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The protective antigen protein, one of the three separate proteins constituting the exotoxin system of Bacillus anthracis, has been crystallized in a form suitable for high resolution structural studies. The crystal form which is most amenable to x-ray analysis is orthorhombic, space group $P2_12_12_1$, a = 101.1 A, b = 95.4 A, c = 87.3 A, with one protective antigen monomer per asymmetric unit.

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Title: Crystallization of the Protective Antigen Protein of *Bacillus Anthracis*

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SUMMARY

The protective antigen protein, one of the three separate proteins constituting the exotoxin system of Bacillus anthracis, has been crystallized in a form suitable for high resolution structural studies. The crystal form which is most amenable to x-ray analysis is orthorhombic, space group $P2_12_12_1$, a = 101.1 Å, b = 95.4 Å, c = 87.3 Å, with one protective antigen monomer per asymmetric unit.

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Virulence of Bacillus anthracis is caused in part by an exotoxin system (1) consisting of three separate proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF). Each individual protein is, by itself, nontoxic. However, PA in combination with EF produces localized edema in the skin of test animals such as guinea pigs and rabbits (2-3), while PA in combination with LF produces death in test animals as quickly as 40 minutes after administration (4,5). The anthrax toxin system thus has two distinct pathological presentations. PA appears to mediate the effects of EF and LF; hence it has been hypothesized that PA binds receptors in sensitive eukaryotic cells (6). EF is an adenylate cyclase that dramatically increases cyclic AMP levels in eukaryotic cells (7). The specific function of LF is currently unknown.

The anthrax exotoxin pathogenic mechanisms appear to be schematically similar to other bacterial exotoxins, such as those of Corynebacterium diphtheriae, Vibrio cholerae, and Pseudomonas aeruginosa, to the extent that (i) a toxin molecule binds a cell surface receptor, and in some cases is internalized by endocytosis; (ii) the catalytic moiety translocates across a membrane into the cell cytoplasm; and (iii) once the catalytic moiety reaches the cytoplasm, the toxic effect is enzymatic. (Strictly speaking, it is currently only an assumption that the toxic effect of LF is enzymatic.) Notably, the EF does not modify a target substrate, but acts by directly increasing the level of cyclic AMP in the cell--thus having the same consequence to a target cell as cholera toxin, which induces increased cyclic AMP concentration by covalent ADP-ribosylation of the regulatory GTP binding protein of the cellular adenylate cyclase system (8).

The three proteins of the anthrax exotoxin system have molecular weights in the range 80,000-90,000 (9). Since neither EF nor LF is toxic in the absence of PA, and neither is covalently bound to PA, a synergystic interaction between PA and either of the other two proteins may be required to translocate them into target cells. Consequently, the anthrax toxin system offers an interesting complement to other bacterial exotoxin systems which are being used to study cellular processes such as receptor-mediated endocytosis and membrane translocation.

In this context, we are initiating x-ray crystallographic work on the exotoxin proteins of B. anthracis, with the intent of solving the three-dimensional molecular structures of the proteins. Our initial efforts have been focused on the PA protein. PA was purified from both the Sterne strain and strain V770-NP1-R by previously published methods (9). The proteins were at least 90% homogeneous when analyzed on SDS gels, where they displayed a molecular weight of 85,000 (9). No evidence is available that PA isolated from Sterne strain differs from that of the V770-NP1-R strain. Crystallization trials were effected with the hanging drop technique, using solutions of PA at approximately 5-10 mg/ml.

Two crystal forms of the PA have been grown and characterized by both precession photography and diffractometry. Both crystal forms grow at 4°C; no crystals have been obtained under any condition at room temperature. The first form grows from 10-20% polyethylene glycol 8,000 as a precipitant, in the pH range 6.5-9.0, with 40-50 mM buffer. (Buffers used, either individually or in mixtures, to give correct pH, were: citrate, pK_{a3} = 6.4; MOPS, pK_a = 7.2; HEPPS, pK_a = 8.0; BICINE, pK_a = 8.4; CHES, pK_a = 9.5.)

Generally, crystals grow as thin needles or bundles of needles; by controlling growth conditions and seeding, it is possible to produce single-crystal rods (Figure 1). The crystals are orthorhombic, space group $P2_12_12_1$, with a = 101.1 Å, b = 95.4 Å, c = 87.3 Å. One PA molecule per crystallographic asymmetric unit gives a computed V_m of 2.6 $Å^3$/dalton, a value typical for protein crystals (10). The crystals diffract to approximately 3.0 Å resolution both on "still" photographs using a 1 kilowatt sealed tube x-ray source, and on oscillation photographs taken on the Cornell High Energy Synchrotron Source (Figure 2), demonstrating that a high resolution structure determination of the PA molecule is feasible.

The second crystal form can be produced using organic solvents as precipitants; the best crystals grow from 20-40% 2-methyl-2,4-pentanediol or dimethylsulfoxide, in the pH range 6.0-8.0, with 40-50 mM buffer. These crystals, being relatively small, have been characterized less precisely: they are orthorhombic, probable space group $P2_12_12_1$, with a = 124 Å, b = 106 Å, c = 76 Å. Assuming one protein monomer per crystallographic asymmetric unit gives V_m equal to 3.1 $Å^3$/dalton, indicating a somewhat higher solvent content than in the first crystal form. Diffraction has been observed to approximately 3.5 Å resolution on still photographs with these crystals.

SDS gels, run with beta-mercaptoethanol present on PA purified from the Sterne strain of B. anthracis, showed significant proteolytic nicking of the protein. Single crystals of both crystal forms of PA, when redissolved and run on gels, displayed the nicked bands in approximately the same proportion, relative to intact protein, as in the starting protein. Crystallization appears there-

fore to neither select for nor exclude the nicked PA protein. A sample of PA purified from the avirulent B. anthracis strain V770-NP1-R had no significant proteolytic nicking. This material failed to crystallize unless seeded with crystals grown from Sterne strain PA. Further, after intentional nicking with chymotrypsin, PA from strain V770-NP1-R still failed to crystallize in the absence of seeding.

Efforts are underway to solve the structure of PA and to crystallize LF in a form suitable for high resolution x-ray crystalographic analysis.

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FIGURE LEGENDS

Figure 1. Crystals of PA, of approximate dimensions 0.075 mm across by 0.5-1.0 mm long.

Figure 2. 2° oscillation photograph of PA crystals, taken on the Cornell High Energy Synchrotron Source. The b* axis is horizontal.

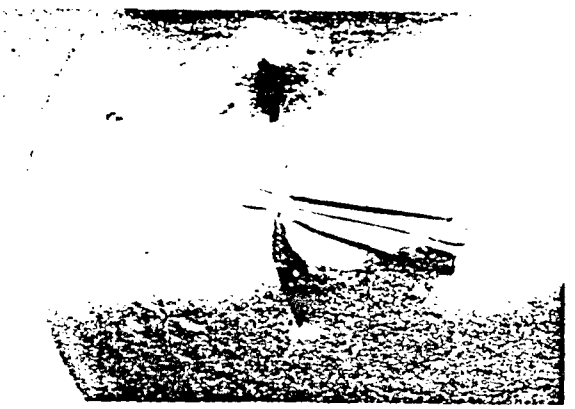

Figure 1.

Figure 2.