Crystal Structure of Bovine Cu,Zn Superoxide Dismutase at 3 Å Resolution: Chain Tracing and Metal Ligands

(erythrocuprein/x-ray crystallography/beta structure)

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ABSTRACT An electron density map at 3 Å resolution has been calculated for Cu⁺⁺,Zn⁺⁺ superoxide dismutase from bovine erythrocytes, and the course of the main chain has been traced. The dominant structural feature is an 8-stranded barrel of antiparallel β-pleated sheet. There is one very short helical section and two long loops of non-repetitive structure. The Cu and Zn are bound between the loops and one side of the β barrel and are about 6 Å apart, with a common histidine ligand. The Cu has four histidine ligands in a somewhat distorted square plane, and the Zn has three histidines and an aspartate in approximately tetrahedral arrangement. The two coppers of a dimer are about 34 Å apart. The two subunits have essentially the same conformation and have an extensive contact area that mainly involves hydrophobic side chain interactions. The overall folding pattern of the polypeptide chain is very similar to that of an immunoglobulin domain. Superoxide dismutases, which catalyze the dismutation of the O₂⁻• radical to molecular oxygen and hydrogen peroxide (1), are found in all of the oxygen-utilizing organisms so far examined (2). These enzymes have been implicated in protection against a variety of types of damage, including the toxicity of hyperbaric oxygen (3), the toxicity of streptonigrin (4), peroxidation of lipids (5, 6), DNA damage (7), erythrocyte lysis (8), etc. The cytoplasmas of eukaryotic organisms contain a superoxide dismutase which is a dimer with a subunit molecular weight of about 16,000 containing one catalytic Cu and one Zn per subunit (2). Both prokaryotes (9) and mitochondria (10) contain Mn superoxide dismutases which are related to each other in amino-acid sequence but unrelated to the Cu,Zn enzyme (11).

The crystallization (12) and low-resolution x-ray structure (13) of the bovine erythrocyte Cu,Zn superoxide dismutase have been reported previously from this laboratory. The crystals have two of the dimeric molecules per asymmetric unit. The current paper describes the production and interpretation of an electron density map at 3 Å resolution.

METHODS

The methods of crystallization, heavy-atom derivative preparation, data collection, data reduction, phasing, and preparation of electron density maps were all as described previously (13) except in the following ways.

Decomposition Scaling. The scaling corrections applied to allow for crystal decomposition were found to be a fairly smooth function of both time and 2θ. In each 2θ range (typically 1°) nine intense reflections, well distributed in φ and χ (φ and χ are Eulerian cradle coordinates for the 4-circle diffractometer), were chosen as decomposition standards. All sets of such standards were measured before and after data collection, and several sets of decomposition standards at appropriate 2θ were measured after each shell of data. If those standards had fallen by more than 10%, data collection skipped to as low a 2θ as necessary to reach a range with acceptable decomposition values.

Backgrounds. For the 3 Å data, correction was made for the φ dependence of the background. Above about 5° in 2θ, the backgrounds were independent of χ, but they showed a systematic φ dependence that approximately followed the φ curve of the absorption correction in shape and position but with an amplitude between 0.2 and 0.5 times as great. Backgrounds were measured as a function of 2θ at φ values near the region of maximum transmission for the absorption curve. Some background measurements were made at other φ values to check the shape of the φ dependence curve and to estimate what fraction (R) it was of the absorption φ dependence. During data reduction, for each reflection a preliminary background was interpolated from the 2θ curve and an empirical absorption correction including projection angle effects (14) was evaluated. The preliminary background was corrected by R times the absorption correction and subtracted from the raw intensity; the full absorption correction was applied to the resulting peak intensity.

Refinement of Heavy Atom Positions. The five heavy atom derivatives used to produce the current 3 Å electron density map are the same ones described for the low-resolution work (13), but at least some additional data were included for each. The outer third of the reflections in this map were phased from the 2-chloromercuri-3-methoxypropyl-urea derivative alone, using anomalous dispersion. Starting with the parameters from the 5.5 Å resolution results, several least squares cycles refining F₀ observed versus F₀ calculated were done on each derivative. One mercuri-urea and one PtCl₆²⁻ crystal were refined separately because of atypical heavy atom occupancies. Table 1 summarizes the range of data included from each derivative, the number of crystals, the number of heavy atom sites, and the residual from refinement.

Photographic Production of Minimaps. The contouring program (George N. Reek's GNRFOUR program), which produces printed output, was set up with successive contour levels alternating very dense characters with blanks or very
light characters, so that the overall appearance resembled drawn contours. The printout for each map section was photographed onto high-contrast 35 mm copy film and enlargements were printed on 8 × 10 inch (20 × 25 cm) sheets of clear Kodakith graphic art film. A slight brownish tinge to the background was removed by washing briefly in a solution of Farmer’s reducer.

The contour sheets were taped by one edge to 8 × 10 inch single-weight window glass. Pieces of 1/16 inch (1.6 mm) wide chart tape were applied to the glass to mark the main chain. After the main chain had been traced, the contour sheets could be flipped to one side and the stack of glass contained a rough backbone model in tape. Fig. 1 shows such a stack for about half the asymmetric unit, with just five of the contour sheets in place at a level where several strands of antiparallel β structure lie approximately in the plane of the sheets for all four subunits.

Averaged, General-Plane Map. About 50 unambiguously equivalent positions for α-carbons, side chains, and the metals were measured from the minimap for all four subunits in the asymmetric unit. The angles of the noncrystallographic 2-fold axes were varied to minimize for each dimer the departure from exact 2-fold relationship of the 50 pairs of points described above. For each subunit, a transformation matrix was determined which placed the dimer center at the origin, the local 2-fold axis along y, and the subunit center on the z axis. The four transformation matrices were applied to the relevant portions of the numerical output from the crystallographic-section electron density map (which is sectioned perpendicular to the crystallographic b axis). After local interpolation to grid points 1 Å apart, the four subunit maps were averaged.

Chain Tracing. The course of the polypeptide chain was first traced on the crystallographic-section minimap, which is at a scale of 0.1 inch/Å. All four noncrystallographically equivalent subunits were compared, especially to resolve ambiguities in the connectivity. For a number of stretches of extended chain a tentative category was assigned to each side group on the basis of its size, shape, and environment. Good matches to each of these lists were located in the amino-acid sequence (15) by checking regions for which β structure was strongly favored in secondary structure predictions§. The intervening portions of the chain were then also matched to the sequence. The chain tracing was confirmed on the averaged electron density map, and several changes were made in assignment of residue position.

RESULTS

The Electron Density Map at 3 Å Resolution. The Cu,Zn superoxide dismutase map is of sufficiently good quality to permit chain tracing at this resolution, although there are a good many noise peaks in the solvent regions. Many of the side groups show characteristic shapes, and some of the carboxyl oxygens show identifiable bumps. The metal sites were identified primarily by their patterns of multiple connectivity to the densities of neighboring chains. The Zn site is the highest peak in each subunit and coincides with the major site of the Hg-for-Zn substitution. The Cu site is the next highest peak, at or above the height of the largest protein peaks. The subunit boundary is clear, even at the contact around the local 2-fold axis. On the averaged map the main chain is much easier to follow, although the side group shapes are not noticeably better.

Within any one subunit there are, on the average, one or two places where the main chain continuity is broken and three or four close contacts where the connectivity is ambiguous; essentially all of these problems were resolvable either by

§ The predictions were made by William Krigbaum and Sara P. Knutton of the Department of Chemistry, Duke University.
comparison among the four subunits or on the averaged map. However, even on the averaged map there are several cross connections at a high contour level, including the disulfide and a salt link between Arg 77 and Asp 99; therefore, it might not have been possible to trace the chain unambiguously at this resolution without knowledge of the amino-acid sequence.

Considering the agreement among noncrystallographically related subunits and the excellent fit to the sequence, the overall folding pattern described here is quite certain; however, there are likely to be local errors because of confusion between the density due to side groups and that due either to carboxyl oxygens, noise, or solvent peaks.

Backbone Coordinates. Coordinates for the α-carbons, metals, and some selected side groups were measured from the averaged map and two complete and two partial sets were measured from the four subunits in the asymmetric unit on the crystallographic-section minimap. All four sets of measurements were transformed to a common coordinate system for comparison. Regions that showed discrepancies were reexamined on the individual maps, and it always proved possible to make a consistent interpretation on all of the maps. For the five final coordinate sets, root mean square errors are 0.9 Å or less. The averaged α-carbon coordinates are being published elsewhere (29).

Identity of the Subunits. The differences between the four noncrystallographically equivalent subunits in the asymmetric unit of the crystal can be tabulated in detail only at higher resolution. However, it is clear at this stage that changes in backbone conformation are minor. It also appears that most, and perhaps all, differences in side chain position are due to nonequivalent crystal packing environments. No differences have been seen at the subunit contact around the local 2-fold axis. Therefore, from examination at this resolution, it seems likely that the two subunits of a Cu²⁺,Zn²⁺ superoxide dismutase dimer in solution would be exactly, or very closely, equivalent in conformation.

Overall Structure. Fig. 2 is a stereo photograph of a wire backbone model of the bovine Cu,Zn superoxide dismutase subunit, and Fig. 3 is a simplified schematic drawing of the structure. The dominant feature is a large cylindrical barrel made up of eight extended chains of entirely antiparallel β-structure; it contains about 75 residues (50% of the backbone). The interior of the β-barrel is packed with hydrophobic side chains. The barrel is somewhat flattened in cross section; the average distance between main chains across it is 12 or 13 Å in the shorter direction and 16 or 17 Å in the longer direction. The half of the β-barrel toward the outside of the subunit is very regular; the four chains on that side have a relatively low amount of twist (about 10° per residue, right handed, as defined along the direction of the chains) and are within hydrogen-bonding distance of one another for as much as nine or 10 residues. The less regular, more twisted, half of the β-barrel is almost entirely internal to the subunit; that side contributes four ligands to the metals and one end to the disulfide bridge. The β structure is diagrammed in Fig. 4.

The rest of the structure of the subunit is made up of two long loops of nonrepetitive structure. Each of the loops begins and ends in two adjacent chains on the less regular side of the β-barrel. The first such loop (residues 48-79) has two distinct halves. The first half, the “disulfide loop”, has its end held back against the barrel by the disulfide bridge and participates extensively in the subunit contact. The second, quite hydrophilic, half makes a figure eight shape and contributes three
ligands to the Zn. The second long loop (residues 119-141), also very hydrophilic, is the exposed "external loop" which was especially prominent at low resolution (13) and can be seen at the far right in Fig. 2. The most certain piece of $\alpha$-helix is in the external loop and is 1 or 1 1/2 turns long. There are two other places in the subunit where there may be a single helical-type hydrogen bond, but at this resolution it is unclear whether the residues involved are really in $\alpha$-helical conformation. The helix content, therefore, is low: between 3% and 8%.

**Subunit Contact.** The relationship of the two subunits to one another is shown in Fig. 5. The contact area across the local 2-fold axis is broad and closely fitted. It involves part of the outside surface of the $\beta$ barrel, the last few residues at the C terminal, and the disulfide loop. It consists principally of hydrophobic side chain interactions: there are at most two main chain and perhaps two or three side chain hydrogen bonds between subunits, while there are 12 to 14 hydrophobic side groups from each subunit that are apparently in van der Waals contact. Val 146 and Ile 111 both interact with their approach between main chains, Gly 49 and Gly 112 of one subunit are opposite Gly 148 of the other subunit.

**Copper and Zinc.** The two Cu atoms on opposite subunits within the superoxide dismutase dimer are approximately 34 Å apart. The Cu and Zn on a single subunit are about 6 Å apart; between them is the imidazole ring of His 61, to which both metals appear liganded. The protein ligands to the Cu are His 44, His 46, His 61, and His 118, arranged in what is probably a slightly distorted square plane. One of the axial directions of the Cu is quite wide open to solvent access. The protein ligands to the Zn are His 61, His 69, His 78, and Asp 81, in approximately tetrahedral arrangement. Almost all the metal ligands have potential hydrogen-bonding groups positioned close enough to form a network of interactions in a second shell out from the Cu and Zn. The ligand geometries around both the metals, and also the histidine ligand shared between them, have extremely close analogs in crystal structures of Cu-imidazole and Zn-imidazole complexes (17).

**Positions of Particular Side Groups and Heavy Atom Sites.** The bovine Cu,Zn superoxide dismutase has a high glycine content (25 out of the 151 residues). Nine of these glycines are at sharp corners, although only one or two of them could be type II tight turns (18) where glycine is essential. Fourteen glycines are in the barrel or its turns, six of them in positions where a side chain would point into the interior of the barrel. There are three glycines at a close approach between main chains across the subunit contact.

The prolines are all at the surface, and all but one are at sharp bends in the backbone direction. The single tyrosine is exposed to solvent. Many of the specific residues inside the $\beta$ barrel can be identified from the diagram in Fig. 4. They include seven of the 15 valines, six of the nine isoleucines, five of the eight leucines, five of the nine alanines, two of the four phenylalanines, the single methionine, and the single free sulphhydril (Cys 6). The SH is not completely inaccessibile, however, because the mercu-urea (as well as the Hg for Zn) derivative has a site that binds at the surface of the barrel in the subunit-contact region, next to the internal SH group.

The disulfide bridge is partially exposed, and one of the PtCl$_4^{2-}$ sites is next to it. His 19 extends into the solvent, and there is a PtCl$_4^{2-}$ site on either side of it, probably with mutually exclusive binding. The major site of the Hg for Zn substitution is less than 1 Å from the Zn position as seen on the electron density map. There is a mercu-urea site next to His 78, one of the Zn ligands. The major IrCl$_6^{3-}$ site apparently includes Thr 56 and Arg 141, two of the side groups lining the cavity that provides solvent access to the Cu.

**DISCUSSION**

**Comparisons with Earlier Work on Superoxide Dismutase.** All of the features of this structure that were interpretable at 5.5 Å resolution (13) have been confirmed. These include the overall shape of the molecule, the location of the local 2-fold axes, the location of the Zn, the large barrel of antiparallel $\beta$ structure, and the absence of any extensive stretches of helix. The agreement of the structure described here with the amino-acid sequence (15) and disulfide location (19) is excellent. However, at this resolution and without a skeletal model having been fit to the map, the chemical sequence evidence should be considered more reliable than the x-ray confirmation of it.

The subunit association is very strong, and its stability is influenced by the disulfide bond although the disulfide does not directly link the subunits (19); in the 3-dimensional structure the subunit contact is seen to be very extensive, and the disulfide is strategically placed to stabilize a loop that contributes a large fraction of the subunit contact area.

Analysis of optical rotatory dispersion, circular dichroism, and infrared spectra (20) has indicated 0-15% $\alpha$-helix and 20-40% $\beta$ structure, probably antiparallel. The best estimate from the x-ray results is that about 5% of the residues are $\alpha$-helical and about 45% are in the $\beta$ conformation, all antiparallel.

The Cu and Zn are bound very tightly, and their presence contributes greatly to the stability of the protein (21); it is, therefore, reasonable to find that the metals are tightly caged by ligands from three strands of the $\beta$ barrel and one of the loops. Electron paramagnetic resonance spectra indicated at least three nitrogen ligands to the Cu in a square plane (22) and photooxidation protection experiments suggested histidine nitrogens (23); four histidine ligands to the Cu are seen in the x-ray structure, approximately square planar. There was
also evidence, from the degree of interaction between the Cu and substitutions at the Zn site, that the metals are five or 6 Å apart (24); the Cu and Zn peaks on the 3 Å electron density map are about 6 Å apart.

When one considers the proximity of the Cu and Zn and the circumstance that they share a ligand, it is surprising that Cu⁺ alone can restore between 20% and 80% of the catalytic activity to the inactive apo-enzyme (1, 25).

**Comparison with Other Protein Structures.** Examples of β sheets (either purely or predominantly antiparallel) that form more or less closed cylinders are common; the classic examples are the two 6-stranded barrels in chymotrypsinogen (26). The structure of prealbumin consists almost entirely of an 8-stranded β barrel which very closely resembles in size and shape the one described above for superoxide dismutase, but the pattern of topological connectivity is completely different (27).

The most striking comparison, however, is with the basic folding pattern of an immunoglobulin structural domain (28), which consists of a 7-stranded barrel (or sandwich) of β structure. If the N-terminal β strand in the superoxide dismutase barrel is ignored, the rest of the topological connectivity is identical for these two functionally different proteins, and the two long loops of the superoxide dismutase structure come at places equivalent to hypervariable region loops in the immunoglobulin structure. (The diagram in Fig. 4 above may be compared with Fig. 1 of ref. 28.) A detailed discussion of this comparison is being published elsewhere.

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