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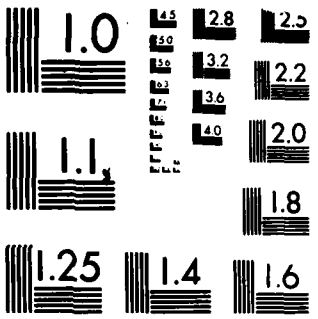

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THE STUDY OF CONFORMATIONAL FLUCTUATIONS IN PROTEINS BY USE OF OPTICAL PROBES

Final Technical Report

by

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January 1984

United States Army
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The purpose of this research was to ascertain the existence and to study the configurational fluctuations in proteins, fluctuations which enable small molecules to penetrate the protein structure. To this end the quenching of the tryptophan phosphorescence in Liver Alcohol Dehydrogenase by oxygen and by iodide ions was studied. The quenching could be interpreted in terms of diffusion of the quenchers through the protein network while its temperature dependence measured the activation energy necessary for the above flucuations to take place. In addition to using tryptophan also the study of porphyrins as

potential optical probes in proteins was undertaken. The excited state electron transfer reactions of the model compound Zn octoethylporphyrin, which constitute the main pathway for its deexcitation, were investigated with a view of using similar redox systems to quench the Zn porphyrin luminescence when incorporated in apomyoglobin instead of the non luminescent heme group. Zn substituted myoglobin was prepared and its optical properties studied.

1. Abstract

The purpose of this research was to ascertain the existence and to study the configurational fluctuations in proteins, fluctuations which enable small molecules to penetrate the protein structure. To this end the quenching of the tryptophan phosphorescence in Liver Alcohol Dehydrogenase by oxygen and by iodide ions was studied. The quenching could be interpreted in terms of diffusion of the quenchers through the protein network while its temperature dependence measured the activation energy necessary for the above fluctuations to take place. In addition to using tryptophan also the study of porphyrins as potential optical probes in proteins was undertaken. The excited state electron transfer reactions of the model compound Zn octoethylporphyrin, which constitute the main pathway for its deexcitation, were investigated with a view of using similar redox systems to quench the Zn porphyrin luminescence when incorporated in apomyoglobin or apohemoglibin instead of the non luminescent heme group. Zn substituted myoglobin was prepared and its optical properties studied.

Key Words: Optical Probes, Protein Phosphorescence, Delayed Fluorescence, Porphyrin Luminescence, Liver Alcohol Dehydrogenase, Zn Octoethylporphyrin, Zn Protoporphyrin.

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2. Introduction

Based on the structures from X-ray diffraction data proteins have for many years been treated as though they were semirigid solids susceptible to conformational rearrangements only upon interacting with small (substrate) molecules. In the last ten years, however, a number of studies using different techniques have indicated that proteins in aqueous solution do not have a well defined rigid structure but that they undergo conformational fluctuations. For example, the results of hydrogen exchange in lysozyme (1) have been interpreted in terms of such fluctuations enabling small molecules to penetrate the protein coil. NMR studies of the aromatic amino acid residues in pancreatic trypsin inhibitor indicate a rotational freedom of the aromatic rings which can only be due to the movement of the polypeptide chains within the protein interior (2). Even the X-ray diffraction data are now reinterpreted to mean that in large parts of the protein structure the amino acid side chains are not rigidly fixed in space but have (a limited) freedom of movement (3). Similarly conformational fluctuations are also predicted by molecular dynamics calculations, lately reviewed by Karplus and Mc Cammon (4), which show that rapid side chain movements are indeed expected to occur in the protein interior.

Of particular relevance for the investigations described here are the studies of Lakowicz and Weber on the fluorescence quenching of the inherent optical probe, the tryptophan residue, in globular proteins (5). These workers were able to show that oxygen diffuses through the protein network almost as readily as through an aqueous solution. Since the tryptophan residues quenched are mostly located in the interior of the protein, where the amino acids are in close Van der Waals contact, the conclusion was reached that rapid conformational fluctuations are necessary in order to create the pathways for oxygen diffusion. On the other hand, Saviotti and Galley have shown that the long lived phosphorescence of tryptophan residues can be observed in aqueous solutions of Liver Alcohol Dehydrogenase (LADH) at room temperature (6). Oxygen quenches this emission with a rate which is by four orders of magnitude smaller than the fluorescence quenching observed by Weber and Lakowicz in other proteins. These findings were interpreted to mean that quenching by oxygen takes place by a mechanism in which large conformational changes expose internal tryptophan residues periodically to the ambient solutions containing oxygen. Lately the room temperature fluorescence and phosphorescence quenching of LADH has been reinvestigated by Calhoun et al. (7). These workers find a rapid quenching of fluorescence and phosphorescence by O_2 but a very much slower quenching rate for the LADH luminescence by I^- and by other small molecules.

The most frequently used inherent optical probe in protein structural research is the tryptophan residue. The difficulty with using this optical probe lies in the fact that most proteins contain a number of such residues. The data obtained in such cases describe therefore the sum of processes for the various tryptophan residues and thus make the interpretation of the results uncertain. The attaching of protein unrelated marker chromophores or fluorophores to a protein suffers from two drawbacks: a) of introducing a foreign molecule into the protein interior and thus possibly influencing its delicate structural balance and b) the uncertainty of the actual location of the marker within the protein. The use of inherent luminescent probes other than the aromatic amino acid residues in protein structural research has not as yet been fully exploited and is one of the objectives of this research.

3. Objectives of Research and Outlay of Report

The objectives of the research described here were:

a) to measure the diffusion of small molecules through a protein made possible by conformational fluctuations of the latter;

b) to evaluate the energetics of the fluctuations from the temperature dependence of the above diffusion;

c) to develop almost - inherent optical probes for structural studies in proteins.

The report deals with structural fluctuations in protein measured over a long time span (miliseconds) by means of quenching of its excited triplet state. For a diffusion limited quenching process the temperature dependence of the quenching enables us to evaluate the energetics of the above conformational fluctuations.

The work describes a) the use of a tryptophan residue as optical probe in the structurally well defined protein system of LADH; b) the study of the optical properties of porphyrins with a view of using them as probes in myoglobin and in hemoglobin.

4. Liver Alcohol Dehydrogenase (LADH)—(submitted for publication)

Phosphorescence of tryptophan residues has long since been observed at room temperature in oxygen free aqueous solutions of LADH (6). The structure of LADH has been reviewed by Branden et al. (8). The enzyme is composed of two subunits each of which contains one tryptophan residue (Trp 15) which is located on its periphery and one tryptophan (Trp 314) which is buried in the area of interaction between the subunits. The room temperature phosphorescence here described stems from Trp 314. The phosphorescence lifetimes reported range from 83 ms (9) through 120 ms (10) to 300 ms (7). Rate constants for the oxygen quenching of this phosphorescence of $k_q = 6 \times 10^8\ M^{-1}sec^{-1}$ and of $7 \times 10^4\ M^{-1}sec^{-1}$ have been found by Calhoun et al. (7) and by Saviotti and Galley (6) respectively. Here we describe the quenching and its temperature dependence of Trp-314 in LADH by oxygen and by potassium iodide.

Methods and Materials

Alcohol Dehydrogenase from horse liver (LADH) was obtained from Böehringer (Mannheim) in 10 ml batches as a suspension in 0.02 M potassium phosphate buffer pH 7 and was stored at 3°C. Its biological activity, as stated by the manufacturer, is about 2.7 units/mg. The enzyme did not deteriorate by more than ten percent during the time one batch of LADH was used. Enzyme solutions which had an activity of less than 2.4 units/mg were discarded. The biological activity of the various enzyme lots used by us was fairly uniform. However, although the fluorescence yield did not change appreciably from one lot to another, the phosphorescence intensity and lifetime in a few of the shipments were up to a factor of two lower than in the majority of the batches. Triply distilled water and reagents of at least analytical grade were used throughout. Solutions were prepared by dissolving 0.2 to 0.5 ml of the LADH suspension in 2×10^{-3} M phosphate buffer, pH 7.5, and precipitating any non dissolved particles by centrifugation. The solution was dialysed overnight at 4°C against the above buffer. Spectroscopic Grade glycerol (Matheson, Coleman and Bell), was added to obtain a final solution of 1:3 glycerol-water with an absorbance of OD ≈ 0.2 at 300 nm.

The glycerol greatly facilitates the degassing procedure used for freeing the solutions from dissolved oxygen (see below). Such solutions when cooled gradually and then stored at 4°C were generally stable for at least two days. Only after prolonged periods at 4°C or if left for a day at room temperature did the enzymatic activity decrease.

The solution was placed in a cell assembly consisting of a 1 cm optical path square specrosil cell with a Dewar-flask-like side arm and fitted with a teflon high vacuum stopcock. This assembly was attached to the vacuum line for dexoygenation. The LADH solution was contained between the walls of the side arm thermos flask and liquid nitrogen was poured into its inner part. When evacuating the assembly, about half the solvent distilled and froze onto the cold surface of the inner part of the above Dewar flask. The LADH solution remained liquid while the oxygen freed during the above distillation was pumped off. When the liquid nitrogen was removed from the thermos flask, the distilled (oxygen free) solvent dropped back into the LADH solution. The solution was gently stirred for about ten minutes with a teflon encased magnetic stirrer in order to equilibrate the vapor phase with the residual oxygen present. By repeating this procedure three to four times, the enzyme solution could be freed from oxygen down to about 10^{-9} M. It was found that by this procedure the LADH did not undergo denaturation and coagulation during deoxygenation. The stopcock closed cell assembly was detached from the vacuum for light emission measurements.

The assembly, with the solution transferred to the optical cell, was placed in a spectrofluoro-phosphorimeter equipped with a light chopper in the path of the exciting Xe arc. Fluorescence was measured by photon counting during the "light on" periods while phosphorescence was measured in the "light off" periods. Phosphorescence lifetimes were determined in the same apparatus without the chopper running. The light illuminating the solution was shut off by a milisecond shutter and the subsequent decay of the emission was recorded on a storage oscilloscope. The temperature dependence of the emission was measured by placing the curvette in an Oxford Instruments cryostat which could be fitted into the cell compartment of our apparatus.

The phosphorescence quenching by oxygen was measured by equilibrating the LADH solution, after dexoygenation and while still attached to the vacuum line, with a predetermined pressure of oxygen. The concentration of oxygen in air saturated 1:3 glycerol-water (2.1×10^{-4} M), and Henry's law were used to calculate the actual O_2 concentration in solution (11). Quenching by I^- ions was measured by adding to the LADH solution the required amount of KI in solution (containing 0.1 mole percent thiosulfate with respect to KI).

Results

The rate constants for the quenching of the Trp-314 phosphorescence in LADH by the apolar oxygen molecule and by the I^- ion were measured in a 1:3 glycerol water solution as a function of temperature. The spectrum of this phosphorescence in the above solvent mixture is given in Figure 1. It is very similar to the spectrum in aqueous solution described before (6). The room temperature phosphorescence yield of LADH, ϕ_p (LADH) = 0.006, was estimated from the phosphorescence to fluorescence emission ratio. The value of ϕ_f(LADH) = 0.12 in turn was based on the quantum yield of the N-acetyl tryptophan amide (NATA) fluorescence ϕ_f(NATA) = 0.16. In the quenching experiments the decrease in phosphorescence intensity and in lifetime were measured at 438 nm. The main difficulty in these experiments lies in the heterogeneity of the LADH samples

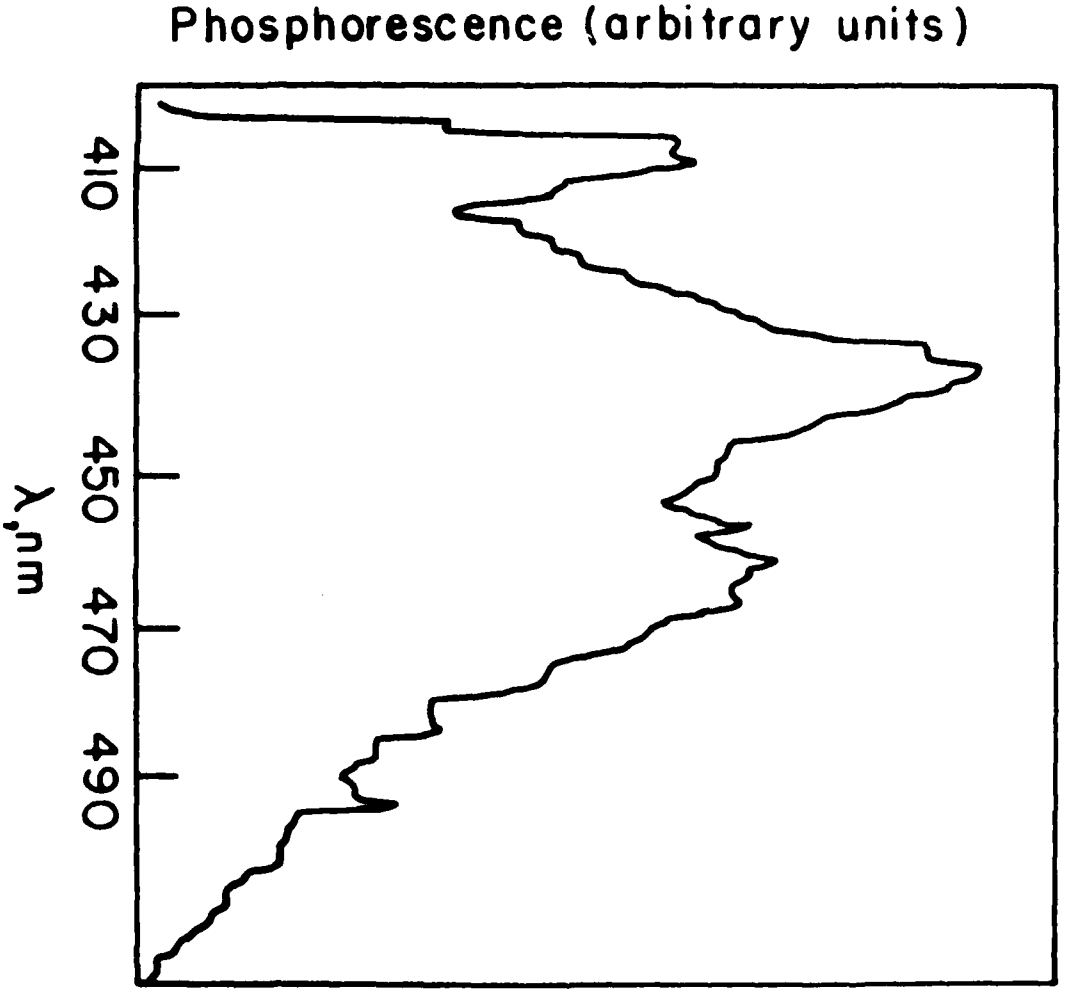


Figure 1

and in the instability of the enzyme solution when cooling it to zero degrees centigrade. Although the biological activity of the LADH was the same, the phosphorescence intensity and lifetime differed sometimes slightly from solution to solution which had been prepared from the same batch. It is possible that this behavior is due to a slight conformational heterogeneity in the particles of the original LADH suspension used to prepare the solution, a heterogeneity which is not reflected in the biological activity. Because of this heterogeneity and lability (see below) of solutions, each experiment had to be done in duplicate. An enzyme solution was prepared and divided into two parts. One part was deoxygenated in the cell assembly and its emission recorded in absence of quencher. The second part in an identical assembly and deoxygenated simultaneously with the first part was measured with a predetermined concentration of quencher added to the solution. Each such pair of solutions was measured at a number of temperatures. The LADH activity was frequently checked before and after experiments and was usually found not to have changed. However, in some cases the clear LADH solution turned turbid in the course of the cooling and reheating cycles indicating that some enzyme had precipitated. Only those experiments in which the cooling and reheating did not visibly affect the solution were retained.

Figure 2 shows the Stern-Volmer plot for quenching of the tryptophan phosphorescence in LADH by oxygen. The excitation wavelength was 300 nm. Data derived from both the decrease in intensity and the decrease in tryptophan triplet lifetime are presented for various oxygen concentrations. It is seen that the same quenching constant $K_{SV} = 1.4 \times 10^7\ M^{-1}$ is obtained from the two sets of data. The corresponding rate constant, k_q, for oxygen quenching at 22°C was obtained from K_{SV} and from the triplet lifetime which at 22°C was found to be τ = 100 ms. The value of the quenching rate constant is therefore $k_q = 1.4 \pm 0.2 \times 10^8\ M^{-1}\ sec^{-1}$. The room temperature data in Fig. 2 were obtained by repeatedly equilibrating solutions with increasing amounts of oxygen. This procedure did not affect the enzyme solution and its activity. At lower temperature the stability of the LADH solutions decreased as described above. It was therefore necessary to prepare fresh solutions for each quencher concentration. Because of the inherent instability and variability in the different solutions as well as the uncertainty in the exact concentration of oxygen dissolved in them, the data are less accurate than those at 22°C. This is reflected in the large error bars in Fig. 3. This Figure shows the temperature dependence of the rate constant. An activation energy of about 14 kcal/mol with an estimated error of ±25% was obtained for the quenching of tryptophan 314 by oxygen. The rate constant for the quenching of this "inner" tryptophan residue by I^- was also determined as a function of temperature. At room temperature a value of $k_q = 1.2 \pm 0.2 \times 10^8\ M^{-1} sec^{-1}$ was obtained. Similar to the quenching by oxygen again an activation energy of about 14±3.5 kcal/mol was found.

Within the experimental error the same quenching rate constant and activation energy was obtained from both the decrease in intensity (or quantum yield (ϕ)) and lifetime (τ), of the tryptophan emission (Figure 2). This means that the value of ϕ/τ is approximately constant for the phosphorescence of the buried tryptophan residue in LADH indicating that the quenching at the very low quencher concentration used here is essentially a dynamic effect and is not due to the formation of ground state complexes.

We did not observe in our experiments illumination dependent effects on the LADH phosphorescence (10). It seems that at the low light intensities (150 W Xe arc, double monochromator, 5 nm band pass slits) and low oxygen concentra-

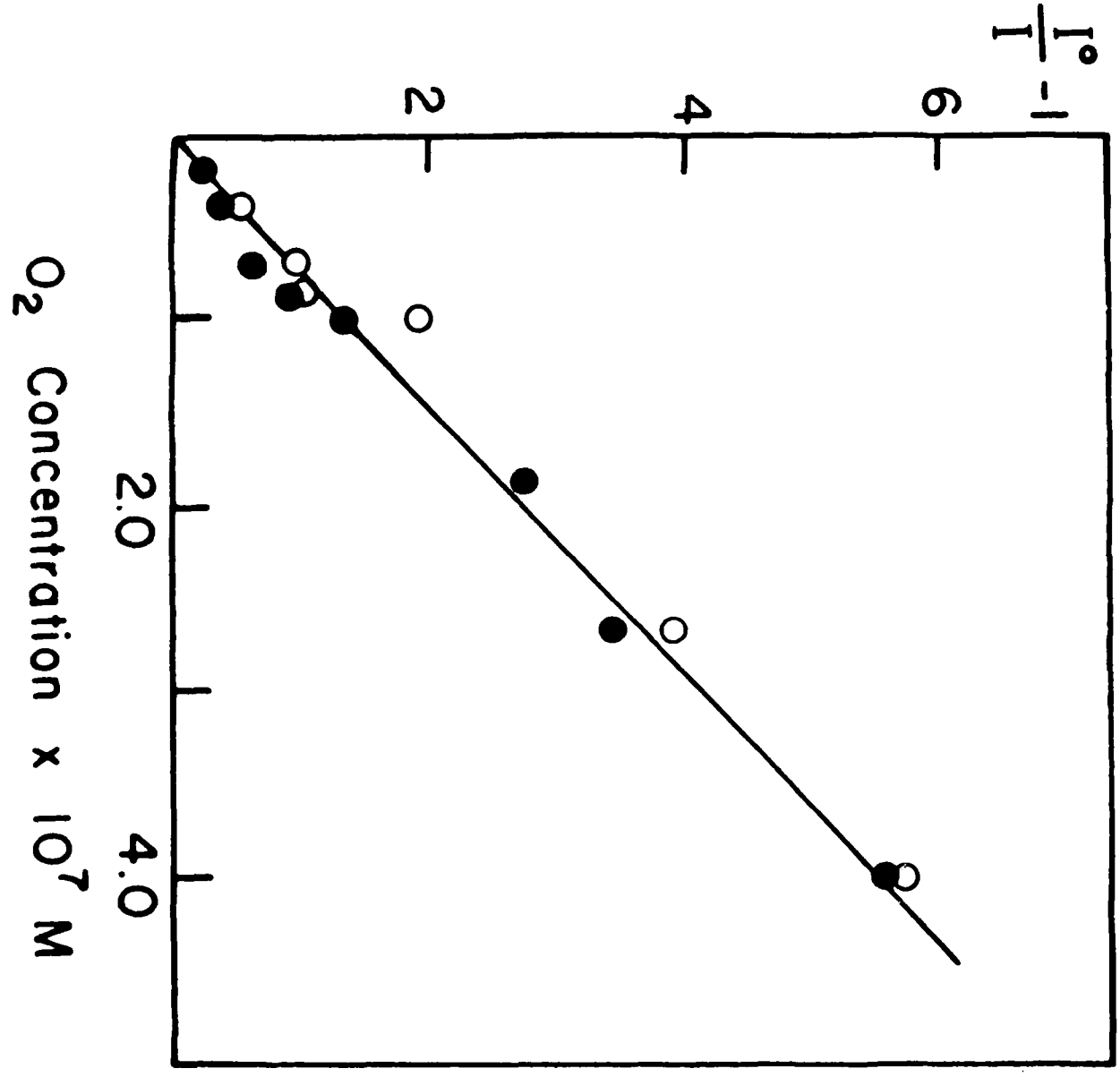


Figure 2

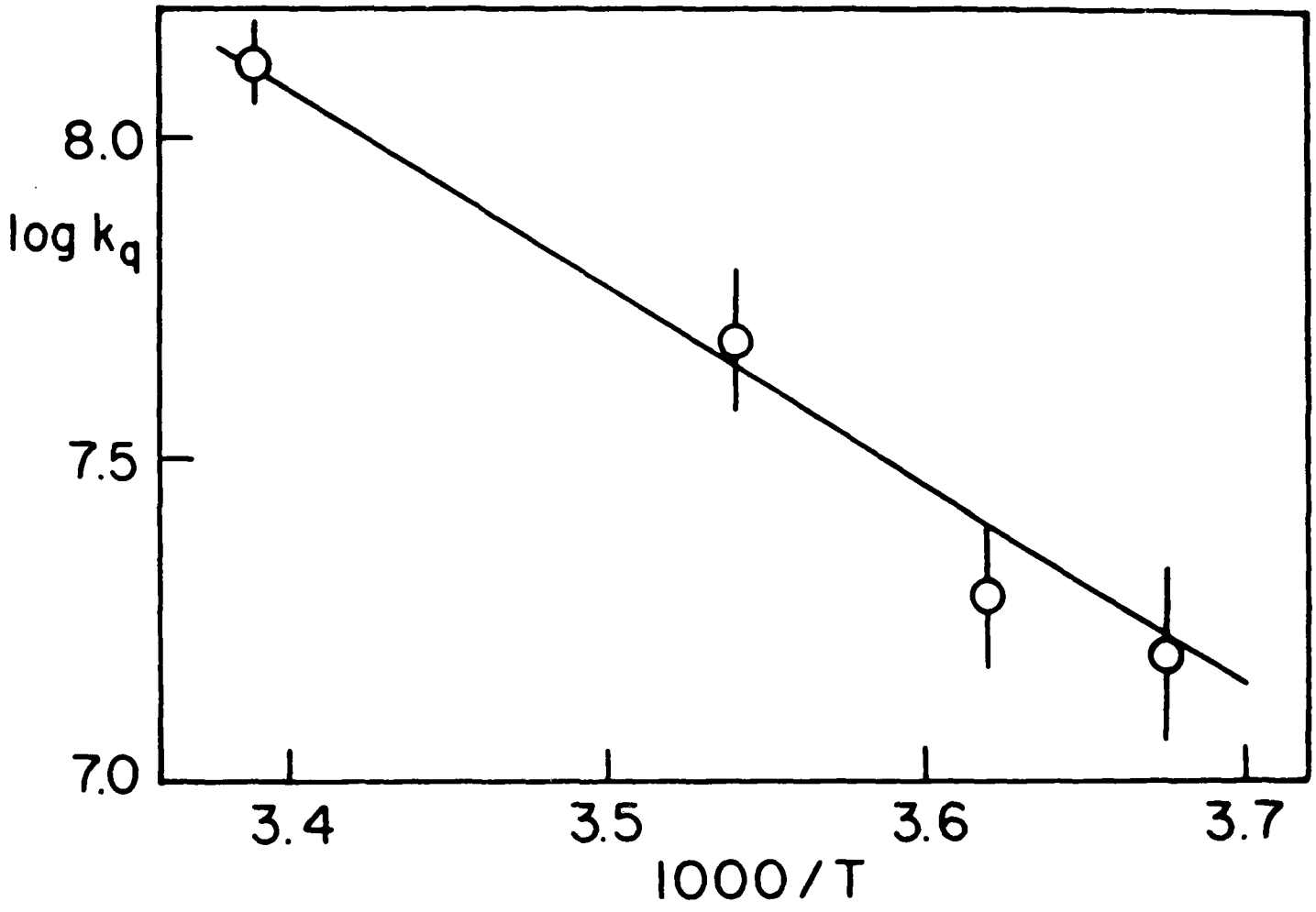


Figure 3

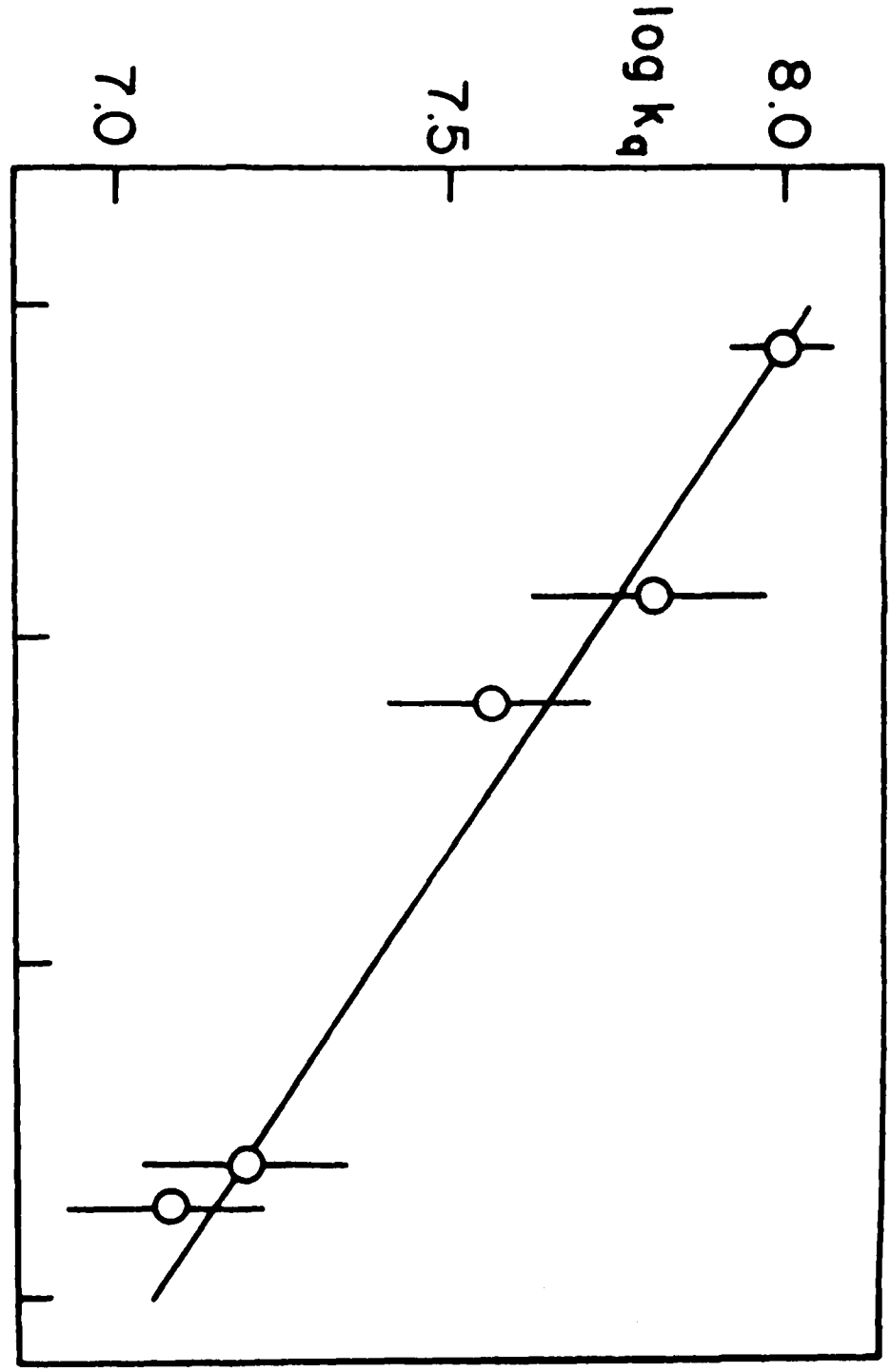


Figure 4

tions employed the rate of photochemical O_2 depletion is very low.

Discussion

Our rate constant for the quenching of the Trp-314 triplet state in LADH is not very different from the value of 6×10^8 $M^{-1}sec^{-1}$ obtained by Calhoun et al. (7). Both indicate an almost free access of the protein interior to O_2. On the other hand, our rate constant for the Trp-314 quenching by I^-, $k_q = 10^8$ $M^{-1}sec^{-1}$, is much larger than the value of $k_q = 10^5$ $M^{-1}sec^{-1}$ found by Calhoun et al. We have no ready explanation for this difference. Both the shorter lifetime and the higher value of k_q indicate a less tight protein structure and hence a less well protected Trp-314 in our system. As mentioned in Methods and Materials, even preparations from the same supplier show some variability in their triplet state properties. It is possible that either the different preparations (Boehringer vs. Sigma) and/or the lower ionic strength (0.0015 M vs. 0.03 M buffer) and presence of glycerol cause the protein structure to become somewhat more penetrable to the quencher. Our main interest however, lies not in the absolute values but in the temperature dependence of the above quenching rate constants.

The activation energy of $E_a \approx 14$ kcal/mole found by us is uncommonly high for a fast process with a rate constant in the 10^8 $M^{-1}sec^{-1}$ range. In terms of ordinary reaction kinetics this would mean an unrealistically large pre-exponential factor $A = 10^{18}$ $M^{-1}sec^{-1}$ in the Arrhenius equation. A high activation energy of 9 to 11 kcal/mol for quenching of the tryptophan fluorescence in proteins by acrylamide has also been reported by Eftink and Ghiron (12). In these experiments the pre-exponential factor reached a value of $A = 10^{15}$ to 10^{16} M^{-1} sec^{-1}. We shall now try to examine this kinetic behavior.

The fluorescence of N-acetyl tryptophan amide, NATA, the model for tryptophan residues in proteins, is quenched by KI in aqueous glycerol (25%) with a rate constant of $k_q = 3 \times 10^9$ $M^{-1}sec^{-1}$ which falls into the range of almost diffusion limited reactions. Hence we attribute this rate constant to the diffusion of I^- ions through the liquid rather than to the deactivation step of the excited singlet. We assume that the same rate constant will also describe the quenching of the NATA triplet state. The diffusion controlled rate constant k_q is proportional to the diffusion constant D. The activation energy of D and hence of k_q can be estimated from the temperature dependence of the solvent viscosity by use of the Einstein-Stokes reciprocal relation $D \propto kT/\eta$. One obtains, from the viscosity data for 1:3 glycerol-water, (Landolt Bornstein Tables) a value of $E_a = 4.5$ kcal/mol. Therefore with $k_q = 3 \times 10^9$ $M^{-1}sec^{-1} = A_d \exp(-E_a/RT)$ the pre-exponent for the diffusion limited rate constant at room temperature is $A_d \approx 10^{13}$ $M^{-1}sec^{-1}$. If, as we assume, also the rate of quenching of the tryptophan residues buried in the interior of the protein is determined by the diffusional movement of the quencher molecules through the tightly packed protein network rather than to the deactivation process, it stands to reason that this diffusion is made possible by temporal displacements of the polypeptide side chains which otherwise are in close Van der Waals contact. Diffusion has been proposed to account for fluorescence quenching of tryptophan residues in a number of proteins (5,12). However, the diffusional mechanism of luminescence quenching in LADH has been questioned and a process has been proposed in which segments of the protein structure collapse providing short-lived "open" gates in the otherwise "closed" protein structure (7). Our results support a model of quencher diffusion through temporary created channels in the protein network.

X-ray diffraction data (3) and a variety of dynamic measurements as well

as molecular dynamics calculations which have been reviewed by Karplus (4), all indicate conformational fluctuations and a certain degree of flexibility, in the structure of proteins. However, mostly the average atomic displacements in the interior part of the protein reach a magnitude of about 0.5Å only. For the diffusion of large ions or small molecules of, say a radius of ∿2Å, through the protein network to take place it is likely that larger displacements of the polypeptide side chains will be required. The activation energy for diffusion within the protein is the energy needed for the quencher molecule to thread its way through the protein. The above flexibility of the structure which allows the amino acid side chains to assume a variety of configuration (at least in the non-helical parts of the protein), facilitates the process. The equivalent of the activated complex in such a diffusion process would be the state at which the local conformation in the protein is sufficiently distorted for the diffusing molecule to pass between the polypeptide side chains. These energy consuming, and hence temperature dependent, polypeptide side chain displacements will be much larger than the above average equilibrium fluctuations in the protein. The displacements can be brought about in a large number of ways if local structures in the protein are temporarily disrupted thus creating channels for the diffusing particle to pass. The number of states such as "disordered" diffusional activated complex could assume is therefore much larger than that of the local average conformations. In terms of the absolute reaction rate theory this effect is probably the source of a positive entropy of activation which would account for the large preexponential factor in the Arrhenius equation mentioned above. We express the quenching rate constant by

$$k_q = A_d \exp(\Delta S^{\ddagger}/R)\exp(-\Delta H^{\ddagger}/RT)$$

where A_d can be assumed to equal the pre-exponential factor characteristic of diffusion in solutions as obtained from the above quenching of N-acetyl tryptophan amide by KI. It is numerically equal to kT/h, the quantity used in the absolute reaction rate theory. $\Delta S^{\ddagger}$ is the entropy of activation characteristic of the protein and $\Delta H^{\ddagger}$ is the enthalpy of activation, as measured by the Arrhenius activation energy. By inserting $k_q = 10^8\ M^{-1}sec^{-1}$, $A_d = 10^{13}\ M^{-1}sec^{-1}$ and $\Delta H^{\ddagger}$ = 14 kcal/mole we obtain for $\Delta S^{\ddagger}$ a value of 23 e.u. This quite large entropy of activation is in part compensated by a large enthalpy of activation. According to our model, this compensation means that the larger the energy required for the disruption of local structures the larger the increase in the number of states which the activated complex can assume.

Finally, if the phosphorescence quenching in LADH is to be attributed to the quencher diffusion it should be reflected both in the quenching of the short-lived fluorescence and of the long-lived phosphorescence. We measured the fluorescence quenching of LADH by KI up to 0.2 M. The exciting wavelength was 303 nm, the emission was measured at 315 nm and the slits of the monochromators had a bandwidth of 2 nm. Under these conditions the fluorescence observed comes almost entirely from the buried tryptophan residue 314. Up to 0.05 M, indeed very little quenching is observed but a small effect, i.e., a decrease in fluorescence intensity by about two percent was found. Such a decrease, together with a lifetime of 3.5 nsec for tryptophan 314 corresponds to a quenching rate constant of about $10^8\ M^{-1}sec^{-1}$ and is therefore consistent with the above phosphorescence data. Eftink and Selvidge (13) found a somewhat smaller quenching rate constant of $k_q \approx 1\text{-}3\times10^7\ M^{-1}sec^{-1}$ for the LADH fluorescence quenching by acrylamide.

Because of the long lifetime, the triplet state is, of course, a much more

sensitive probe than the excited singlet when rate constants in the 10^7 to 10^8 $M^{-1}sec^{-1}$ range are being measured.

5. Porphyrins as Optical Probes in Proteins

Metal containing porphyrins appear at the active site of many proteins. Mostly these porphyrins do not show any light emission upon illumination into their absorption bands. Substitution of fluorescent Zn or Mg porphyrins for the native iron porphyrins in myoglobin and in hemoglobin has been shown not significantly to influence the structure of the protein (14). We studied first the optical properties of fluorescent porphyrins and their interactions with small quencher molecules in solution in order subsequently to substitute them for the native ones into the above proteins. These luminescent porphyrins were to be used as optical probes to follow structural fluctuation in the globin proteins.

1) Zn Octaethylporphyrin (ZnOEP) was used as a model compound to study the quenching (by electron transfer) rate and mechanism of excited luminescent porphyrins.

2) The optical properties of Zn protoporphyrin were studied and it was subsequently substituted for the iron containing heme in myoglobin.

6. Zn Octoethylporphyrin (ZnOEP) — Excited State Electron Transfer (in press)

It has been found that porphyrins in the excited singlet and triplet states transfer electrons to electron acceptors — notably quinones (15,16). The fluorescence quenching of ZnOEP seems to follow the reduction and oxidation potentials of substances known to be electron donors and acceptors respectively in a way which suggests that charge transfer might be responsible for the loss of its excitation energy in both polar and apolar media.

Materials and Methods

Zn-Octaethyl Porphyrin was obtained from Porphyrin Products (Utah) and was used without further purification. Toluene, Analar grade, was distilled from molecular sieves (4Å) directly prior to preparing the solutions to be measured. Acetonitrile, MC & B, was first distilled from P_2O_5 and redistilled from molecular sieves immediately before being used. A stock solution of about 10^{-4} M ZnOEP in toluene was kept in the dark under refrigeration. 150 µl of this solution were diluted with 4 ml of solvent in a glass stoppered spectrosil fluorescence cell. For quenching experiments 4 ml of solvent containing the quenching substance were used. As a rule, quencher concentrations did not exceed 0.15 M so as to minimize impurity and other high concentration side effects. All quenching substances used were of Fluka purissimum (>99%) or Analar grade except for Laboratory reagent Acrylamide (>98.5%), 3,4-Dimethylphenol and 9,10-Anthraquinone (>98%). Diethylaniline was redistilled under N_2 and Benzaldehyde was purified chromatographically. The fluorescence was measured in a conventional fluorimeter with a R-928 (Hamamatsu) photomultiplier and a Brookdeal-Ortec photon counting system (PC-5). The ZnOEP was excited in its 533 nm absorption band.

The fluorescence lifetimes were measured on a SLM phase fluorimeter at 18 MHz and at 30 MHz modulation.

Results

The fluorescence lifetimes of ZnOEP in presence of oxygen in toluene and in acetonitrile were found to be 1.9 ns and 2.1 ns, respectively. The standard deviation was 0.15 ns. Lifetimes were obtained both from phase shift and from intensity demodulation. The difference in the value obtained by the two methods was found to lie within the above error range. These lifetimes of ZnOEP in the absence of quenchers were used to calculate the quenching rate constants, k_q, from the Stern-Volmer quenching constants. The latter were generally obtained by steady state measurements. In some cases the quenching constants were also determined by lifetime measurements. Because of the inherently short lifetime of ZnOEP* these measurements yield k_q values which are less accurate (±30%) than those from steady state measurements. Within the estimated error range the k_q values from lifetime measurements agreed with the steady state results. The quenching rate constants in the polar solvent acetonitrile and in the nearly apolar toluene, k_q, for a number of electron acceptors and electron donors together with their polarographic halfwave reduction and oxidation potentials, respectively, are presented in Table 1. The values obtained from lifetime measurements are given in parentheses. In all cases studied the fluorescence spectrum of ZnOEP did not change in the presence of quencher. Even in the apolar toluene no new emission band, indicative of an excited state complex, was observed.

The number of electron acceptors and donors, used in the quenching experiments, was severely limited by two factors: a) the solubility of the solutes in at least one of the solvents used—acetonitrile and/or toluene; b) the requirement that specific interactions and complex formation in the ground state between ZnOEP and the quenching substance do not take place. Particularly in the non polar solvent toluene, the absorption spectra showed that with many known electron donors and acceptors ZnOEP formed ground state complexes. Some potential quenchers which caused changes in the ZnOEP absorption spectrum were: Benzophenone in acetonitrile and in toluene; Phenylenediamine and α-Naphthylamine in toluene, Fumaric and Maleic acid in acetonitrile. In some cases in which no ground state complex is observed, the Stern-Volmer quenching plots did not obey the usual linear relation (for example, indole gave an upward curvature). This might be due to pre-existing weak molecular interactions not revealed by the absorption spectrum. Quenchers which showed such specific interactions were not used in this study. The quenching substances in Table 1 are ordered according to their decreasing polarographic half-wave reduction potentials. It is seen that good electron acceptors, the quinones ($E_{\frac{1}{2}}$(Red) > -1.0 V with respect to the Standard Calomel Electrode (SCE)), quench the ZnOEP fluorescence with a rate which is, or is almost, diffusion controlled. The quenching rate decreases by a factor of more than thirty for substances with reduction potentials of $E_{\frac{1}{2}}$(Red) ≈ -2.0 V with respect to the SCE.

Discussion

It has been experimentally found that the difference between the first polarographic half-wave oxidation potential and the first reduction potential of ZnOEP, $E_{\frac{1}{2}}(Ox) - E_{\frac{1}{2}}(Red) = 0.63\ V - (-1.61\ V) = \delta$ nearly equals the lowest excitation energy of the molecules, $\Delta E_{00} = 2.15$ eV. In simple molecular orbital language, this might be taken to mean that the energy needed to transfer an electron from one porphyrin molecule to another, i.e., the standard free energy for the reactions $2P \rightarrow P^+ + P^-$, is given by the difference between the highest occupied (HOMO) and the lowest unoccupied molecular orbital (LUMO) of the porphy-

rin. In fact, the near equality of δ and ΔE_{00} is probably due to the mutual cancellations of effects since such an approach neglects conformational entropy changes, solvation energies and charge interactions.

On the other hand, it has been found experimentally that in polar solvents the fluorescence quenching of excited electron donors, D* in the presence of acceptors, A, or of excited electron acceptors, A*, in presence of donors, D, is often accompanied by the formation of the corresponding ions D^+ and A^- (17). The standard free energy for the transfer of an electron in these systems

$$D^* + A \rightleftarrows D^*\cdots A \text{ (encounter cage)} \rightleftarrows D^+\cdots A^- \text{ (solvated ion pair)} \rightarrow$$

at the distance where this transfer takes place can be described by

$$G^\circ = E(D/D^+) - E(A^-/A) - \Delta E_{00} - e^2/\epsilon r_{DA} \qquad (1)$$

where $E(D/D^+)$ and $E(A^-/A)$ are the oxidation potential of the donor and the reduction potential of the acceptor respectively (17,18). They are approximated by the corresponding polarographic half-wave potentials $E_{\frac{1}{2}}(Ox)$ and $E_{\frac{1}{2}}(Red)$. ΔE_{00} is the lowest electronic excitation energy of the donor or acceptor and ϵ is the dielectric constant of the solution. The last term in equation (1) represents the stabilization due to the Coulomb interaction of the solvated D^+ and A^- ions formed at the distance of electron transfer. It has been found by Weller and coworkers, for a variety of donor-acceptor pairs in polar solvents that as long as the value of ΔG° is less than $\simeq$ -0.4 eV (-10 kcal/mole) the electron transfer rate is determined by the rate of diffusion and takes place with a rate constant of $k \simeq 2\times10^{10}\ M^{-1}s^{-1}$. For more positive ΔG° values it was found that the above rate constants decrease. Below $k_q = 10^8\ M^{-1}s^{-1}$ a linear dependence of log k_q on ΔG° was observed.

Such a behavior can quite generally be expected for reactions which do not involve complex molecular bond rearrangements. Proton transfer is an example of such a process. More relevant to the present discussion, is the transfer of charge in the excited singlet state of molecules whose valence electrons are located in extended π or peripheral nonbonding orbitals. If for such a donor-acceptor system the equilibrium strongly favors the transfer of charge, i.e., $\Delta G^\circ < 0$ and no chemical bond breakage is involved, one can expect that the process will proceed with no appreciable activation energy and that electron transfer will take place on every encounter. In the range of positive ΔG° values, a free energy of activation, which at least equals ΔG°, will be experienced. In this range of slowed down charge transfer other processes which quench the excited state might compete with the transfer of electric charge. In polar solvents porphyrins, such as ZnOEP, in the excited triplet state are known to interact with similar molecules in their ground state. By the transfer of an electron, a positive and a negative ion are formed in the process (19). If such a process were also to take place between excited singlet and ground state molecules of ZnOEP it follows from equation (1) that, because of the near-equality of $E_{\frac{1}{2}}(Ox) - E_{\frac{1}{2}}(Red) = \delta$ (2.24 eV for ZnOEP) and the excitation energy ΔE_{00} (2.15 eV), the free energy change for the reaction:

$$ZnOEP^* + ZnOEP \rightarrow ZnOEP^+ + ZnOEP^-$$

would be determined by the Coulomb interaction at the distance of charge transfer, i.e., $\Delta G^\circ \simeq 0.09 - e^2/\epsilon r_{DA}(eV)$.

Mauzerall (20) has shown that for various metal porphyrins, electron transfer probably takes place over relatively large distances by a tunneling mechanism. The average charge separation for tunneling in the singlet state is esti-

mated at a ring to ring distance of 5Å or a molecular center to center distance of about 10Å. This would yield a value of $\Delta G^\circ \approx 0.01$ to 0.05 eV, for the above charge transfer reaction in the polar solvent acetonitrile.

It is proposed here that the fluorescence quenching of ZnOEP by electron acceptors or donors other than porphyrins in polar solvents also take place by electron transfer from or to the excited ^{1}ZnOEP*. Such a quenching mechanism is indicated by the dependence of the quenching rate constant k_q on ΔG°, the standard free energy change for electron transfer as calculated from the reduction or oxidation potentials of the quenchers (equation 1).

Our data in Table 1 show that in CH_3CN variour quinones, which are good electron acceptors, and phenylendiamine, an electron donor, all quench the ZnOEP fluorescence with a rate constant in the $k_q = 1.5 \pm 0.5 \times 10^{10} M^{-1}s^{-1}$ range as can be expected if their $E_{\frac{1}{2}}$(Red) or $E_{\frac{1}{2}}$(Ox) values, respectively, are inserted in equation 1, (see Figure 1). We used a dielectric constant of $\varepsilon = 37$ and an average ZnOEP to donor (or acceptor) distance for electron transfer of $r_{DA} = 7$Å. This yields a Couloumb stabilization term of $e^2/\varepsilon r_{DA} \approx 0.06$ eV.

It seems, therefore, that for processes in which the electrochemical considerations predict a zero free energy of activation for the electron transfer step, fluorescence quenching takes place upon every donor-acceptor encounter.

When electron acceptors with reduction potentials similar to or more negative and electron donors with oxidation potentials near or more positive than the corresponding $E_{\frac{1}{2}}$ values for ZnOEP are used, a decrease in the quenching rate constant is observed as must be expected if the electron transfer reaction has a free energy of activation. Because of the short lifetime of ZnOEP the decrease in the quenching rate constant in our experiments could not be measured in the range in which a linear dependence of log k_q on ΔG° has been found in other systems (17). However, even so, the scatter in data points is larger than warranted by the experimental error. Moreover, in many cases the decrease in log k_q is much smaller than can be expected from the above electrochemical considerations. The broken line in Figure 5 roughly indicates the decrease in log k_q with ΔG° for the quenching by electron transfer as found by Rehm and Weller (17). It seems that in the range where our rate constants decrease to values of $k \approx 10^9\ M^{-1}s^{-1}$ or less, other quenching processes are more rapid than the transfer of an electron. The quenching rate constants measured then represent the fastest deactivation pathway of ZnOEP and would be specific for each quencher molecule. This, for example, seems to occur with the hydroxylated aromatics 14, 15 and 16 in Figure 5. In such cases the rate constants need not, of course, follow the dependence expected for the transfer of an electronic charge.

The accuracy of the ΔG° values in Figure 5 is limited by the uncertainty in the quantities $E(D/D^+)$ and $E(A^-/A)$ (equ. 1). The polarographic half-wave potentials were in most cases determined in acetonitrile as solvent. However, the concentration and the kind of supporting electrolyte used seem to affect the value of $E_{\frac{1}{2}}$ obtained. Moreover, even under the same experimental conditions, different $E_{\frac{1}{2}}$ values are often reported for the same substance (see, for example, ref. 21, data for benzoquinone).

Fluorescence quenching by electron donors or acceptors in non polar solvents often proceeds via intermediary excited dimers – heteroexcimers or exciplexes – with their own characteristic emission (18). Similar to the solvated ion pairs formed in polar solution the quenching by heteroexcimer formation

$$A^* + D \rightleftarrows A^* \cdots D \text{ (encounter cage)} \rightleftarrows (AD)^* \text{ (heteroexcimer)} \rightarrow$$

TABLE 1: Quenching of ZnOEP Fluorescence by Electron Acceptors and Donors in Acetonitrile and in Toluene; Halfwave Potential and Quenching Rate Constants k_q

	Acceptor	$E_{\frac{1}{2}}$(Red) in CH_3CN	$k_q \times 10^{-9}$	
			CH_3CN	Toluene
1.	p-Benzoquinone	-0.48	18 (23)	22 (17)
2.	1,4-Naphthoquinone	-0.82	20	17
3.	Duroquinone	-0.84	16	10
4.	9,10-Anthraquinone	-0.98		20
5.	Benzaldehyde	-1.58*	1.6 (1.8)	1.0
	ZnOEP	-1.61*		
6.	Benzoic acid	-1.94*	0.7	
7.	Acrylamide	-1.94	0.57	
8.	Naphthalene	-2.63	~0.01	

	Donor	$E_{\frac{1}{2}}$(Ox) in CH_3CN	$k_q \times 10^{-9}$	
			CH_3CN	Toluene
9.	Phenylenediamine	+0.6	10	
10.	o-Aminophenol	+0.4*	3.5	
11.	α-Naphthylamine	+0.62	2.5	
12.	3,4-Dimethylphenol	+0.51*	1.8	
	ZnOEP	+0.63*		
13.	N-diethylaniline†	+0.76	0.7	0.6
14.	α-Naphthol	+1.14	1.3	
15.	1,4-Hydroquinone	+1.16	1.7	
16.	Phenol	+1.47	0.33	

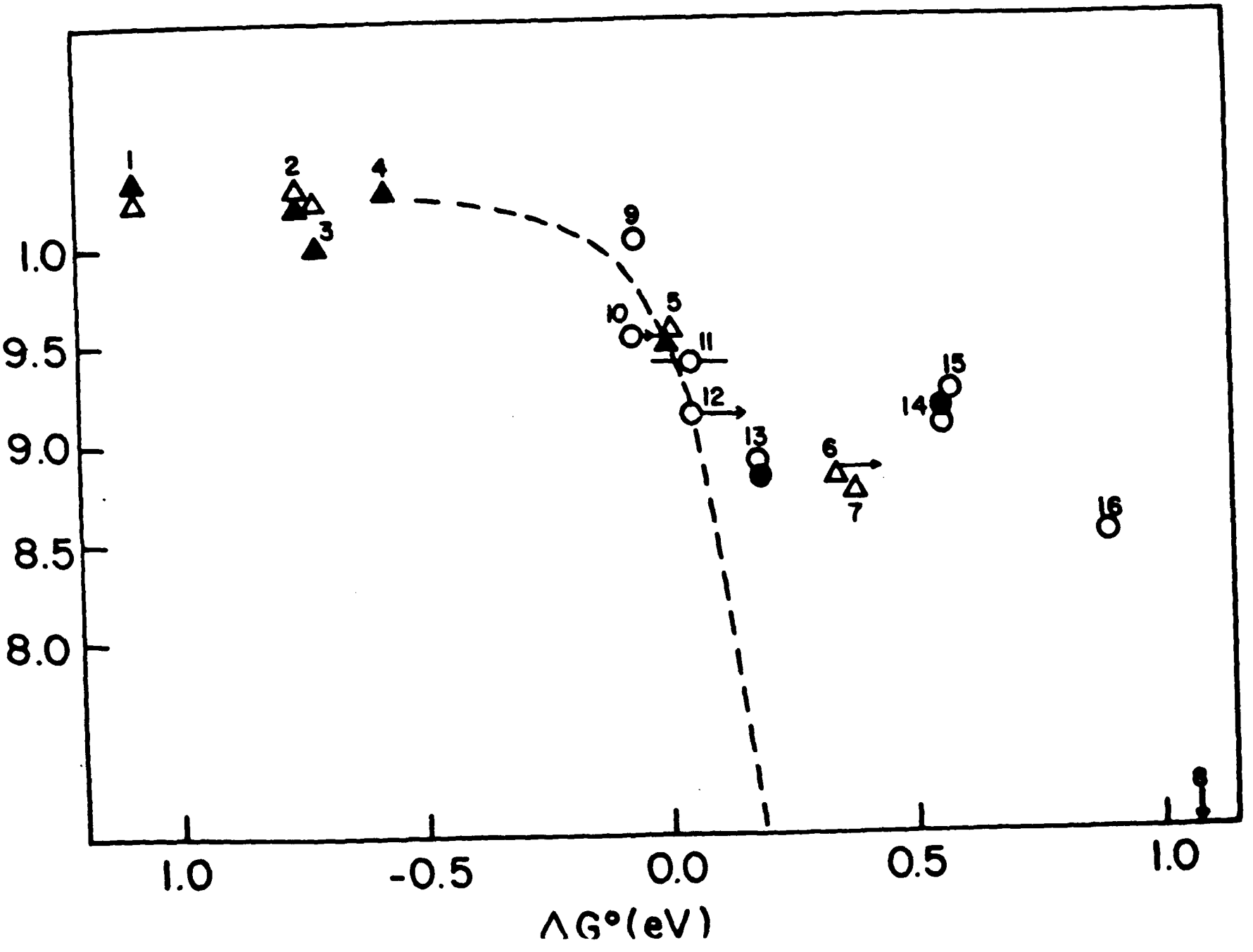
Halfwave potentials $E^{\frac{1}{2}}$ against the S.C.E. in acetonitrile were taken from ref. 13 except where indicated by an asterisk

† Reference 17

* Benzaldehyde in 1:1 Benzene MeOH, ref. 23, Benzoic acid in 75% Dioxane, ref. 23, o-Aminophenol in aqueous buffer, ref. 23, 3,4-Dimethylphenol in 1:1 isopropanol-ac.ac. buffer, ref. 24, ZnOEP in DMSO, ref. 25

k_q values in parenthesis are from lifetime measurements. Estimated error is ±30%

Figure 3



is also accompanied by the transfer of an electronic charge. The exciplex finally reverts to the initial ground state molecules (18). For electron donor acceptor pairs which yield a significantly negative ΔG° value in equation 1, it has been found that the rate of heteroexcimer formation is diffusion limited (22). That no change in the fluorescence spectrum of ZnOEP and hence no exciplex fluorescence was observed in any of the molecular systems in Table 1 does not mean that a short lived nonfluorescent complex is not involved as intermediary in the quenching process. Indications also exist that even in apolar solvents electron transfer within the photochemical cage yields a small amount of free ions in solution (19). In any case we cannot estimate with any confidence the free energy change of the quenching process in non polar solvents like toluene. Strictly speaking, equation 1 might not be applicable if the electron transfer step in the reaction chain does not result in (almost) separated ionic species but in a heteroexcimer of partial charge transfer character. Even if that equation does constitute a good approximation to ΔG° for the quenching process, data for oxidation and reduction potentials are not available in toluene. As a rule, reduction potentials become more negative and oxidation potentials more positive with decrease in solvent polarity (21), i.e., the transfer of charge becomes more difficult. On the other hand, the Coulomb stabilization, $e^2/\varepsilon r_{DA}$ in equation 1, will also increase in non polar solvents. Though the two effects cancel each other to some extent, still one cannot predict the free energy change for the quenching process in toluene based on its value in a polar solvent. Nevertheless, for lack of the correct ΔG° values, $\log k_q$ in toluene in Figure 5 was plotted against ΔG° as calculated for acetonitrile. In view of this crude representation the similarity in the quenching rate constants in the two solvents is quite remarkable. For quenchers with very negative ΔG° values for electron transfer, i.e., good electron donors, in acetonitrile it stands to reason that also in toluene the free energy change for the process will still remain negative. The data points ought perhaps be shifted along the ΔG° axis but would still remain in the $\Delta G^\circ < 0$ range. The measured reaction rates in toluene in these cases show that the process here too is encounter limited. A mechanism invoking charge transfer to account for the fluorescence quenching of ZnOEP by electron donors and acceptors in both polar and non polar solvents agrees well with the observations of Ballard and Mauzerall (19) who found by conductometric measurements that the rate of the ZnOEP triplet – ZnOEP ground state reaction yielding positive and negative ions is very similar in CH_3CN and in toluene. Of course, the free ion yields differ vastly in the two solvents.

It is more astonishing that also when the electrochemically determined ΔG° values (equation 1) in acetonitrile become positive and when they might have a quite different value in toluene, the quenching constants for ZnOEP in the two solvents still resemble each other. The similar position of the break in the log versus ΔG° curve leads us to assume that the contribution to the true ΔG° values due to electron transfer in toluene and in acetonitrile are not very different. The $\log k_q$ values in the two solvents in the range where $\log k_q < 10^{10}$ indicates that the quenching mechanisms which might differ for different quenching substances are apparently similar for a given quencher in the two solvents. This is brought out by the data for substances 14, 15 and 16 in Figure 1.

In conclusion, we feel it is very likely that as long as the electrochemically determined ΔG° value is negative, the quenching of ZnOEP fluorescence both in polar and in apolar media takes place via electron transfer. For quenching substances with redox potentials such that $\Delta G^\circ > 0$ for the transfer of an electron, other quenching routes might become more efficient. However, in such

cases the rate determining step in the reaction between ZnOEP and the quencher seems to be the same in both the polar acetonitrile and in the apolar toluene.

7. Zn Protoporphyrin (ZnPP) —Spectral Properties and Incorporation into Myoglobin. (Preliminary results).

ZnPP with two carboxyl groups on the periphery of the aromatic ring is one of the few water soluble porphyrins. It is also the porphyrin which has been successfully substituted for the native heme in hemoglobin (14). As a first step its spectroscopic properties were studied in solution.

It was found that even in alkaline solution ZnPP undergoes association as seen from its absorption spectrum. In 50% ethanol/water, however, only the monomeric form exists in solution. Consequently this solvent was used. To study the long-lived triplet state of ZnPP its delayed fluorescence was measured. ZnPP, when excited into its singlet ^1S state, crosses, with a yield of about 60%, into the triplet state. Because of the comparatively small singlet-triplet gap ($\sim$3000 cm^{-1}) a small part of the triplets are thermally back-excited to ^1S and subsequently they emit the ^1S fluorescence. The time dependence of this "delayed fluorescence" is naturally determined by the triplet state lifetime. The advantage of using the delayed fluorescence lies in the fact that, contrary to the phosphorescence, its intensity increases with increase in temperature due to the above thermal repopulation of the ^1S state.

Materials and Methods

ZnPP was obtained from Porphyrin Products Inc. (Utah) and dissolved in 1:1 aqueous ethanol of Fluka Spectroscopic grade. The solution in a square spectroscopic cell capped by a rubber septum was purged by bubbling prepurified nitrogen through it. It was illuminated by a Molectron 400 nitrogen—dye laser in its 545 nm absorption band and the emission observed at 600 nm by an R928 Hamamatsu photomultiplier. The signal was fed into a Biomation 8100 digitiser and hence into a Nicolett signal averager.

Myoglobin was obtained from Sigma Chemical Co. The apomyoglobin was prepared and subsequently ZnPP substituted by the procedures of Teale (26) and Lamola 14) respectively.

Results

Only some preliminary results can be reported at this stage. When illuminating the ZnPP solution at 545 nm by the laser flash we were able to observe the delayed fluorescence which displays the regular fluorescence spectrum peaking at 600 nm. The lifetime of the emission at room temperature was about τ = 200 μs.

The ZnPP substituted myoglobin was soluble in aqueous solutions. The absorption and emission spectrum showed that it is the ZnPP monomer which is bound by the protein. It is apparently well protected within the pocket of the protein. This is seen from the long measured lifetime, $\tau \approx 5$ ms, of the delayed fluorescence and hence of its triplet state.

8. Conclusions

The preliminary results quoted at the end of the report indicate the potential of using fluorescent porphyrins as almost-inherent optical probes in heme proteins. The accessibility of the heme pocket to small (substrate) molecules and its temperature dependence will be investigated if and when financial support for this project is obtained.

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