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The Crystal Structure of Cytochrome *c* Peroxidase*

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The three-dimensional conformation of yeast cytochrome *c* peroxidase has been determined from a 2.5 Å electron density map computed with phases obtained from two isomorphous mercury derivatives. Partial sequence information that has recently become available aided in completion of the tracing of the polypeptide backbone, confirmed the presence of a proximal histidine heme ligand and aided in identification of tryptophan, histidine, and arginine side chains close to the sixth coordination position of the heme.

Ten helical segments, helices A through J, containing about half the residues in our current model are the most prominent features of the cytochrome *c* peroxidase structure. The model also contains three antiparallel β pairs but no parallel β structure.

Cytochrome *c* peroxidase is folded into two clearly defined domains. The heme occupies a crevice between domains, sandwiched between helix B in Domain I and helix F in Domain II, with only one pyrrole ring edge exposed. The crevice is lined with aliphatic and aromatic side chains, several of which are in contact with the heme. An additional interaction holding the heme in place is a hydrogen bond between one of the heme propionates and a threonine side chain extending from one strand of an antiparallel β pair.

The proximal histidine heme ligand extends from the COOH terminus of helix F with NE2 bonded to the iron atom. A water molecule serves as the sixth heme ligand. The iron atom is displaced from the plane of the heme approximately 0.3 to 0.5 Å toward the proximal histidine.

A histidine, an arginine, and a tryptophan side chain extend from helix B and lie close to the sixth coordination position on the distal side of the heme. The distal tryptophan indole ring makes contact with and is nearly parallel to the most buried surface of the heme, while the distal arginine side chain is in contact with the most exposed surface of the heme, with its guanidinium group pointing toward the entrance to the heme crevice. Both NE2 of the distal histidine and the indole ring nitrogen of the distal tryptophan are hydrogen-bonded to the axial water ligand.

Structural comparisons between myoglobin and cytochrome *c* peroxidase in the immediate vicinities of their hemes reveal both similarities and difference. Both proteins have, in the Fe^{3+} state, a water ligand at the axial sixth coordination position which is in turn hydrogen-bonded to a distal histidine side chain. However, the presence of a tryptophan and an arginine side chain close to the sixth coordination position is unique to cytochrome *c* peroxidase.

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Yeast cytochrome *c* peroxidase is a mitochondrial enzyme that catalyzes the 2-electron reduction of hydroperoxides by ferrocytochrome *c* in the following reaction:

$$2 \text{ cyt } c\ (Fe^{2+}) + ROOH + 2H^+ \rightarrow 2 \text{ cyt } c\ (Fe^{3+}) + ROH + H_2O$$

The reaction occurs in three steps. In Step 1, hydroperoxide rapidly removes two reducing equivalents from cytochrome *c* peroxidase to produce Compound I, alternatively designated complex $E \cdot S$ (Yonetani *et al.*, 1966; Yonetani, 1976). In Step 2, one reducing equivalent is then transferred from ferrocytochrome *c* to Compound I following formation of a specific 1:1 complex (Kang *et al.*, 1977; Mochan and Nicholls, 1971; Nicholls and Mochan, 1971), converting Compound I to Compound II. Finally a second reducing equivalent is transferred from a second ferrocytochrome *c* molecule to Compound II, regenerating the native cytochrome *c* peroxidase.

The three-dimensional structure of cytochrome *c* peroxidase is of interest for several reasons. Until now, no detailed crystal structure analysis has been reported for any heme-containing enzyme, leaving a considerable gap in our understanding of this ubiquitous and widely studied class of biochemical reactions. Moreover, cytochrome *c* peroxidase is ideally suited as a relatively simple model for studying the interaction between cytochrome *c* and its membrane-bound oxidase, a much more complicated molecule. In contrast to the oxidase, cytochrome *c* peroxidase is a readily purified water-soluble monomeric protein consisting of 293 amino acid residues and a single noncovalently bound heme. Cytochrome *c* peroxidase can be readily purified and crystallized (Yonetani and Ray, 1965; Yonetani *et al.*, 1966) and thus constitutes one of nature's rare gifts to the biochemists.

Cytochrome *c* peroxidase has been the subject of a wide range of physical and chemical analyses (Yonetani, 1976) including x-ray crystallography (Poulos *et al.*, 1978; Hagman *et al.*, 1969). We recently reported our initial analysis of the crystal structure of cytochrome *c* peroxidase based on a 2.5 Å electron density map phased with two isomorphous mercury derivatives (Poulos *et al.*, 1978). The location and orientation of the heme was reported and the fifth axial heme ligand was identified as the side chain of a histidine residue. In addition, we described changes occurring in the heme crevice when cyanide binds at the sixth axial coordination position.

We have since completed tracing the polypeptide chain aided in part by the acquisition of partial sequence informa-

tion and in part by improvement of the electron density map by an electron density modification procedure. Moreover, the sequence data have enabled us to confirm identification of residues located on the distal side of the heme lying close to the sixth axial coordination position.

INTERPRETATION OF ELECTRON DENSITY MAP

Preparation of crystals, heavy atom derivatives, and collection of intensity data have been described previously (Poulos *et al.*, 1978).

Initial attempts were made to follow the course of the polypeptide backbone with the aid of "mini" electron density maps displayed on a scale of 0.25 cm/Å (Poulos *et al.*, 1978). Several ambiguous regions were encountered, the most troublesome appearing as three breaks in the main chain electron density. Except for these areas, the conformation of the backbone was clear, especially near the heme, but it soon became evident that an unambiguous tracing of the whole chain in a mini map was precluded by the presence of these breaks. The difficulty was resolved by constructing a Kendrew-Watson model as well as by two new developments: 1) a density modification procedure yielded a new electron density map that was somewhat improved over the original multiple-isomorphous-replacement phased map; and 2) partial sequence data became available.

1. Freer[1] has developed a simple computational procedure for improvement of electron density maps. This procedure is based on the knowledge that the true electron density function being sought cannot contain any negative regions. Freer's computer program in effect processes the initial electron density map in such a way as gradually to impose this nonnegativity condition in cycles of successive approximation. Happily, the procedure actually did result in some small but significant improvement in approximately half of the poorly defined connections between helices.

2. Sequence information in the form of amino acid sequences for 25 peptide fragments was acquired while we were in the process of interpreting the density-modified map.[2] These sequences enabled us to identify seven continuous chain sections comprising 60 residues. The seven continuous segments were located in helices A, B, D, E, F, a 3-residue segment between helices A and B, and a second 3-residue segment containing the single cysteine residue near the COOH terminus of helix D. Although 60 residues out of a total of 293 represents only 20% of the cytochrome *c* peroxidase molecule, nevertheless one particular 7-residue sequence enabled us to disentangle an especially troublesome intersection of three segments of electron density. In consequence, we were then able to trace the entire backbone chain without ambiguity.

Partial segments of a Kendrew-Watson model were constructed in small but otherwise conventional optical comparators. The electron density map was interpreted in segments of 20 to 30 residues and coordinates of the atoms in each segment were measured using the device constructed by Salemme and Fehr (Salemme and Fehr, 1972). This procedure avoided the cumbersome problems often encountered when the entire model is fitted to the electron density before the coordinates are measured. Another advantage of this method is that several smaller and inexpensive optical comparators can be utilized simultaneously by a number of people, thereby shortening the total time required to construct a complete model.

The polarities of the 10 main chain helical segments were readily apparent in the electron density map, but the direction of the main chain was not so obvious in certain elongated stretches of the backbone, in some loops on the surface and in one of the β pairs. For this reason the electron densities corresponding to the helical regions were interpreted first, beginning at the proximal histidine near the COOH terminus of helix F.

Once the 10 helical segments were constructed and partial sequence data were incorporated, it became clear how the helices had to be connected to give a stereochemically sensible model consistent with the electron density. In those parts of the molecule for which sequence data were unavailable, identification of side chains was made, insofar as possible, from the appearance of the electron density alone.

Subsequently, the model was further adjusted with the aid of an Evans and Sutherland Picture System to improve its fit to the electron density map and to idealize the geometry of its component parts, particularly in and around the heme crevice.

OVERALL DESCRIPTION OF THE MOLECULE

The resulting model contained 266 of the expected 293 amino acid residues, leaving 27 residues that have not been accounted for. Fifteen of the missing residues are contained in an uninterpretable region of the electron density map which appears to represent the NH_2-terminal tail of the molecule. The remainder of the missing residues are probably associated with two loops on the surface of the molecule which will require modification as incorporation of sequence data and crystallographic refinement progresses. Fortunately, changes in these regions are not likely to alter the essential features of our current model.

The overall shape of the cytochrome *c* peroxidase molecule approximates a compact ellipsoid with about half of the residues distributed among 10 helical segments and with little β structure. We have identified only three clearly defined antiparallel β pairs lying on the surface of the molecule. These β pairs account for about 14% of the residues we have located, including the turns connecting the antiparallel strands. The model contains no parallel β structure.

Three stereoscopic views of the α carbon backbone chain of cytochrome *c* peroxidase are shown in Figs. 1 to 3. One of the most striking features of cytochrome *c* peroxidase is the division of the molecule into two distinct halves. This division is most clearly seen in Fig. 1. Domain I contains helices A through D, a short antiparallel β pair at the COOH terminus of helix B, and a long stretch of backbone chain extending from the COOH terminus of helix D to the NH_2 terminus of helix E. Helix E constitutes the principal connection between domains and is located approximately in the middle of the sequence. Domain II contains helices F through J and the remaining β structure. The final COOH-terminal stretch of polypeptide chain extends from the COOH-terminal end of helix J and lies in Domain I, placing the NH_2 and COOH termini on the same side of the molecule. The first β pair encountered in the molecule begins near the COOH terminus of helix B and is most easily viewed in Fig. 1. A second β pair begins at the COOH terminus of helix F with one strand of the β pair lying just under and close to the heme propionates (Fig. 2). The final β pair begins at the COOH terminus of helix G (Fig. 2). This third β pair clearly exhibits a more pronounced right-handed twist than do the other two.

HEME ENVIRONMENT

The heme is bound in a crevice between the two domains, sandwiched between helix B in Domain I and the COOH terminus of helix F in Domain II. Only an edge of pyrrole IV is accessible to the external milieu. Extending from the upper strand of the β pair at the COOH terminus of helix F is a side

[1] S. T. Freer, unpublished results.
[2] K. Takio and T. Yonetani, unpublished results.

FIGS. 1 to 3. **Three stereoscopic views of the α carbon backbone of cytochrome *c* peroxidase.** The NH_2 and COOH termini are circled, except in Fig. 2 where the COOH terminus is hidden from view. The 10 helices are labeled A through J. The domain structure is emphasized in Fig. 1 by representing the backbone in Domain I as a *heavier broken line,* and the backbone in Domain II as a *thinner solid line.* All views also show the heme and both the proximal and distal histidines. The distal arginine and distal tryptophan are also represented in Fig. 2.

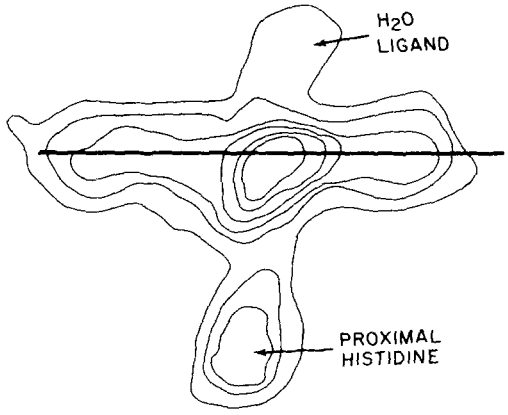

FIG. 4. **The electron density map viewed parallel to the plane of the heme.** The plane of the heme is indicated by a *thick straight line*; electron density corresponding to the proximal histidine and the axial water ligand is also shown.

chain, probably threonine as judged by current sequence data, which can hydrogen bond to the propionate of pyrrole IV. The electron density map shows continuous density between this heme propionate and the side chain, clearly indicating a strong interaction. That this interaction is important to heme binding is evidenced by experiments of Asakura and Yonetani (Asakura and Yonetani, 1969), who found that hemes containing either esterified propionates or ethyl groups in place of the propionates have considerably reduced affinities for apocytochrome *c* peroxidase.

Earlier we identified histidine as the fifth axial or proximal heme ligand with NE2 bonded to the iron atom (Poulos *et al.*, 1978). This identification has now been confirmed by the sequence data. The imidazole ring of the proximal histidine extends from the COOH terminus of helix F (Figs. 1 to 3).

The heme iron atom is displaced 0.3 to 0.5 Å below the plane of the heme toward the proximal histidine. This displacement is most easily seen by viewing the electron density in a direction parallel to the plane of the heme, as shown in Fig. 4. It should be cautioned, however, that we have as yet not accounted for possible distortions of the heme from planarity which could significantly alter our estimate of the displacement of the iron atom. Nevertheless, the electron density map is sufficiently clear in this region for us to conclude that the iron atom is in fact displaced toward the proximal histidine. In addition, displacement of the heme iron atom is consistent with what has been observed in high spin ferric globins and metalloporphyrins (Hoard, 1971; Hoard, 1975).

An additional feature to be noted in Fig. 4 is the finger of electron density extending above the iron atom in the sixth coordination position. In a previous study, we noted this feature of the electron density map and tentatively concluded that an aquo ligand is bound at the sixth coordination position in cytochrome *c* peroxidase (Poulos *et al.*, 1978). We can now be more definite about this conclusion since the electron density modification procedure employed in the present study has clarified this feature of the map. Moreover, a very similar weak finger of electron density extending into the sixth coordination position was observed in the first electron density maps of metmyoglobin where a water molecule is known to be the sixth ligand.[3]

Three residues extend from helix B and lie close to the sixth axial coordination position: histidine, tryptophan, and arginine. Previously, we had correctly identified the tryptophan residue but misinterpreted the electron density of the arginine as a tyrosine (Poulos *et al.*, 1978).

[3] M. Perutz, personal communication.

Fig. 5 shows the fit to the electron density of a short section of helix B containing these 3 distal residues in the known sequence Arg-Leu-Ala-Trp-His.[2] This stretch of 5 residues lies approximately in the middle of helix B with the carbonyl oxygen of the arginine hydrogen bonded to the amido nitrogen of the histidine. The electron density corresponding to helix B is especially clear, enabling us accurately to position the side chains, especially those of the distal tryptophan and arginine. Nevertheless, there remains some doubt as to the correct orientation of the distal imidazole ring. The electron density corresponding to the distal imidazole is rather spherical, making it difficult to decide how to orient the imidazole ring around the CB—CG bond. However, a slight flattening of the electron density indicates that the orientation of the imidazole ring shown in Fig. 5 is probably correct. Moreover, as discussed below, stereochemical requirements for hydrogen bonding between the distal histidine and the axial water ligand are satisfied by this orientation of the ring.

Fig. 6 shows the relationship between the heme, the 3 distal residues, the proximal histidine and the axial water ligand. These residues are also shown in the α-carbon backbone model of cytochrome *c* peroxidase in Fig. 2. Both the distal tryptophan and arginine side chains lie about 3.6 Å above the distal face of the heme. The indole ring is nearly parallel to the most buried surface of the heme while the arginine side chain lies above its most exposed surface with the guanidinium group extending toward the entrance to the heme crevice. As we noted earlier (Poulos *et al.*, 1978), the most direct route for ligands entering this pocket is through an opening near the distal arginine guanidinium group.

The guanidinium group of the distal arginine is also about 3.6 Å from the carboxylate side chain of the heme propionate on pyrrole IV, which as mentioned earlier, is the heme propionate that is hydrogen-bonded to the protein via a probable threonine side chain. However, the guanidinium group is not quite close enough, nor is it correctly oriented to form hydrogen bonds with the propionate. Nevertheless, the close proximity of these two oppositely charged but partially buried groups must serve to stabilize the local geometry.

The distal histidine and tryptophan side chains are only 3.1 Å apart and therefore well within van der Waal's contact distance of one another. These side chains form a tight pocket around the sixth coordination position of the heme. Moreover, as shown in Fig. 6, both NE2 of the histidine and the ring nitrogen NE1 of the tryptophan are in position to hydrogen bond to an axial water ligand.

Steric crowding around the ligation pocket prevents the water ligand from assuming ideal axial geometry. This could be the reason the epr signal seen in high spin ferric peroxidases exhibit a larger rhombic component, and thus greater deviation from ideal axial geometry, than do globins in the high spin ferric state (Peisach and Blumberg, 1971).

COMPARISON WITH MYOGLOBIN

Among heme proteins for which crystal structures have been determined, cytochrome *c* peroxidase most closely resembles the globin family.

Both cytochrome *c* peroxidase and myoglobin contain a noncovalently bound heme embedded in a hydrophobic crevice formed by a system of helices. Although both can exist in either the Fe^{2+} or Fe^{3+} state, their midpoint potentials are quite different, $E'_0 = -194$ mV for cytochrome *c* peroxidase (Conroy *et al.*, 1978) compared with +50 mV for myoglobin (Cassatt *et al.*, 1975), and correspondingly, the physiologically functional state is Fe^{3+} for cytochrome *c* peroxidase, but Fe^{2+} for myoglobin. Additionally, both cytochrome *c* peroxidase

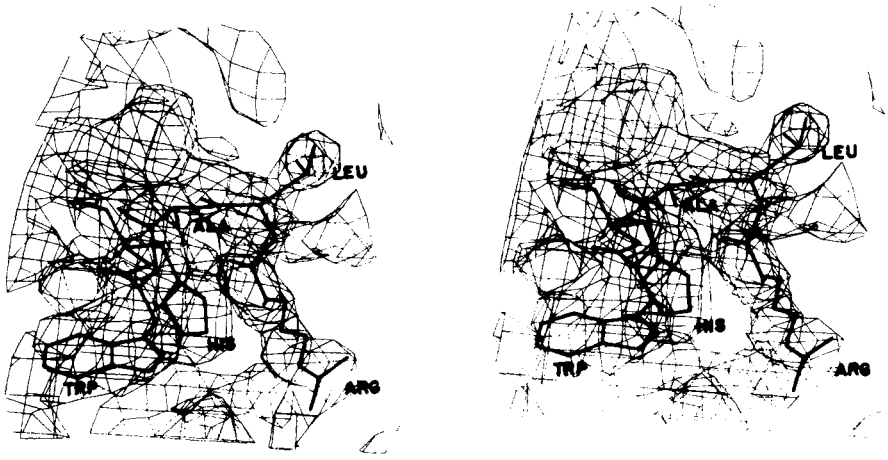

FIG. 5. **Stereoscopic view of the sequence Arg-Leu-Ala-Trp-His, located in the approximate center of helix B, superimposed on the electron density map.**

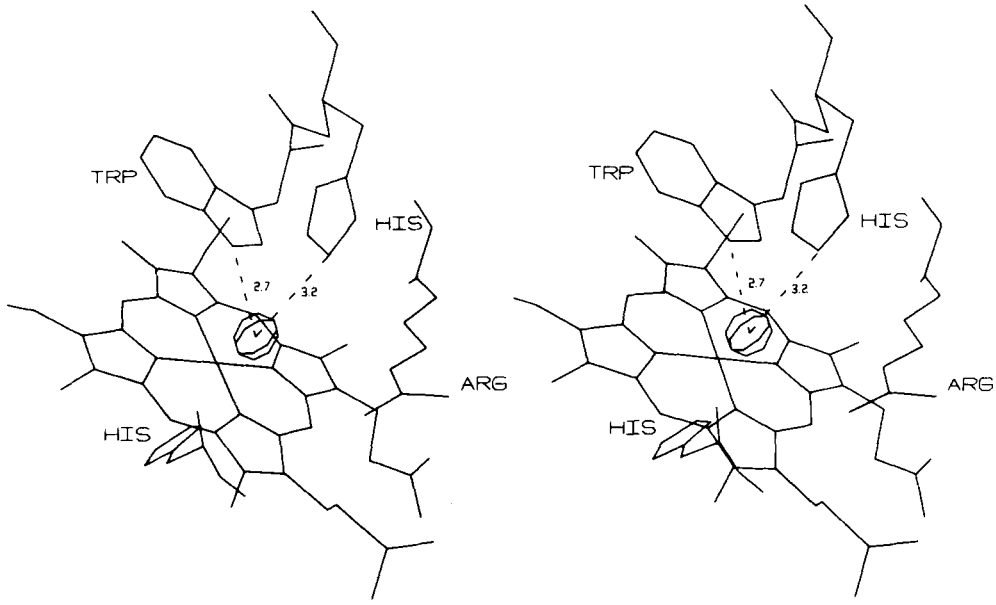

FIG. 6. **Stereoscopic view of the heme crevice showing interactions between the heme, its axial ligands and the 3 distal residues, arginine, tryptophan, and histidine.**

and myoglobin can be oxidized to the Fe^{4+} (ferryl) state (George and Irvine, 1952), and indeed the Fe^{4+} state is involved in the catalytic reaction cycle of cytochrome *c* peroxidase (Lang *et al.*, 1976).

In both cytochrome *c* peroxidase and myoglobin, the sixth coordination position of the heme is not occupied by a protein-linked group. Rather, in the Fe^{3+} state the sixth ligand is a water molecule in both proteins, and additionally, this water molecule is hydrogen-bonded to a distal histidine side chain. In the Fe^{2+} state of myoglobin the sixth position is unoccupied, and by analogy we anticipate that the same will be true of cytochrome *c* peroxidase. Further, the fifth heme ligand in both cytochrome *c* peroxidase and myoglobin is a proximal histidine side chain which extends from the vicinity of the COOH terminus of helix F.

Finally, the heme in both cytochrome *c* peroxidase and myoglobin forms similar high and low spin complexes with ligands like fluoride and cyanide.

Despite their similarities, however, cytochrome c peroxidase and myoglobin carry out entirely different functions. Clearly these functional differences must be closely correlated with structural differences around the sixth coordination site.

A comparison between cytochrome *c* peroxidase and myoglobin in the immediate vicinity of their respective hemes reveals several such structural differences. The comparison is shown in the stereoscopic models in Fig. 7, *A* and *B*. The two models were oriented with their distal and proximal histidines and the most buried pyrrole of their respective heme rings in corresponding positions. The most buried pyrrole is II in cytochrome *c* peroxidase and I in myoglobin. As can be seen in Fig. 7, the geometrical relationship between the 2 histidines and the heme is nearly the same in both proteins.

Beyond this similarity, however, the structural analogy breaks down. The position occupied by the distal tryptophan side chain in cytochrome *c* peroxidase is occupied instead by the side chain of Val-E11 in myoglobin. Moreover, it should be noted that in no known globin structure is there an aromatic residue at the position of Val-E11. The distal tryptophan in cytochrome *c* peroxidase allows two types of interactions which are not possible in the globins: 1) the indole ring is nearly co-planar with the heme, allowing interactions of the heme and indole π orbitals, and 2) the indole ring nitrogen in cytochrome *c* peroxidase can interact with ligands at the sixth heme coordination position.

Another important point of contrast between cytochrome *c* peroxidase and myoglobin is the relative positioning of the

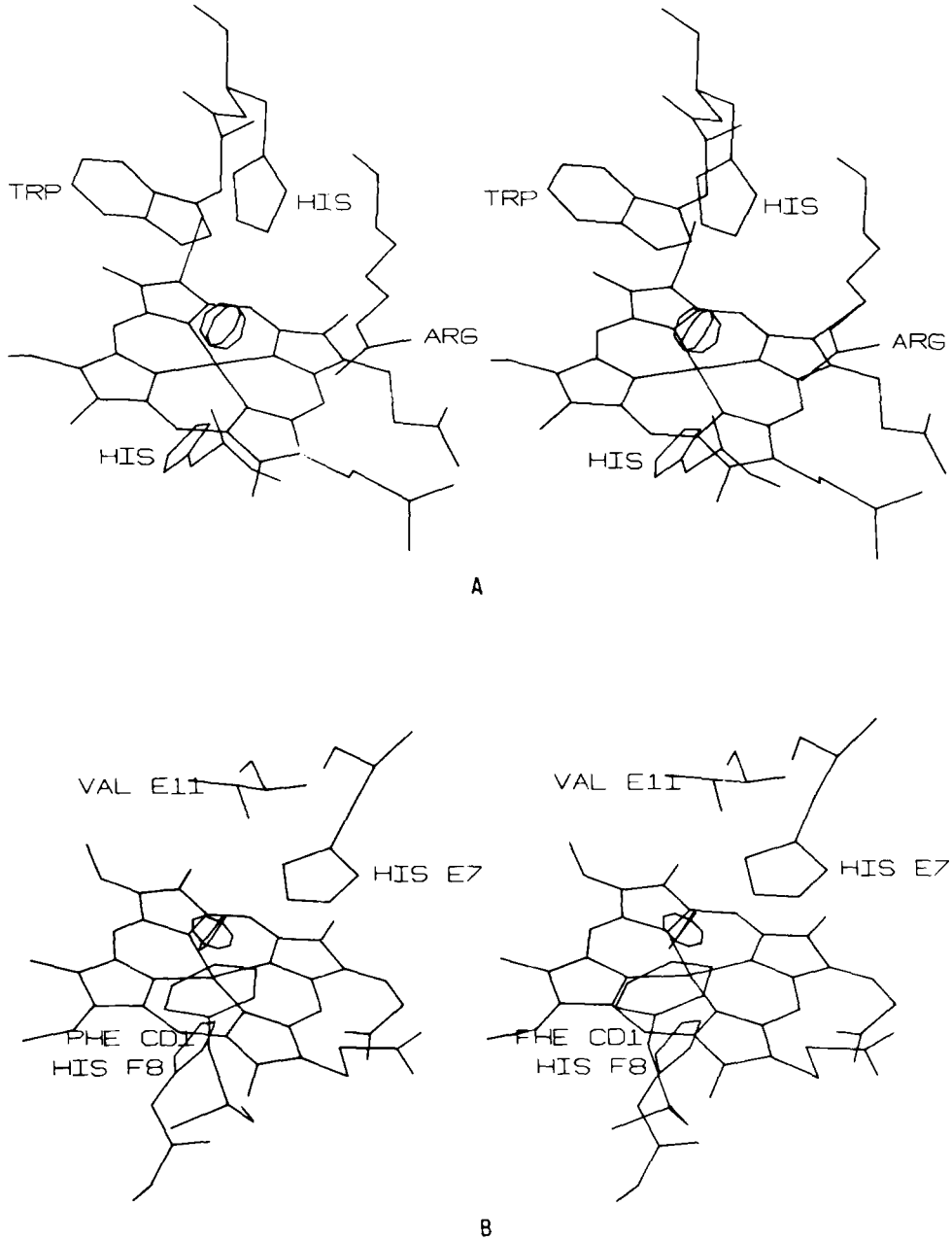

FIG. 7. **Stereoscopic views of *A*, cytochrome *c* peroxidase, and *B*, myoglobin in the vicinity of their respective sixth coordination positions.** These views allow direct visual comparisons between cytochrome *c* peroxidase and myoglobin.

distal arginine side chain in cytochrome *c* peroxidase compared with the Phe-CD1 side chain in myoglobin, which can be seen in Fig. 7. The presence of a phenylalanine at position CD1 is apparently essential for proper functioning of hemoglobin (Dacie *et al.*, 1967). We believe the distal arginine in cytochrome *c* peroxidase is also essential for its catalytic activity, a topic we will consider in detail in a later paper.

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