 9 to 4.7. This experiment shows that [^{3}H]STX binding is essentially constant in the range of pH 9–7. Further acidification to pH 4 results in a progressive inhibition of [^{3}H]STX binding to a level near the limit of detectibility. Fitting of the pH titration in the presence of 200 mM NaCl to the Hill equation, $y = B_{max}K_{0.5}^n/([H^+]^n + K_{0.5}^n)$, gives a value of n = 1.0 for the Hill coefficient and p$K_{0.5}$ = 5.7. Similar behavior (n = 1.0; p$K_{0.5}$ = 5.4) with a small shift of the titration curve to lower pH is observed at 600 mM NaCl (Figure 4A). This comparison shows that the pH dependence is rather insensitive to ionic strength in this range of NaCl concentration. The data of Figure 4A also indicate that the effect of pH is not due to the ionization state of the ligand. STX has two cyclized guanidinium groups (Figure 2). The C-2 guanidinium group of the six-membered ring has a pK_a of 11.3, and the C-8 guanidinium group of the five-membered ring has a pK_a of 8.2 (Rogers & Rapoport, 1980; Shimizu et al., 1981). Since both of these guanidinium groups are completely protonated over the range of pH 7–4, inhibition of [^{3}H]STX binding by decreasing pH is most likely due to protonation of saxiphilin. The lack of a significant decrease in the level of [^{3}H]STX binding in the range of pH 8–9 also implies that protonation of the C-8 guanidinium of STX is not required for high-affinity binding to saxiphilin.

The dependence of the rate of [^{3}H]STX association and dissociation on [H^+] (Figure 3) requires that both the STX-bound and STX-free states of saxiphilin are sensitive to protonation. A Hill coefficient of n = 1 for the effect of [H^+] on the fractional occupancy of saxiphilin by [^{3}H]STX (Figure 4A) further suggests that this pH dependence is mediated by a single titratable group of saxiphilin. The simplest model that can explain these observations is a negative allosteric interaction between the protonation of a single amino acid residue of saxiphilin and the STX-binding site. According to

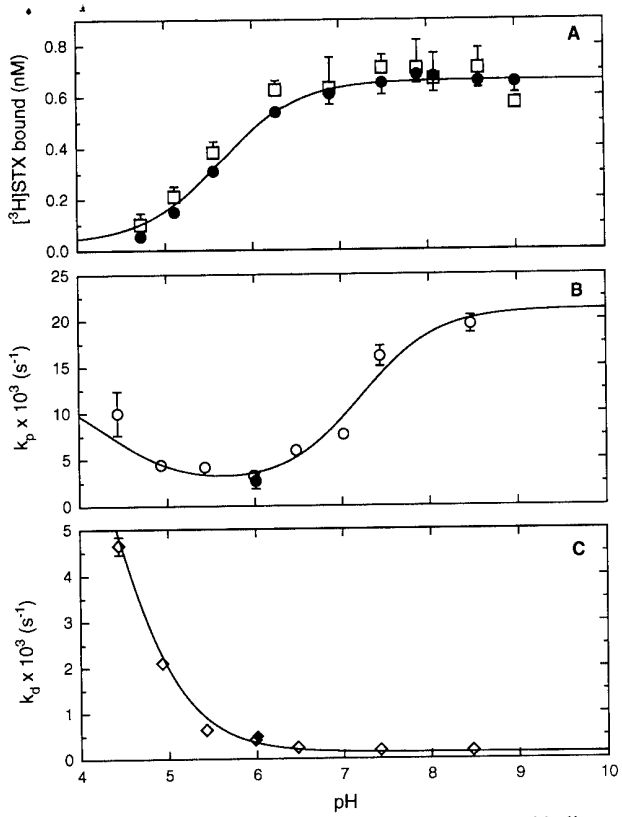
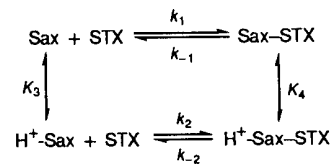


FIGURE 4: Analysis of the pH dependence of [³H]STX binding to saxiphilin. (A) Equilibrium measurement of bound [³H]STX after incubation of 5 nM [³H]STX at pH 9–4 in the presence of 200 mM NaCl (●) and 38 ng/mL (~0.42 nM) saxiphilin. The pH buffer was 20 mM Tris/10 mM Mes/10 mM acetic acid. Results for a similar experiment at higher ionic strength (600 mM NaCl) are also shown (□). (B) Apparent pseudo-first-order rate constant for association (k_p) of [³H]STX as a function of pH. k_p (○) was measured at various pH values as described in Figure 3A. (C) Apparent rate constant for dissociation (k_d) of [³H]STX as a function of pH. k_d (◇) was measured at various pH values as described in Figure 3B. Errors were evaluated from the fitting statistics of a single time course (B and C) or from the standard error of the mean of three experiments (A). Solid lines correspond to a theoretical fit obtained by simultaneous fitting of the data sets from A (200 mM NaCl), B, and C to eqs 1–3 derived from the scheme as discussed in the text. The best-fit parameters are listed in Table 1. Solid symbols in B and C correspond to measurements made on a different saxiphilin preparation.

this model, a certain residue of saxiphilin can be protonated in either the STX-free or STX-bound form. Protonation of the STX-free form results in an H^+-form of the protein that has lower affinity for STX and a slower apparent association rate. Similarly, protonation of the STX-bound form of saxiphilin also destabilizes STX binding and results in an H^+-saxiphilin–STX ternary complex that has a faster dissociation rate of STX, giving rise to pH-dependent dissociation of [³H]STX. Thus, the pH dependence of [³H]STX binding can be explained by the following four-state scheme, where Sax refers to the unprotonated form of saxiphilin and H^+-Sax refers to the protonated form:



In the above scheme, k_1 and k_2 are the bimolecular association rate constants for STX binding to the unprotonated and protonated forms of saxiphilin, respectively. The dissociation constants k_{-1} and k_{-2} are the respective rate constants for STX dissociation. K_3 and K_4 are the equilibrium dissociation constants for protonation of the STX-free and STX-bound forms of saxiphilin, respectively. To investigate whether this scheme is sufficient to describe the kinetics and pH dependence of STX binding, pseudo-first-order association (k_p) and dissociation (k_d) rate constants for [³H]STX were measured at various pH values from 8.5 to 4.5 from the time course of binding as illustrated in Figure 3. Results shown in Figure 4B,C indicate that k_p follows a biphasic dependence on pH with an apparent minimum near pH 5.5 and that k_d increases in a monotonic fashion with decreasing pH.

Assuming that the K_3 and K_4 protonation reactions are in rapid equilibrium compared to the slower STX-binding steps, the dependence of k_p on $[H^+]$ can be derived from the rate expression for association of [³H]STX to the combined unliganded states, Sax and H^+-Sax, to obtain

$$k_p = [^3\text{H-STX}]\left(\frac{k_1 + k_2[H^+]/K_3}{1 + [H^+]/K_3}\right) + \left(\frac{k_{-1} + k_{-2}[H^+]/K_4}{1 + [H^+]/K_4}\right) \quad (1)$$

Similarly, the dependence of k_d on $[H^+]$ as derived from the rate expression for the dissociation of [³H]STX from the combined states, Sax–STX and H^+-Sax–STX, is given by

$$k_d = \frac{k_{-1} + k_{-2}[H^+]/K_4}{1 + [H^+]/K_4} \quad (2)$$

Since the equilibrium concentration of [³H]STX bound has also been measured as a function of pH (Figure 4A), the scheme can be used to predict this relationship. The following equation derived from the scheme expresses the concentration of bound [³H]STX ([bound]) as a function of the total concentration of binding sites ($[\text{bound}]_{max}$), the free [³H]-STX concentration, and the equilibrium constants (K_3, K_4, and $K_1 = k_{-1}/k_1$):

$$[\text{bound}] = \frac{[\text{bound}]_{max}[^3\text{H-STX}]}{[^3\text{H-STX}] + K_1(1 + [H^+]/K_3)/(1 + [H^+]/K_4)} \quad (3)$$

For the purpose of fitting the data in Figure 4 to eqs 1–3, the equilibrium constant K_3 can be eliminated from eqs 1 and 3 by making use of the microscopic reversibility relation, $K_3 = K_1K_4/K_2$. Equations 1–3 thus describe the theoretical behavior of the data in Figure 4 on the basis of the above scheme and five independent kinetic parameters (k_1, k_{-1}, k_2, k_{-2}, and K_4). To obtain estimates for these parameters, the data in Figure 4A–C were simultaneously fit to eqs 1–3 using a nonlinear least-squares procedure. The resulting best-fit parameters are summarized in Table 1, and theoretical curves using these values are shown as solid lines through the data in Figure 4A–C. According to the scheme and the parameters of Table 1, the unprotonated form of saxiphilin has a high affinity for STX with an equilibrium constant of $K_1 = 0.12$ nM, whereas the protonated form has a low affinity of $K_2 = 102$ nM. The amino acid residue that determines the observed pH dependence is expected to have a pK_a (pK_3) of 7.22 in the STX-free form and 4.29 (pK_4) in the STX-bound form.

Effect of Temperature upon [³H]STX Binding. The temperature dependence of [³H]STX binding was investigated

Table 1: Binding Parameters for a Four-State Model of pH-Dependent Binding of STX to Saxiphilin[a]

parameter	value	units
k_{-1}	$1.3 \pm 1.0 \times 10^{-4}$	s^{-1}
k_1	$1.1 \pm 0.1 \times 10^{6}$	$s^{-1}\ M^{-1}$
k_{-2}	$1.1 \pm 0.9 \times 10^{-2}$	s^{-1}
k_2	$1.1 \pm 0.3 \times 10^{5}$	$s^{-1}\ M^{-1}$
K_1	1.2×10^{-10}	M
K_2	1.0×10^{-7}	M
K_3	6.0×10^{-8}	M
K_4	$5.1 \pm 0.6 \times 10^{-5}$	M

[a] Data in Figure 4A–C were simultaneously fit to eqs 1–3 derived from the scheme using k_{-1}, k_1, k_{-2}, k_2 and K_4 as independent variables. Error estimates correspond to standard errors obtained from the fit.

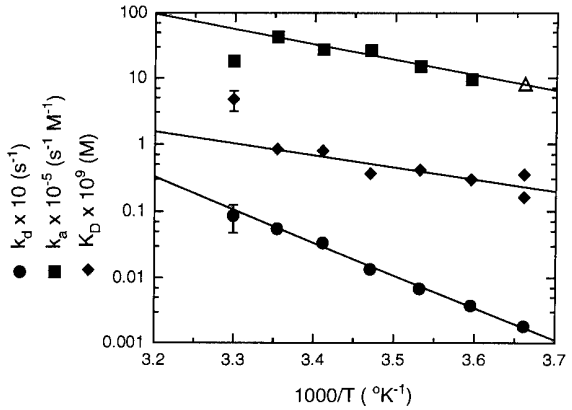


FIGURE 5: Arrhenius and van't Hoff plots of [^{3}H]STX binding to saxiphilin at pH 7.4. K_D values (◆) were determined from Scatchard analyses performed at 5 °C intervals between 0 and 30 °C. k_d (●) values were similarly determined from the dissociation time course at various temperatures. k_a values (■) were calculated from the measured k_d and K_D values according to $k_a = k_d/K_D$. One k_a value (△) at 0 °C was also measured experimentally from the association time course.

by measuring the dissociation rate constant, k_d, and the equilibrium K_D at 5° intervals over the temperature range of 0–30 °C at pH 7.4. The apparent bimolecular association rate constant at these temperatures was obtained from the relationship $k_a = k_d/K_D$. The results are presented as combined van't Hoff ($\ln K_D$ vs T^{-1}) and Arrhenius plots ($\ln k$ vs T^{-1}) in Figure 5. These plots are well-described by linear functions in the temperature range of 0–25 °C. At 30 °C, the measured K_D deviated from linear behavior, suggesting either a temperature-dependent transition or possibly thermal inactivation. Of the three parameters, k_d exhibited the highest temperature dependence with a 4.3-fold increase from 0 to 10 °C. For this same temperature increase, k_a and K_D increased by 1.7- and 2.2-fold, respectively. The solid line through the K_D values in Figure 5 is a fit to the equation $\ln K_D = \Delta H°/RT - \Delta S°/R$, using $\Delta H° = -8.3 \pm 2.0$ kcal mol^{-1} and $\Delta S° = 13.8 \pm 7.2$ cal mol^{-1} K^{-1}. The solid lines through the k_d and k_a values are fit to the equation $\ln k = \ln A - E_a/RT$ using $E_a = 22.5 \pm 1.0$ kcal mol^{-1} for k_d and 11.1 ± 2.1 kcal mol^{-1} for k_a. At 0 °C, $T\Delta S°$ is equal to 3.8 kcal mol^{-1}, which indicates that the enthalpic term predominants over the entropic term in the free energy change of binding ($\Delta G° = \Delta H° - T\Delta S°$). However, as noted by Dickinson et al. (1993), it is difficult to interpret such parameters mechanistically since absolute values of binding free energy and entropy depend on the choice of a reference state, normally 1 M concentration.

Inhibition of [^{3}H]STX Binding by Divalent Metal and Lanthanide Cations. By all indications, binding of [^{3}H]STX to saxiphilin is a chemically specific interaction. The only class of organic molecules found to competitively inhibit this binding reaction is STX and naturally occurring or synthetic derivatives of STX that differ by small chemical substituents (Mahar et al., 1991). In the course of the present experiments, we found that a variety of other molecules that structurally resemble STX, such as adenosine, 8-aminoguanine, 8-aminoguanosine, creatinine, folic acid, uric acid, and xanthine, do not affect [^{3}H]STX binding to saxiphilin when tested at concentrations in the range of 1–10 mM (L. Llewellyn, unpublished results). Such observations emphasize that the structural requirements for ligand binding to saxiphilin are stringent.

Since saxiphilin is homologous to transferrin, an important question is whether it can bind metal ions. Thus far, attempts to directly demonstrate transferrin-like binding of $^{55}Fe^{3+}$ have been unsuccessful (Li et al., 1993). To further examine this question, various divalent metal and lanthanide cations were tested for their ability to inhibit binding of [^{3}H]STX both in the presence and absence of $NaHCO_3$. As summarized in Figures 6 and 7, a variety of such metal ions display inhibition in the millimolar range. For example, Figure 6A shows that Zn^{2+} inhibits [^{3}H]STX binding with a $K_{0.5}$ of 67 mM. Such inhibition is not merely an effect of ionic strength since $MgCl_2$ tested up to 200 mM (Figure 6A) and NaCl tested up to 1 M (not shown) have no effect on the control level of [^{3}H]STX binding. Furthermore, the data of Figures 6 and 7 show that certain lanthanide cations such as Eu^{3+}, Tb^{3+}, and Nd^{3+} are more effective inhibitors of [^{3}H]STX binding than the tested transition metals (Co^{2+}, Ni^{2+}, and Zn^{2+}) with a $K_{0.5}$ of ~6 mM. This inhibition is not strictly dependent on added HCO_3^-, as noted by the similar $K_{0.5}$ values measured with or without 100 mM $NaHCO_3$ (Figure 7). However, several of the lanthanides such as Tb^{3+}, Pr^{3+}, and La^{3+} do show a 2–10-fold enhancement of affinity in the presence of HCO_3^-. Some of the metal ions exhibited Hill coefficients (n) greater than 1.0 in displacement titrations, suggesting the participation of more than one ion (e.g., $n = 1.5$ for $ZnCl_2$; Figure 6A). However, it is possible that such behavior may also be related to slow equilibration as described below for Pr^{3+}.

In the case of Pr^{3+}, an unusual phenomenon was observed. If saxiphilin is incubated for 1 h with [^{3}H]STX and increasing concentrations of Pr^{3+}, practically no binding inhibition is observed with up to 100 mM $PrCl_3$ in the absence of added HCO_3^- (Figure 6B). However, effective displacement by Pr^{3+} ($K_{0.5} = 6$ mM) is observed in the presence of 100 mM HCO_3^- (Figure 6A). When assayed after equilibration for 1 h, $NaHCO_3$ promotes the inhibition of [^{3}H]STX binding in the presence of 100 mM Pr^{3+} in a concentration-dependent fashion (Figure 6C). The unusually steep nature of the HCO_3^- titration curve (Figure 6C) suggested that the effect of HCO_3^- might reflect a synergistic effect on the rate at which Pr^{3+} is able to displace [^{3}H]STX rather than highly cooperative binding of HCO_3^-. Indeed, if Pr^{3+} is incubated for 6 h with saxiphilin and [^{3}H]STX in the absence of HCO_3^-, the titration curve for Pr^{3+} approaches that observed for a 1 h incubation with 100 mM HCO_3^- (Figure 6B). These results indicate that equilibration of Pr^{3+} with saxiphilin is slow in the absence of HCO_3^- and that HCO_3^- acts synergistically to enhance the rate of equilibration of the metal ion.

In the presence of 10 mM $PrCl_3$ and 100 mM $NaHCO_3$, Scatchard analysis revealed a low-affinity K_D of 4.6 nM for [^{3}H]STX binding without a change in B_{max}, indicative of a competitive interaction between the toxin and the lanthanide cation (data not shown). To further examine the mechanism of inhibition of [^{3}H]STX binding by lanthanide cations, the effect of 10 mM Pr^{3+} plus 100 mM $NaHCO_3$ on the time

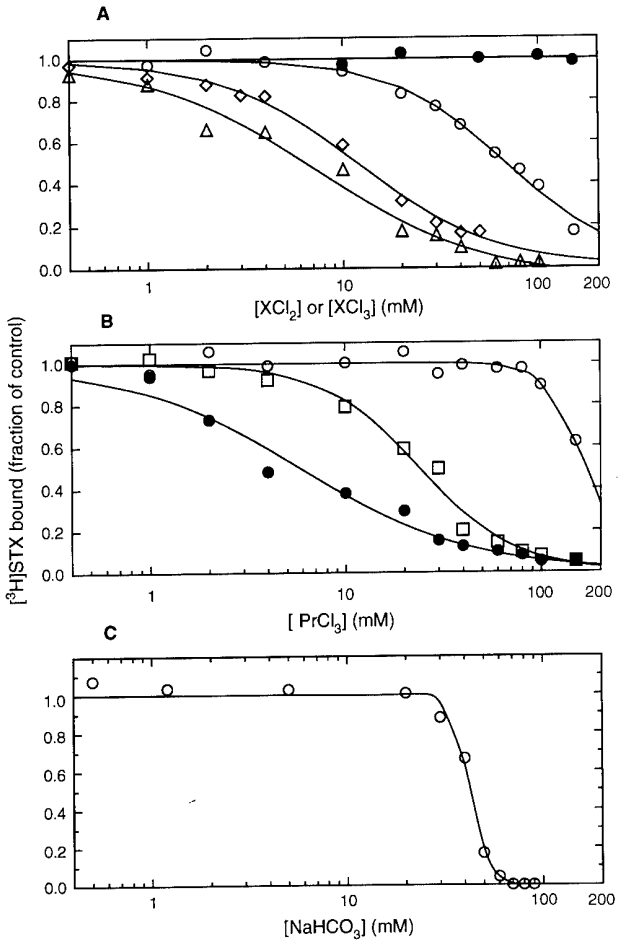


FIGURE 6: Inhibition of [^{3}H]STX binding to saxiphilin by various divalent and trivalent cations. (A) Effect of (△) $EuCl_3$, (◇) $TbCl_3$, (○) $ZnCl_2$, and (●) $MgCl_2$ on equilibrium binding of [^{3}H]STX. Saxiphilin (20 ng/mL, ~0.22 nM) was preincubated for 1 h in the presence of 4.8 nM [^{3}H]STX, 100 mM NaCl, 100 mM $NaHCO_3$, 100 mM Mops-NaOH (pH 7.45), and the indicated concentrations of di(tri)valent cations. [^{3}H]STX binding is normalized to the control value in the absence of metal ions. Except for $MgCl_2$, the solid lines are fit to the Hill equation, $y = K_{0.5}^n/([X]^n + K_{0.5}^n)$, using n = 1.10 ($EuCl_3$), 1.24 ($TbCl_3$), and 1.50 ($ZnCl_2$) and $K_{0.5}$ = 6.0 ± 0.7, 11.7 ± 0.6, and 66.6 ± 2.6 mM, respectively. (B) Effect of $PrCl_3$ measured after a 1 h incubation (○), effect of $PrCl_3$ measured after a 6 h incubation (□), and effect of $PrCl_3$ plus 100 mM $NaHCO_3$ measured after a 1 h incubation (●). For $PrCl_3$ plus $NaHCO_3$, the solid line fit to the Hill equation is drawn according to n = 1.0 and $K_{0.5}$ = 5.8 ± 0.7 mM. (C) Titration of $NaHCO_3$ in the presence of 100 mM $PrCl_3$ assayed after 1 h incubation.

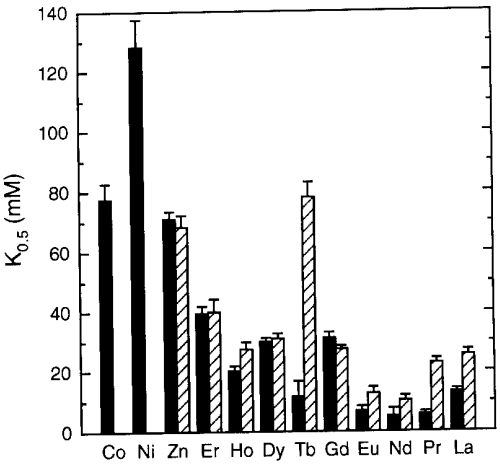


FIGURE 7: The concentration ($K_{0.5}$) of various divalent metal and lanthanide cations that produced 50% inhibition of [^{3}H]STX binding as determined in Figure 6. Solid bars correspond to results obtained in the presence of 100 mM $NaHCO_3$, and cross-hatched bars were obtained in the absence of $NaHCO_3$. Binding was measured after equilibration for 1 h except in the case of $PrCl_3$ which was equilibrated for 6 h. The effect of bicarbonate was not tested for Co^{2+} and Ni^{2+}.

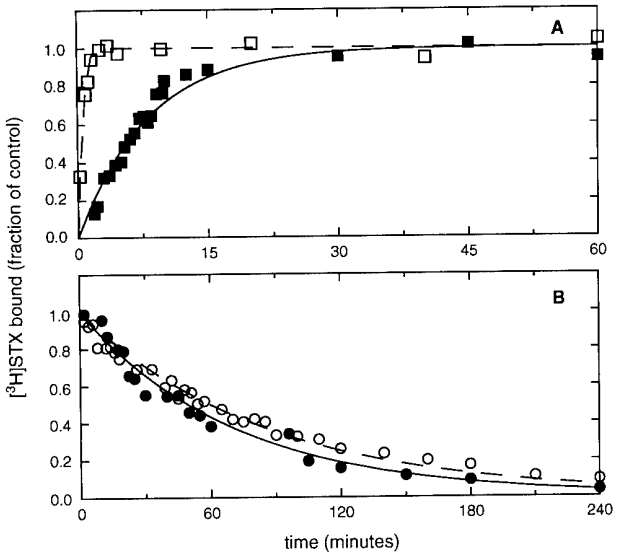


FIGURE 8: Effect of $PrCl_3$ plus $NaHCO_3$ on the kinetics of [^{3}H]STX binding to saxiphilin. The association time course (A) and the dissociation time course (B) of [^{3}H]STX was determined in the absence (open symbols) or presence (filled symbols) of 10 mM $PrCl_3$ plus 100 mM $NaHCO_3$. Other conditions: 19.2 nM [^{3}H]STX, 34 ng/mL saxiphilin (~0.38 nM), 100 mM NaCl, 100 mM Mops-NaOH (pH 7.4). Binding data are normalized to values at long times (A) or initial values (B). Dashed lines (control) and solid lines ($PrCl_3$ plus $NaHCO_3$) are fits to a single exponential using rate constants of (A) 13.4 × 10^5 (□) and 1 × 10^5 s^{-1} M^{-1} (■) and (B) 2.07 × 10^{-4} (○) and 2.7 × 10^{-4} s^{-1} (●). Some overlapping data points have been omitted to relieve crowding.

course of [^{3}H]STX association and dissociation was studied. The results of Figure 8 show that the reduced affinity for [^{3}H]STX in the presence of Pr^{3+}/HCO_3^- is due to a large decrease in the toxin association rate with virtually no effect on the toxin dissociation rate. Thus, inhibition of [^{3}H]STX binding by lanthanides is very different from that of H^+, which greatly accelerates the dissociation rate as well as slows the association of [^{3}H]STX. The results of Figure 8 are consistent with a model in which a low-affinity metal cation-binding site is formed by amino acid residues that directly participate in STX binding. If binding of a lanthanide ion and STX are mutually exclusive, Pr^{3+} would only affect the association rate of [^{3}H]STX and not the toxin dissociation rate, since the two ligands would never be simultaneously bound.

Inhibition of [^{3}H]STX Binding by TMO Modification. Trimethyloxonium is a protein modification reagent that methylates carboxyl groups of aspartate and glutamate residues in a rather specific manner (Parsons et al., 1969; Raber et al., 1979). As shown in Figure 9, treatment of saxiphilin for 10 min with increasing concentrations of TMO resulted in the complete inhibition of [^{3}H]STX binding. However, when the prebound complex of saxiphilin and [^{3}H]-STX was treated with TMO in the same fashion, virtually complete protection was observed (Figure 9). These results suggest that carboxyl groups of saxiphilin are involved in STX binding. It is likely that the observed inhibition of [^{3}H]STX binding by TMO results from the methylation of aspartate and/or glutamate residues of saxiphilin that participate in hydrogen bonds with the bound toxin molecule.

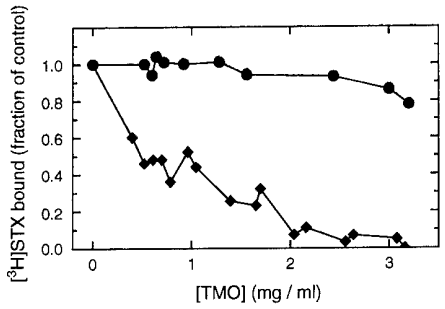


FIGURE 9: Effect of chemical modification by TMO on [^{3}H]STX binding to saxiphilin. Saxiphilin (91 ng/mL, ~1 nM) was pre-exposed to the indicated concentrations of TMO for 10 min in the absence (◆) or presence (●) of 19.2 nM [^{3}H]STX before assay of [^{3}H]STX binding. Other conditions: 100 mM NaCl, 100 mM Tris-HCl, pH 8.6. Binding data are normalized to the value in the absence of TMO (0.46 pmol of [^{3}H]STX bound).

DISCUSSION

This paper describes the functional characteristics of ligand binding to a newly recognized member of the transferrin family. Saxiphilin was the first protein to be identified that is homologous to transferrin (Morabito & Moczydlowski, 1994) but does not contain at least one fully intact Fe^{3+}/HCO_3^--binding site formed by the five highly conserved signature ligands of Asp63(392), Tyr95(426), Arg124(456), Tyr188-(517), and His249(585) as numbered according to the sequence of human transferrin (Yang et al., 1984) in the N(C)-lobes. As noted in Table 2, saxiphilin contains only one of these known Fe^{3+} ligands at position Asp60, which is equivalent to Asp63 of human transferrin. Two other transferrins, human melanotransferrin (Rose et al., 1986; Baker et al., 1992) and a transferrin from the tobacco hornworm (Bartfield & Law, 1990), have both lost one functional Fe^{3+} site in the C-lobe. However, it is not known whether the loss of Fe^{3+} binding in one lobe of these latter two proteins corresponds to a new function such as the binding of a different ligand. Recently, a 79 kDa monomeric protein purified from pig plasma has also been characterized as a novel member of the transferrin family that does not bind iron (Wuebbens et al., 1994). This latter protein was originally described as a specific inhibitor of the CAII isozyme of carbonic anhydrase (Roush & Fierke, 1992) and later found to be related to transferrin after purification and sequencing. The example of saxiphilin and the porcine inhibitor of carbonic anhydrase support the possibility that transferrins may comprise a diverse protein family. This leads to an important biochemical question that has only begun to be addressed—how many members of the transferrin family are there and what are their functions?

In the case of saxiphilin, STX may be potentially considered as a substitute ligand for Fe^{3+}; however, much less is known about the physiological function of saxiphilin in comparison to transferrin and the transferrin receptor cycle of Fe^{3+} metabolism (Crichton, 1991; Dautry-Varsat, 1986). Besides the lack of Fe^{3+}-binding residues, a unique function for saxiphilin is also suggested by the presence of a 144-residue insertion that has not been found in other members of the transferrin family (Morabito & Moczydlowski, 1994). Although saxiphilin and transferrin are clearly divergent members of a gene family, several of the functional characteristics of saxiphilin described in this paper are reminiscent of transferrin behavior. Most notable among these similarities is the pH dependence of [^{3}H]STX binding (Figure 4A) which occurs over a pH range that is about 0.5 pH unit more basic than that observed for the displacement of Fe^{3+} from human serum transferrin (Princiotto & Zapolski, 1975; Lestas, 1976; Mazurier & Spik, 1980; Baker et al., 1992). This finding supports the hypothesis that saxiphilin may also function in the internalization of a ligand by endocytosis, since a decrease in pH is a common physiological mechanism by which receptor-bound ligands are released in endosomes (Dautry-Varsat, 1986).

The 1:1 stoichiometry and the monoexponential kinetics of the [^{3}H]STX-binding reaction indicate that saxiphilin has only one STX-binding site in comparison to the two Fe^{3+}-binding sites of most known transferrins. [^{3}H]STX binding to saxiphilin also readily occurs without the addition of a cofactor such as HCO_3^- which is required for physiological binding of Fe^{3+} to transferrins (Schlabach & Bates, 1975; Harris & Aisen, 1989). The study of the kinetics of Fe^{3+} exchange to or from transferrin proteins generally requires a synergistic anion (e.g., HCO_3^- and nitrilotriacetic acid) and a chelator or Fe^{3+} acceptor (e.g., EDTA and desferrioxamine) to observe Fe^{3+} release. The one-site system and the absence of a cofactor greatly simplify quantitative analysis of the pH dependence of [^{3}H]STX binding to saxiphilin in comparison to transferrins.

Considering the difference between a one-site and two-site system, the equilibrium pH dependence of [^{3}H]STX binding to saxiphilin (Figure 4A) most closely resembles the experimentally resolved pH dependence of Fe^{3+} binding to the N-terminal lobe of human serum transferrin (Lestas, 1976). The pH dependence of Fe^{3+} binding to native diferric–transferrin titrates in the range of pH 6–4, but displays a characteristically biphasic titration curve that is known to reflect slightly higher affinity of Fe^{3+} binding to the "acid-stable" C-lobe as compared to the "acid-labile" N-lobe (Aisen et al., 1978). In contrast to serum transferrin and saxiphilin, the pH dependence of Fe^{3+} binding to human lactoferrin occurs over a pH range that is approximately 2 pH units more acidic (Mazurier & Spik, 1980; Baker et al., 1992; Day et al., 1992). The greater acid stability of Fe^{3+} binding to lactoferrin has been proposed to reflect a primary function of maintaining a low concentration of free Fe^{3+} in secretions such as milk to limit the growth of microorganisms rather than a role in Fe^{3+} delivery to cells (Dewan et al., 1993). Studies of the pH dependence of Fe^{3+} dissociation from transferrin have previously suggested that protonation of a functional group with a pK_a of ~7 serves as a triggering mechanism in the release of Fe^{3+} (Chasteen & Williams, 1981; Thompson et al., 1986). In the case of lactoferrin, protein–protein interactions between the N-lobe and the C-lobe are also important in stabilizing the release of Fe^{3+} to pH (Legrand et al., 1990; Day et al., 1992).

Chemical modification studies of serum transferrin have shown that ethoxyformylation of histidine residues with diethylpyrocarbonate markedly stabilizes serum transferrin to the pH-dependent dissociation of Fe^{3+} (Thompson et al., 1986). Comparison of various transferrin sequences led Thompson et al. (1986) to implicate the homologous residues His207 in the N-lobe and His535 in the C-lobe of human transferrin as the histidine residues that trigger the release of Fe^{3+} from each respective lobe upon protonation. These residues are generally conserved in both lobes of several serum transferrins but are replaced by Glu in the N-lobe and Asp in the C-lobe of the more acid-stable human lactoferrin (see Table 2). Noting that these residues are located near the Fe^{3+}-binding site and the hinge region involved in domain closure, Anderson et al. (1989) also suggested that they may play a role in pH dependence. Melanotransferrin, which

Table 2: Sequence Comparison Adjacent to Residues That Bind Fe^{3+} and HCO_3^- in Transferrins for Human Serum Transferrin (Stf),[a] Human Lactoferrin (Ltf),[b] and Saxiphilin (Sax)[c]

N-lobe[d]	Asp63	Tyr95	Arg124	Tyr188	His207	His249
	*	*	! ! !!	*	+	*
Stf	DAVTLDAG	TFYYAVA	HTGLGRSAG	YFGYSGAF	DVAFVKHSTI	YPSHTVVA
Ltf	DAVTLDGG	THYTAVA	HTGLRRTAG	YFSYSGAF	DVAFIRESTV	VPSHAVVA
Sax	DAMFLDSG	TCHYTVA	HSGVSKTDG	YYGNYGAF	DVAFLRSTAL	VPAGTVVT
C-lobe[d]	Asp392	Tyr426	Arg456	Tyr517	His535	His585
	*	*	! ! !!	*	+	*
Stf	DAMSLDGG	AGYFAVA	HTAVGRTAG	YYGYTGAF	DVAFVKHQTV	APNHAVVT
Ltf	DAMSLDGG	EGYLAVA	HTAVDRTAG	YYGYTGAF	DVAFVKDVTV	APNHAVVS
Sax	DAVKLEVQ	GTLRAVA	HTGVGDIAG	YYGNQGAF	DVAFVPHTVV	IPPPAIVT

[a] Human transferrin sequence (Yang et al., 1984). [b] Human lactoferrin sequence (Rey et al., 1990). [c] Saxiphilin sequence (Morabito & Moczydlowski, 1994). [d] Functionally significant residues are numbered from the amino terminus of human transferrin. * = Residues that directly coordinate Fe^{3+} in human lactoferrin (Anderson et al., 1989) and rabbit transferrin (Bailey et al., 1988). ! = Residues that hydrogen bond with HCO_3^- in lactoferrin (Anderson et al., 1989). + = Histidine residues proposed to mediate the pH dependence of human transferrin (Thompson et al., 1986).

contains only one functional Fe^{3+}-binding site in its N-lobe, exhibits a transferrin-like pH dependence in the range of pH 6–5 (Baker et al., 1992) and also has a conserved His residue homologous to His207 of transferrin. Table 2 shows that saxiphilin has a Ser residue (N-lobe) and a His residue (C-lobe) in this position. The pH-dependent kinetics of [^{3}H]-STX binding to saxiphilin (Figure 4) are consistent with a pK_a of 7.22 for the residue that mediates inhibition in the toxin-free state. This pK_a value is typical of the histidine imidazole group. If the hypothesis of Thompson et al. (1986) is correct, then the corresponding His679 residue in the C-lobe of saxiphilin may be the functional group responsible for the distinctive transferrin-like pH dependence of saxiphilin. Dewan et al. (1993) have also identified a unique interaction between two highly conserved lysine residues in the crystal structure of the N-lobe of chicken transferrin that is proposed to act as a pH-sensitive triggering mechanism for opening of the binding cleft and Fe^{3+} release. Saxiphlin is one of the few transferrins that lack these two lysine residues and also an analogous Lys–Asp–Arg interaction in the C-lobe. These particular sequence differences may also be important in determining the pH dependence of the different members of the transferrin family.

According to our analysis of the data in Figure 4, the pH dependence of STX binding to saxiphilin may be regarded as an example of an antagonistic ligand interaction (Weber, 1975) between the binding of STX and H^+ at distinct sites on saxiphilin. Using the parameters of Table 1, the coupling free energy between STX and H^+ for this interaction ($\Delta\Delta G^\circ = RT \ln(K_2/K_1) = RT \ln(K_4/K_3)$) is calculated to be 3.9 kcal/mol. This is one of the largest values of coupling free energy reported for experimentally accessible ligand-binding interactions of soluble proteins, which generally fall in the range of ±2 kcal/mol (Cantor & Shimmel, 1980). Since Fe^{3+}-binding transferrins are known to undergo large conformational changes identified as open and closed forms of the two domains that form the Fe^{3+}-binding site (Anderson et al., 1990; Grossmann et al., 1992), it is conceivable that this large coupling energy reflects a similar conformational change of saxiphilin. In this case, the reversible protonation of free saxiphilin (K_3 reaction in the above-mentioned scheme) might actually represent a more complex equilibrium of closed and open conformations of the STX-binding site, which exist in both protonated and unprotonated forms.

The enthalpy change ($\Delta H^\circ = -8.3$ kcal mol^{-1}) measured from the temperature dependence of the K_D of [^{3}H]STX binding (Figure 5) indicates that the forward binding reaction of the toxin to saxiphilin is exothermic. Direct calorimetric studies of the binding of the Fe–NTA complex to chicken transferrin in the presence of bicarbonate have shown that ligand binding to transferrin is a complex process that occurs in two distinct stages (Lin et al., 1991). The first rapid stage is an exothermic contact binding of the Fe–NTA complex analogous to that inferred here for STX binding to saxiphilin, and the second stage reflects more complex thermodynamic behavior of the slower exchange of NTA and bicarbonate. With respect to activation energy, the temperature dependence of the first-order dissociation rate constants for Fe^{3+} removal from each lobe of human transferrin is very similar to that measured here for dissociation of STX from saxiphilin. Kretchmar and Raymond (1986) found that dissociation of Fe^{3+} from the C-lobe has an activation energy of 20 kcal mol^{-1}, nearly identical to the 22.5 kcal mol^{-1} value found for saxiphilin. The temperature dependence of Fe release from the N-lobe of transferrin is more complex and exhibits a discontinuity with a value of 21 kcal mol^{-1} below 12 °C and 15 kcal mol^{-1} above 20 °C (Kretchmar & Raymond, 1986). Comparison of the present data with the temperature dependence of [^{3}H]STX binding to the guanidinium toxin receptor site of sodium channels also shows very similar behavior and absolute values of binding enthalpy and activation energies for dissociation and association. For example, binding of [^{3}H]STX to sodium channels from rat brain synaptosomes is characterized by $\Delta H^\circ = -6.0$ kcal mol^{-1}, $E_a = 18.8$ kcal mol^{-1} for k_d, and $E_a = 10.9$ kcal mol^{-1} for k_a, as calculated from the data of Weigele and Barchi (1978). In this regard, it is interesting that two structurally distinct proteins exhibit similar absolute binding affinity and binding thermodynamics

for the same neurotoxin. It remains to be seen whether this coincidence reflects similar molecular interactions in the mechanism of binding.

The low affinity for various metal ions detected by inhibition of [^{3}H]STX binding (Figure 7) is quite distinct from the behavior of transferrins, which have been found to tightly bind a variety of transition metals and lanthanides (Harris & Aisen, 1989). If we hypothesize that the evolutionary process that led to saxiphilin involved the transformation of an Fe^{3+}-binding site of an ancestral transferrin into a binding site for STX, then inhibition by metal ions may reflect the remaining vestige of a formerly functional Fe^{3+} site. The competitive kinetics of [^{3}H]STX binding to saxiphilin observed in the presence of Pr^{3+} (Figure 8) is consistent with an overlapping location of a low-affinity site for Pr^{3+} and a high-affinity site for STX. The curious effect of HCO_3^- on the equilibration of Pr^{3+} with saxiphilin (Figure 6B) is also vaguely reminiscent of the synergistic effect of HCO_3^- and other anions in promoting the binding of metal ions to transferrin. The phenomenon of anion-assisted binding of metal ions is one of the unique characteristics of transferrins. It is possible that this phenomenon also reflects the evolutionary relationship of saxiphilin and transferrin. The observations on metal ion inhibition and the evidence from TMO modification that carboxylate groups form part of the STX-binding site (Figure 9) together suggest that one or several glutamate or aspartate residues form hydrogen bonds with STX and alternatively serve as ligands to form a weak metal ion-binding site in the absence of STX. An analogy to the sodium channel binding site for STX can also be drawn for this finding, since TMO modification is known to abolish guanidinium toxin binding to sodium channels (Reed & Raftery, 1976; Doyle et al., 1993). STX binding in sodium channels is also known to behave competitively with respect to monovalent alkali cations and various divalent metal ions (Schild & Moczydlowski, 1991; Doyle et al., 1993).

Crystallographic analysis of lactoferrin has identified a large internal cavity adjacent to the Fe^{3+}-binding site with an approximate diameter of 10 Å (Anderson et al., 1989). Anderson et al. (1989) proposed that this cavity allows for the binding of larger organic anions that are known to substitute for HCO_3^- in transferrin. In principle, a cavity of this size would be large enough to accomodate STX (approximate size, 8.5 Å × 4.4 Å × 6.9 Å) and may be the analogous location of the toxin-binding site in saxiphilin. On the basis of the available data and homology to transferrin, we propose that the C-lobe cavity of saxiphilin is the most probable location of the STX-binding site. The N-lobe is an unlikely candidate because the 144-residue insertion unique to saxiphilin in this lobe occurs directly in the hinge region shown to mediate the opening and closing reaction of the Fe^{3+}-binding site in lactoferrin (Anderson et al., 1989, 1990). A large insertion at this location would be expected to perturb the conformational dynamics of the N1 and N2 subdomains necessary for ligand binding within the interdomain cleft. As noted above, the presence of the conserved His679 residue in the C-lobe of saxiphilin (Table 2) provides a plausible candidate residue for the pH dependence of [^{3}H]STX binding. In considering the various substitutions of Fe^{3+}-site residues (Table 2), the C-lobe of saxiphilin has an Asp residue in place of the Arg456 residue of transferrin that hydrogen bonds with HCO_3^- (Anderson et al., 1989). Saxiphilin also has a conservative substitution of Glu for Asp392 in the C-lobe of transferrin that coordinates directly with Fe^{3+}. The presence of these latter carboxyl ligands in the binding site for STX could explain the sensitivity of [^{3}H]STX binding to modification by TMO (Figure 9) and may also account for the ability of lanthanide cations to bind weakly and competitively displace [^{3}H]STX (Figure 7). With respect to the lanthanides, it is interesting that only the C-lobe of transferrin is capable of binding Nd^{3+} and Pr^{3+} (Luk 1971; Harris et al, 1981). Such clues lead us to suspect that the C-lobe of saxiphilin contains the STX-binding site. This hypothesis can now be tested by site-directed mutagenesis of recombinant saxiphilin which is currently in progress.

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Expression of Saxiphilin in Insect Cells and Localization of the Saxitoxin-Binding Site to the C-Terminal Domain Homologous to the C-Lobe of Transferrins†

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ABSTRACT: Saxiphilin is a plasma protein from the bullfrog (*Rana catesbeiana*) that is homologous to transferrin. Most known transferrins contain two binding sites for Fe^{3+}/HCO_3^-, one in each of two homologous domains called the N-lobe and C-lobe. However, native saxiphilin does not bind Fe^{3+} but stoichiometrically binds one molecule of the neurotoxin saxitoxin (STX) with a dissociation constant (K_D) of ~0.2 nM. To pursue structural analysis of the STX binding site, cDNA encoding saxiphilin was used to construct a baculovirus expression vector that directs synthesis and secretion of a ~92-kDa recombinant saxiphilin protein (R-sax) in cultured insect cells. Culture medium harvested from infected cells contained 25−67 pmol of [^{3}H]STX binding sites/mL with a K_D of 0.22 nM. The kinetics and pH dependence ($pK_{0.5}$ = 5.4) of [^{3}H]STX binding to R-sax are similar to native saxiphilin, implying proper folding and functional activity. Another baculovirus expression vector was constructed to encode a deletion mutant of saxiphilin consisting of the first 20 N-terminal residues containing the secretory signal sequence spliced to the C-terminal, 361-residue fragment homologous to the C-lobe domain of transferrins. This vector directed the secretion of a ~38-kDa derivative of saxiphilin (C-sax) that was recognized by anti-saxiphilin antibody. C-sax also exhibited [^{3}H]STX binding activity with a lower affinity K_D of ~0.9 nM, a 4-fold faster dissociation rate for [^{3}H]STX than native saxiphilin, and a pH dependence ($pK_{0.5}$ = 5.7) similar to R-sax ($pK_{0.5}$ = 5.4). These results establish that the binding site for STX and residues that determine the pH dependence of toxin binding are located within the C-lobe domain of saxiphilin.

Saxiphilin is a soluble protein found in plasma and tissues of various animals that is characterized by high affinity for saxitoxin (STX),[1] a tricyclic neurotoxin that blocks voltage-sensitive sodium channels (Doyle et al., 1982; Mahar et al., 1991). Purification and partial sequencing of the native protein (Li & Moczydlowski, 1991) led to the isolation of cDNA encoding saxiphilin from the bullfrog (*Rana catesbeiana*) (Morabito & Moczydlowski, 1994, 1995). Surprisingly, the primary sequence of saxiphilin was found to be homologous to transferrins, a family of high-affinity (K_D ~ 10^{-20} M) Fe^{3+}-binding proteins [reviewed in Crichton (1991) and Welch (1992)]. For example, the amino acid sequence of saxiphilin is 51% identical to serum transferrin of *Xenopus laevis* (African clawed frog) and 42% identical to human lactoferrin. However, saxiphilin differs from the transferrin family by substitutions in 9 of 10 highly conserved residues directly involved in binding of Fe^{3+} and an anion cofactor (HCO_3^-) in the two metal-binding sites of transferrins (Anderson et al., 1989; Bailey et al., 1988). These latter differences account for the inability of saxiphilin to bind Fe^{3+} (Li et al., 1993). Bullfrog saxiphilin is also distinguished by a unique insertion of 143 residues composed of a tandem duplication which contains two copies of a recognized protein module known as a type 1 thyroglobulin domain (Malthiery & Lissitzky, 1987; Morabito & Moczydlowski, 1994, 1995).

In humans, Fe^{3+}-binding members of the transferrin family include serum transferrin, melanotransferrin, and lactoferrin. Serum transferrin is the major iron carrier and transport protein in vertebrates and is an important determinant of cell growth. It is internalized by binding to a cell surface receptor followed by endocytosis, acidification of the endosome compartment, release of Fe^{3+}, and recycling of apotransferrin to the cell surface (Dautry-Varsat, 1986; Thorstensen & Romslo, 1990). Melanotransferrin is abundantly expressed on the surface of melanoma cells (Rose et al., 1986) and contains only one functional Fe^{3+}-binding site in its N-terminal domain (Baker et al., 1992). Melanotransferrin does not appear to mediate uptake of Fe^{3+} (Richardson & Baker, 1992). Its exact function is unknown. Lactoferrin is present in milk and other secretions and is also found within secondary granules of neutrophils (Bullen, 1987). Along with transferrin, lactoferrin inhibits microbial infections by limiting the availability of free iron (Griffiths & Bullen, 1987). These two proteins are also considered to provide an important protection against the potential toxicity of free Fe^{3+}/Fe^{2+} ions, which mediate the production of hydroxyl free radical via the Fenton reaction (Crichton, 1991). Lactoferrin appears to possess a variety of other regulatory

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; pfu, plaque-forming units; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; STX, saxitoxin; Tris, tris(hydroxymethyl)aminomethane.

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activities that include modulation of the immune system. Recently, lactoferrin has been documented to bind to specific DNA sequences and activate the transcription of a reporter gene (He & Furmanski, 1995).

The structural homology between saxiphilin and transferrins leads to the question of whether any of the known or proposed paradigms of transferrin function apply to saxiphilin. For example, does saxiphilin function as a toxin defense mechanism, growth factor, intracellular ligand-delivery vehicle, transcription factor, etc.? To address these questions, it is necessary to establish methods for production of the recombinant protein. This would provide a readily available source of saxiphilin for structural analysis, investigation of its cellular physiology, and possible application in detection assays for STX. For this purpose, we have constructed a baculovirus expression vector that can be used for the production of recombinant saxiphilin by cultured insect cells. In this paper, we describe the [^{3}H]STX binding activity of the recombinant protein. In order to define the location of the STX binding site, this system was used to express a deletion fragment of saxiphilin consisting of only the C-terminal domain homologous to the C-lobe of transferrins. This truncated protein binds [^{3}H]STX in a manner similar to that of the whole protein, demonstrating that the C-terminal half of the molecule contains the toxin binding site. A preliminary report of this work has been published in abstract form (Morabito et al., 1995).

MATERIALS AND METHODS

Materials. High Five insect cells, Sf9 insect cells, the pBlueBac III baculovirus transfer vector, and the pCR II vector were purchased from Invitrogen (San Diego, CA). Grace's insect cell medium, Sf-900 serum-free insect cell medium, heat-inactivated fetal bovine serum, and 4% agarose gel with Bluo Gal were from GibcoBRL (Grand Island, NY). The BaculoGold insect cell transfection kit was obtained from Pharmingen (San Diego, CA). [^{3}H]STX and the ECL Western blot detection kit were purchased from Amersham (Arlington Heights, IL). *Taq* polymerase (Gene Amp) was from Cetus (Norwalk, CT). Plasmid constructs were propagated in *Escherichia coli* strains DH5α (GibcoBRL) or SURE (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by the Yale Protein and Nucleic Acid Chemistry Facility.

Sequence Information. In the course of this study, a few sequencing errors were discovered in the originally reported cDNA sequence of saxiphilin (Morabito & Moczydlowski, 1994). In the revised translation, the secreted form of saxiphilin is 825 residues in length instead of 826 residues as reported previously. Residue numbers of saxiphilin given here refer to the corrected sequence summarized in Morabito and Moczydlowski (1995) and updated in the Genbank data base (Accession Number U05246).

Baculovirus Expression Vector for Recombinant Saxiphilin. Excision of a pBluescript SK− plasmid containing saxiphilin cDNA was accomplished by coinfection of *E. coli* with Lambda ZAP II phage containing the previously isolated clone (Morabito & Moczydlowski, 1994) and helper phage according to the Strategene protocol. The pBluescript SK−/saxiphilin plasmid was digested with *Pvu*I, which does not cut the saxiphilin insert. This linearized DNA was used as a template to amplify saxiphilin cDNA by PCR using a T3 sense primer (5′-ATTAACCCTCACTAAAG-3′) and an antisense primer, anti-XP (5′-CTGCAGTCTAGAGAAGATCAACGTGCCA-3′), that was designed to match a 16-nucleotide sequence in the 3′ untranslated region of saxiphilin cDNA linked to restriction sites for *Xba*I and *Pst*I. The PCR contained ~0.1 μg of template DNA, 1 μM 5′ and 3′ primers and was carried out for 30 cycles (1 min at 94 °C, 2 min at 50 °C, and 2.5 min at 72 °C). The ~2.7-kb PCR product was digested with *Pst*I and subcloned into the *Pst*I-cut pBlueBac III transfer vector and also back into *Pst*I-cut pBluescript SK−. The latter saxiphilin cDNA in pBluescript SK− was completely sequenced by the method of Sanger et al. (1977) to determine whether any errors were introduced by PCR. Insect Sf9 cells were coinfected with the pBlueBac III/saxiphilin vector and modified linear baculovirus DNA according to directions of the Pharmingen BaculoGold transfection kit to generate infective recombinant baculovirus containing saxiphilin cDNA inserted downstream of the polyhedrin promoter. A single virus plaque was isolated and propagated by conventional methods (Summers & Smith, 1987; O'Reilly et al., 1994).

Baculovirus Expression Vector for the C-Lobe of Saxiphilin. The *Pvu*I-cut pBluescript SK−/saxiphilin plasmid was first used as a template to amplify saxiphilin cDNA coding for residues 466−825 by PCR. This reaction used a sense primer (5′-CATCTTCCATCCAAAAATAAAGTGCGG-3′) corresponding to the cDNA sequence of saxiphilin residues 466−474 and a T7 antisense primer (5′-AATACGACTCACTATAG-3′). Using the product of this reaction as a template, two consecutive rounds of PCR (1 min at 94 °C, 2 min at 42 °C, and 2 min at 72 °C, 30 cycles) were then performed to add the N-terminal secretory signal sequence of native saxiphilin. The first round of PCR used a sense primer, 5′-CTTGAGCTTTGCGGCAGCACATCTTCCATCC-3′, and the anti-XP antisense primer described above. The second round of PCR used the sense primer 5′-CGATCTGCAGATGGCTCCGACTTTCCAAACAGCTCTGTTTTTCACCATCATTAGCTTGAGCTTTGCGGC-3′ and the anti-XP antisense primer. The resulting product consisted of cDNA coding for amino acid residues −19 to 1 linked to 465−825 of native saxiphilin that is flanked by restriction sites for *Pst*I. The final PCR product was subcloned into the TA cloning site of pCR II and completely sequenced. This confirmed an open reading frame with one nonsilent, PCR-generated mutation (corresponding to mutation of Ser616 in native saxiphilin to Pro). The final cDNA coding for saxiphilic C-lobe was excised from pCR II with *Pst*I and inserted into the *Pst*I cloning site of the pBlueBac III vector. This construct was used to generate an infectious recombinant baculovirus as described above for recombinant saxiphilin.

Insect Cell Culture and Production of Recombinant Saxiphilin. For routine production of baculovirus, insect Sf9 cells (derived from *Spodoptera frugiperda* ovarian cells) were grown as adherent cells in Grace's medium supplemented with 10% fetal bovine serum. To isolate recombinant virus, agarose plugs from single plaques were resuspended in 1 mL of FBS-supplemented Grace's medium and used to infect ~2 × 10^6 Sf9 cells. Virus-containing medium was titered by plaque assay (Summers & Smith, 1987) using 4% agarose gel with Bluo Gal to reveal blue-colored plaques of recombinant virus. Recombinant virus stock containing ~5 × 10^7 to 5 × 10^8 pfu/mL was produced by amplification in

adherent cultures of Sf9 cells. For small-scale production and characterization of secreted recombinant saxiphilin, adherent cultures of ~5 × 10^6 insect High Five cells (derived from *Trichoplusia ni* ovarian cells) were grown in Sf-900 serum-free medium and infected with virus stock at a multiplicity of ~5 pfu/cell. The cell culture medium was collected 3 days after infection, supplemented with protease inhibitors (1 μM leupeptin, 1 μM pepstatin, and 5 mM EDTA), and clarified of cell debris and virus by microcentrifugation (5 min at 16000*g*). The resulting supernatant was directly analyzed by SDS−PAGE, immunoblot assay, and [^{3}H]STX binding assays. Large-scale production of recombinant saxiphilin was performed by the National Cell Culture Center (Minneapolis, MN). One-liter suspension cultures of High Five cells were grown to a density of ~1 × 10^6 cells/mL in serum-free HyQ CCM3 medium (Hyclone) and infected with recombinant baculovirus at a multiplicity of 2−5 pfu/cell. Three days after infection, the cell culture medium was harvested, centrifuged, supplemented with inhibitors (0.01% phenylmethanesulfonyl fluoride, 3 mM NaN_3, and 5 mM EDTA), and shipped on ice to our laboratory.

SDS−PAGE and Immunoblot Analysis. Samples (15 μL) of serum-free cell culture medium were subjected to SDS−PAGE (Laemmli, 1970) using a 10% polyacrylamide gel. The slab gel was electroblotted onto a nitrocellulose membrane (0.2 μm) using a Sartoblot IIS semidry electroblotting apparatus (Sartorius). The membrane was preincubated for 10 min with Blotto (5% nonfat dry milk, 20 mM Tris-HCl, pH 7.3, 150 mM NaCl, and 0.1% Tween 20) and then incubated for 1 h at 22 °C with 1:100 dilution (in Blotto) of affinity-purified rabbit antibody against native saxiphilin (Li & Moczydlowski, 1993). Following three consecutive 5-min washes in Blotto, the membrane was incubated with 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody for 1 h at 22 °C. After two consecutive 10-min washes in Blotto, followed by two consecutive washes in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$, immunoreactive protein complexes were visualized by chemiluminescence detection according to instructions of the ECL Western blotting kit (Amersham).

[^{3}H]STX Binding Assays. Measurements of equilibrium [^{3}H]STX binding, Scatchard analysis, association and dissociation kinetics, and assay of the pH dependence of [^{3}H]-STX binding to recombinant saxiphilin were performed essentially as previously described for native saxiphilin (Llewellyn & Maczydlowski, 1994). Bound [^{3}H]STX was separated from free toxin by rapid passage of 100-μL aliquots over minicolumns of AG50W-X2 resin (100−200 mesh, Tris+ form) that were preequilibrated with 100 mM Tris-HCl, pH 7.4, and 10 mg/mL bovine serum albumin. The standard incubation buffer for the binding assay was 20 mM Mops-NaOH, pH 7.4, and 200 mM NaCl. In the binding experiments of Figures 2, 3, 5, and 6, the assay mixture contained either 5 μL (R-sax) or 8 μL (C-sax) of undiluted cell culture supernatant containing the respective forms of recombinant saxiphilin in 250 μL of assay volume. The pH titration of Figure 6 was performed using a buffer containing 200 mM NaCl and 20 mM Tris/10 mM Mes/10 mM acetic acid adjusted with tetramethylammonium hydroxide or HCl as designed to maintain a relatively constant ionic strength in the range of pH 4−9 (Ellis & Morrison, 1982). Data were corrected for nonspecific [^{3}H]STX binding by subtraction of blank assays carried out in the presence of 10 μM nonradioactive STX. All [^{3}H]STX binding assays were performed at 0 °C by sample incubation on ice. The working specific activity of [^{3}H]STX used in these experiments was 37 700 cpm/pmol.

RESULTS

Predicted Domain Structure of Saxiphilin. The primary sequence of saxiphilin was aligned with that of human lactoferrin using the GAP program (gap weight 3.0, length weight 0.1) of the GCG Wisconsin sequence analysis package (version 8). As described previously (Morabito & Moczydlowski, 1994), this alignment is characterized by homology throughout the two sequences (42% identity, 63% similarity) except for an insertion of 143 residues in saxiphilin. The alignment was used to predict structural domains of saxiphilin (Figure 1) corresponding to those of lactoferrin previously identified in an X-ray crystal structure (Anderson et al., 1989). The residue numbering in Figure 1 reflects recent corrections to the saxiphilin sequence (Morabito & Moczydlowski, 1995). Saxiphilin is composed of an N-terminal secretory signal peptide (residues −19 to −1) followed by 825 residues of the mature protein. Saxiphilin can be subdivided into an N-lobe domain (residues 1−461) and a C-lobe domain (residues 473−825), which coincides with an internal duplication (~39% identity) that is found in all members of the transferrin family. These two lobes correspond to distinct globular domains with a similar fold in the lactoferrin crystal structure (Anderson et al., 1989). The presumed N- and C-lobe domains are linked by a short connecting peptide (residues 462−472 in saxiphilin). In lactoferrin, this connecting peptide is an α-helical segment that connects the N- and C-lobes. From the known pattern of disulfide bonding in transferrins, saxiphilin is expected to contain at least six conserved disulfide linkages in the N-lobe and eight in the C-lobe that are numbered a−f and a′−h′, respectively, in Figure 1 at the position of corresponding cysteine residues. Structural analysis of lactoferrin has also identified two globular subdomains in each lobe, known as N1/N2 and C1/C2, that appose to form a cleft where each of the two Fe^{3+}/HCO_3^- binding sites is located. The presumed linear sequence map of these subdomains in saxiphilin is also identified in Figure 1 on the basis of their homologous location in lactoferrin. In addition, saxiphilin contains a unique 143-residue insertion that occurs at a position between subdomains N1 and N2, which forms a hinge region in lactoferrin. This saxiphilin insertion is itself an internal duplication, containing two homologous modules (labeled Thyr-1A and Thyr-1B in Figure 1) that each contain a type 1 repetitive element of thyroglobulin (Mathiery & Lissitzky, 1987).

Previous work showed that equilibrium binding of [^{3}H]-STX to saxiphilin exhibits a pH dependence similar to that of Fe^{3+} binding to transferrins (Llewellyn & Moczydlowski, 1994). This apparently conserved aspect of the ligand-binding mechanism suggested that STX may bind in a cleft between subdomains N1/N2 or C1/C2 in an aqueous cavity analogous to the location of one of the two Fe^{3+}/HCO_3^- sites in transferrins. Since the opening and closing of this cleft in lactoferrin is mediated by the hinge region (Gerstein et al., 1993), the obstructing presence of the large 143-residue insertion at this location in the N-lobe of saxiphilin suggested that the C-lobe of saxiphilin is the most likely candidate for the STX binding site. Alternatively, it might be supposed

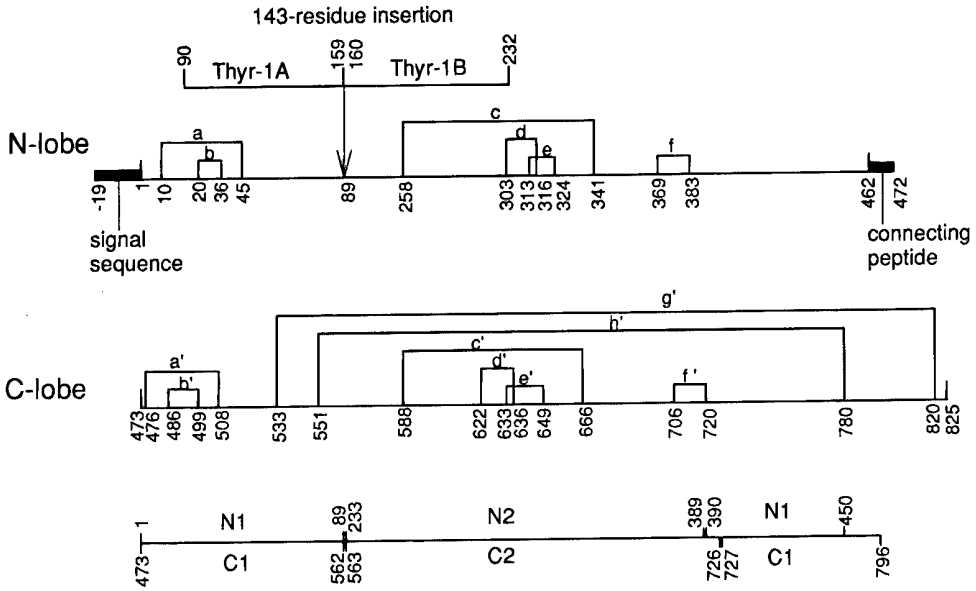

FIGURE 1: Diagram of the linear sequence of bullfrog saxiphilin showing the location of conserved disulfide bonds and N- and C-lobe structural domains predicted on the basis of sequence alignment with human lactoferrin. Numbers refer to the saxiphilin amino acid sequence (Morabito & Moczydlowski, 1994, 1995) and residues pairs a−f and a′−h′ indicate predicted disulfide bonds. Saxiphilin residues 90−232 containing two type 1 thyroglobulin domains (Thyr-1A and 1B) are shown as an insertion in the N-lobe. The bottom line shows the relative location of saxiphilin sequences corresponding to predicted N1/N2 and C1/C2 subdomains of the N-lobe and C-lobe, respectively.

that the 143-residue insertion in the N-lobe defines the STX binding site. To test these hypotheses, we engineered expression vectors for production of whole recombinant saxiphilin and a truncated form of saxiphilin corresponding to the presumed C-lobe domain in Figure 1.

Construction of Baculovirus Expression Vectors. To construct a baculovirus expression vector for recombinant saxiphilin (R-sax), the previously cloned, full-length saxiphilin cDNA sequence was amplified by PCR from the original clone in pBluescript SK− and inserted into the baculovirus transfer vector pBlueBacIII. Insect Sf9 cells were cotransfected with this transfer vector and modified linear baculovirus DNA. Clones of the recombinant, infectious virus were isolated and purified as described in Materials and Methods. Construction of a baculovirus expression vector for recombinant C-lobe of saxiphilin (C-sax) likewise involved PCR amplification of a DNA sequence composed of a synthetic oligonucleotide sequence encoding amino acid residues −19 to +1 of saxiphilin linked in frame to cDNA encoding saxiphilin residues 465−825. The amplified C-sax DNA sequence was inserted into pBlueBacIII, and recombinant virus was isolated by the same cotransfection procedure used for R-sax.

Sequencing of the PCR product showed that no errors had occurred in the coding sequence for R-sax; however, two base changes were observed for C-sax. One of these changes was a C → T substitution in the 3′ position of the TCC codon, corresponding to Ser-469 in native saxiphilin, which did not change the amino acid translation. The second change was a T → C substitution in the 5′ position of the TCC codon corresponding to Ser-616 in native saxiphilin, which resulted in a mutation of this residue to Pro. Since the baculovirus vectors for R-sax and C-sax were designed to include the native secretory signal sequence of the bullfrog protein (residues −19 to −1 of saxiphilin), we tested for expression by assaying the culture medium of Sf9 and High Five insect cells infected with both types of recombinant virus for [^{3}H]-STX binding activity.

Expression of Recombinant Saxiphilin and the C-Lobe of Saxiphilin. There was no detectable [^{3}H]STX binding activity in control medium taken from culture flasks of the uninfected insect cell lines, Sf9 and High Five, but such activity was observed within 1 day after infection with recombinant baculovirus coding for R-sax and C-sax. The [^{3}H]STX binding activity measured in the infected cell medium increased as a function of time and reached a maximal level at approximately 3 days after infection (not shown), when characteristic cell lysis occurs in the baculovirus infection cycle (Summers & Smith, 1987; O'Reilly et al., 1994). Figure 2A shows an example of a binding titration performed with a fixed amount of medium collected from Sf9 cells infected with R-sax baculovirus. In this experiment, increasing concentrations of [^{3}H]STX up to 10 nM were incubated with 5 μL of culture medium in a standard assay volume of 250 μL and bound [^{3}H]STX was separated from the free toxin on small cation-exchange columns. The data indicates the presence of a saturable component of [^{3}H]STX binding in the culture medium. In contrast, only a small, linear component corresponding to nonspecific background in the column assay was observed in the presence of excess unlabeled STX. Scatchard plots of specific [^{3}H]STX binding to culture medium from cells expressing R-sax and C-sax (Figure 2B) were consistent with a single class of sites with K_D values of 0.22 ± 0.01 nM and 0.93 ± 0.11 nM for R-sax and C-sax, respectively. Taking into account assay dilution factors, the maximal binding activity in the experiment of Figure 2B is equivalent to 25 pmol of [^{3}H]STX binding sites/mL of culture medium for R-sax and 21 pmol/mL for C-sax. For 13 different large-scale trials of R-sax expression in 1-L suspension cultures of High Five cells, the mean [^{3}H]STX binding activity of the harvested culture medium was 40 ± 13 pmol/mL (±SD). Using a value of 91 000 for the protein molecular weight of saxiphilin, the observed range of binding activity corresponds to a potential yield of 2.2−6.1 mg of saxiphilin/L.

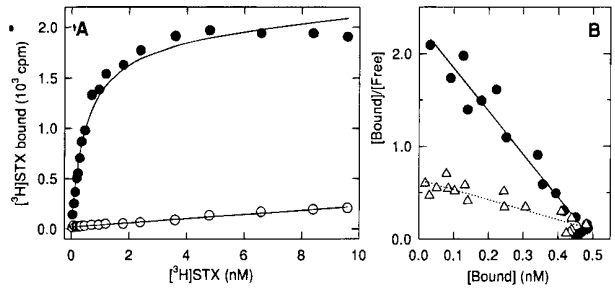

FIGURE 2: [^{3}H]STX binding titration of culture medium from baculovirus-infected insect cells. (A) Raw data for binding of [^{3}H]-STX to medium collected from High Five cells expressing R-sax assayed in the absence (●) and presence (○) of 10 μM unlabeled STX to assess nonspecific binding. Data points are the mean of duplicate samples. (B) Scatchard plots of [^{3}H]STX binding to medium from High Five cells expressing either R-sax (●, K_D = 0.22 nM) or C-sax (△, K_D = 0.93 nM).

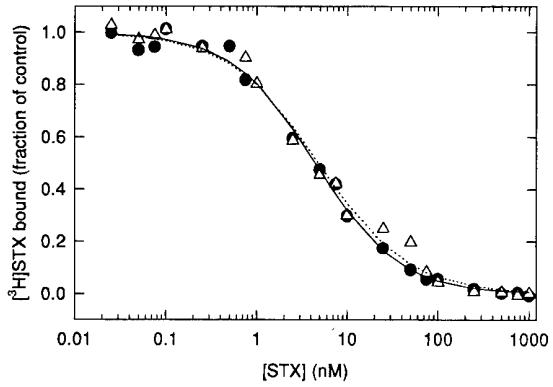

FIGURE 3: Competition of bound [^{3}H]STX with unlabeled STX. An aliquot of culture medium from High Five cells expressing R-sax (●) or C-sax (△) was assayed for specific binding in the presence of 5 nM [^{3}H]STX and various concentrations of STX. Specific binding is expressed as a fraction of the control value in the absence of unlabeled STX. Data points are the mean of duplicate samples. Data are fit to the equation $y = K_{0.5}{}^{n'}/(K_{0.5}{}^{n'} + [STX]^{n'})$ using the parameters, for R-sax (solid line), $K_{0.5}$ = 4.4 nM and n' = 0.94, and for C-sax (dotted line), $K_{0.5}$ = 4.8 nM and n' = 0.88.

To further characterize the homogeneity of the binding activity, a ligand competition assay was performed by titration of unlabeled STX in the presence of a fixed concentration of [^{3}H]STX. For medium taken from cells expressing C-sax and R-sax, the ligand competition assay was consistent with one class of STX binding sites as indicated by Hill coefficients close to 1.0 (Figure 3, legend). In such experiments, the K_D of the competitor ligand (STX) can be estimated from the concentration of STX at 50% displacement, $K_{0.5}$, according to the relationship $K_{0.5} = K_D(1 + [STX^*]/K_D^*)$, where [STX*] is the free concentration of [^{3}H]STX and K_D* is the equilibrium dissociation constant for [^{3}H]STX independently determined by the direct Scatchard analysis of Figure 2B. This use of this equation gives K_Ds of 0.18 nM and 0.75 nM for STX binding competition to R-sax and C-sax, respectively. Aside from demonstrating a single class of binding sites, the close agreement between the calculated K_D values for unlabeled STX and those for [^{3}H]STX measured by Scatchard analysis confirms that the concentration and specific activity of [^{3}H]STX used in these experiments are well calibrated.

To examine the size of the recombinant saxiphilin proteins, 15 μL of culture medium from cells expressing R-sax and C-sax was subjected to SDS−PAGE and immunoblot

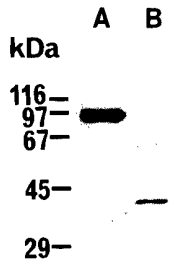

FIGURE 4: Immunoblot of cell culture medium from High Five cells infected with baculovirus expression vectors. An aliquot (15 μL) of medium from cells expressing R-sax (lane A) or C-sax (lane B) was subjected to SDS−PAGE and electroblotted onto a nitrocellulose membrane. The electroblotted membrane was exposed to anti-saxiphilin antibody and processed for chemiluminescence detection. Positions of standard molecular weight markers are shown at the left.

analysis. In this assay, affinity-purified polyclonal antibodies to native bullfrog saxiphilin (Li et al., 1993) were used to detect the recombinant forms of saxiphilin. This experiment resolved a single immunoreactive band in each of the two samples. As shown in Figure 4, culture medium from cells infected with baculovirus coding for R-sax exhibited a band with an apparent molecular mass of 92 kDa, and the corresponding sample for C-sax exhibited a band of 38 kDa, relative to protein molecular weight standards. These values are in close agreement to the theoretical protein molecular masses of R-sax (90.9 kDa) and C-sax (39.6 kDa), calculated from the respective primary sequences, assuming removal of the 19-residue, N-terminal signal sequence. To further characterize the recombinant R-sax protein, [^{3}H]STX binding activity was purified from High Five cell culture medium using a procedure similar to that described for the purification of native saxiphilin from bullfrog plasma (Li & Moczydlowski, 1991). The resulting preparation consisted of a predominant component with a molecular mass of 92 kDa corresponding to the band identified in the immunoblot of Figure 4 (not shown). When this sample was subjected to automated amino acid sequencing, a major sequence corresponding to the first 21 residues of native saxiphilin was evident, although there also appeared to be lower levels of an unrecognized sequence contaminating the sample. Despite the impurity, these data indicate that the native signal sequence of saxiphilin is correctly cleaved en route to secretion by the cultured insect cells.

Kinetics and pH Dependence of [^{3}H]STX Binding to R-sax and C-sax. To investigate the basis for the ~4-fold difference in the K_D of [^{3}H]STX for the whole recombinant saxiphilin protein vs that for the C-lobe, we measured the kinetics and pH dependence of [^{3}H]STX binding. Panels A and B of Figure 5 show the time course of association and dissociation, respectively, of [^{3}H]STX for both R-sax and C-sax in culture medium from infected insect cells. The time course of association measured in the presence of 10 nM [^{3}H]STX was virtually identical for the two proteins and was well described by an exponential function, as expected for pseudo-first-order kinetics. The derived bimolecular association rate constants for [^{3}H]STX binding were (1.8 ± 0.1) × 10^6 s^{-1} M^{-1} and (1.7 ± 0.1) × 10^6 s^{-1} M^{-1} for R-sax and C-sax, respectively. However, the time course of [^{3}H]-STX dissociation as measured by the rate of exchange with excess unlabeled STX was significantly faster for the C-sax

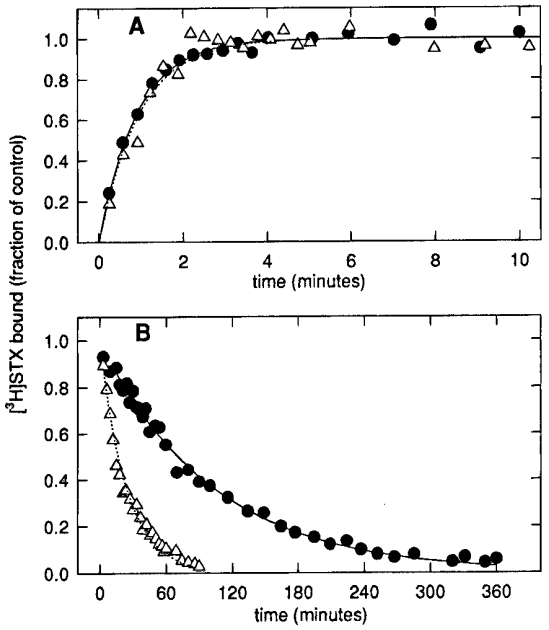

FIGURE 5: Kinetics of [^{3}H]STX binding to recombinant saxiphilin. (A) Association time course of [^{3}H]STX (10 nM) binding to an aliquot of medium from High Five cells expressing R-sax (●) or C-sax (△). Data points are normalized to the equilibrium level of binding and fit using a pseudo-first-order rate constant of 0.0187 s^{-1} (solid line, R-sax) or 0.0176 s^{-1} (dotted line, C-sax). (B) Time course of [^{3}H]STX dissociation from R-sax (●) and C-sax (△). Data points are normalized to [^{3}H]STX bound before addition of excess unlabeled STX and fit to a first-order rate constant of 1.64×10^{-4} s^{-1} (solid line, R-sax) and 7.02×10^{-4} s^{-1} (dotted line, C-sax).

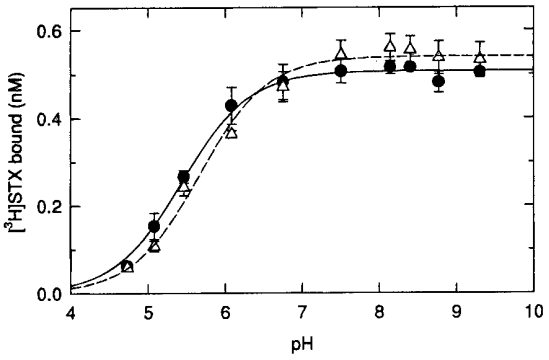

FIGURE 6: pH dependence of [^{3}H]STX binding to recombinant saxiphilin. An aliquot of medium from High Five cells expressing R-sax (●) or C-sax (△) was assayed for equilibrium binding in the presence of 5 nM [^{3}H]STX at various pH values. Data points are the mean of duplicate samples and are expressed as nanomolar bound [^{3}H]STX corrected for nonspecific binding in the presence of 10 μM STX. Data are fit to the equation $y = B_{max}\, K_{0.5}/(K_{0.5} + [H^+])$, using $pK_{0.5} = 5.44$ and $B_{max} = 0.51$ nM (R-sax, solid line) or $pK_{0.5} = 5.66$ and $B_{max} = 0.54$ nM (C-sax, dotted line).

protein compared to R-sax (Figure 5B). In both cases the time course was well described by a single-exponential decay. The best-fit values of the corresponding dissociation rate constants were $(1.64 \pm 0.03) \times 10^{-4}$ s^{-1} for R-sax and $(7.02 \pm 0.16) \times 10^{-4}$ s^{-1} for C-sax. Figure 6 shows the pH dependence of equilibrium binding of [^{3}H]STX to R-sax and C-sax. The data conform to a one-site H^+ titration curve as previously described for native saxiphilin (Llewellyn & Moczydlowski, 1994). The $pK_{0.5}$ values derived for the two proteins were very similar, 5.44 ± 0.04 and 5.66 ± 0.05 for R-sax and C-sax, respectively, as compared to $pK_{0.5} = 5.70$ for native saxiphilin assayed under the same conditions.

DISCUSSION

In this study, baculovirus-mediated expression was used to successfully produce recombinant saxiphilin and a deletion mutant of saxiphilin consisting of the predicted C-lobe domain. To our knowledge, this is the first report of expression of a vertebrate relative of the transferrin family in insect cells. It was expected that invertebrate cells would be suitable for expression of saxiphilin since endogenous Fe^{3+}-binding transferrin proteins have been previously identified in insects (Bartfeld & Law, 1990; Jamroz et al., 1993; Kurama et al., 1995). Recombinant human serum transferrin and human lactoferrin have been expressed in the mammalian BHK cell line (baby hamster kidney cells) using the pNUT expression vector (Mason et al., 1993; Stowell et al., 1991). This latter system was also used to express functionally active forms of the half-molecule N-lobe of both human transferrin and human lactoferrin (Funk et al., 1990; Mason et al., 1991; Day et al., 1992). Previous attempts to express transferrin in procaryotic cells such as *E. coli* have not yielded functionally active protein with Fe binding activity, presumably due to improper folding (Funk et al., 1990; Ikeda et al., 1992). The protein folding and/or secretion apparatus of eucaryotic cells appears to be necessary for functional expression of secreted forms of whole and N-lobe and C-lobe domains of transferrin, lactoferrin, and their relatives such as saxiphilin.

Equilibrium binding analysis (Figure 2) indicates that recombinant saxiphilin (R-sax) binds [^{3}H]STX with virtually the same affinity ($K_D \simeq 0.2$ nM) as native saxiphilin from bullfrog plasma (Llewellyn & Moczydlowski, 1994). Native and recombinant saxiphilin appear to have the same molecular mass (~91 kDa) as judged by virtual comigration of the respective bands on SDS−PAGE (data not shown). The kinetics and pH dependence of [^{3}H]STX binding to R-sax are also very similar to that described for native saxiphilin (Llewellyn & Moczydlowski, 1994). At pH 7.4 and 0 °C, the observed bimolecular association rate constants are 8.0×10^5 s^{-1} M^{-1} for native saxiphilin and 18.0×10^5 s^{-1} M^{-1} for R-sax, whereas the measured dissociation rate constants are 1.44×10^{-4} s^{-1} and 1.64×10^{-4} s^{-1}, respectively. Thus, by the criteria of molecular size and ligand-binding properties, recombinant saxiphilin produced in insect cells so closely resembles the native bullfrog protein that it should be useful for the further analysis of its structure and cellular function.

It was previously deduced that the native 91-kDa saxiphilin protein contains one high-affinity binding site for [^{3}H]STX (Llewellyn & Moczydlowski, 1994). The new finding of [^{3}H]STX binding by the ~40-kDa C-terminal domain, C-sax, establishes that the STX binding site is located in this portion of the protein. The similar pH dependence of [^{3}H]STX binding to C-sax and R-sax (Figure 6) further implies that the protonatable protein residue responsible for modulating the kinetics of ligand binding is also located in the C-lobe. Since there is one mutation (corresponding to Ser-616 to Pro in native saxiphilin) in the expressed form of C-sax that was inadvertently introduced by PCR, we cannot unambiguously attribute the slightly lower [^{3}H]STX binding affinity and faster dissociation rate of C-sax to the loss of interlobe interactions that may result from deletion of the N-lobe of saxiphilin. On the basis of sequence alignment to lactoferrin, this mutated residue is predicted to lie with a short 3_{10} turn

(Anderson et al., 1989). Since this turn lies on the exterior surface of lactoferrin distant from the Fe binding site in the C-lobe, it seems likely that this mutation would not have a major impact on the structure of the [^{3}H]STX binding site that we hypothesize to be located within the interdomain cleft between subdomains C1 and C2. However, this proline residue may partially disrupt local secondary structure and be the basis for the observed 4-fold faster rate of [^{3}H]STX dissociation from C-sax vs R-sax. With the baculovirus expression system described here, the effect of altering this residue and other residues on the kinetics of STX binding can now be addressed by site-specific mutagenesis.

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Phylogenetic survey of soluble saxitoxin-binding activity in pursuit of the function and molecular evolution of saxiphilin, a relative of transferrin

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SUMMARY

Saxiphilin is a soluble protein of unknown function that binds the neurotoxin, saxitoxin (STX), with high affinity. Molecular characterization of saxiphilin from the North American bullfrog, *Rana catesbeiana*, has previously shown that it is a member of the transferrin family that does not bind Fe^{3+}. In this study, we surveyed various animal species to investigate the phylogenetic distribution of saxiphilin as detected by the presence of soluble [^{3}H]STX binding activity in plasma, hemolymph or tissue extracts. We found that saxiphilin activity is readily detectable in a wide variety of arthropods, fish, amphibians, and reptiles. The pharmacological characteristics of [^{3}H]STX binding activity in phylogenetically diverse species indicates that a protein homologous to bullfrog saxiphilin is likely to be constitutively expressed in many ectothermic animals. The results suggest that the saxiphilin gene is evolutionarily as old as an ancestral gene encoding bi-lobed transferrin, a Fe^{3+}-binding and transport protein which has been identified in several arthropods and all vertebrates that have been studied. Two plausible hypotheses for the biological function of saxiphilin are discussed: a role in the detoxification of saxitoxin acquired from microorganisms and/or a role in binding an unidentified endogenous ligand that regulates voltage-sensitive Na^+ channels.

1. INTRODUCTION

The neurotoxin, saxitoxin (STX), and a large array of STX derivatives are produced by certain species of dinoflagellates in the marine environment and cyanobacteria in the freshwater ecosystem (Carmichael et al., 1990; Hall et al., 1990; Schantz, 1986). Consumption of toxic phytoplankton by filter feeding organisms results in the accumulation and dispersal of STX through the food chain to animals that have been reported to include an ascidian, annelids, molluscs, crabs, fish, and ultimately mammals (Anderson and White, 1992; Geraci et al., 1989; Gessner, et al., 1996; Llewellyn and Endean, 1989; Nagashima et al., 1984; Yasumoto et al., 1986). The human intoxication syndrome of paralysis and death resulting from unwitting consumption of STX-contaminated shellfish is commonly known as "paralytic shellfish poisoning" or PSP. The neurotoxic action of STX is due to potent blockade of voltage-sensitive Na^+ channels that mediate nerve and muscle action potentials (Ritchie & Rogart, 1977). STX exerts half-maximal block of Na^+ current at a concentration of 2-100 nM STX, depending on the particular Na^+ channel isoform (Guo et al., 1987). At the molecular level, the STX-binding site on Na^+ channels has been localized to residues within a conserved sequence motif in four homologous domains of the α-subunit that forms part of the ion-selective pore (Terlau et al., 1991).

While the molecular pharmacology of STX related to the Na^+ channel is well characterized, our group has been attempting to uncover the biological significance of a different high-affinity binding site for STX that is located on a soluble protein named saxiphilin. This site was first recognized by the finding of high-affinity binding activity for [^{3}H]STX in tissue extracts and plasma of frogs and toads (Doyle et al., 1982; Mahar et al.,

1991). A component exhibiting soluble [^{3}H]STX-binding activity was purified from plasma of the bullfrog, *Rana catesbeiana*, and found to correspond to a 91 kDa protein related to the transferrin family of Fe^{3+}-binding proteins (Li and Moczydlowski, 1991). Structural similarity of saxiphilin to transferrin is indicated by a high level of sequence similarity; e.g., 51% identity to *Xenopus laevis* transferrin and 44% identity to human serum transferrin (Morabito & Moczydlowski, 1994).

Molecular characterization of saxiphilin has revealed interesting details of its structure and biochemical properties but has not yet defined its function. Its well-known relatives, serum transferrin and lactoferrin, have a bi-lobed structure owing to the presence of two internally homologous domains of ~340 residues, the N-lobe and C-lobe, that each contain a high affinity site for Fe^{3+} ($K_D \simeq 10^{-20}$ M) and the synergistic anion cofactor, HCO_3^- (Baker & Lindley, 1992). Bullfrog saxiphilin has the same internal sequence duplication as transferrins but has substitutions in nine of ten highly conserved Fe^{3+}-site residues, accounting for its lack of demonstrable Fe^{3+}-binding activity (Li et al., 1993; Morabito & Moczydlowski, 1994). The larger molecular mass of saxiphilin (91 kDa) vs. transferrins (~78 kDa) is due to the presence of an insertion of 144 residues in the N-lobe that contains two thyroglobulin type-1 domains (Morabito & Moczydlowski, 1995). The single high affinity binding site for STX ($K_D \simeq 0.2$ nM) in bullfrog saxiphilin has been localized to the C-lobe as determined by assay of a recombinant form of the protein in which the N-lobe has been deleted (Morabito et al., 1995). [^{3}H]STX binding to saxiphilin is also inhibited at low pH in a manner reminiscent of the pH-dependent release of Fe^{3+} by transferrin (Llewellyn & Moczydlowski, 1994), a process that is important in the delivery

of iron to eukaryotic cells by transferrin receptor-mediated endocytosis (Thorstensen & Romslo, 1990).

Aside from the recognized role of serum transferrin in iron transport, transferrin and lactoferrin are also responsible for maintaining low levels of free Fe^{3+} in biological fluids, which inhibits the growth of Fe^{3+}-requiring microorganisms and protects against the potential toxicity of Fe^{2+}/ Fe^{3+} in the generation of hydroxyl free radical (Crichton, 1991). By analogy to this latter chemical defense function, it may be hypothesized that saxiphilin functions as a defense mechanism against STX intoxication, by sequestering STX that an animal might acquire from microbial sources. Although comparatively little is known about the chemical ecology of STX in the freshwater environment, an argument for this hypothesis can be drawn from previous observations of tadpole mortality associated with STX production by the cyanobacterial species, *Aphanizomenon flos-aquae* (Ikawa et al., 1982), and the occurrence of saxiphilin in Ranid tadpoles and frogs (Mahar et al., 1991).

In addition to the problem of its function, another interesting question concerns the molecular evolution of saxiphilin. Transferrins or Fe^{3+}-binding, transferrin-like proteins have thus far been identified in all classes of vertebrates, several insect species and an ascidian (Bartfeld & Law, 1990; Jamroz et al., 1993; Kurama et al., 1995; Martin et al., 1984; Welch, 1990). Sequence data imply that the gene duplication underlying the two-lobed structure of modern transferrin occurred before the evolutionary divergence of insects and the chordate lineage (Bowman et al., 1988; Bartfeld & Law, 1990). Since saxiphilin contains this same internal duplication, a transferrin gene may have been the direct ancestral precursor of the saxiphilin gene. Evidence has recently emerged that saxiphilin is not the only example of a non-Fe^{3+}-binding member of the transferrin family.

Fierke and coworkers have identified a protein inhibitor of carbonic anhydrase in the pig (PICA) that is homologous to transferrin but has substitutions of several key Fe^{3+}-site residues (Roush & Fierke, 1992; Wuebbens et al., 1994). The examples of saxiphilin and PICA suggest that transferrin-like proteins may reflect a family of genes with diverse biological functions.

To pursue the function and evolution of saxiphilin, we conducted a phylogenetic survey of soluble [^{3}H]STX-binding activity. The known pharmacological characteristics of this activity in the bullfrog provide a unique set of criteria to identify the presence of saxiphilin-related proteins in other species. We hypothesized that if saxiphilin functions primarily in a defensive capacity against STX intoxication from microbial sources, it ought to be commonly expressed by animal species inhabiting ecosystems known to harbor STX-producing phytoplankton. Unexpectedly, we found putative saxiphilin-like activity in terrestrial arthropods such as a centipede and a spider and in a wide variety of ectothermic vertebrates including fish and amphibians from the aquatic environment as well as reptiles indigenous to semi-arid locales. The data imply that saxiphilin has an ancient origin in animal evolution and that it may function in process(es) of broadly-based biological significance.

2. METHODS

(a) Materials. [^{3}H]STX was purchased from Amersham, purified, and standardized according to Moczydlowski et al. (1988). Several different lots of [^{3}H]STX used in this study had specific activites in the range of 20,200 -35,100 cpm/pmol. STX was purchased from Calbiochem (La Jolla, CA) and the following STX derivatives:

decarbamoylsaxitoxin (dcSTX), neosaxitoxin (neoSTX), B1 and C1 (see Fig. 2a for structures) were generously provided by Dr. Sherwood Hall (US Food and Drug Administration). Stock solutions of these toxins were diluted in 1 mM citrate buffer, pH 5.0. The common buffers, Mops, Mes, Hepes, and Tris and the anesthetics, tricaine methanesulfonate and sodium brevital were from Sigma (St. Louis, MO). The cation exchange resin, AG50W-X2, H^+ form, 100-200 mesh, was obtained from Bio-Rad (Richmond, CA). Other chemicals were reagent grade from commercial sources.

(b) Sources of animals and sample preparation. Some species used in this study were purchased from Connecticut Valley Biological (Southampton,MA), Carolina Biological (Burlington, NC), and Charles Sullivan Co. (Nashville, TN). Various animals were collected in the vicinity of Mount Desert Island Biological Laboratory (Salisbury Cove, ME) and the Australian Institute of Marine Science (Townsville, Queensland Australia). Numerous professional colleagues listed in the acknowledgements generously donated plasma and tissue samples from animals used in their own studies. Plasma samples from several species of sharks, dolphins and whales were obtained from the New England Aquarium (Boston, MA) through the assistance of Dr. Don Anderson at the Woods Hole Oceanograghic Institute (Woods Hole, MA). Lyophilized plasma from terrestrial mammals, birds, Thailand cobra (*N. n. kaouthia*), and liver extract of the African lungfish were purchased from Sigma.

Live animals were handled humanely according to guidelines of the Yale University Animal Care and Use Committee. Live amphibians and reptiles studied in the laboratory were anesthetized with tricaine methanesulfonate or sodium brevital and

exsanguinated via the aortic arch with a syringe containing 100 µl of 0.1 mg/ml heparin sulfate. Whole blood was centrifuged in an Eppendorf microfuge, plasma was removed, and stored frozen at -80° C for later assay. In our experience, saxiphilin-like [^{3}H]STX binding activity in whole plasma is very stable over the course of a year when stored frozen or lyophilized. This is in contrast to dilute solutions of purified bullfrog saxiphilin which are labile to repeated freeze-thawing (Llewellyn & Moczydlowski, 1994). Animals too small for convenient collection of blood or hemolymph were anesthetized by hypothermia and processed for tissue extraction. Whole animal extracts were prepared by homogenization on ice with a Tissumizer homogenizer (Tekmar, Cincinnati, OH) using two 10 s bursts at 8,000 rpm followed by two 10 s bursts at 24,000 rpm in a buffer consisting of 10 mM Mops-NaOH, pH 7.4, 0.3 M sucrose, 5 mM EDTA, 1 µM pepstatin, 1 µM aprotinin, and 100 µM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 x g to ensure complete removal of solid debris and particulate membranes that might contain STX-binding sites associated with Na^+ channels. The supernatant was decanted for assay and stored frozen at -80° C.

(c) Measurement of [^{3}H]STX binding. All measurements of [^{3}H]STX binding were carried out at ~0° C by continuous incubation of assay solutions on ice. In the standard survey for soluble [^{3}H]STX binding, 50-100 µl aliquots of plasma samples or extracts were added to a solution with a final concentration of 20 mM Mops-NaOH, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, and approximately 5 nM [^{3}H]STX in a volume of 250 µl and incubated for at least 1 h. Control samples for determination of the non-specific blank also contained 10 µM STX. Duplicate aliquots of 100 µl were processed for

separation of bound and free [^{3}H]STX on small columns of AG50W-X2 cation exchange resin and quantitated by liquid scintillation counting as previously described (Llewellyn & Moczydlowski, 1994).

Assay of saturable binding behavior for selected species was similarly performed by varying the concentration of [^{3}H]STX in the assay from 0.1 to 26 nM. Data from such experiments were fit to a 1-site binding model using the EBDA and LIGAND equilibrium binding analysis programs from Biosoft (Cambridge, UK). Structure-activity relationships for STX derivatives were investigated by competitive binding titrations in which [^{3}H]STX was held constant at 4.4 nM and the concentration of various unlabeled STX derivatives was varied. Data from such experiments were fit to the logistic equation: $f = [toxin]^{n'} / ([toxin]^{n'} + K_{0.5}^{n'})$, where f is the ratio of [^{3}H]STX bound in the presence of competitor toxin to that without competitor, $K_{0.5}$ is the competitor concentration at 50% inhibition, and n' is a pseudo-Hill coefficient. Non-linear least squares fitting was performed using the curve fitting utility of Sigmaplot (Jandel, San Rafael, CA). Error estimates for $K_{0.5}$ given in Table 3 were obtained from the Sigmaplot fitting routine.

Dependence of binding on pH was studied by similar assay using 4.4 nM [^{3}H]STX in buffers ranging from pH 4 -9. The pH was buffered with 20 mM Tris, 10 mM Mes, and 10 mM acetic acid adjusted with tetramethylammonium hydroxide or HCl at nearly constant ionic strength as described by Ellis & Morrison (1982). H^+ titration data were analyzed by fitting to the following logistic function similar to that used for toxin competition: $f = [H^+]^{n'} / ([H^+]^{n'} + [H^+]_{0.5}^{n'})$ where $[H^+]_{0.5}$ is the proton concentration at 50% inhibition, and n' is a pseudo-Hill coefficient.

Dissociation kinetics of [^{3}H]STX were followed by pre-equilibration of samples with [^{3}H]STX and assay of bound radioligand at various times after addition of 10 μM STX. Similarly, association kinetics under pseudo-first order conditions were followed by assay of bound radioligand at various times after addition of 4.4 or 8.8 nM [^{3}H]STX. Kinetic data were fit either to a single-exponential function of time or a sum-of-two-exponentials function of time as described in the text. Protein assays were performed according to the method of Cabib & Polachek (1984).

3. RESULTS

From previous biochemical characterization in the bullfrog, saxiphilin may be recognized by soluble high-affinity binding of [^{3}H]STX that is specifically displaced by excess unlabelled STX (Mahar et al., 1991; Llewellyn & Moczydlowski, 1994). Binding of [^{3}H]STX to saxiphilin is readily discriminated from such binding to voltage-sensitive Na^+ channels by its solubility in the absence of detergents, its presence in plasma and extracts of non-electrically excitable tissues, lack of binding competition by 100 μM tetrodotoxin, and a distinctively slow time course of dissociation. Thus, to screen various animal species for expression of saxiphilin we assayed soluble [^{3}H]STX binding in plasma or hemolymph samples where possible, or in whole tissue extracts of small vertebrates and invertebrates. Species selected for this study included representatives of all major vertebrate classes and invertebrate phyla. The survey sample consisted of animals indigenous to five continents (N. America, S. America, Europe, Asia, Australia) with diverse habitats including species that have been previously documented to acquire STX from blooms of toxic dinoflagellates.

Table 1 summarizes the effective concentration and specific activity (per mg protein) of soluble [^{3}H]STX binding sites in samples from species that tested positive for saxiphilin. The level of detectable activity ranged from 0.2 nM effective concentration (pmol sites per ml plasma or g tissue extract) in an unidentified Australian cockroach to ~5,000 nM in the plasma of one individual specimen of the wood frog, *Rana sylvatica.* Positive species include animals belonging to the major classes of ectothermic vertebrates and arthropods. The list of positive vertebrates include teleost fish, amphibians (frogs, toads, salamanders and newts) and reptiles (lizards and snakes). The low levels of activity measured in the water flea, *Daphnia*, and an unidentified Australian cockroach might be questionable since these measurements were near the lower limit of detection (~0.1 nM [^{3}H]STX binding sites), but the finding of robust activity (~80 nM) in an Australian centipede (*Ethmostigmus rubripes*) and in a North American orb weaving spider (*Araneus c.f. cavaticus*) point to the occurrence of saxiphilin in invertebrates. Since this study was completed, one of us has also observed saxiphilin-like activity in the hemolymph of several species of marine Xanthid crabs indigenous to the eastern coast of Australia (L. E. Llewellyn, personal communication), extending the finding of arthropod saxiphilin to crustaceans.

Table 2 is a listing of species that were tested by the same method and found not to exhibit any detectable saxiphilin activity. We did not find evidence of saxiphilin-like activity in endothermic vertebrates as represented by various domesticated birds or mammals including dolphins and whales. Negative reptiles included various turtles, crocodilians, a tiger snake, and the primitive tuatara of New Zealand. The common laboratory species, *Xenopus laevis* (African clawed frog), is the only amphibian tested to

date that does not appear to express saxiphilin. Negative vertebrate marine species also include several fish, four sharks, the evolutionarily primitive coelocanth, and a lamprey. Of particular note, the bivalve mollusks, *Saxidomus giganteus* and *Mytilus edulis*, are known to accumulate STX from toxic marine dinoflagellates (Shimizu et al., 1978; Schantz, 1986), but they do not appear to produce saxiphilin. Other tested invertebrates that lack detectable saxiphilin include various marine and terrestrial creatures: an ascidian, echinoderms, annelids, mollusks, insects, crustaceans, and a soft coral.

To test the presumption that soluble [^{3}H]STX-binding activity found across this diverse phylogenetic spectrum has properties similar to that of the previously characterized saxiphilin from *Rana catesbeiana*, samples from the following representative species were selected for further characterization: plasma of *Bufo marinus* (marine or cane toad), plasma of *Thamnophis sirtalis* (garter snake), lyophilized plasma of *Naja naja kaouthia* (Thailand cobra), an extract of whole *Gambusia affinis* (mosquito fish), and hemolymph of *Ethmostigmus rubripes* (an Australian centipede). Appropriate dilutions of these samples were titrated with various concentrations of [^{3}H]STX in the range of 0.1 to 26 nM and assayed for binding in the absence and presence of excess unlabelled STX (10 μM) to assess total and non-specific binding, respectively. Samples from all five of these species exhibited a component of saturable, high-affinity binding and a linear component of non-specific binding [^{3}H]STX-binding (Fig. 1). The binding titration data were analyzed with the EBDA and LIGAND programs to derive an apparent equilibrium dissociation constant (K_D) for [^{3}H]STX based on the assumption of a single class of binding sites. The K_D values estimated by this approach were: 210 ± 29 pM (*B. marinus*), 15 ± 6 pM (*N. n. kaouthia*), 240 ± 60 pM (*T. sirtalis*), 39 ± 7 pM (*G. affinis*), and 10 ± 6 pM (*E. rubripes*).

These K_D values, all less than 1 nM, fall in a range where the accuracy of equilibrium analysis is limited by the ability to accurately determine extremely low concentrations of free [^{3}H]STX ligand. However, the values indicate an equivalent or higher affinity than that previously reported for purified native (K_D = 350 $\pm$ 20 pM) or recombinant (K_D = 220 $\pm$ 10 pM) saxiphilin from *Rana catesbeiana*.

Although numerous organic molecules have been tested, derivatives of STX are the only class of compounds that we have ever observed to competitively displace [^{3}H]STX binding to bullfrog saxiphilin. To investigate whether the soluble STX-binding sites of the five test species share a common structure-activity relationship, a competition displacement assay was performed for four STX derivatives: neoSTX, dcSTX, B1, and C1 (Fig. 2a). As illustrated in Fig. 2, samples from all five test species exhibited monotonic displacement titration curves with Hill coefficients close to 1.0, characteristic of a single site or a homogeneous class of STX-binding sites. In each case, the concentration of free STX required for 50% displacement of specific [^{3}H]STX binding was very close to the concentration of free [^{3}H]STX in the assay (~4 nM), in accordance with the relationship of Cheng and Prusoff (1973) relationship for one-site binding. With respect to displacement by the four STX derivatives, the highest and lowest affinity was observed for dcSTX and C1, respectively. For the snake, fish and centipede species, neoSTX exhibited higher affinity than B1 (Table 3). However, for the toad species, B1 exhibited slightly higher affinity than neoSTX. These results are in contrast to saxiphilin activity from Ranid frogs, where neoSTX has 550-fold lower affinity than STX (Mahar et al., 1991). It thus appears that the low intrinsic affinity for neoSTX in bullfrog saxiphilin is an exception rather than the rule and that saxiphilin homologs from many species bind neoSTX nearly as well as

STX. For [^{3}H]STX binding activity examined in this study, in no case did we observe competitive displacement by 100 μM tetrodotoxin, which is a Na^+ channel blocker with a very different structure from STX (Ritichie & Rogart, 1977). This emphasizes the unambiguous pharmacological discrimination of saxiphilin from STX-binding sites of voltage-sensitive Na^+ channels, which have been universally found to exhibit competitive binding between STX and tetrodotoxin.

Previous studies showed that the pH-dependence of [^{3}H]STX binding to bullfrog saxiphilin resembles the pH-dependence of Fe^{3+}-binding to serum transferrin (Llewellyn & Moczydlowski, 1994). We previously suggested that this coincidence may reflect a common structural-functional relationship or a shared aspect of cellular physiology. To investigate whether this biochemical property of saxiphilin activity is conserved in other species, equilibrium binding of [^{3}H]STX was measured as a function of pH for the five test species (Fig. 3). The pH titration curves of the two snake species, *N. n. kaouthia* and *T. sirtalis*, exhibited a similar pH dependence as that of *Rana catesbeiana* with half-maximal inhibition occuring near pH ~5.5 (Table 3). However, the Hill coefficient (n') derived for H^+ was higher than that previously observed for the bullfrog protein (n' = 1.0), with n equal to 1.4 and 2.1 for *T. sirtalis* and *N. kaouthia*, respectively. [^{3}H]STX-binding activity from *B. marinus* and *G. affinis* was less sensitive to H^+-inhibition. Complete titration curves for these latter two species could not be obtained due to known limitations of the cation-exchange column assay at pH values less than 4.5. The available data suggest $pH_{0.5}$ values of ~4.4 and ~4.7, for *B. marinus* and *G. affinis*, respectively. The putative saxiphilin activity of the centipede species, *E. rubripes*, exhibited a $pH_{0.5}$ of 5.4

and a Hill coefficient of ~3.2, corresponding to a steeply sensitive inhibition with respect to $[H^+]$ that may indicate more than one site of H^+ modulation.

The kinetics of dissociation and association of [^{3}H]STX was also investigated. Figure 4 shows representative data collected in these experiments for *B. marinus*, *N. n. kaouthia*, *G. affinis* and *E. rubripes*. In the case of the toad and centipede, both the dissociation and association kinetics were well described by a simple exponential time course. The derived dissociation rate constant for *B. marinus* from the experiment of Fig. 4A at pH 7.4 was $k_{off} = 1.1 \pm 0.1 \times 10^{-5}\ s^{-1}$. The rate of [^{3}H]STX dissociation measured for plasma samples from two other specimens of *B. marinus* was $1.2 \pm 0.1 \times 10^{-5}\ s^{-1}$ and $1.3 \pm 0.2 \times 10^{-5}\ s^{-1}$, illustrating the consistency of these kinetic determinations from individual to individual. The association rate of the *B. marinus* sample was measured in the presence of 4.4 nM [^{3}H]STX under near pseudo-first order conditions, where the concentration of ligand was about seven-fold greater than the number of total sites. Under these conditions, the time course was so rapid that the early portion could not be resolved with the present methodology. However, the data can be used to calculate a lower limit for the bimolecular association rate constant of $k_{on} \geq 9.8 \times 10^6\ s^{-1}M^{-1}$. The ratio of k_{off} / k_{on} provides an upper limit estimate for the equilibrium K_D for *B. marinus* of 1.1 pM, which is an order of magnitude lower than that estimated (15 pM) from the equilibrium titration data of Fig. 1. As noted above, equilibrium K_D measurements in the range of 1 pM by standard Scatchard analysis with the available specific activity of [^{3}H]STX are technically unattainable. Thus, the K_D values estimated here by the kinetic approach are more likely to be closer to the true value.

The corresponding rate constants for *E. rubripes* were $k_{off} = 4.3 \pm 0.3 \times 10^{-6}$ s^{-1} and $k_{on} = 5.2 \pm 0.7 \times 10^{6}$ $s^{-1}M^{-1}$, giving an estimated K_D of 0.8 pM. Figure 4h shows that the time course of association for the centipede sample was fairly well resolved, being slower than that of the other species in Fig. 4. The dissociation rate of [^{3}H]STX from the centipede hemolymph sample corresponds to a halftime of 1.9 days, which is extraordinarily slow for the dissociation of a small organic molecule from a protein acceptor site.

The kinetic behavior of the fish and snake samples was more complex than that of the toad and centipede. For these species, the time course of [^{3}H]STX dissociation was better fit by a sum-of-two exponentials rather than one exponential (Fig. 4). However, a major slow kinetic component of the dissociation process comprised 77-92% of the decay for these three species, corresponding to the following k_{off} rate constants: $9.8 \pm 1.5 \times 10^{-6}$ s^{-1} for *G. affinus*, $1.1 \pm 0.1 \times 10^{-4}$ s^{-1} for *N. n. kaouthia*, and $1.3 \pm 0.1 \times 10^{-5}$ s^{-1} for *T. sirtalis*. These latter slow components correspond to dissociation halftimes of 19.6, 1.7 and 14.8 hours, respectively, all slower than the halftime of 1.3 hours previously measured for bullfrog saxiphilin. The association time course for the two snake species conformed well to a single exponential process, but the fish sample was better described by two components (Fig. 4g). For the cobra, *N. n. kaouthia*, and the fish, *G. affinis*, the association time course was faster than that of a system governed by a bimolecular k_{on} of 10^7 $s^{-1}M^{-1}$. Curiously, the association rate observed for the garter snake, *T. sirtalis*, was very well resolved since it was slower than that of any of the other species (data not shown), corresponding to a k_{on} of $3.2 \pm 0.2 \times 10^5$ $s^{-1}M^{-1}$. If we use a one-site equilibrium to approximate the behavior of these systems and consider only the major slow component

of the dissociation time course, then the ratio of k_{off} / k_{on} for the two snake species and the fish species yield the following effective K_D estimates: 6 pM, *N. n. kaouthi*; 40 pM, *T. sirtalis*; and, 0.6 pM *G. affinis*. Surprisingly, the approximate K_D values for the five test species investigated most thoroughly in this study are all substantially lower than the K_D of ~200 pM previously measured for bullfrog saxiphilin.

With respect to the pH dependence discussed above, STX binding to bullfrog saxiphilin has also been shown to undergo allosteric modulation by H^+ as manifested by an increased rate of ligand dissociation at low pH (Llewellyn & Moczydlowski, 1994). To investigate whether this functional property is conserved, we have also measured the dissociation time course at pH 4.3-4.5 for three species. As shown in Figs. 4a-4c, samples from *B. marinus*, *N. n. kaouthia*, and *G. affinis* all exhibited a substantially faster dissociation time course at the lower pH. This effect is equivalent to an 3.6-fold, 5.6-fold and 9.1-fold enhancement of the slowest component of the dissociation reaction for *G. affinis*, *B. marinus* and *N. n. kaouthia*, respectively.

4. DISCUSSION

(a) Saxiphilin has an ancient origin in animal evolution.

The properties of soluble [^{3}H]STX-binding activity characterized for the cane toad (*B. marinus*) , garter snake (*T. sirtalis*), Thailand cobra (*N. n. kaouthia*), mosquito fish (*G. affinis*) , and centipede (*E. rubripes*) leave little doubt that all five of these species contain a protein homologous to bullfrog saxiphilin. Although there are some subtle differences in binding kinetics, pH-dependence, and structure-activity relationships of STX

derivatives among the five species, these parameters have the characteristic biochemical and pharmacological signature of the purified saxiphilin protein from *Rana catesbeiana* (Mahar et al., 1991; Llewellyn & Moczydlowski, 1994). The results thus imply that an STX-binding site like that present in bullfrog saxiphilin is conserved in phylogenetic groups as diverse as arthropods and reptiles. This leads us to conclude that a gene coding for the saxiphilin protein is present and functionally active in both the arthropod and chordate phyla.

Of the major vertebrate classes, saxiphilin activity is readily detected in numerous fish, amphibians and reptiles. The biogeographic distribution of animals found to express saxiphilin includes representatives from diverse habitats in Asia (e.g., *N. n. kaouthia*), Australia (e.g., *V. rosenbergii*), Europe (e.g., *R. temporaria*), Africa (e.g., *P. aethiopicus*), and the Americas (*R. sylvatica*), indicating that this phenotypic characteristic is not confined to a particular climatic zone. The probable existence of saxiphilin in a myriapod (e. g., the centipede *E. rubripes*), arachnids (*Araneus c. f. cavaticus*), crustaceans (e. g., *Daphnia*; Xanthid crabs, L. Llewellyn, unpublished data) and some insects (e.g., an unidentified cockroach) further suggests that it may have first appeared during the emergence of invertebrates some 800 million years ago. Evolutionary speculations on its origin may be considered in light of the fact that bi-lobed, Fe^{3+}-binding, transferrin-like proteins are also present in insects (Bartfeld & Law, 1990; Jamroz et al., 1993; Kurama et al., 1995). The occurrence of saxiphilin in arthropods is consistent with the possibility that it arose directly from an ancestral bi-lobed transferrin precursor and later acquired an insertion of 144-residues that is present in bullfrog saxiphilin (Morabito & Moczydlowski, 1994; 1995). Alternatively, it is possible that bi-lobed transferrin and

saxiphilin both arose independently by gene duplication from a single-lobed, ~40 kDa transferrin precursor, such as that previously described in the urochordate ascidian, *Pyura stolonifera* (Martin et al., 1984).

The most striking finding of our characterization of [^{3}H]STX-binding activity from evolutionarily diverse species is the extraordinarily high affinity of the soluble STX-binding site in some animals. While K_D's in the range of 10^{-12} M are not that uncommon for bioactive peptides, binding affinity in this range is relatively rare for small organic molecules; e.g., one of the strongest known interactions is that of biotin-avidin, $K_D \simeq 10^{-15}$ M (Gitlin et al., 1987). The binding energy for such protein-ligand interactions is generally derived from multiple non-covalent interactions between the functional groups of protein residues and atoms of ligand subsitutent groups. The STX molecule, with its divalent positive charge and six guanidino nitrogen atoms, offers a rigid scaffold with the potential ability to form multiple electrostatic and hydrogen-bonding interactions within a protein binding site. Another factor that may contribute to this picomolar affinity is the mechanical ability of transferrin proteins such as lactoferrin to capture their ligands (i.e., Fe^{3+}/HCO^{3-}) within a cavity that closes in the bound state like a hinged jaw (Anderson et al., 1990), thus utilizing a protein conformational change to stabilize the bound ligand. Whatever the mechanism, the chemical specificity and evolutionary conservation of the STX-saxiphilin interaction makes it difficult to argue that this association is a completely fortuitous molecular affiliation unrelated to biological function. In this regard, it is evident that the STX-saxiphilin interaction in some species may be at least three orders of magnitude stronger than the typical nanomolar affinity of STX binding to the Na^+ channel (Ritchie & Rogart, 1977), the site that mediates biological toxicity of STX.

(b) Constitutive expression of saxiphilin is exhibited by certain ectothermic animal species.

The inference that a saxiphilin gene is widely distributed in the arthropod and vertebrate genomes leads to a series of questions raised by the negative observations of Table 2, which imply the apparent absence of saxiphilin in sister taxa and possibly in whole classes of the vertebrate sub-phylum. For example, among the Anuran sub-class of Amphibia, why is saxiphilin readily detected in particular frogs and toads (e.g., *Rana catesbeiana, R.. sylvatica, R. temporaria, Bufo marinus*), but not in the African frog, X*enopus laevis*? Similarly, among the class Reptilia, why is saxiphilin present in the sub-class (Lepidosauria) containing the orders of lizards and snakes, but apparently absent in both the sub-class (Archosauria) that includes alligators and crocodiles and in the sub-class (Anapsida) that includes turtles? One explanation may be that saxiphilin is a non-essential protein or that its function is readily served by other proteins. Alternatively, the pattern of disparate expression could indicate selective loss of a once functional gene. Another possible interpretation of the data of Tables 1 and 2 is that saxiphilin expression is a character specific to certain ectothermic animals. One might speculate that the saxiphilin gene is preferentially utilized by cold-blooded animals that do not have metabolic control of their body temperature and was deactivated or lost in the evolutionary lineages leading to birds and to mammals.

Along these lines, it may be noted that the highest level of saxiphilin-like activity was observed in the wood frog, *Rana sylvatica* (having a mean activity of 1600 pmole/ml plasma). This small frog species has the northern-most habitat range in North America

and has developed special mechanisms to survive prolonged periods hibernating under a superficial layer of ground cover in a frozen state during the winter (Storey, 1990). Although we certainly have not surveyed a sufficient number of species to permit a general conclusion, the present data lead us to wonder whether there may be a functional correlation between high levels of saxiphilin activity and particular species of ectothermic animals that tolerate a wide variation in body temperature.

On the other hand, an apparent lack of saxiphilin activity as measured by the present methodology does not necessarily prove that the saxiphilin gene is absent in the negative species of Table 2. Indeed, it is possible that a functional gene is present and that we have failed to detect soluble [^{3}H]STX activity for a number of reasons. For example, our survey results may be a reflection of the underlying biochemical pharmacology of the STX-binding site. If for instance, there existed an endogenous ligand that binds to saxiphilin in a competitive manner with STX, the failure to detect [^{3}H]STX-binding in a crude extract could be due to pre-existing saturation of the STX-binding site with such a ligand. Alternatively, it may be that transcription of the gene is inducible and turned off in most animals under normal physiological conditions. Other possibilities are that saxiphilin is present in amounts below the detection limit of our assay, or that it is expressed only in certain tissues or cell types that are not well sampled by our typical assay of plasma or whole animal extracts.

Previous work showed that saxiphilin mRNA is transcribed in bullfrog liver and several other tissues (Morabito & Moczydlowski, 1994). However, from the present results, saxiphilin does not appear to be an ubiquitous component of animal plasma like serum transferrin in the vertebrates, which is found at a relatively constant concentration

of approximately 2.5 mg/ml (~30 μM) in all adult specimens of vertebrate species that have been examined, excepting humans with the debilitating mutation of atransferrinanemia (Welch, 1990; Welch, 1992). Saxiphilin expression in some animals may be more like that of the locally expressed transferrin homologs, melanotransferrin or lactoferrin, in vertebrates. The expression and secretion of these latter proteins in humans is mostly limited to the surface of melanoma cells and several other cell types in the case of melanotransferrin (Rose et al., 1986; Kennard et al., 1995); or, to neutrophils and particular fluid secretions such as milk in the case of lactoferrin (Lönnerdal & Iyer, 1995). The curious pattern of saxiphilin expression reflected in our results merits further investigation to determine what transcriptional and translational regulatory mechanisms may control its synthesis.

(c) What is the biological function of saxiphilin?

The simplest hypothesis for the function of saxiphilin is that it plays a defensive role against STX intoxication. However, this idea must be questioned since the most dramatic example of microbial STX production in nature occurs in conjunction with sporadic blooms of toxic marine dinoflagellates, and we did not find a consistent association of saxiphilin with species that may directly or indirectly be exposed to dinoflagellate toxins. In particular, mollusks such as *Mytilus edulis* and *Saxidomus giganteus* are known to bioaccumulate various STX derivatives (Shimizu et al., 1978; Schantz, 1986), but do not exhibit evidence of saxiphilin-like activity. The Atlantic mackerel, *Scomber scombrus*, has been found to contain STX in its viscera and such mackerel have previously been linked to the deaths of humpback whales (Geraci et al.,

1989). However, neither plasma from mackerel nor two whale species contained detectable saxiphilin activity (Table 2). Similarly, most other marine invertebrates, fish, and mammals that we sampled (albeit a limited selection) tested negative for saxiphilin-like activity. Two interesting exceptions are high levels of saxiphilin activity found in the eel, *Anguilla rostrada* (Table 1) which can adapt from saltwater to freshwater, and the recent finding of saxiphilin-like activity in particular species of marine Xanthid crabs from Australia (L. Llewellyn, personal communication). Aside from these latter two possible cases, the present data do not indicate that saxiphilin plays a universal or even a common role in animal encounters with toxic marine dinoflagellates.

In the freshwater ecosystem, STX and various STX derivatives have thus far been found to be produced by three genera of cyanobacteria: *Aphanizomenon*, *Anabaena* and *Lyngbya* (Ikawa et al., 1982; Carmichael et al., 1990; Negri et al., 1995; Carmichael, W., Bell, P. and Moczydlowski, E, unpublished results). The occurrence of saxiphilin in many amphibians and small freshwater fish suggests that it may directly function or may be secondarily recruited as a mechanism of STX detoxification in some of these species. A previous report of tadpole mortality in association with lake blooms of toxic *Aphanizomenon* blue-green algae (Ikawa et al., 1982) has prompted us to investigate the effect of STX on various species of frog tadpoles in the laboratory. This work, still in progress, does suggest that there is a correlation between a given tadpole species' resistance to STX and expression levels of saxiphilin (P. Bell and E. Moczydlowski, unpublished results).

Nevertheless, Table 2 shows that saxiphilin occurs in several animal species that would not be expected to be exposed to cyanobacterial toxins. For example, the lizard

Sceloporus poinsetti feeds on insects, spiders and vegetation and acquires moisture from condensation. Likewise, there is currently little reason to suspect that animals such as the centipede, garter snake, cobra or goanna lizard would encounter toxic levels of STX via water or food sources. Nevertheless, it may be that large gaps in our knowledge of STX production in microorganisms currently prevent an adequate evaluation of the role of STX/saxiphilin in the natural history of these animals. Aside from the finding that arginine may be a precursor to STX in *Aphanizomenon flos-aquae* (Shimizu et al., 1984), there is virtually no information on the enzymes or genes involved in STX biosynthesis. The mechanism of STX production by dinoflagellates is also obscure, although there is a report that endosymbiotic bacteria may be involved (Kodama et al., 1990). If certain pathogenic bacteria share the genes for STX production with cyanobacteria and dinoflagellates, saxiphilin may potentially represent a mechanism to counter the potential threat of STX-induced paralysis during a microbial infection.

Given numerous inconsistencies of the toxin defense hypothesis, a conservative interpretation of our data is that saxiphilin may be recruited as an anti-toxin defense mechanism in some animals (e.g., *Ranid* frogs), but that it has another more general function. Since the only known ligands that bind to saxiphilin are neuroactive STX derivatives, we continue to suspect that the voltage-sensitive Na^+ channel may be involved. Until recently, it could be argued that the guanidinium toxins, STX and tetrodotoxin, target a functionally essential structure of vertebrate Na^+ channels. However, the results of recent mutagenesis studies of mammalian Na-channels (Terlau et al., 1991) have identified certain amino acid residues that can be conservatively mutated to greatly reduce sensitivity to these toxins without seriously impairing the

electrophysiological function of the channel (i.e., voltage-dependent gating and ionic selectivity). When it is considered that binding of STX and tetrodotoxin to nerve Na^+ channels with nanomolar affinity is evolutionarily conserved from *Drosophila* to humans, we are led to the suggestion that the STX binding site on Na^+ channels may be conserved for a physiological reason-- such as the necessity of maintaining a binding site for a ligand that regulates Na^+ channel activity. Therefore, we speculate that in some animals saxiphilin may function in the recovery or sequestration of an endogenous ligand that regulates Na^+ channel activity. Given the widespread occurrence of STX and tetrodotoxin in nature, there may be other biological roles for these molecules such as the possibility that tetrodotoxin may act as a pheromone in the puffer fish (Matsumura, 1995).

In conclusion, this study has determined that soluble [^{3}H]STX binding activity characteristic of saxiphilin has a broad phylogenetic distribution among arthropods and vertebrates whose nervous systems rely on STX-sensitive Na^+ channels that function prominently in electrical excitability. The existence of these two distinct high-affinity binding sites for STX in a given organism raises suspicion of possible physiological relationship. Many questions regarding the function, expression and molecular evolution of saxiphilin as a transferrin-related protein remain to be resolved. Further exploration of the evolutionary and biological significance of saxiphilin may eventually unravel this biological mystery.

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FIGURE LEGENDS

Figure 1. Demonstration of saxiphilin-like activity in toad plasma, plasma from two different snake species, fish extract, and centipede hemolymph by titration of [^{3}H]STX binding. A constant amount of soluble protein from various animal species was assayed for binding at the indicated concentrations of [^{3}H]STX as described in Methods. Plasma samples and extracts were pre-equilibrated with [^{3}H]STX for 1-2 h before assay, except for *E. rubripes* hemolymph which was pre-equilibrated for 8 h. Total binding in cpm (○). Non-specific binding in the presence of 10 μM STX (●). Data points are the mean of duplicate determinations. Non-specific binding was fit by a simple linear regression and total binding was fit to a one-site model with K_D values given in the text. (a) *Bufo marinus* (cane toad) plasma, 72 μg protein/ml. (b) *Naja naja kaouthia* (Thailand cobra) plasma, 74 μg protein/ml. (c) *Thamnophis sirtalis* (garter snake) plasma, 103 μg protein/ml. (d) *Gambusia affinis* (mosquito fish) extract, 180 μg protein/ml. (e) *Ethmostigmus rubripes* (centipede) hemolymph, 360 μg protein/ml.

Figure 2. Competitive inhibition of [^{3}H]STX binding by unlabelled STX and four different STX derivatives. (a) Chemical structure of naturally occurring STX derivatives used in this study: STX (○), $R_1 = CONH_2$, $R_2 = H$, $R_3 = H$; dcSTX (◆), $R_1 = H$, $R_2 = H$, $R_3 = H$; neoSTX (Δ), $R_1 = CONH_2$, $R_2 = OH$, $R_3 = H$; B1 (▼), $R_1 = CONHSO_3^-$, $R_2 = H$, $R_3 = H$; C1 (❑), $R_1 = CONHSO_3^-$, $R_2 = H$, $R_3 = OSO_3^-$. (b) *Naja naja kaouthia* plasma, 40 μg protein/ml. (c) *Gambusia affinis* extract, 180 μg protein/ml. (d) *Ethmostigmus rubripes* hemolymph 180 μg protein/ml. Data points are the mean of duplicate determinations.

Figure 3. pH dependence of [^{3}H]STX binding. Panel (a) shows results for *Bufo marinus* plasma (●, 72 μg protein/ml), *Naja naja kaouthia* plasma (▼, 74 μg protein/ml), and *Gambusia affinis* extract (Δ, 180 μg protein/ml). Panel (b) shows results for *Thamnophis sirtalis* plasma (◆, 41 μg protein/ml) and *Ethmostigmus rubripes* plasma (○, 360 μg protein/ml). Data points and error bars are the mean ± SEM of three experiments. Solid and dashed curves represent fits of the data to a logistic function of [H^+] given in Methods. Best-fit values of $pH_{0.5}$ and n' are listed in Table 3.

Figure 4. Time course of dissociation and association for [^{3}H]STX binding compared for different species. (a, e) *Bufo marinus* plasma. (b, f) *Naja naja kaouthia* plasma. (c, g) *Gambusia affinis* extract. (d, h) *Ethmostigmus rubripes* hemolymph. Dissociation (a, b, c, d) or association (e, f, g, h) time course of specific [^{3}H]STX binding was measured as described in Methods. Data were obtained at pH 7.4 except for a, c and e, where dissociation at pH 7.4 (◆) is compared to that at various acidic pH values (○) as follows: (a) pH 4.3, (b) pH, 5.3, and (c) pH 4.5. Data points are fit (solid lines) to a single exponential function (a, d, e, f, h) or to a sum of two exponentials (b, c, g) as described in the text.

Table 1. [^{3}H]STX binding in species found to exhibit saxiphilin-like activity. Data are results of a standard assay for binding of 5 nM [^{3}H]STX as described in Methods. Effective concentration of [^{3}H]STX binding sites per ml plasma or g tissue is reported as the mean ± SD (n) where n is the number of determinations. Abbreviations: P, plasma; H, hemolymph; E, extract; F, freshwater species; M, marine species; n. d., not determined.

Species (common name and geographical origin)	[^{3}H]STX binding sites pmol / ml plasma or g tissue	pmol / mg protein
Teleost Fish (except lungfish)		
Hypostomus plecostomus (catfish, S. America) E, F	20 ± 3 (4)	6.7
Poecilia reticulata (guppy, Americas) E, F	72 ± 9 (4)	15.8
Anguilla rostrata (eel, N. America) E, M, F	2.5 ± 0.8 (4)	n. d.
Gambusia affinis (mosquito fish, N. America) E, F	72 ± 8 (4)	3.2
Pomacentrus sp. (damselfish, N. America) E, M	1.9 ± 0.1 (2)	0.1
Apogon sp. (cardinalfish, N. America) E, M	29 ± 1.3 (3)	1.0
Danio rerio (zebrafish, Asia) E, F	6.3 ± 0.3 (3)	0.3
Protopterus aethiopicus (lungfish, Africa) E, F	0.5 (1)	0.02
Amphibians		
Notopthalamus viridescens (Eastern newt, N. America) E	1.5 ± 0.5 (11)	0.2
Ambystoma tigrinum (tiger salamander, N. America) P	76 ± 2 (3)	3.3
Rana sylvatica (wood frog, N. America) P	1590 ± 440 (18)	68
Rana temporaria (grass frog, Europe) P	669 ± 18 (3)	34
Bufo marinus (cane toad, S. America) P	49 ± 5 (8)	1.9
Reptiles		
Varanus rosenbergii (goanna monitor lizard, Australia) P	326 ± 26 (4)	5.0
Sceloporus poinsetti (crevice spiny lizard, N. America) P	1100 ± 109 (6)	491
Naja naja kaouthia (Thailand cobra, Asia) P	223 ± 20 (3)	7.6
Crotalus viridus viridus (rattlesnake, N. America) P	2.4 ± 0.5 (3)	0.1
Thamnophis ordinoides (garter snake, N. America) P	133 + 89 (4)	10.7
Thamnophis sirtalis (garter snake, N. America) P	486 ± 322 (6)	19.8
Arthropods		
Daphnia sp. (waterflea, N. America) E	0.8 ± 0.2 (2)	n. d.
unidentified cockroach (Australia) E	0.2 ± 0.03 (3)	0.02
Ethmostigmus rubripes (centipede, Australia) H	79 ± 18 (3)	2.2
Araneus c. f. cavaticus (orb web spider, N. America) E	2.9 ± 0.7 (7)	n. d.

Table 2. List of tested species that did not contain detectable [^{3}H]STX binding activity in plasma (P), hemolymph (H), or soluble extracts (E).

Vertebrata (Super-class Agnatha)

Mordacia mordax (lamprey) E

Vertebrata (Super-class Gnathostomes)

Squalus acanthius (dogfish shark) P
Ginglymostoma cirratum (nurse shark) P
Negaprion brevirostrio (lemon shark) P
Somniosus microcephalus (Greenland shark) P
Raja erinacea (little skate) P
Scomber scombrus (Atlantic mackerel) P
Cyclopterus lumpus (lumpfish) P
Pseudopleuronectes americanus (flounder) P
Makaira indica (marlin) P
Arothron manilensis (puffer fish) E
Latimeria chalumnae (coelocanth) E

Vertebrata (Class Amphibia)

Xenopus laevis (African clawed frog) P

Vertebrata (Class Reptilia)

Elseya dentata (snapping tortoise, Australia) P
Dermochelys coriacea (leatherback turtle) P
Pseudemys scripta (red ear turtle, N. America) P
Caretta caretta (loggerhead turtle) E
Sphenodon punctata (tuatara, New Zealand) P
Notechis scutatus (Australian tiger snake) P
Alligator mississippiensis (alligator, N. America) P
Crocodylus porosus (crocodile, Australia) E

Vertebrata (Class Aves)

Gallus gallus (chicken) P
Anser anser (domestic goose) P
Anas sp. (duck) P
Columba lives (domestic pigeon) P
Meleagris gallopavo (common turkey) P

Vertebrata (Class Mammalia)

Equus caballus (horse) P
Oryctolagus sp. (rabbit) P
Ovis aries (domestic sheep) P
Homo sapiens (human) P
Rattus norvegicus (rat) P
Bos taurus (cow) P
Sus scrofa (pig) P
Lagenorhynchus acutus (whitesided dolphin) P
Globicephala malaena (pilot whale) P
Delphinus delphis (common dolphin) P
Balaenoptera acutorostrata (minke whale) P

Sub-phylum Urochordata (Class Asidiacea)

Polycarpa sp. (ascidian) E

Phylum Echinodermata

unidentified sea urchin (Australia) E
Strongylocentrotus droebachiensis (sea urchin) E
Acanthaster planci (crown-of-thorns starfish) E
Asterius forbesii (starfish) E
Asterius vulgaris (starfish) E
Solaster endeca (starfish) E
Cucumaria frondosa (sea cucumber) E
Echinarachnius parma (sand dollar) E
Tunica mogula (tunicate) E

Phylum Arthropoda

Penaeus monodon (tiger prawn) H
Cancer borealis (Jonah crab) H
Pagurus sp. (hermit crab) H
Homarus americanus (lobster) H
Ocypode corimana (ghost crab)
Artemia salina (brine shrimp) E
unidentified millipede (Australia) E
Manduca sexta (tobacco hornworm moth) H
Blaberus sp. (cockroach) H, E
Drosophila melanogaster (fruit fly) E
Cicada sp. (cicada) E

Phlyum Mollusca

Mytilus edulis (blue mussel) H, E
Saxidomus giganteus (butter clam) H, E
Aplysia californica (sea hare) H, E
Spisula solidissma (clam) E
Acmaea c. f. testudinalis (limpet) E
Littorina litorea (periwinkle) E

Phylum Platyhelmintes

Pseudoceros sp. (flatworm) E

Phylum Cnidaria

Sarcophyton elegans (soft coral) E

Phylum Brachiopoda

Lingula sp. (brachiopod) E

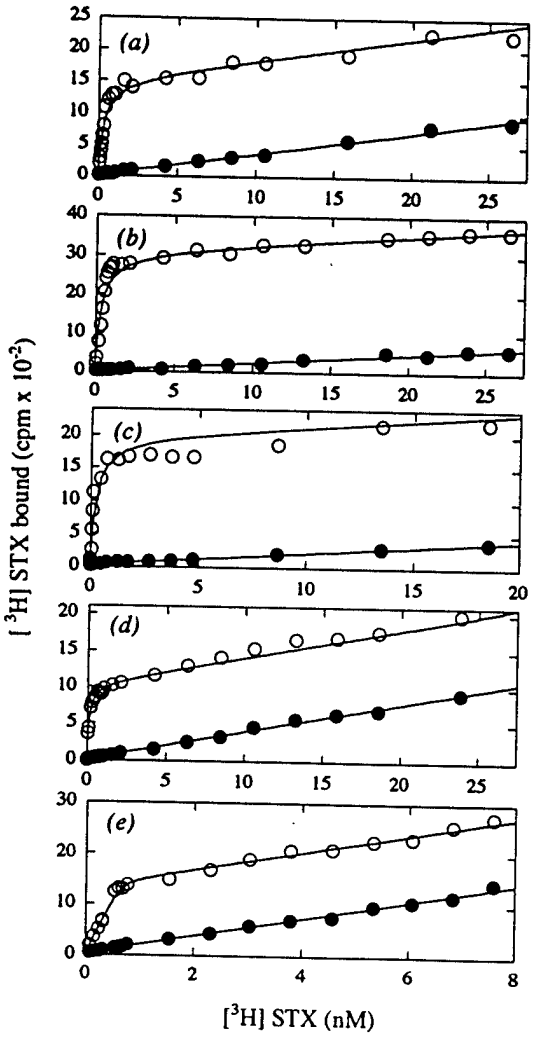
Phylum Annelida

Gastrolepida clavigea (annelid) E
Glycera dibranchiata (bloodworm) H, E

Table 3. Comparison of structure-activity relationships and pH dependence of [^{3}H]STX binding for various species. [^{3}H]STX binding to plasma or extracts was titrated with unlabelled STX derivatives or H^+ as in Figs. 2 and 3. IC_{50} values for STX derivatives, pH values for 50% inhibition and pseudo Hill (n') coefficients were determined by fitting data to logistic functions given in Methods. Values in parentheses are ratios relative to STX for each species. Data for *Rana catesbeiana* are taken from Mahar et al. (1991) and Llewellyn and Moczydlowski (1994).

	IC_{50} (nM)					pH dependence	
Species	STX	dcSTX	NEO	B1	C1	$pH_{0.5}$	n'
Rana catesbeiana	4.7 ± 0.7 (1.0)	11.1 ± 1.2 (2.4)	2640 ± 180 (560)	4.5 ± 0.4 (1.0)	730 ± 160 (155)	5.7	1.0
Bufo marinus	5.3 ± 0.9 (1.0)	10.8 ± 1.6 (2.0)	32.3 ± 2.4 (6.1)	19.0 ± 2.1 (3.6)	7540 ± 620 (1420)	4.4	1.1
Thamnophis sirtalis	4.0 ± 2.1 (1.0)	1.4 ± 0.2 (0.4)	6.0 ± 1.7 (1.5)	253 ± 25 (63)	3480 ± 190 (870)	5.5	1.4
Naja naja kaouthia	4.0 ± 0.4 (1.0)	1.7 ± 0.3 (0.4)	20.8 ± 1.7 (5.2)	118 ± 5 (30)	7540 ± 460 (1890)	5.4	2.1
Gambusia affinis	5.0 ± 0.3 (1.0)	7.7 ± 0.7 (1.5)	11.8 ± 0.8 (2.4)	179 ± 10 (36)	63000 ± 2500 (12600)	4.7	2.0
Ethmostigmus rubripes	5.0 ± 0.3 (1.0)	6.1 ± 0.9 (1.2)	8.5 ± 0.4 (1.7)	20.3 ± 1.2 (4.1)	2830 ± 120 (566)	5.4	3.2

FIGURE 1



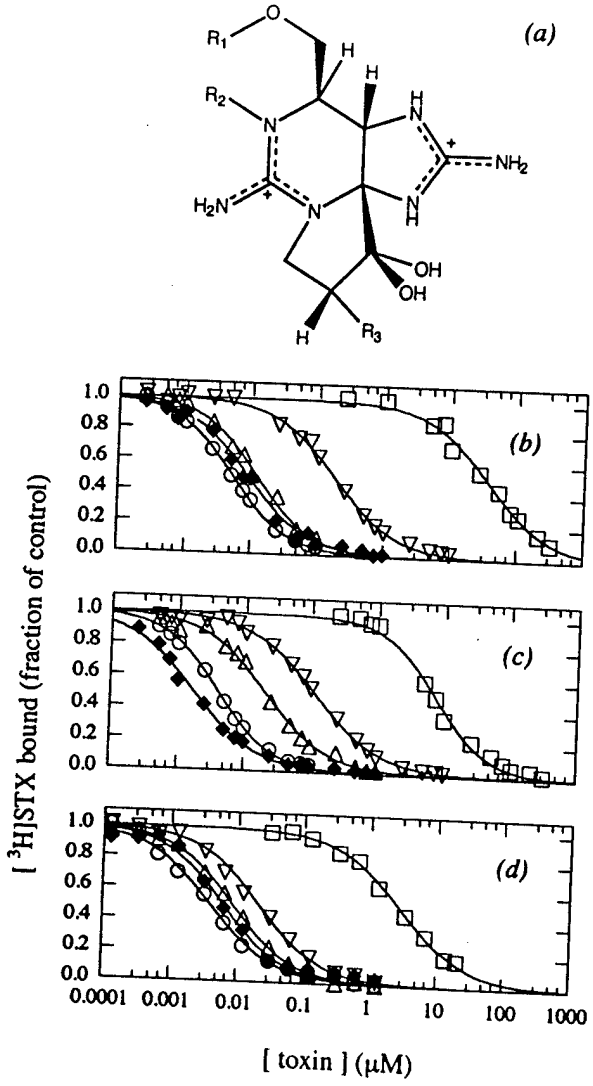

(a)
R1
O
H
H
R2
N
H
N
+
NH2
H2N
+
N
N
H
OH
OH
H
R3
(b)
(c)
(d)
1.0
0.8
0.6
0.4
0.2
0.0
[3H]STX bound (fraction of control)
0.0001
0.001
0.01
0.1
1
10
100
1000
[toxin] (μM)

FIGURE 3

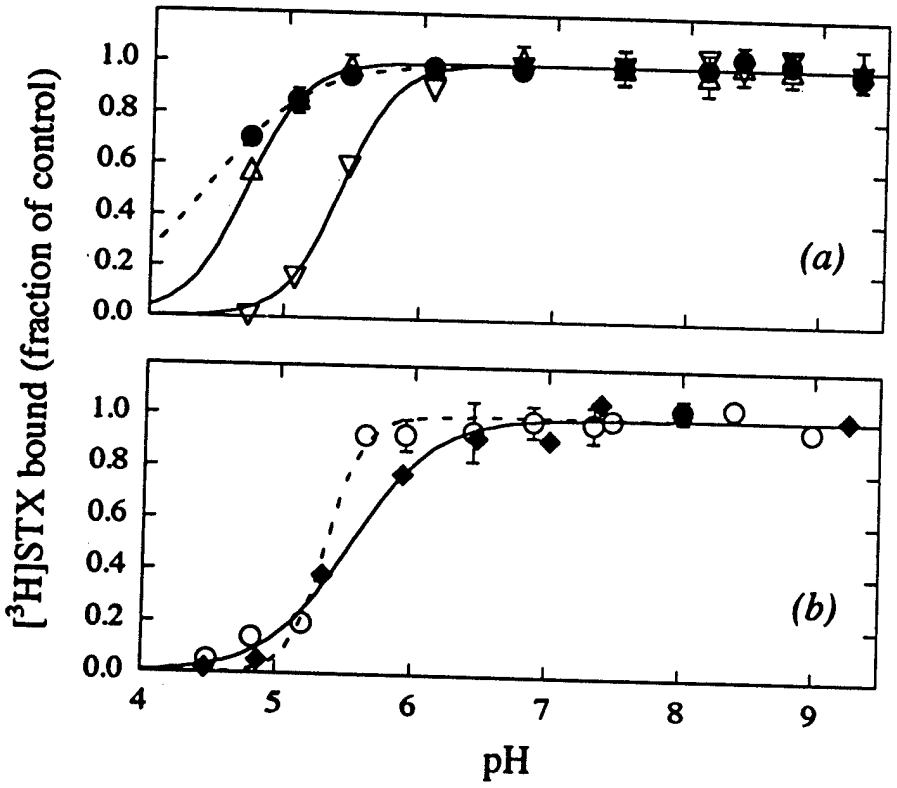


FIGURE 4