A Hypothetical Model of the Cytochrome c Peroxidase-Cytochrome c Electron Transfer Complex*

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A hypothetical three-dimensional model of the cytochrome c peroxidase-tuna cytochrome c complex is presented. The model is based on known x-ray structures and supported by chemical modification and kinetic data.

Cytochrome c peroxidase contains a ring of aspartate residues with a spatial distribution on the molecular surface that is complementary to the distribution of highly conserved lysines surrounding the exposed edge of the cytochrome c heme crevice, namely lysines 13, 27, 72, 86, and 87. These lysines are known to play a functional role in the reaction with cytochrome c peroxidase, cytochrome oxidase, cytochrome c, and cytochrome b.

A hypothetical model of the complex was constructed with the aid of a computer-graphics display system by visually optimizing hydrogen bonding interactions between complementary charged groups. The two hemes in the resulting model are parallel with an edge separation of 16.5 Å. In addition, a system of inter- and intramolecular ξ-ξ and hydrogen bonding interactions forms a bridge between the hemes and suggests a mechanism of electron transfer.

Peroxidases catalyze the following sequence of reactions.

\[
P + \text{ROOH} \rightarrow \text{P(I)} + \text{ROH} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{P(I)} + \text{D} + \text{H}^+ \rightarrow \text{P(II)} + \text{D}^+ \quad (2)
\]

\[
\text{P(II)} + \text{D} + \text{H}^+ \rightarrow \text{P} + \text{D}^+ \quad (3)
\]

where P, P(I), and P(II) represent the native enzyme, Compound I, and Compound II, and D is a suitable electron donor.

Recently, we have proposed that all peroxidasues utilize the same mechanism in Step 1 of the reaction cycle (Poulos and Kraut, 1980). This conclusion was based on the recently determined x-ray structure of cytochrome c peroxidase and an already existing body of work on peroxidase biochemistry. The essential features of the mechanism are acid-base catalysis by the invariant distal histidine (His-52 in cytochrome c peroxidase), stabilization of a developing negative charge in the transition state by an invariant arginine residue (Arg-48), and stabilization of the Fe(IV) oxidation state. Acid-base catalysis and charge stabilization promote heterolysis of the RO—OH bond to produce an oxidized enzyme 2 eq above the ground state (Compound I). In all peroxidasues 1 electron is removed from the iron atom generating a ferryl, Fe(IV), iron atom (Lang et al., 1976; Maeda and Morita, 1967; Moss et al., 1969; Schulz et al., 1979). In plant peroxidasues the 2nd electron is probably removed from the heme to give a heme radical (Dolphin et al., 1971), while in cytochrome c peroxidase, the 2nd electron is probably abstracted from an aromatic side chain (Yonetani et al., 1966), most likely the indole ring of Trp-51 (Poulos and Kraut, 1980).

Where peroxidasues differ most notably is in the types of substrates utilized as electron donors in Steps 2 and 3 of the reaction cycle. Horseradish peroxidase oxidizes small aromatic molecules like indole acetic acid (Yamazaki et al., 1968) while cytochrome c peroxidase preferentially oxidizes ferrocytochrome c (Yonetani, 1976). The purpose of the present communication is to offer a three-dimensional model for the cytochrome c peroxidase-cytochrome c complex occurring in these last two steps of the cytochrome c peroxidase reaction cycle, that is, in the electron transfer reaction between cytochrome c peroxidase and ferrocytochrome c.

**Comparison Between Cytochrome c Peroxidase and Cytochrome Oxidase**

Because cytochrome c peroxidase is a relatively simple molecule compared to the multi-subunit, membrane-bound oxidoreductases that interact with cytochrome c, and, most importantly, because the x-ray structure of cytochrome c peroxidase is now known (Poulos et al., 1980a), the cytochrome c peroxidase-cytochrome c reaction is a particularly attractive system for investigating the structural basis of biological electron transfer reactions. In addition, cytochrome c peroxidase and cytochrome oxidase are very similar in their reactions with cytochrome c, and thus, the cytochrome c peroxidase-cytochrome c interaction may help to elucidate some of the principles involved in membrane-bound electron transport.

The three most general properties shared by cytochrome c peroxidase and cytochrome oxidase are: 1) both enzymes react with cytochrome c to form a specific 1:1 complex with approximately the same binding constant (\(10^6 \text{ M}^{-1}\)); 2) both enzymes react with cytochrome c with approximately the same second order rate constant (\(10^6 \text{ M}^{-1} \text{s}^{-1}\)); and 3) the kinetics of both enzymes with cytochrome c is highly sensitive to ionic strength (Nicholls, 1974; Smith and Conrad, 1956; Davies et al., 1964; Mochan, 1970; Mochan and Nicholls, 1971; Nicholls and Mochan, 1971; Leonard and Yonetani, 1974). The ionic strength dependence of these reactions and their inhibition by polycations (Smith and Minnaert, 1965; Nicholls and Mochan, 1971) indicate that positive charges on the surface of cytochrome c are involved in forming electron transfer complexes with both cytochrome c peroxidase and cytochrome oxidase. Several years ago, Salemme et al. (1973) suggested that a ring of highly conserved lysine residues surrounding the heme crevice

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of cytochrome c is crucial to the formation of ionic complexes with its physiological oxidation-reduction partners. More recently, Margoliash and co-workers showed that specific chemical modification of these lysines, especially lysines 13, 72, and 87, inhibits reaction with both cytochrome c peroxidase and the oxidase (Ferguson-Miller et al., 1978; Kang et al., 1978). Furthermore, this same group of cytochrome c lysines is involved in the reaction with cytochromes b5 (Siong et al., 1977) and c1 (Ahmed et al., 1978; Speck et al., 1979; Reider and Bosshard, 1980). It appears, therefore, that cytochrome c peroxidase, oxidase, c1, and b5 all share a common binding region on cytochrome c, and that electrons are both donated and accepted via the exposed heme edge.

Another property shared by cytochrome c peroxidase and the oxidase is their pattern of cross-reactivity with cytochrome c from different species. In general, if any of the highly conserved lysines around the heme crevice in eukaryotic cytochrome c are missing from a given prokaryotic cytochrome c, that prokaryotic cytochrome c is an inferior substrate for both the peroxidase and the oxidase (Yamanaka, 1975; Errede and Kamen, 1978).

For these reasons, we believe the model presented here has implications that extend well beyond the area of immediate concern in this paper, namely the stereochemistry of the cytochrome c peroxidase-cytochrome c interaction.

Rationale Behind Model-Building Experiments

With the x-ray structure of cytochrome c peroxidase now known, we are in a position to investigate the stereochemistry of the cytochrome c peroxidase-cytochrome c complex by model-building experiments. A similar model-building approach was taken by Salemme (1976) in describing the complex formed between cytochromes c and b5. Salemme’s model showed that carboxylate groups surrounding the exposed heme edge of b5 can interact with the ring of highly conserved lysine residues around the exposed edge of the cytochrome c heme. The two hemes are then parallel and 8.4 Å apart with no intervening protein groups, suggesting that electron transfer occurs directly between exposed heme edges. The results of subsequent chemical modification experiments on the carboxylates of b5 correlate well with Salemme’s model (Dailey and Strittmatter, 1979). As we shall see, interactions between charged residues in the cytochrome c peroxidase-cytochrome c complex are very similar to the b5-c interactions, although the two hemes in the former model are much further apart. An important attraction of the cytochrome c peroxidase-cytochrome c system, however, is that cytochrome c and cytochrome c peroxidase are physiological oxidation-reduction partners while cytochromes b5 and c are not. Even so, cytochrome b5 does reduce cytochrome c at a substantial rate in vitro (Strittmatter, 1964), and the close similarity between the amino acid sequences of b5 and the heme binding domain of yeast cytochrome b5 (Guiard and Lederer, 1976) suggests that Salemme’s model may in fact be relevant to the in vivo transfer of electrons from cytochrome b5 to cytochrome c.

Our approach is similar to Salemme’s. We have simply examined the structures of both proteins to see if there exists an obviously complementary distribution of charged residues on their respective surfaces. (There does, of course, or this report would not have been written.) Then, by model-building experiments, we can determine the probable structure of the electron transfer complex and ask how well the model agrees with known solution properties of the complex.

Complementarity Between Surface Charged Groups in Cytochrome C Peroxidase and Cytochrome C

The model of cytochrome c peroxidase used in the present study was updated from our earlier version (Poulos et al., 1980a) by incorporating the recently completed primary sequence (Takio et al., 1980) into the already existing 2.5 Å electron density map. Out of a total of 293 residues in the molecule, 288 are presently included in our current model. The locations of residues 1 to 5 are still uncertain but fortunately these have no bearing on the present study. There are few significant changes from our original model other than, of course, the indentification of many more side chains. Most important to the present study, all charged residues have been located. Only the side chains conformations of 1 aspartate, 6 glutamate, and 5 lysine residues remain uncertain, although their a-carbon positions are known, but none of these residues are in the cytochrome c binding region.

After the primary sequence of cytochrome c peroxidase was incorporated into our model, it was immediately apparent that the surface of the molecule surrounding the edge of the heme to which the heme propionates are attached contains a preponderance of negatively charged residues, while there are few positively charged residues in this area, as shown in Figs. 1 and 2. Particularly noteworthy are the negatively charged regions circled in Fig. 1. Fig. 2 shows that positively charged residues are located primarily on the “back” surface of the molecule.

This distribution of charges on the surface of cytochrome c peroxidase is complementary to the distribution found in cytochrome c where the exposed edge of the heme crevice is surrounded by a ring of positively charged lysine residues. The most significant observation, however, is that aspartates

![Fig. 1](image) The cytochrome c peroxidase molecule with backbone amido nitrogen position of all glutamate and aspartate residues labeled. Key acidic regions on the “front” surface of the molecule are circled.
interface, effectively excluding solvent. The only unfavorably displayed simultaneously on an Evans and Sutherland Picture System and oriented so as to optimize hydrogen bonding distances and geometries between aspartates 37, 79, and 216 in forming the cytochrome c peroxidase-cytochrome c complex.

MODEL-BUILDING EXPERIMENTS

Coordinates for the cytochrome c model used in the present study were taken from the 2.0 Å structure of tuna cytochrome c reported by Swanson et al. (1976) and were obtained from the Brookhaven Protein Data Bank. Coordinates for b6 (Matthews et al., 1972) were also obtained from the Brookhaven Protein Data Bank. All inter- and intramolecular distances were measured between atomic centers.

In order to construct a hypothetical cytochrome c peroxidase-cytochrome c complex, both protein molecules were displayed simultaneously on an Evans and Sutherland Picture System and oriented so as to optimize hydrogen bonding distances and geometries between aspartates 37, 79, and 216 in the peroxidase and lysines 13, 27, and 72 in cytochrome c. We then observed that four additional hydrogen bond interactions could be formed between Asp-34, Asp-37, Gln-86, and Asn-87 in the peroxidase and Lys-87, Lys-86, Gln-16, and Gln-12 in cytochrome c. Moreover, the heme planes of the two molecules were found to be parallel to within about 10° and when the two molecules were slightly adjusted to make the hemes exactly parallel, hydrogen bonding geometries actually improved.

As a general observation, it appears that cytochrome c peroxidase and cytochrome c fit together with remarkable precision. Essentially no gaps at all exist at the intermolecular interface, effectively excluding solvent. The only unfavorably close contact occurs between His-180 in cytochrome c peroxidase and the carbonyl oxygen atom of Ile-81 in cytochrome c, a distance of 2.0 Å, and as will be seen below, the side chain of His-180 is readily repositioned.

Additionally, we have compared the structure of the cytochrome c peroxidase-cytochrome c complex with that of the cytochromes c-b6 complex by re-examining Salemme's hypothetical model on the Picture System. We find essentially the same interactions described by Salemme (1976), although the overall fit between cytochromes c and b6 is not as good as the fit between cytochrome c and its peroxidase. In particular, the distance of 4.5 Å between Lys-72 in cytochrome c and Asp-60 in cytochrome b6 is too long for the predicted hydrogen bond, and Gln-16 in cytochrome c is too close to Glu-44 in cytochrome b6, a distance of about 2 Å. That a better fit is found between the peroxidase and cytochrome c is undoubtedly a consequence of the fact that these two proteins are physiological oxidation-reduction partners while cytochromes c and b6 are not.

DESCRIPTION OF THE MODEL COMPLEX

The resulting hypothetical structure of the cytochrome c peroxidase-cytochrome c complex is shown in Figs. 3 and 4. In addition, Table I lists all groups found to be involved in intermolecular hydrogen bonding and Fig. 5 depicts the geometry of these interactions. All hydrogen bonding distances (2.7 to 3.0 Å) and geometries are normal and all side chains, except for Lys-72 and Lys-87 in cytochrome c, are in a fully extended conformation (Fig. 5).

An additional important feature of the complex is the approximately antiparallel positioning of the NH2-terminal helix in cytochrome c, composed of residues 2 to 18, with respect to the 85 to 95 helical stretch in cytochrome c peroxidase (Fig. 3). This arrangement of helices is particularly stable since the positive, NH2-terminal end of the cytochrome c peroxidase helical dipole lies near the negative, COOH-terminal end of the cytochrome c helical dipole. The closest approach between backbone atoms in the two helices is 6.2 Å, between CA of Glu-12 in cytochrome c and CA of Asn-87 in cytochrome c peroxidase. Two glutamine side chains extending from the NH2-terminal cytochrome c helix, Gln-12 and Gln-16, hydrogen bond with Gln-86 and Asn-87, respectively, the side chains of which extend from the cytochrome c peroxidase helix. Gln-12 and Gln-16 in cytochrome c have not been implicated previously in the function of cytochrome c, although the probable importance of Gln-16 was mentioned by Ferguson-Miller et al. (1979), who noted that this residue is highly conserved in eukaryotic cytochromes c and is positioned near the entrance to the heme crevice.

Other close approaches include the following nonpolar contacts: CG2 of Ile-81 in cytochrome c to CB of Asp-216 in peroxidase (4 Å); CG2 of Ile-81 in cytochrome c to the peptide oxygen of His-58 in peroxidase (3.4 Å); and CG of Lys-86 in cytochrome c to CE1 of Tyr-39 in peroxidase (4.3 Å). The interactions involving Ile-81 of the cytochrome provide a
**Model of the Cytochrome c Peroxidase-Cytochrome c Complex**

**TABLE I**

Hydrogen-bonding interactions in the cytochrome c peroxidase-
tuna cytochrome c complex  

| Tuna cytochrome c | Corresponding residues in yeast iso-1-cytochrome c | Cytochrome c peroxidase |
|------------------|--------------------------------------------------|------------------------|
| Lys-13           | Arg-18                                           | Asp-37                 |
| Lys-27           | Lys-32                                           | Asp-70                 |
| Lys-72           | Lys-77                                           | Asp-216                |
| Lys-96           | Lys-91                                           | Asp-37                 |
| Lys-87           | Lys-92                                           | Asp-34                 |
| Gln-12           | Thr-17                                           | Asn-87                 |
| Gln-16           | Gln-21                                           | Gln-86                 |

particularly good set of hydrophobic contacts because the Ile-81 side chain is exposed on the surface of cytochrome c, and therefore, an entropically favored desolvation of Ile-81 results upon formation of the complex. Additionally, Ile-81 aids in sealing off the intermolecular interface from the external solvent. Finally, because a hydrophobic residue is conserved at position 81, usually isoleucine in animals and valine in plants (Dickerson and Timkovich, 1975), it seems reasonable to suggest that this region of the cytochrome c molecule is designed to serve as a hydrophobic anchor in forming electron transfer complexes.

One objection that may be raised regarding the physiological significance of the proposed cytochrome c peroxidase-cytochrome c complex is that obviously the substrate for yeast cytochrome c peroxidase is not tuna cytochrome c. However, in light of the extensive sequence homologies among all eu- karyotic cytochromes we expect the structure of yeast and tuna cytochromes c to be nearly identical (Timkovich, 1979). Moreover, for comparison we have constructed a hypothetical yeast iso-1-cytochrome c model by making the necessary amino acid substitutions in the tuna cytochrome c structure. Only two substitutions are relevant to formation of this com-
cytochrome the hemes in cytochrome an edge-to-edge distance of 16.5.

They reasoned that if, in the 1:1 complex, the cytochrome the two hemes are parallel, although perpendicularly dis-rearrangement of the two polypeptide backbone chains.

The most notable structural feature of our proposed model is that the two hemes are also parallel in Salemme (1976) Fig. 5.

The iron-to-iron distance is 24.6 while the closest heme edge-heme edge distance is 16.5 Å between pyrrole IV in cytochrome c peroxidase and pyrrole II in cytochrome c. These two pyrrole rings are also the most exposed portions of the hemes in cytochrome c peroxidase and cytochrome c and an edge-to-edge distance of 16.5 Å is the closest approach that can be achieved without invoking considerable conformational rearrangement of the two polypeptide backbone chains.

The probable importance of parallel heme planes in an electron transfer complex is underscored further by recalling that the two hemes are also parallel in Salemme's (1976) proposed model for the b2-c complex. In the latter, however, the edge-to-edge distance is only 8.4 Å and the hemes are almost co-planar. We believe it is unlikely to be merely coincidental that parallel heme planes have turned up in these two independent investigations, as will be discussed shortly.

**Correlation of the model with solution studies**

Before proceeding we wish to emphasize that the observed surface complementarity between cytochrome c peroxidase and cytochrome c and the resulting excellent stereochemical fit obtained from model-building experiments are quite striking. Therefore, we believe that the present hypothetical model is in fact a true representation of the actual electron transfer complex. Unfortunately, there are few data available that rigorously test our model other than a small number of experiments which have sought to determine the distance between hemes in the 1:1 complex. We will now briefly review the results of these experiments.

Gupta and Yonetani (1973) used nuclear magnetic resonance techniques to estimate the distance between hemes. They reasoned that if, in the 1:1 complex, the cytochrome c heme is relatively close to the heme iron atom in cytochrome c peroxidase, then changing the spin state of the latter by addition of F⁻ or CN⁻ should perturb the nuclear magnetic resonance signals generated by the cytochrome c heme methyl groups. No such effect was observed and it was concluded that the two hemes must be at least 25 Å apart. The closest cytochrome c peroxidase heme iron atom to cytochrome c heme methyl distances in our model is 19.3 Å and the greatest is 24 Å giving an average distance of about 21.6 Å, which agrees reasonably well with the estimate of Gupta and Yonetani.

A second approach, taken by Leonard and Yonetani (1974), was to replace the cytochrome c peroxidase heme with iron-free porphyrin and then determine the distance between the porphyrin in cytochrome c peroxidase and the heme in cytochrome c by measuring the quenching of porphyrin fluorescence due to the cytochrome c heme. The estimated cytochrome c peroxidase porphyrin to cytochrome c heme distance was 14.3 Å, which is in adequate agreement with the heme edge-heme edge distance of 16.5 Å measured from our model.

The last set of experiments to be considered were not directed toward estimating the heme-heme distance but are nevertheless relevant to the present discussion. Mochan and Nicolls (1971) found that the rate of reaction of cytochrome c peroxidase with H2O2 is unaffected in the presence of cytochrome c. Thus, in the cytochrome c peroxidase-cytochrome c complex, a channel leading from the external medium to the cytochrome c peroxidase heme crevice must remain open. As we reported previously (Poulos et al., 1978), the route for ligands to and from the heme crevice lies at the opening above pyrrole IV of the heme. Figs. 3 and 4 show that this channel remains open in our proposed model complex, enabling hydroperoxides to react with cytochrome c peroxidase even while it is bound to cytochrome c.

Perhaps the most direct support for our model comes from recent cross-linking experiments carried out by Bisson and Capaldi. They found that cytochrome c containing a single photoactive arylazido group attached to lysine 13 specifically cross-links with a region on cytochrome c peroxidase containing residues 32 to 48. This is precisely the region of interaction we would have predicted on the basis of our model since in our model Lys-13 of cytochrome c hydrogen bonds with Asp-37 of the peroxidase.

1 R. Bisson and R. Capaldi, manuscript submitted for publication.
MECHANISM OF ELECTRON TRANSFER

The occurrence of parallel heme planes in both the present model for the cytochrome c peroxidase-cytochrome c complex and in Salemme's model for the cytochrome b$_5$-cytochrome c complex (Salemme, 1976) suggests that the electron transfer mechanism may well involve a supramolecular conduction orbital composed of $\pi$ orbitals from both hemes. However, because of the large distance between hemes in the peroxidase-cytochrome c complex, 15.5 Å edge-to-edge, it is likely that intervening protein groups must also make a contribution (although in the b$_5$-c model, with an edge-to-edge distance of only 8.4 Å, direct electron transfer between hemes may be possible). In fact, examination of the model does reveal an intricate bridge of $\pi$-$\pi$ interactions, ionic interactions, and hydrogen bonds connecting the two hemes and suggests a possible path for transfer of electrons. We shall now describe these interactions in some detail.

The geometry of the proposed heme-heme bridge is illustrated in Fig. 6. Fig. 6, A and B, differ only in the orientation of the His-180 side chain in cytochrome c peroxidase. In Fig. 6A, the side chain of His-180 is shown as we have located it in the peroxidase electron density map. However, if maintained in this orientation in our model of the complex, His-180 would make an unfavorable close contact with the carbonyl oxygen of Ile-81 in cytochrome c and it must therefore move when the complex forms. Owing to steric restraints, there are only a limited number of orientations available and Fig. 6B shows the most likely one. What is most striking about the geometrical arrangement of residues and hemes in Fig. 6B is the number of planar conjugated and aromatic groups, including the hemes, which are parallel to one another. The His-180 imidazole ring is parallel to and 4.1 Å below the side chain of Phe-82 in cytochrome c. In turn, the benzene ring of Phe-82 is parallel to and 5 Å below the cytochrome c heme. Additionally, both the indole ring of Trp-51 and the guanidinium group of Arg-48 in cytochrome c peroxidase are parallel to and 3.6 Å above the distal surface of the heme. Finally, the Ile-81-Phe-82 peptide group in cytochrome c is approximately parallel to the His-180 imidazole ring in the peroxidase. Incidentally, it will be recalled that Phe-82 is conserved in all mitochondrial cytochromes c (Fergusson-Miller et al., 1979).

We suggest that the postulated conduction orbital is composed of overlapping $\pi$ orbitals contributed by the hemes and by the other parallel conjugated groups listed above and depicted in Fig. 6. In addition to these $\pi$-$\pi$ contacts, the heme-heme bridge also contains a system of hydrogen bonds and ionic interactions. First, observe that NE2 of His-180 in the peroxidase forms a 2.6 Å hydrogen bond with the peptide amido nitrogen of Ala-83 in cytochrome c. His-180 is then positioned with ND1 at a distance of 3.5 Å from the side chain oxygen atom of Thr-179 in the peroxidase for a possible intramolecular hydrogen bond. Meanwhile, the propionate group of pyrrole IV forms two strong hydrogen bonds with the side chain oxygen of Thr-179 and is also about 3.5 Å from the positively charged guanidinium group of Arg-48. The geometry of this last interaction does not, in fact, suggest hydrogen bonding but rather a charge-charge interaction (Poulos et al., 1980a). The Thr-179-propionate-Arg-48 interactions are probably quite strong since the propionate is well shielded from solvent by the side chains of Thr-179, His-180,

**Fig. 6. Proposed electron-proton bridge in the cytochrome c peroxidase-cytochrome c complex.** Cytochrome c peroxidase is to the right and cytochrome c to the left. Dashed lines indicate hydrogen bonds. Views A and B differ in the position of His-180 in cytochrome c peroxidase with A showing the position of His-180 in the native enzyme.
and Leu-181, while the Arg-48 guanidinium group is sequestered within the heme crevice and cannot be fully solvated.

Overall, the entire heme-heme bridge consists of a compact set of interactions with essentially no gaps. The largest distance between any two groups in the bridge is between the phenyl ring of Phe-82 and the heme ring in cytochrome c, a distance of about 5 Å.

Even if our current picture of the heme-heme bridge is somewhat imprecise or incomplete, it is nevertheless clear that the imidazole ring of His-180 is the only cytochrome c peroxidase side chain capable of interacting directly with the cytochrome c heme or with groups within the cytochrome c heme crevice. Therefore, we will now consider how His-180 might mediate the transfer of an electron from cytochrome c to cytochrome c peroxidase.

The first step in the reaction is the oxidation of cytochrome c peroxidase by a hydroperoxide molecule to produce Compound I which contains both a ferryl, Fe(IV), iron, and a radical probably centered on the indole ring of Trp-51 (Poulos and Kraut, 1980). We suggest that the presence of this "free" radical in Compound I plays a crucial role in the electron transfer mechanism. Three observations suggest that the radical readily communicates with other groups in the protein: 1) an electron spin resonance signal associated with Compound I is not indefinitely stable but decays with a rate constant of 9.3 × 10⁶ s⁻¹ (Erman and Yonetani, 1975a); 2) the spontaneous decay of Compound I results not only in the partial destruction of a tryptophan residue, probably Trp-51, but also in the destruction of tyrosine and phenylalanine residues as well (Erman and Yonetani, 1975b); and, 3) in Compound II, that is the enzyme at only one oxidation equivalent above the resting enzyme, a radical and the ferryl iron are in rapid equilibrium (Coulson et al., 1971). Therefore, we propose that the radical is able to communicate with the surface of the protein via the overlapping system of π orbitals and ionic interactions just described, generating an electron-deficient center at the cytochrome c peroxidase-cytochrome c intermolecular interface near His-180. Juxtaposition of the now electron-deficient His-180 imidazole ring and the electron-rich cytochrome c heme plus Phe-82 ring systems then creates a charge transfer complex that significantly lowers the energy barrier to transfer of an electron from the cytochrome c heme to His-180. The electron is then funneled into the peroxidase heme crevice via the system of overlapping π orbitals, ionic and hydrogen bonding interactions connecting His-180 with the peroxidase heme.

Thus far, we have described only a 1-electron reduction of cytochrome c peroxidase Compound I, but in fact, 2 electrons are required to regenerate the native enzyme. Initially, one might predict that the first and second electron transfer steps proceed by different mechanisms, especially since Kang et al. (1977) report the existence of a second kinetic process distinguishable binding site for cytochrome c on the peroxidase (although this second site has a 280-fold lower affinity for yeast iso-2-cytochrome c in Tris buffers). However, two observations lead us to suspect that the same mechanism operates in both electron transfer steps. First, as already mentioned, the radical and the ferryl iron are in rapid equilibrium in Compound II (Coulson et al., 1971). Therefore, the radical would also be able to play a fundamental role in the second electron transfer step by communicating with the intermolecular interface, just as in the first reduction. Second, Hayashi and Yamazaki (1979) have recently found that horseradish peroxidase Compounds I and II exhibit nearly the same midpoint potential. If in fact the corresponding states of cytochrome c peroxidase are also isopotential, then there need be no fundamental differences between the first and second electron transfer steps. In order to achieve this equivalence some readjustment in the local heme environment would probably accompany the transfer of an electron to Compound I. Exactly what the proposed conformational rearrangement might be is open to speculation, but its nature is suggested by our recent observation (Poulos and Kraut, 1980) that an unusual set of interactions exists in the internal pocket occupied by the proximal histidine, His-174. Specifically, His-174 appears to hydrogen bond with Gin-239 which in turn hydrogen bonds with a buried glutamate side chain, Glu-187. We suggested that the His-174-Gln-239-Glu-187 interactions impart some degree of anionic character to His-174, thereby stabilizing higher oxidation states of the iron atom during the catalytic reduction of hydroperoxides. It is conceivable that the dynamics of these interactions in the "proximal pocket" contribute to modulation of the enzyme’s oxidation-reduction potential during the electron transfer reaction as well.

CORRELATION OF THE MECHANISM WITH SOLUTION STUDIES

Fortunately, certain features of the proposed electron transfer mechanism can be tested. In particular, chemical modification experiments should aid in further elucidating the role of the pyrrole IV propionate, His-180, and the aspartate residues on the front surface of the cytochrome c peroxidase molecule. In fact, Asakura and Yonetani (1969) and Mochan (1970) have already shown that the cytochrome c peroxidase heme propionates are important in the electron transfer reaction. These investigators found that replacement of the heme in cytochrome c peroxidase with modified hemes containing esterified propionates: 1) does not prevent the reaction with hydroperoxides to give Compound I; 2) dramatically lowers V₅₀ in the reaction with ferrocyanochrome c but has little effect on Kₘ; and 3) does not prevent isolation of a 1:1 cytochrome c peroxidase-cytochrome c complex by gel filtration chromatography. Two general conclusions can be drawn from these results that are relevant to the present discussion. First, Result 1 indicates that esterification of the heme does not cause significant alteration in the geometry at the active site. Second, Results 2 and 3 indicate that cytochrome c can bind to cytochrome c peroxidase containing esterified hemes but that electron transfer is inhibited. In light of our proposed model, it is likely that esterification of the pyrrole IV propionate disrupts an important link in the circuit connecting the cytochrome c peroxidase and cytochrome c hemes.

In a preliminary set of experiments, we have already found that treatment of cytochrome c peroxidase with the carboxyl group specific reagent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Hoare and Koshland, 1967), rapidly eliminates the enzyme’s ability to oxidize ferrocytochrome c, most probably by blocking the essential carboxylate groups required to form the electron transfer complex (Poulos et al., 1980b). Although these results do support our proposed model, a more rigorous test would be to prepare a number of cytochrome c peroxidases containing selectively modified carboxyl groups and check the various derivatives for cytochrome c peroxidase activity and their ability to bind cytochrome c.

FEASIBILITY OF THE PROPOSED MECHANISM

Next we consider the feasibility of the three essential features of the proposed mechanism: 1) the participation of hydrogen bonding; 2) electron transfer through aromatic and conjugated systems; and 3) the formation of aromatic radicals. Hydrogen bonding is thought to play an important role in inorganic outer sphere electron transfer mechanisms (Reynolds and Lunry, 1966). Moreover, Horne (1963) concluded that hydrogen atom transfer could be achieved over distances as large as 100 Å between iron atoms via a system of hydrogen-
bonded water molecules. In addition, it appears to be generally agreed that a hydrogen-bonded polypeptide backbone provides a suitable semiconducting pathway in proteins (Boguslavski and Vannikov, 1970; Eley, 1968; Kopple et al., 1962).

Essential as the conductivity of specific amino acid side chains is concerned, Mayahi and Habboush (1965) found that the imidazole heterocycle should provide a good conducting pathway owing to its hydrogen-bonding capabilities and relatively low specific resistance. These latter two observations are particularly noteworthy in view of the proposed role of Thr-179 and His-180 in the cytochrome c peroxidase-cytochrome c electron transfer reaction. Because hydrogen bonding serves as an important link between the peroxidase and cytochrome c hemes and because hydrogen bonding can apparently serve to transfer hydrogen atoms, it is conceivable that His-180 mediates the transfer of a proton as well as an electron.

Electron transfer through aromatic and conjugated systems has long been thought to play an essential role in biological electron transfer reactions (Winfield, 1965; Dickerson et al., 1972). Thus, it is important to note that semiconduction (Katon, 1968), photoinduced conductivity (Kepler, 1960) and electron transfer between a mononegative aromatic radical and an uncharged aromatic ring (Ward and Weissman, 1956; McConell, 1961) are well known phenomena. However, a serious objection to any mechanism involving aromatic groups in biological electron transfer reactions is that a large free energy barrier must be overcome initially to form the aromatic radical (Ewall and Bennett, 1974). Indeed, a possible objection to our mechanism is that both Phe-82 in cytochrome c and His-180 in cytochrome c peroxidase might acquire, at least transiently, some radical character. Nevertheless, electron transfer through aromatic or peptide groups is not considered to present a significant energy barrier once the energetically costly step of removing or introducing an electron is achieved (Kopple et al., 1961). Therefore, it is important to re-emphasize that cytochrome c peroxidase Compound I already contains a radical probably localized on Trp-51. Furthermore, we have postulated that the radical communicates with the cytochrome c peroxidase-cytochrome c interface, thereby considerably lowering the free energy barrier to the most energetically difficult step of the reaction, namely the transfer of an electron from the cytochrome c heme to the cytochrome c peroxidase His-180 imidazole ring. The remaining steps in the reaction, namely the transfer of an electron and possibly a proton from His-180 to the cytochrome c peroxidase heme, should not present a significant energy barrier.

CONCLUSION

In conclusion, we briefly consider those features of our model which we believe may be generally relevant to other biological electron transfer systems.

First, the anticipated existence of a specific set of interactions between oxidation-reduction partners involving complementary charged groups is confirmed by the present study taken together with Salemme's cytochromes c-b model (Salemme, 1976). Similar ionic interactions may well play an important role in a wide variety of electron transport systems.

Second, we suggest, in accordance with Winfield's earlier ideas (Winfield, 1965) that protein radicals play an important role in biological electron transfer reactions. This is especially true for enzymes like cytochrome c peroxidase where a reactive oxygen intermediate generates a protein radical or electron "hole," thereby significantly lowering the free energy barrier to the electron transfer reaction. Furthermore, we expect protein radicals to participate in other biological electron transfer reactions even where activated oxygen intermediates are not involved, especially if the oxidation-reduction center itself is buried and not readily accessible to electron donors or acceptors. Validation of this prediction must, of course, await further information on the geometry of the oxidation-reduction centers in electron transfer proteins like the respiratory cytochromes c₇, b, and aa₃.

Third, we would predict that in systems where highly reactive intermediates are formed, the oxidation-reduction center must, as in cytochrome c peroxidase, be well sequenced by the polypeptide chain in order to protect the active center from nonspecifically discharging via random collisions with cellular oxidants and reductants. This requirement also demands that the protein itself participate in the electron transfer reaction by serving as a link between donor and acceptor. In this regard it is interesting to note that the distance between oxidation-reduction centers in the bacteriochlorophyll-cytochrome c complex (Dutton et al., 1979) and the cytochrome oxidase-cytochrome c₃ complex (Vanderkooi et al., 1977) is estimated to be ~25 Å. Thus, as in cytochrome c peroxidase, the bacteriochlorophyll and oxidase reactive centers must not be readily accessible because, in accordance with the above discussion, it is necessary to protect the reactive intermediates. In the case of bacteriochlorophyll, the reactive intermediate is a high energy radical (Dutton et al., 1979) while in cytochrome oxidase, the reactive intermediate resembles a peroxyl-iron intermediate (Chance et al., 1979).

Fourth and last, it is most unlikely that the finding of parallel hemes in both our model and in Salemme's cytochrome c-b model is an artifact of the model-building experiments. Moreover, fluorescent polarization experiments of cytochrome c bound to oriented membranes suggest that the cytochrome c heme and the oxidase heme are approximately parallel to one another (Vanderkooi et al., 1977; Leigh and Harmon, 1977; Erecinska et al., 1977). Therefore, it seems reasonable to propose as a working hypothesis that, in electron transfer reactions between heme proteins generally, the interactions between complementary charged groups are specifically designed to bring the hemes into parallel alignment. Such an arrangement enables the system to achieve physiological electron transfer rates by maximizing overlap between π orbitals and, as in the case of cytochrome c peroxidase, by optimizing interactions between mediating protein groups and the hemes.

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