The Cytochrome c Peroxidase-Cytochrome c Electron Transfer Complex

THE ROLE OF HISTIDINE RESIDUES*

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The histidine-selective reagent diethyl pyrocarbonate and dye-sensitized photooxidation have been used to study the functional role of histidines in cytochrome c peroxidase. Of the 6 histidines in cytochrome c peroxidase, 5 are modified by diethyl pyrocarbonate at alkaline pH and 4 by photooxidation. The sixth histidine serves as the proximal heme ligand and is unavailable for reaction. Both modification reactions result in the loss of enzymic activity. However, photooxidized peroxidase retains its ability to react with H2O2, and for form a 1:1 cytochrome c peroxidase-cytochrome c complex. It is, therefore, concluded that the extra histidine modified by diethyl pyrocarbonate is the catalytic site distal histidine, His 52.

In the presence of cytochrome c, no enzymic activity is lost by photooxidation and a single histidine, His 181, is protected from oxidative destruction. This finding provides strong support for the hypothetical model of the cytochrome c peroxidase-cytochrome c complex in which His 181 lies near the center of the intermolecular interface where it seems to provide an important link in the electron transfer process.

Cytochrome c peroxidase from yeast which catalyzes the oxidation of ferrocytochrome c is rapidly becoming one of the more thoroughly studied heme enzymes. Ever since Yonetani and Ray (1965) worked out a simplified method of purifying cytochrome c peroxidase, the enzyme has been studied by a number of sophisticated methods (Yonetani, 1976). These include recent determination of the amino acid sequence (Takio et al., 1980), the nucleotide sequence of the corresponding gene (Kaput et al., 1982), and the three-dimensional crystal structure (Poulos et al., 1980). For these reasons, cytochrome c peroxidase offers a particularly good model for understanding the mechanism of heme enzyme-catalyzed cleavage of O—O bonds. In addition, cytochrome c, which is the natural electron donor of the peroxidase reaction, is one of the best studied heme proteins with respect to both structural and electron transfer function (Margoliash and Bosshard, 1983). Therefore, the cytochrome c peroxidase-cytochrome c complex offers itself as an excellent probe to study the mechanisms of interprotein electron transfer.

In a preliminary attempt to correlate the wealth of biochemical and physical data available on both cytochrome c peroxidase and cytochrome c with the x-ray structure of these two heme proteins, one of us has proposed hypothetical stereochemical mechanisms for both the formation of compound I, a stable reaction intermediate of cytochrome c peroxidase with H2O2, and the cytochrome c peroxidase-cytochrome c electron transfer reaction (Poulos and Kraut, 1980a, 1980b; Poulos, 1982a, 1982b). In the cytochrome c peroxidase-cytochrome c complex, it has been found that surface carboxylates on cytochrome c peroxidase recognize and interact with the essential and highly conserved lysines lining the exposed heme edge of cytochrome c (Poulos and Kraut, 1980b; Bisson and Capaldi, 1981; Waldmeyer et al., 1982; Bechtold and Bosshard, 1982). Our present work has been designed to clarify the role played by histidines in the formation of the cytochrome c peroxidase-cytochrome c complex as well as in the proposed mechanism of electron transfer.

A particularly important feature of the proposed electron transfer complex is the prediction that the heme edges cannot be much less than 16–18 Å apart owing to the position of the cytochrome c peroxidase heme well within the heme crevice. This latter structural limitation eliminates the possibility of direct electron transfer between heme edges. However, the refined crystal structure1 of cytochrome c peroxidase shows that the imidazole group of His 181 is situated between both hemes near the center of the postulated interaction domain and may serve as a bridging group in the electron transfer reaction (Poulos, 1982b; Poulos and Finzel, 1984). His 181 also plays an important structural role in that the imidazole ring hydrogen bonds with one of the heme propionates and an aspartate residue (Asp 37). Asp 37 also forms a ionic link with Lys 13 of cytochrome c in the hypothetical model. Direct experimental support for the importance of the Asp 37 region in formation of the complex was provided by Bisson and Capaldi (1981) who demonstrated that a photoactive Lys 13 derivative of cytochrome c specifically cross-links with the 32–48 tryptic peptide of cytochrome c peroxidase.

A second histidine, His 52, is also thought to play a crucial role in the cytochrome c peroxidase catalytic cycle by serving as an acid/base catalyst in the formation of compound I (Poulos and Kraut, 1980a; Poulos, 1982a, 1982b). Coulson

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1 B. C. Finzel and T. L. Poulos, unpublished results.
and Yonetani (1972) proposed a similar role for histidine based on photooxidation experiments of apocytochrome c peroxidase.

Here we present chemical modification experiments using diethyl pyrocarbonate and dye-sensitized photooxidation to study the role of histidines in cytochrome c peroxidase catalysis. Briefly, we find that modification of only 2 histidines inhibits cytochrome c peroxidase activity, that His 181 is indeed at the intermolecular interface, and that His 52 is probably involved in the formation of compound I.

EXPERIMENTAL PROCEDURES

RESULTS

Inactivation by Diethyl Pyrocarbonate—Initial experiments were designed to learn under what conditions, if any, diethyl pyrocarbonate would inactivate cytochrome c peroxidase by ethoxyformylation of the imidazole side chain and pH was found to be the most sensitive variable. As shown in Fig. 1, diethyl pyrocarbonate modifies 3 of the 6 histidines of cytochrome c peroxidase at pH 7 within 15 min with no loss in peroxidase activity. At pH 8, however, 5 out of 6 histidines are modified with only a trace of activity remaining. Control experiments showed that cytochrome c peroxidase does not lose activity in the phosphate/KCN buffer over the time course of the reaction (data not shown).

Fig. 2 demonstrates that when the modification reaction with diethyl pyrocarbonate is run at pH 8, 2-3 histidines react before any loss of activity, again emphasizing that cytochrome c peroxidase has two slowly reacting histidines, the modification of which correlates well with the loss in activity.

Besides histidine, other nucleophilic groups may react with diethyl pyrocarbonate, in particular tyrosine, lysine, and cysteine (Miles, 1977). Tyrosine seems not to be modified appreciably since no change in optical density at 280 nm accompanied chemical modification with diethyl pyrocarbonate (Miles, 1977). Modification of lysine and cysteine cannot be ruled out. However, acetylation of lysines on cytochrome c peroxidase has only a negligible effect on activity (Waldmeyer et al., 1982), while the single cysteine residue of cytochrome c peroxidase can be blocked by p-mercuribenzoate without effect on enzymic activity (Yonetani, 1976). Therefore, it appears that the loss in activity results mainly, if not exclusively, from the modification of histidine residues.

In order to determine the effect of pH more carefully, the ethoxyformylation was carried out at pH values between 7 and 8. The results are shown in Fig. 3. These data indicate that modification of 1 or both of those histidines which are accessible to diethyl pyrocarbonate only at alkaline pH is responsible for the loss in activity. Alternatively, Fig. 3 could reflect a pH-induced conformational change in which the high pH histidines become available for reaction only at alkaline pH.

Cytochrome c peroxidase in the form of the cytochrome c peroxidase-cytochrome c complex was inactivated to the same degree as cytochrome c peroxidase alone, indicating that cytochrome c does not prevent diethyl pyrocarbonate from reacting with the crucial histidine residue(s). This result is in marked contrast to what we observe when the cytochrome c peroxidase-cytochrome c complex is photooxidized where cytochrome c is able to fully protect the enzyme from inactivation (see below).

Since (ethoxyformyl)histidine residues are unstable, it is not feasible to isolate peptides with modified histidines, and hence the 3 fast and the 2 slow reacting residues cannot be localized applying conventional protein chemistry. Therefore, photooxidation was employed to further test the role of histidines in the peroxidase reaction.

Inactivation by Photooxidation—Photooxidation in the presence of the anionic aromatic dye rose bengal partly inactivates cytochrome c peroxidase, while no inactivation occurs in the presence of the cationic aromatic dye methylene blue (Fig. 4). The rate of inactivation exhibits apparent first order kinetics. When the enzyme is present as the cytochrome c peroxidase-cytochrome c complex, no inactivation takes place (Fig. 4). Also, the extent of inactivation is pH-independent between pH 5 and 7 (not shown).

\[\text{FIG. 2. The number of histidine residues modified at pH 8 as a function of enzyme activity. Reaction conditions were as described in the legend to Fig. 1.}\]
Cytochrome c Peroxidase-Cytochrome c Complex

Photooxidation of histidine and tryptophan in cytochrome c peroxidase and in the cytochrome c peroxidase-cytochrome c complex

Numbers of amino acid residues destroyed are based on 6 histidines and 7 tryptophans in native cytochrome c peroxidase (CCP). Histidine was determined by amino acid analysis and tryptophan from the spectrophotometrically measured ratio of tyrosine to tryptophan, assuming 14 tyrosines in native cytochrome c peroxidase and no photooxidative destruction of tyrosine (see "Experimental Procedures"). Values are the average from two determinations. Numbers in parentheses are percentage residual activity determined immediately after photooxidation.

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<tr>
<td>120</td>
<td>4.0 (15)</td>
<td>2.9 (95)</td>
</tr>
</tbody>
</table>

*ND, not determined.

Substrate Binding and Steady State Kinetics—Binding of cytochrome c to native and photooxidized cytochrome c peroxidase was evaluated by gel permeation chromatography (not shown) and spectrophotometrically (Fig. 5). The stoichiometry of binding was 0.9 ± 0.1 cytochrome c/cytochrome c peroxidase which had been photooxidized for 120 min (15% residual activity). The dissociation constant was unchanged at about 10^{-5} M. Therefore, modification of the single histidine and the 2 tryptophans has no influence on the formation of the enzyme-substrate complex.

In order to further characterize the nature of inactivation, the initial velocities for the oxidation of ferrocytochrome c by H$_2$O$_2$ have been measured as a function of both the ferrocytochrome c and H$_2$O$_2$ concentrations, using native, ethoxyformylated, and photooxidized cytochrome c peroxidase (Fig. 6 and Table III). Both modification reactions slow down the apparent maximum turnover number for the two substrates, but the apparent $K_a$ values are unchanged. The reaction with ferrocytochrome c is not of the simple Michaelis-Menten type (Kang et al., 1978; Kang and Erman, 1982), but this can only be detected when the substrate concentrations span a wider range than used in the experiments of Fig. 6.

Compound I Formation—Cytochrome c peroxidase reacts with a stoichiometric amount of H$_2$O$_2$ to form a stable reaction intermediate known as compound I (Abrams et al., 1942; Yonetani, 1976). Compound I is a 2 oxidizing equivalents above the original ferric enzyme with the heme iron in the form of Fe(IV) and a stable free radical on some protein group (reviewed in Yonetani, 1976; Poulos and Finzel, 1984). This property confers to compound I a characteristic absorption spectrum which was used to titrate the number of H$_2$O$_2$ molecules required to oxidize one molecule of native and modified cytochrome c peroxidase. Cytochrome c peroxidase ethoxyformylated to below 5% residual activity fails to react with H$_2$O$_2$ to give compound I. The situation is more complex with photooxidized cytochrome c peroxidase. Here, addition of H$_2$O$_2$ still generates the characteristic absorption spectrum (Fig. 7), but the extent of reaction is below 1 mol of H$_2$O$_2$/mol of photooxidized cytochrome c peroxidase. In a typical example 0.65 mol of H$_2$O$_2$ reacts with photooxidized cytochrome c peroxidase having 30% residual activity. Therefore, photooxidation seems to interfere with, but not to abolish, the enzyme's ability to form compound I and, hence the

![Graph showing the activity of cytochrome c peroxidase over time](image-url)

**Fig. 4.** Photooxidation of cytochrome c peroxidase. Sixty μM enzyme in 15 mM sodium phosphate buffer, pH 6, was irradiated in the presence of 28 μM rose bengal. Samples were withdrawn at the time points indicated and assayed for peroxidase activity (○). C, control without irradiation; □, irradiation in the presence of 61 μM methylene blue. Photooxidation of the cytochrome c peroxidase-cytochrome c complex was carried out by irradiation of a solution containing 15 μM cytochrome c peroxidase and 30 μM cytochrome c (■).

Residual peroxidase activity was determined either immediately after photooxidation or else after addition of an excess of bovine serum albumin to the photooxidized probe followed by chromatography on Bio-Gel P-100. In the latter procedure, albumin competes for some rose bengal which otherwise remains tightly bound to the photooxidized cytochrome c peroxidase where it is revealed through an absorbance centered at 545 nm. The residual activity of samples treated in this way was occasionally higher than that of samples which were assayed without pretreatment. The extent of recovery of activity, however, varied between experiments and seemed to depend on pH. The variability in the pH-dependent recovery of activity was more pronounced after prolonged photooxidation. Recovery of peroxidase activity was at most 20%. The phenomenon was not investigated further.

Although photooxidation is not a very specific reaction, destruction of histidine occurs with some selectivity in the presence of the anionic dye rose bengal which binds to the protonated imidazole group at slightly acidic pH (Means and Feeney, 1971; Forman et al., 1973; Etoh et al., 1982). Indeed, the amino acid composition of cytochrome c peroxidase which had been irradiated for 120 min does not change within the error of the analysis except for a marked loss of histidine (Table I). In particular, neither tyrosine nor methionine is being destroyed to any appreciable extent in the presence of rose bengal.

 Destruction of tryptophan cannot be detected by conventional amino acid analysis. A spectroscopic method (Servillo et al., 1982) was, therefore, used to determine tryptophan. Up to 2 tryptophan residues are destroyed during prolonged photooxidation. Therefore, histidine and tryptophan are the 2 residues photooxidized under our experimental conditions.

Table II compares the number of histidines and tryptophans destroyed in cytochrome c peroxidase and in the cytochrome c peroxidase-cytochrome c complex. Very clearly, cytochrome c protects a single histidine from photooxidation, and none of the tryptophans is destroyed in the complex. Thus, it appears that the loss in peroxidase activity results from photooxidation of a single histidine and/or 1-2 tryptophan residues.

![Table II: Numbers of amino acid residues destroyed are based on 6 histidines and 7 tryptophans in native cytochrome c peroxidase (CCP). Histidine was determined by amino acid analysis and tryptophan from the spectrophotometrically measured ratio of tyrosine to tryptophan, assuming 14 tyrosines in native cytochrome c peroxidase and no photooxidative destruction of tyrosine (see "Experimental Procedures"). Values are the average from two determinations. Numbers in parentheses are percentage residual activity determined immediately after photooxidation.](table-url)

<table>
<thead>
<tr>
<th>Time of irradiation</th>
<th>Histidine</th>
<th>Tryptophan</th>
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</tr>
</tbody>
</table>

*ND, not determined.
The decrease of the maximum turnover number with $H_2O_2$ (Table III) cannot be fully explained by a lack of formation of compound I. Rather, photooxidation seems to inhibit preferentially some subsequent step of the reaction pathway.

Localization of the Protected Histidine—Since cytochrome c protects at the same time cytochrome c peroxidase from inactivation and a single histidine residue from oxidative destruction, we have attempted to localize this possibly crucial residue. To this end, photooxidized cytochrome c peroxidase was cleaved at methionine residues with cyanogen bromide, by cleavage of cytochrome c peroxidase, but none after photooxidation of the cytochrome peroxidase during photooxidation (Fig. 9, top, and Table IV). Native and photooxidized cytochrome c peroxidase yield the same set of CNBr fragments in comparable yield, indicating once more that methionine residues are not destroyed by photooxidation.

Of the 6 histidines of cytochrome c peroxidase, 4 are in fragment 1-119 and 2 in fragment 173-230/231 (Takio et al., 1980; Kaput et al., 1982). None of the histidines of fragment 1-119 is protected by cytochrome c since the histidine content of this fragment decreases similarly in the cytochrome c peroxidase-cytochrome c complex and in free cytochrome c peroxidase during photooxidation (Fig. 9, top, and Table IV).

In fragment 173-230/231, however, about 0.8 histidine is missing after a 120-min irradiation of cytochrome c peroxidase, but none after photooxidation of the cytochrome c peroxidase-cytochrome c complex (Fig. 9, bottom, and Table IV). The single protected histidine residue must therefore reside in this fragment. Since His 175 is the proximal heme ligand completely sequestered in the protein and therefore unavailable for chemical modification reagents, the single protected histidine must be His 181.

**DISCUSSION**

Of the 6 histidines in cytochrome c peroxidase, 5 are available to chemical agents, while the proximal histidine heme ligand (His 175) is sequestered within the proximal pocket and is unavailable for reaction (Fig. 10). Dye-sensitized photooxidation modifies 4 of the 5 available histidines, while at alkaline pH diethyl pyrocarbonate modifies all 5. Both reactions result in the loss of enzymic activity. Based on our fragmentation experiments, we conclude that the single histidine which is not photooxidized but is ethoxyformylated at alkaline pH is located in fragment 1-119. Photooxidized inactivated cytochrome c peroxidase still reacts with $H_2O_2$ to give the characteristic compound I spectrum, demonstrating that the catalytic machinery required for compound I formation remains virtually intact. Since His 52 is expected to play a role as an acid/base catalyst in the reaction with $H_2O_2$ (Poulos and Kraut, 1980a; Poulos, 1982a, 1982b), His 52 cannot be 1 of the histidines subject to photooxidation. On the other hand, ethoxyformylation at alkaline pH disrupts the ability of the enzyme to generate the characteristic compound I spectrum upon reaction with $H_2O_2$, leading us to conclude that His 52 is the residue which is not photooxidized but ethoxyformylated at alkaline pH. One possible reason why His 52 is available to diethyl pyrocarbonate but not to photooxidation is that the much larger rose bengal molecule used in the photooxidation experiments cannot penetrate through the access channel connecting the surface of the enzyme with the distal side of the heme.

**Fig. 9.** Histidine content of CNBr fragments 1-119 (top) and 173-230/231 (bottom) of cytochrome c peroxidase which was photooxidized for the periods indicated either as the free enzyme (solid line), or in the form of the cytochrome c peroxidase-cytochrome c complex (dashed line).

Ethoxyformylation is pH-dependent. At pH 7 or lower, only 3 of the 5 available histidines are ethoxyformylated with no loss in activity, while at alkaline pH, all 5 react, resulting in >95% loss in activity. Careful consideration of the ionic environment surrounding each histidine, chemistry of the diethyl pyrocarbonate reaction, and the stability of cytochrome c peroxidase at alkaline pH gives some clues as to which histidines are ethoxyformylated at the different pH values and why. Ethoxyformylation of histidine requires that the imidazole group be a neutral nucleophile. Therefore, we can expect the reaction between diethyl pyrocarbonate and any cationic histidine in cytochrome c peroxidase to be much slower than with a neutral histidine. Examination of the refined cytochrome c peroxidase structure shows that only His 181 resides in an environment conducive to a cationic histidine at neutral pH. His 181 is bracketed by and hydrogen bonds with two carboxylates, Asp 37 and a heme propionate. Both theoretical calculations (Matthew et al., 1979) and titration experiments (Ho and Russu, 1978) demonstrate that histidine juxtaposed to a carboxylate in this way will exhibit a pK $>8$. Therefore, we expect His 181 to remain protonated and relatively unreactive towards diethyl pyrocarbonate at
neutral pH, indicating that 1 of the 2 histidines ethoxyformylated at alkaline pH is His 181.

As we discussed earlier, His 52 is also ethoxyformylated at alkaline pH, but because no activity is lost when the diethyl pyrocarbonate reaction is run at acidic pH, His 52 is probably one of the "high pH" histidines. However, unlike His 181, His 52 is not expected to exhibit an unusually high pK (Poulos and Finzel, 1984), so the explanation for why His 52 reacts only at alkaline pH lies elsewhere. We suggest that His 52 becomes available for reaction at elevated pH levels owing to an alkaline-induced structural change which loosens the access channel leading from the enzyme surface to the active site, thereby rendering His 52 available to a diethyl pyrocarbonate molecule. In support of this hypothesis, we note that cytochrome c peroxidase irreversibly inactivates upon standing at alkaline pH unless a ligand like cyanide is present (Yonetani, 1976). The inactivation process is illustrated in the following simple scheme.

Low pH \rightarrow high pH \rightarrow inactive

The precise mechanism by which the high pH conformer is "loosened" is not clear, although we suggest that the low to high pH transition is intimately associated with deprotonation of His 181 for the following reasons. His 181 initiates an \( \alpha \)-helical turn enabling the side chains of His 181, Asn 184, and Ser 185 to provide a hydrogen bonding pocket for the pyrrole IV heme propionate (Poulos and Finzel, 1984). Moreover, the 181-185 helical turn forms one corner of the ligand access channel leading from the molecular surface to the active site. Deprotonation of the His 181 imidazolium group will disrupt the propionate-His 181-Asp 37 hydrogen bonds leading to a loosening of the access channel. As a result, a diethyl pyrocarbonate molecule can more readily approach His 52 in the active site. Unfortunately, both His 52 and 181 appear to react with diethyl pyrocarbonate at alkaline pH, we cannot directly test the functional role of His 181 using diethyl pyrocarbonate alone.

The photooxidation experiments, however, do suggest a crucial role for His 181. In the presence of cytochrome c, only His 181 is protected from photooxidation, indicating that His 181 is situated at the cytochrome c peroxidase-cytochrome c intermolecular interface as predicted by the hypothetical model (Poulos and Finzel, 1984). In addition, no activity is lost when the photooxidation experiment is carried out in the presence of cytochrome c, indicating a possible functional role for His 181. However, 2 tryptophan residues are also protected from photooxidation by cytochrome c, so we must consider the possibility that destruction of tryptophan is responsible for the loss in activity. Only 2 tryptophans are near the heme, 51 and 191; but since both are buried and extremely well ordered in the crystal structure, it is unlikely that either of these is appreciably oxidized. Trp 211 and 223, however, reside in a \( \beta \)-pair region which exhibits relatively high temperature factors in the crystal structure, indicating considerably more disorder or "floppiness" in this region of the molecule (Finzel, 1983). Furthermore, Trp 211 and 223 are situated on the surface of cytochrome c peroxidase to which cytochrome c binds, although the hypothetical model does not indicate a masking of either tryptophan when the complex forms. Nevertheless, formation of the complex may induce significantly greater order in this particular region of the cytochrome c peroxidase molecule, rendering Trp 211 and 223 unavailable for photooxidation.

Aside from Trp 51 and 191, none of the tryptophans in cytochrome c peroxidase, including 211 and 223, are near the heme nor do any of the tryptophans provide an obvious link between cytochrome c peroxidase and cytochrome c. Therefore, we suggest that photooxidation of tryptophan is not responsible for the loss in activity. Alternatively, we suggest in accord with the proposed role of His 181 in the electron transfer mechanism of Poulos and Finzel (1984) and Poulos and Kraut (1980b) that photooxidation of His 181 destroys an important link in the aromatic-ionic conduit which assists and directs the transfer of an electron from cytochrome c to cytochrome c peroxidase. Our observation on the steady state kinetic properties of photooxidized cytochrome c peroxidase gives additional support to the proposed function of His 181 since photooxidation decreases the rate of electron transfer, while the apparent \( K_m \) values for cytochrome c and H\(_2\)O\(_2\) remain unchanged.

Also consistent with the hypothetical model of the cytochrome c peroxidase-cytochrome c complex are our findings that cytochrome c does not protect cytochrome c peroxidase from inactivation by diethyl pyrocarbonate. In the hypothetical model, the access channel leading to His 52 remains open so that cytochrome c is not expected to interfere with ethoxyformylation of His 52. Since His 181 is buried in the cytochrome c peroxidase-cytochrome c complex and is most likely not ethoxyformylated, we attribute the loss in activity to ethoxyformylation of His 52. This is the one explanation most consistent with x-ray structures and the hypothetical model.

In conclusion, our present experimental findings lend fur-
ther support to the proposed role of His 52 as an acid/base catalyst in the formation of compound I, to the location of His 181 at the cytochrome c peroxidase-cytochrome c intermolecular interface, and to the possible role played by His 181 in the electron transfer reaction.

Acknowledgments—We thank B. Waldmeyer and R. Bechtold for help with some experiments and for fruitful discussions. The technical assistance of L. Braun is gratefully acknowledged.

REFERENCES

Cytochrome c Peroxidase-Cytochrome c Complex

Amino acid composition of cyto c peroxidase before and after 120 min irradiation in the presence of 20 μM benzalkonium. Values are the average of at least 2 determinations. No correction was made for the loss of benzalkonium and destruction of some amino acids during acid hydrolysis. Cys and Trp are not determined.

Table 1

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(a) no irradiation; (b) irradiated with 0.2% benzalkonium; (c) instrument correction was made for the loss of benzalkonium.

Table 2

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Table 3

| Amino acid composition of CNB-fragments 1-119 and 127-210 of native and photooxidized cyto c peroxidase. No correction was made for the loss of tyrosine, serine, and threonine by acid hydrolysis during acid hydrolysis. Cysteine, tryptophan, histidine, and tyrosine are not determined. Numbers in parentheses based on the known amino acid sequence (Taklo et al., 1982). Values are quoted from the work of H. Inoue et al. (1968).

Fragment 1-119

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Fragment 127-210

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(a) no irradiation; (b) irradiated with 0.2% benzalkonium; (c) instrument correction was made for the loss of benzalkonium.

Figure 1: Reaction of cyto c peroxidase with benzalkonium as a function of pH. The conditions were as described in Figure 1, except that the benzalkonium concentration was 0.2% at room temperature. The reaction was allowed to proceed for 30 min after which enzyme activities were determined.
Figure 5. Spectroscopic titration of cytochrome c of 0.17 mM phototreated cytochrome c peroxidase (100) and of 5.28 mM native cytochrome c peroxidase (1000) in 10 mM sodium phosphate, pH 6, according to Rees and Vitelo (1986). The arrows indicate the concentration of cytochrome c, and the numbers on the abscissa are the concentration of native cytochrome c peroxidase (1000). Titration of cytochrome c with peroxidase. The second slope of cytochrome c peroxidase at its is due to increasing gray light and light matter at high amounts, and is not due to a second 

Figure 7. Spectroscopic evidence for compound I formation. Top panel: Spectrum of phototreated cytochrome c peroxidase (1000) (solid line) and of native 

Figure 8. Chromatography of C66-fragments from phototreated cytochrome c peroxidase on a column (15 x 100 cm) of Sephadex G-200 (100-200 mesh) eluted with 10 mM phosphate buffer (pH 7.4) at 25°C. Fractions were collected and analyzed for protein content. The samples were analyzed by SDS-PAGE and Coomassie Blue staining. The protein bands were visualized using a gel imaging system. The gel was stained with Coomassie Blue, destained in 10% acetic acid, and imaged using a gel imaging system. The gels were scanned and the bands were quantified using ImageJ software. The results were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. The significance level was set at p < 0.05. The data are presented as mean ± SEM. The differences were considered significant if p < 0.05.
The cytochrome c peroxidase-cytochrome c electron transfer complex. The role of histidine residues.

H R Bosshard, J Bänziger, T Hasler and T L Poulos