Manganese and Iron Superoxide Dismutases Are Structural Homologs*

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The crystal structure of a tetrameric manganese superoxide dismutase from a thermophilic bacterium, *Thermus thermophilus* HB8, has been determined at 4.4-Å resolution by local averaging of electron density maps calculated by isomorphous replacement. The spatial arrangement of the principal secondary structural features of iron superoxide dismutase is conserved in manganese dismutase. The structural homology is displayed by orienting the polypeptide chain of *Escherichia coli* Fe dismutase in the electron density map of Mn dismutase. Densities corresponding to bound Mn⁴⁺ occur at locations equivalent to the Fe²⁺ positions in iron dismutase, indicating one metal binding site per chain, or four sites per tetramer. The Mn tetramer, with 222 symmetry, is approximately rectangular in shape and appears to be constructed with only two unique interfaces. One set of interchain contacts closely resembles the dimer interface of Fe dismutase, but the other interface utilizes an inserted polypeptide segment that has no equivalent in Fe dismutase.

The enzymes observed to catalyze dismutation of superoxide (Reaction 1) have been classified according to the nature of the metal ion(s), Cu/Zn, Fe, or Mn, required for catalysis (1, 2).

\[ 2O_2 + 2H^+ \rightarrow H_2O_2 + O_2 \]  

(1)

The structural basis for the metal selectivity exhibited by these enzymes is emerging as the several classes are studied by crystallographic methods. X-ray structures of Fe dismutases from *Escherichia coli* (3) and *Pseudomonas ovalis* (4) and of Cu/Zn dismutase from bovine erythrocytes (5, 6) have shown that the folds of the Fe and Cu/Zn proteins are unrelated. The full details of the ligand geometry in Fe dismutase remain to be elucidated, but the polypeptide conformations in the vicinity of the metals are not the same in Cu/Zn and Fe dismutases. In this paper, we describe a structure of the third type of dismutase, a tetrameric manganese superoxide dismutase from *Thermus thermophilus* HB8, at 4.4-Å resolution, and demonstrate, in accord with predictions from partial amino acid sequences (7), that the highly helical fold of Fe dismutase (Fig. 1) recurs in a manganese-containing dismutase. The similarity of the Fe and Mn dismutase structures, along with recent reports that either Fe or Mn can be incorporated in certain of these proteins (8, 9), raises intriguing questions about the evolution of the Fe and Mn enzymes.

Superoxide dismutases have also been distinguished from one another according to their modes of subunit aggregation. Cu/Zn dismutases are invariably observed to be dimeric species (1). Most Fe dismutases are also dimers (2), but a tetrameric iron protein has been isolated from *Methanobacterium bryantii* (10). Three-dimensional structures have shown that the Cu/Zn and dimeric Fe proteins dimerize differently (3-6), with the metal sites near the dimer interface in Fe dismutases, and distant from the interface in the Cu/Zn enzyme. Mn dismutases occur as dimers or tetramers in prokaryotic and eukaryotic cells. Although the eukaryotic Mn dismutases are usually tetrameric and the prokaryotic, dimeric, exceptions are the tetrameric Mn dismutases from the extreme thermophiles, *Thermus thermophilus* HB8 (11) and *Thermus aquaticus* YT-1 (12). The present study of dismutate from *T. thermophilus* HB8 is the first report concerning the arrangement of chains in a tetrameric superoxide dismutase.

**EXPERIMENTAL PROCEDURES**

The structure of *T. thermophilus* Mn dismutase was first determined at pH 5.7 by single isomorphous replacement and subsequently at pH 7.0 by multiple isomorphous replacement.

Crystalization—Crystals (13) of tetrameric manganese superoxide dismutase, isolated from *T. thermophilus* HB8 (11), were grown by vapor diffusion in the presence of ~50% saturated ammonium sulfate;
buffers were 50 mM acetate at pH 5.7 and 5 mM phosphate at pH 7.0. Crystals were maintained in 60% saturated ammonium sulfate at pH 5.7 or 7.0. The crystals are tetragonal, space group P4_2_2_1, with a = 146.6 and c = 55.6 Å. Earlier measurements of the crystal densities gave values intermediate between those expected for two and four chains per asymmetric unit (13), but the maps reported here show that two subunits constitute the crystallographic asymmetric unit.

Single Isomorphous Replacement Maps—A Pt derivative was prepared by soaking crystals overnight in 1 mM K_2PtCl_4 and 60% saturated ammonium sulfate, pH 5.7. X-ray intensities, including anomalous scattering contributions from the Pt derivative, were measured to 4.4-Å resolution at 4 °C using an automated four-circle diffractometer with Ni-filtered CuKα radiation. Coordinates of the Pt binding sites, one per subunit, were determined from a difference Patterson synthesis, and refined using an FHLE refinement scheme (14) to an R_{FHLE} of 0.42. Several cycles of phase refinement (15) were used to improve the estimates of the scale factor, occupancies, and thermal parameters. Phases were calculated from the two enantiomorphous arrangements of heavy atom binding sites; the mean figure of merit, <$m$>, was 0.64 and the Kraut residuals (16) for the correct (see below) and incorrect phase sets were 0.064 and 0.067, respectively. The subsequent <$m$> weighted Fourier maps were calculated with 3813 unique reflections measured to 4.4-Å resolution. We examined single isomorphous replacement maps based on phases from the two alternative sets of Pt coordinates and selected one as more likely to be correct, because it contained connected densities corresponding to helices.

Multiple Isomorphous Replacement Maps—Difficulties in the preparation of heavy atom derivatives at low pH, where some heavy atom

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**FIG. 2.** The correspondence of the polypeptide folds of Fe and Mn superoxide dismutases. Views show the backbone model of Fe dismutase superimposed on the 4.4-Å electron density map of Mn dismutase. Electron densities are the result of averaging four images of the chain (cf. "Experimental Procedures"). Residue numbers are for Fe dismutase and are preliminary, based on a model built into a 3.1-Å map (3) without the full chemical sequence. a, stereo comparison of the N-terminal domains of Mn and Fe dismutases. Density corresponding to residues inserted between helices A and B can be seen at the lower right (indicated with an arrow). Density at the lower left belongs to the adjoining subunit and to residues near position 150, in the C-terminal domain. To assist in interpretation, a separate drawing of residues 5-77 of iron dismutase, in the same orientation, is shown in the right panel. b, stereo comparison of the C-terminal domains of Mn and Fe dismutases, showing homologies in the positions of the antiparallel sheet and in the helical regions. In both structures, helices begin near positions 90, 100, 155, and 170. Density nearest the viewer is part of the second helix (B) of domain 1. The disparity between the density and the model at position 120 probably represents a real difference in conformation; this region contacts another chain in tetrameric Mn dismutase.

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Tetrameric Mn Dismutase at 4.4 Å Resolution

Fig. 3. A stereo comparison of the N-terminal domains of Mn and Fe dismutases (rotated from Fig. 2A) to display in more detail the correspondence of the helices and the variations produced by the insertion between them. To simplify the view, densities farther than 6 Å from the backbone of Mn dismutase have been deleted.

Fig. 4. The electron density near the Mn positions in the averaged MIR map (pH 7.0). The superimposed contours are from sections z = -0.0535 to z = -0.1615, a slab 6.0 Å in depth. The model is a representation of the C₆ coordinates of one chain of Fe dismutase, oriented in the equivalent region of Mn dismutase. Mn B is related to Mn A by the local molecular dyad.

Reagents are relatively insoluble, prompting us to attempt crystallization and derivative preparation at pH 7.0. Crystals grown at this pH actually proved to be larger and more regular in form than those obtained at pH 5.7. The Pt derivative was prepared, as at pH 5.7, by soaking crystals of the native protein in 1 mM K₂PtCl₄ overnight. The mercurial, 2,6-dichloromercuri-4-nitropheno1, was added to crystals at saturating concentrations (<3 mM) after equilibration with buffered 95% saturated Li₂SO₄.

X-ray intensities were measured to a resolution of 4.4 Å at room temperature from native crystals and from crystals soaked in the Pt salt and the dimercurial, but anomalous, scattering differences were not determined. Difference Fourier syntheses, using the SIR phases, served to locate the derivative binding sites. The major peaks in the Pt maps were found to be equivalent to the sites determined from the pH 5.7 experiments; their magnitudes eliminated the possibility that they were artifacts of the phasing. An FHLE refinement (14) of the centric data from the Pt derivative produced an R_mIR of 0.53.

The difference Fourier for the dimercurial derivative indicated four major sites and a substantial number of possible minor binding sites. These possibilities were analyzed by computer graphics to check their conformity to the noncrystallographic symmetry which we determined both from analysis of the Rossmann-Blow rotation function (see below) and from the SIR map. Fourteen binding sites were selected as candidates for FHLE refinement; they accounted for all of the major and a number of the minor peaks in the dimercurial difference map. At the end of the FHLE refinement, with R_mIR = 0.41, we had eliminated 3 of the 14 sites on the basis of low occupancy or high thermal parameters.

The parameters from FHLE refinement were used to calculate initial phases which we employed in a number of rounds of phase refinement. A Pt residual map, calculated with Fourier coefficients, ([F_Pt-cal] - |F_Pt-obs|), and phases, 9F_Pt, indicated two additional Pt binding sites (one per subunit). Both phase refinement and the calculation of a residual map from the dimercurial derivative data were consistent with the final set of mercurial binding sites selected from the FHLE refinement. The last cycle of phase refinement, including the four Pt binding sites, produced an R_mIR of 0.16 for the Pt derivative; the corresponding value for the dimercurial was 0.17. The parameters were used to calculate MIR phases with an average figure of merit, <m>, of 0.64.

Determination of Noncrystallographic Symmetry—Analysis of the Rossmann-Blow rotation function (17) had indicated the orientation of two mutually perpendicular dyads and suggested that a third molecular symmetry axis coincided with a crystallographic dyad. The major Pt binding sites (two per asymmetric unit) were used to locate the approximate intersection of the three dyads. The initial approximation of the coordinate transformation relating the two subunits in the asymmetric unit was refined by least squares fitting of the electron density in the 4.4-Å SIR map; to do this, we used the program LSQRHO.2 As in our refinement of the local 2-fold axis in E. coli Fe dismutase (3), the calculation was carried out by comparing boxes of electron density with volumes of 8 × 10⁴ Å³ located near the centers of the subunits. Conversion to the equivalent screw transformation (18) verified that the local axes were simple 2-folds intersecting the crystallographic dyad, consistent with the expected 222 molecular symmetry.

—W. Hendrickson, private communication.

2 W. Hendrickson, private communication.
Fig. 5. A model of the Mn dismutase tetramer, built from the chain of iron dismutase (see "Experimental Procedures"). Interfaces between north and west and between south and east units are like the dimer interface of Fe dismutase. Additional residues not found in Fe dismutase are involved in the northeast and south-west interfaces and provide interchain contacts. (cf. Fig. 3) The subunits are disposed about the 222 symmetry origin in a flattened arrangement with their centers of mass approximating a rectangle.

Averaging—Local averaging was performed with LSQRHO. Averaging of the SIR map using the refined local symmetry operation produced a map in which the fold of iron dismutase could immediately be recognized; this same fold was obvious in an unaveraged MIR map. The effect of averaging was certainly to simplify the interpretations, especially of the SIR map, but in retrospect we could recognize the major secondary structural elements of the molecule even in the unaveraged SIR map; hence, much of the improvement is attributed to clarification of the molecular boundaries. For display of the polypeptide fold, we averaged four independent images of the chain, two from the map at pH 5.7 and two from the map at pH 7.0. The resulting electron density is illustrated in Fig. 2.

Use of the Fe Dismutase Model to Interpret the Mn Dismutase Maps—The structural similarities of iron and manganese dismutases were examined by orienting the model of iron dismutase (Fig. 1) in an averaged Mn dismutase density map (Figs. 2 and 3). The transformation relating the structures was first approximated from equivalent features including helix termini and the Fe and Mn centers; it was then refined using LSQRHO, fitting the Fe dismutase electron density (3.1-Å resolution) to that of Mn dismutase. A model of the Mn dismutase tetramer was constructed with the monomer backbone of iron dismutase (3). α-Carbon coordinates for the first subunit were calculated from the refined transformation described above. Coordinates for the remaining three subunits were then generated using the refined local symmetry operation and the crystallographic dyad.

RESULTS AND DISCUSSION

Fig. 2 illustrates Cα backbone positions from E. coli Fe dismutase maneuvered into the Mn dismutase map using the transformation found by fitting of the Fe to the Mn dismutase electron density. The correspondence of the iron dismutase model with the experimental density for Mn dismutase provides compelling evidence for the homology of the two folds. The spatial distribution of secondary structures is essentially the same in both proteins. In particular, regions equivalent to the helices of Fe dismutase possess the typical thickness, grooves, and side chain projections of helices. The fit of the experimental density to the two crossing helices (A and B of Fig. 1) of the N-terminal domain of iron dismutase is best seen in Fig. 3 and the matching of the four helices of the C-terminal domain in Fig. 2B. The three strands of the β-sheet of iron dismutase lie in density with a typical β-sheet twist and the crossover connection between strands 2 and 3 (residues 130-144 in Fe dismutase) is well defined (Fig. 2B).

The largest deviation between the model of Fe dismutase and the experimental density is in the region connecting the two helices of the N-terminal domain, where the manganese dismutase chain is lengthened by an insertion (Fig. 3) which stabilizes the tetramer, as described below. More detailed comparison of the iron dismutase model with the manganese dismutase density reveals displacements of the model from the center of the electron density in several regions including the helix containing residue 105 (helix D) and the curved chain segment (near residue 85) through which residues near the C terminus are tightly threaded. These kinds of local variations have been encountered in other studies of homologous proteins (19-24) and discussed in relation to internal packing (25, 26).

The electron density in the vicinity of the Mn sites is displayed in Fig. 4. The densities at the Mn positions are maxima for the entire map, exceeding the typical backbone densities by factors of about 1.2. The metal positions are equivalent to those of Fe in iron dismutase and the maps indicate that ligands are contributed by the crossing helices of the N-terminal domain and by residues near position 150 in the C-terminal domain. The enzyme used for crystallization contained about 2.4 g atoms of Mn and 0.3 g atoms of Zn per tetramer, in agreement with other analyses for this dismutase (12). Although reports of metal to subunit ratios for Mn dismutase of less than one have led to suggestions that the Mn binding sites are constructed with residues from two subunits (12), the Mn positions in the present structure clearly shows that there must be one metal binding site per subunit. Accurate estimates of the metal occupancy in the crystals, which can be obtained by integrating the electron density, will require higher resolution data.

Recent reports indicate that selectivity of dismutase apoproteins for metals is not absolute, at least for the enzymes that usually contain Mn or Fe. An iron enzyme isolated from *Bacteroides fragilis* (8) can be reconstituted as an active manganese enzyme; Zn inhibits reconstitution, presumably by competing for the metal site. Evidence that identical polypeptides may incorporate either Mn or Fe comes from studies of the dismutases of *Nocardia asteroides* (27) and is especially convincing for the enzyme(s) from *Propionibacterium shermanii* (9), which are isolated as Mn or Fe proteins, depending on the metal composition of the growth medium. Fe dismutase from Ps. *ovalis* has been converted to apoprotein and reconstituted with manganese (28) and the enzyme from *T. thermophilus* has been reconstituted with iron, in both cases the reported enzymatic activity is very low. Interchangeability of Fe and Mn is consistent with the considerable structural homology shown by the present studies at 4.4 Å resolution, although it is not clear why some proteins show dismutase activity with both metals while others do not. Detailed comparisons of the ligand environments will be essential for a full

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3 Determined by J. A. Fee.
4 D. Goodin, M. Klein, and J. A. Fee, private communication.
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assessement of the basis of metal selectivity in Fe and Mn dismutases.

Fig. 5 illustrates the Mn dismutase tetramer viewed along a noncrystallographic 2-fold axis. The northwest and southwest monomer-monomer interfaces are equivalent to the dimer interface in Fe dismutase. The correspondence of these Mn and Fe interfaces was established by showing that rigid body fitting of the iron and manganese dismutase chains also superimposes the local dyads. The northeast and

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Note Added in Proof.—We have calculated a map of T. thermophilus manganese dismutase at a resolution of 2.4 Å, using data from the multiwire detector facility at UCSD, La Jolla. Tracing of the chain in the higher resolution map corroborates the interpretations based on the map at 4.4 Å. The appearance of the electron density in the vicinity of the Mn, combined with examination of the sequence (29) of B. stearothermophilus Mn dismutase, indicates that the metal ligands are His, His, Asp, and His, corresponding to positions 26, 81, 163, and 167 in the sequence of the B. stearothermophilus enzyme.

The three-dimensional structures of Fe dismutases at about 3-Å resolution (3, 4) have suggested the possibility that a previously undetected cofactor, not covalently attached to the protein, was bound in the active center. In our 2.4-Å electron density map of Mn dismutase from T. thermophilus, we see an equivalent feature which is housed at the active center but connected to the protein backbone at a position corresponding to Trp 130 in the B. stearothermophilus Mn dismutase sequence. This observation eliminates the possibility that Mn dismutase contains an active center cofactor and suggests that the discontinuity in the Fe dismutase electron density may be an artifact of the lower resolution of the maps.

REFERENCES