Purification, Crystallization, and Preliminary X-ray Diffraction Studies of the Flavoenzyme Mercuric Ion Reductase from *Bacillus* sp. Strain RC607*

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The flavoenzyme mercuric ion reductase from *Bacillus* sp. strain RC607 was purified by dye-ligand affinity chromatography. The protein was crystallized from solutions of high ionic strength, and one of the two crystal forms obtained has proven suitable for x-ray diffraction studies. Preliminary analysis showed that these crystals belong to the tetragonal space group 1422. The unit cell dimensions are a = b = 180.7 Å; c = 127.9 Å. The diffraction pattern extends to better than 3 Å resolution. Crystal density measurements are consistent with one enzyme dimer of 2 × 69,000 Da comprising the asymmetric unit. Trypsin treatment of the native enzyme resulted in the removal of 157 amino acids at the N terminus. After purification, the remaining fragment (amino acids 158–631), which is still fully active in vitro, could be crystallized under the same conditions as native enzyme. Twinning problems, however, did not allow complete analysis of these crystals.

The molecular basis of bacterial resistance to mercurials is the enzymatic reduction of mercuric ions to elemental mercury. This two-electron reduction is catalyzed by the homodimeric enzyme, mercuric ion reductase, using NADPH as the electron source. Mercuric reductase contains both a flavin (FAD) and an active site redox-active disulfide, making it a member of the disulfide oxidoreductase family that includes glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase (1, 2). Of these latter enzymes, x-ray crystal structures have been determined for both glutathione reductase from human erythrocytes (3) and lipoamide dehydrogenase from *Azotobacter vinelandii* (4), with glutathione reductase being one of the structurally best characterized enzymes to date.

Although protein and DNA sequencing have confirmed that the disulfide oxidoreductases and mercuric reductase are indeed homologs (5, 6), the latter is the only family member thus far identified which performs its redox chemistry on a fragment (amino acids 158–631), which is still fully active in vitro, could be crystallized under the same conditions as native enzyme. Twinning problems, however, did not allow complete analysis of these crystals.

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80–85-amino acid domain is either totally absent or present in one or two tandemly repeated copies (15, 16). In the Tn501 enzyme, which has one such domain, in vitro proteolysis experiments have shown that it can be removed with no loss in mercuric reductase activity (5); thus, the role of this segment remains unclear. The recent construction of a good overproducer of the enzyme from Bacillus sp. strain RC607 with a subunit molecular weight of 69,000 (it has the N-terminal domain repeated twice) has now facilitated its isolation in useful quantities (16). In this paper, we report the detailed large scale purification and successful crystallization of the Bacillus mercuric ion reductase as well as initial x-ray characterization of these crystals.

**EXPERIMENTAL PROCEDURES**

**Enzyme Purification**—The purification of Bacillus mercuric reductase from Escherichia coli JM83 (pYW40) was as described by Wang et al. (16) but with several modifications. Cells were grown at 37°C in LB medium, harvested at 10^9 cells/ml, and stored as pellets at −70°C. The pellets were resuspended in purification buffer (20 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, 0.1% β-mercaptoethanol), lysed by two passages through a French pressure cell, and the lysate clarified by centrifugation for 45 min at 27,000 × g. After an initial 0–55% ammonium sulfate precipitation, the bulk of the enzyme was precipitated by the addition of ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation and then dialyzed against this buffer to remove residual ammonium sulfate. The enzyme concentration was estimated spectrophotometrically using an extinction coefficient of ε_{540} = 11.3 mM^-1 cm^-1 for enzyme-bound flavin (assumed to be the same as that of Tn501 enzyme, 18). Crystals were grown using the hanging drop vapor-diffusion method (19), and varying concentrations of ammonium sulfate or sodium citrate as precipitants were used. Hanging drops were prepared by mixing 5 μl of the concentrated enzyme stock with 5 μl from the corresponding reservoir containing 1000 μl of the desired precipitant solution. Crystal densities were measured in a gradient of carbon tetrachloride and toluene calibrated with CsCl solutions of known density.

**RESULTS AND DISCUSSION**

**Protein Purification and Partial Proteolysis—**Bacillus sp. strain RC607 mercuric reductase was purified from E. coli JM83 cells harboring plasmid pYW40 (16). This plasmid (a pUC9 derivative, 22) directs constitutive overexpression of the enzyme to the extent that 10 g (dry weight) of cells grown in a fermenter yielded about 100 mg of pure mercuric reductase. Fig. 1, lane C, shows an SDS-polyacrylamide gel electrophoresis of purified protein. Although this enzyme does not seem to be as susceptible to N-terminal domain proteolysis as the Tn501 and Tn21 mercuric reductases (5, 16, 23), there were several bands of lower molecular weight present in the preparation, presumably proteolytic products. Efforts made to remove these smaller proteins either by DEAE ion exchange chromatography or 2',5'-ADP-Sepharose affinity chromatography (more specific for nucleotide-binding pockets than RedA) have proven unsuccessful (data not shown). At any rate, the initial protein preparation crystallized readily (see below); and when crystallized protein was redissolved and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1, lane B), the smaller molecular weight bands were still present, indicating that proteolytic nicking must not cause significant structural disruption of this enzyme.

The N-terminal domains of mercuric reductase could be completely removed by partial digestion with serine proteases. Partial trypsin digestion of the enzyme yielded a fragment with a subunit molecular weight of 54,000 (Fig. 1, lane E) as compared with 69,000 for the unproteolysed enzyme (Fig. 1, lane F).

**Crystallization**—The ammonium sulfate paste was redissolved in 25 mM HEPES pH 7.0, 3 mM NaN_3, 1 mM EDTA and dialyzed against this buffer to remove residual ammonium sulfate. The enzyme solution was then concentrated by ultrafiltration (Centricon 30, Amicon) to about 20–30 mg/ml protein, if necessary preceded by exchange of buffer by repeated ultrafiltration in 25 mM MES/TRIS (pH 5.0–6.8), 3 mM NaN_3, 1 mM EDTA, or 25 mM HEPES (pH 7.2–8.0), 3 mM NaN_3, 1 mM EDTA.

**Data Collection**—For X-ray studies, the crystals were mounted in thin walled quartz capillaries in the usual way. Cell constants, symmetry, and systematic absences were determined from x-ray precession photographs that were taken with nickel-filtered double-focused CuKα radiation produced by a fine focus rotating anode generator (GX18, Elliott-Marconi) operating at 35 kV and 50 mA. Native data sets were collected on an area detector (Siemens/Nicolet, Madison, WI) and reduced with the help of the program package XDS (21).

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**Simplified version of the proposed EH₂/EH₄ catalytic cycle employed by the flavin disulfide oxidoreductases.**

**Scheme 2.** Minimal EH₂/EH₄ catalytic cycle employed by mercuric reductase (adapted from Ref. 14).

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**Crystallization of Flavoenzyme Mercuric Ion Reductase from Bacillus**

**Scheme 1.** Minimal EH₂/EH₄ catalytic cycle employed by the flavin disulfide oxidoreductases.
mized conditions. Crystals could also be grown in the presence of 2 mM Hg(CN)₂ after the enzymic cysteines had been prerduced by a short incubation with dithiothreitol (as in 14).

Crystals of trypsin-cleaved Bacillus mercuric reductase grew from various ammonium sulfate solutions. Typically they were rod shaped (0.6 x 0.15 x 0.15 mm³); sometimes small parallelepips (0.3 x 0.2 x 0.2 mm³) or needles were obtained. Initial crystallographic analysis revealed a high degree of twinning. Given the weaker affinity of the proteolyzed enzyme for FAD, additional cofactor was added to later crystallization setups. However, although this did improve the size of the crystals, the twinning problem was not overcome.

X-ray Diffraction Analysis—Native Bacillus mercuric ion reductase crystals diffract to better than 3 Å resolution. They are stable in the x-ray beam for several days at room temperature, making them suitable for analysis of the three-dimensional structure of the enzyme. Symmetry and systematic absences on precession photographs of the hk0 and 0kl reciprocal lattice planes are consistent with the tetragonal space group I422. Fig. 3 shows a 9° precession photograph of the hk0 reciprocal lattice plane of one of these crystals. The axes of the unit cell are a = b = 180.7 Å and c = 127.9 Å. Determination of the crystal density gave an average value of 1.31 g/cm³ consistent with two monomers per asymmetric unit (Matthews parameter Vm = 1.9 Å³/dalton; 24). This is well within the range observed for protein crystals and indicates a solvent content of 55%. A search for isomorphous heavy atom derivatives is in progress.

REFERENCES

Fig. 2. Crystals of native Bacillus sp. strain RC607 mercuric ion reductase. The crystals were grown using the hanging drop method. The reservoir conditions were: 1.8 M ammonium sulfate, 25 mM MES-Tris, pH 6, 10 mM dithiothreitol, 3 mM NaN₃, 1 mM EDTA. The crystals grew to their final size within about a week. The maximum dimensions are 1.3 x 1.1 x 0.6 mm³.

Fig. 3. X-ray precession photograph of a crystal of native Bacillus sp. strain RC607 mercuric ion reductase. The photograph of the hk0 plane was taken with a precession angle of µ = 9° and a crystal to film distance of 100 mm. The exposure time was 16 h on a fine focus rotating anode x-ray generator (CuKα) operated at 35 kV and 50 mA.