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Proton NMR and Electrophoretic Studies of the Covalent Complex Formed by Cross-Linking Yeast Cytochrome *c* Peroxidase and Horse Cytochrome *c* with a Water-Soluble Carbodiimide†

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ABSTRACT: The 1:1 covalently cross-linked complex between horse cytochrome *c* and yeast cytochrome *c* peroxidase (ccp) has been formed by a slight modification of the method of Waldmeyer and Bosshard [Waldmeyer, B., & Bosshard, H. R. (1985) *J. Biol. Chem. 260*, 5184–5190]. This earlier study has been extended to show that efficient cross-linking of the two proteins can occur in a variety of buffers over a broad ionic strength range. The substitution of ferrocytochrome *c* for ferricytochrome *c* in the cross-linking studies resulted in an increased yield of 1:1 complex (~10–20%) under the conditions studied. An improved method for purifying the covalent complex in relatively large quantities is presented here as are the results of electrophoresis and proton NMR studies of the complex. Both electrophoresis and NMR studies indicate modification of some surface acidic amino acids in the covalent complex by the carbodiimide. The proton hyperfine-shifted resonances of cytochrome *c* are broadened in the covalent complex relative to free cytochrome *c*, and the resonances corresponding to the cytochrome *c* heme 3-CH_3 and 8-CH_3 groups are shifted closer together in the complex. Integration of NMR resonances confirms a 1:1 complex as the primary cross-linking reaction product. However, we also demonstrate that the covalent complex can be further coupled to ccp and to cytochrome *c* to form higher molecular weight aggregates.

Cytochrome *c* peroxidase (ferrocytochrome *c*:hydrogen peroxide oxidoreductase, EC 1.11.1.5; ccp)[1] from yeast catalyzes the hydrogen peroxide oxidation of ferrous cytochrome *c* (Yonetani, 1965; Conroy & Erman, 1978; Yonetani & Ray, 1966; Nicholls & Mochan, 1966; Kang et al., 1977; Kang & Erman, 1982) by a mechanism that is believed to involve specific complex formation between the two proteins (Mochan,

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[1] Abbreviations: ccp, cytochrome *c* peroxidase; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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1970; Mochan & Nicholls, 1971; Erman & Vitello, 1980; Poulos & Kraut, 1980; Kraut, 1981; Leonard & Yonetani, 1974; Bisson & Capaldi, 1981; Waldmeyer & Bosshard, 1985; Waldmeyer et al., 1982, 1980; Pettigrew & Seilman, 1982; Gupta & Yonetani, 1973). The concept of a catalytically required molecular docking complex between cytochrome *c* and cytochrome *c* peroxidase has therefore evolved on the basis of the structures of the individual proteins (Poulos & Kraut, 1980; Kraut, 1981; Poulos et al., 1980; Takano & Dickerson, 1981; Dickerson et al., 1971; Finzel et al., 1984).

Here we report results of a detailed electrophoresis study of the products of the chemical cross-linking reaction between horse cytochrome *c* (both ferri and ferro forms) and yeast cytochrome *c* peroxidase. The purified cytochrome *c*–ccp covalent complex was also studied by proton NMR spectroscopy. The cross-linking procedure that we adopted was that of Waldmeyer and Bosshard (1985) employing the so-called "zero-length" cross-linker EDC. It has been demonstrated in this previous work that the two proteins in the covalent complex are present in 1:1 stoichiometry and that the cross-linking occurs between lysine-13 and lysine-86 in the cytochrome *c* primary sequence and acidic residues on the peroxidase surface at primary sequence positions 32–37 (Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985). Furthermore, in combination with chemical modification studies (Bechtold & Bosshard, 1985), these results support the hypothetical structure for this molecular redox complex that was predicted on the basis of structural comparisons (Poulos & Kraut, 1980).

Experiences in our laboratories[2,3] have demonstrated that UV–visible (Erman & Vitello, 1980) and, so far, Raman[2] spectroscopies are nearly insensitive to formation of the noncovalent ccp–cytochrome *c* complex. For example, UV–visible extinction coefficients change by only about 2% upon complex formation. In contrast, large spectroscopic changes for the interaction between horse cytochrome *c* and cytochrome *c* peroxidase have been observed with proton NMR spectroscopy (Gupta & Yonetani, 1973; Satterlee et al., 1987). The changes observed by proton NMR are unambiguous and involve, in some instances, chemical shift changes of 288 Hz at 361 MHz. To observe them we take advantage of the fact that both high-spin (ccp) and low-spin (ferric cytochrome *c*) heme proteins exhibit proton hyperfine NMR shifts of heme substituent groups as a result of the heme-centered paramagnetism associated with the ferric ion (Satterlee, 1986).

The cross-linking studies described here were undertaken for several reasons. We are currently performing a systematic proton NMR investigation of the noncovalent interaction of cytochrome *c* and cytochrome *c* peroxidase under a variety of conditions.[3] These include variations in the type of buffer as well as the pH and ionic strength of the medium. By simulating the conditions of the NMR experiments in the cross-linking studies, we have attempted to assess the relative tendency of the two proteins to associate under a given set of conditions. In addition, it has been reported that the activity of the peroxidase (Kang et al., 1977; Kang & Erman, 1982) as well as the ability of the enzyme to bind cytochrome *c* at two separate sites (Kang et al., 1977; Kornblatt & English, 1986) is extremely sensitive to buffer conditions. By demonstrating that the covalent complex can be formed under a variety of conditions (i.e., where binding of cytochrome *c* at a single high-affinity site is favored or where the cytochrome *c* is thought to be able to bind at two separate sites) and purified in relatively large quantities, we provide evidence for the feasibility of spectroscopic and kinetic studies of the covalent complexes formed under these various conditions. Covalent complexes of cytochrome *c* and ccp prepared in our laboratory have recently been employed in kinetic studies, which include measurements of the rate of intramolecular oxidation of ferrocytochrome *c* by ccp compound I (Erman et al., 1987).

Experimental Procedures

Protein Preparation. Cytochrome *c* peroxidase was isolated and purified as previously described (Yonetani & Ray, 1965a; Erman & Vitello, 1980). Cytochrome *c* (horse heart type VI) was purchased from Sigma and used without further purification, although it was completely oxidized to the ferric state with potassium ferricyanide prior to its use in the cross-linking reaction. Following oxidation, the ferricytochrome *c* was passed through a small Dowex 1-X8 (Bio-Rad) column equilibrated with 0.1 M potassium phosphate, pH 7.0, in order to remove unreacted ferricyanide ion and its reaction products. The protein was then dialyzed against distilled deionized water and lyophilized. Ferrocytochrome *c* was prepared by the method of Yonetani and Ray (1965b) except that the Sephadex column was equilibrated in the buffer that was to be used in the cross-linking reaction.

Cross-Linking Conditions and SDS–PAGE Analysis of the Cross-Linking Reaction Products. The cross-linking reactions were performed by the method of Waldmeyer and Bosshard (1985) with minor modifications. The cross-linking reaction was carried out at 25 °C for 30 min in buffer (see Figure 2) in the presence of 10 mM EDC. The proteins were generally present in the reaction mixture in concentrations of 150 μM (cyt *c*) and 50 μM (ccp) (unless otherwise stated), and the reaction was quenched by the addition of solid sodium acetate to a concentration of 200 mM.

Cross-linking reactions (Figures 1 and 2) were analyzed by SDS–PAGE carried out on a Bio-Rad Protean II apparatus according to the procedure of Laemmli (1970). All SDS–PAGE samples were dialyzed against 0.01% NaCl prior to electrophoresis. The integrated intensities of the individual protein bands were estimated with a Zeineh soft laser scanning densitometer. Percentages of covalent complex estimated from gels were calculated relative to the amount of un-cross-linked ccp, taking into account the molecular weights of both species.

Purification of the 1:1 Covalent Complex. The 1:1 ccp–cytochrome *c* complex was isolated in high purity (greater than 98% pure) by a combination of gel filtration and ion-exchange chromatography. The addition of the ion-exchange column improved the purification of the previous study and enabled us to obtain relatively large quantities of purified 1:1 complex. In the first step, the reaction mixture was concentrated by ultrafiltration (Amicon, YM-5) to 5-mL total volume. Subsequently, it was loaded onto a Sephacryl S-200 column (2.5 × 140 cm) that had been equilibrated with 0.1 M ammonium acetate, pH 5.0, and washed with the same buffer at a rate of 15 mL/h. All fractions containing 1:1 complex (with the exception of fractions contaminated with higher molecular weight protein aggregates) were combined and dialyzed extensively against 10 mM sodium cacodylate, pH 6.0. The sample was then loaded on to a CM-Sepharose (Sigma) column (1.0 × 7 cm) equilibrated with the same buffer. Under these conditions, most of the complex adhered to the column while ccp flowed through. The column was then washed sequentially with approximately 500 mL of 10 mM sodium cacodylate, pH 6.0, and 100 mL of 50 mM sodium cacodylate,

[2] R. Alden, S. J. Moench, M. R. Ondrias, J. D. Satterlee, J. E. Erman, unpublished data.

[3] S. J. Moench, J. D. Satterlee, J. E. Erman, unpublished data.

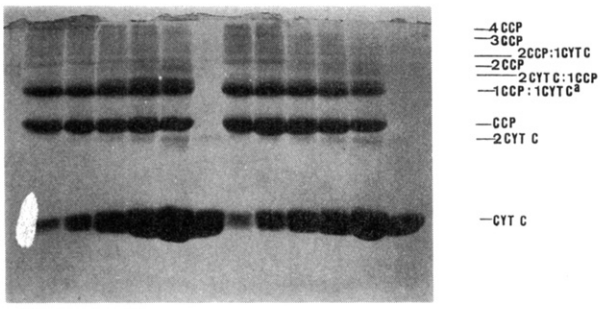

FIGURE 1: SDS (15%) gel comparing ccp cross-linked with ferro- and ferricytochrome *c* in 10 mM sodium cacodylate, pH 6.0. (Lanes 1–5) ccp cross-linked with ferrocytochrome *c*. The ratios of cytochrome *c* to ccp are 1:1 (lane 1), 2:1 (lane 2), 3:1 (lane 3), 5:1 (lane 4), and 10:1 (lane 5). (Lanes 7–11) ccp cross-linked with ferricytochrome *c* (cytochrome *c*:ccp ratio also varied between 1:1 and 10:1). Percentage of 1:1 complex formed: (lane 1) 43%; (lane 2) 40%; (lane 3) 43%; (lane 4) 39%; (lane 5) 43%; (lane 7) 39%; (lane 8) 35%; (lane 9) 35%; (lane 10) 36%; (lane 11) 34%. (Lane 6) Ferrocytochrome *c* + EDC; (lane 12) ferricytochrome *c* + EDC. (Superscript a) Splitting of the 1:1 complex band at higher ratios of cytochrome *c* to ccp was a reproducible phenomenon and may be due to the presence of different conformational populations of 1:1 complex.

pH 6.0. The column was not developed further. Rather, the resin containing the 1:1 complex was removed from the top of the column, placed in another small column, and eluted with 500 mM sodium cacodylate, pH 6.0. All purification steps described above were carried out at 4 °C.

NMR Measurements. Proton NMR spectra were obtained on a GE spectrometer operating at a proton frequency of 361 MHz. The details of spectral acquisition are identical with those previously described (Satterlee et al., 1983) except that active probe temperature control at 23 ± 1 °C was maintained (unless otherwise stated) and a recycle time of 800 ms was employed. Sample pH was monitored before and after data acquisition and found to be between 6.6 and 6.8 (uncorrected meter reading). Sample solutions were made up to be 2.8 mM for cytochrome *c* and 1.4 mM for the complex, which is a constant heme concentration.

Results and Discussion

Effect of Cytochrome c Oxidation State on the Yield of 1:1 Complex. Figure 1 presents the results of SDS–PAGE analysis of the cross-linking reaction carried out on ccp in the presence of both ferrocytochrome *c* and ferricytochrome *c* at cytochrome *c* to ccp ratios from 1.0 to 10. Although most studies involving the interaction of ccp with cytochrome *c* have been carried out with ferricytochrome *c*, ferrocytochrome *c* is the actual substrate of ccp while ferricytochrome *c* is a competitive inhibitor of the enzyme.

Upon integrating the intensity of the 1:1 complex band relative to the intensity of the free (unreacted) ccp present, it was observed that approximately 10–20% (depending upon conditions) less complex formed when ferricytochrome *c* was used in the reaction (see Figure 1 legend). This yield difference was most pronounced at cytochrome *c* to ccp ratios of 10:1. This result is consistent with the fluorescence study of Leonard and Yonetani (1974), who found a slightly greater dissociation constant for the ferricytochrome *c* complex than for the ferrocytochrome *c* complex.

Identification of Higher Molecular Weight Protein Aggregates by SDS–PAGE. In addition to the bands corresponding to unreacted ccp and cytochrome *c* and the 1:1 complex of these two proteins, five distinct higher molecular weight protein aggregates can be seen on SDS gels of the unpurified reaction mixtures. These have been identified (as labeled in Figure 1) by means of experiments in which cytochrome *c* and ccp were reacted alone with EDC and also individually combined with purified 1:1 complex and reacted with EDC.

In this study, very little dimerization of cytochrome *c* is detected while ccp is shown to readily dimerize as well as to form higher molecular weight aggregates. Dimerization of ccp by EDC was previously shown by Waldmeyer and Bosshard (1985) as was the formation of the 2:1 cytochrome *c*–ccp complex. However the 2:1 cytochrome *c*–ccp complex identified in the previous study was observed only when the cross-linking reaction was carried out at ratios of cytochrome *c* to ccp of 5:1 or greater. In contrast, we have detected 2:1 cytochrome *c*–ccp complex formation at cytochrome *c* to ccp ratios as low as 2:1. It is clear from Figure 1 that the amount of 2:1 cytochrome *c*–ccp complex increases as the ratio of cytochrome *c* to ccp is increased in the reaction mixture. This result is obtained regardless of whether ferro- or ferricytochrome *c* is present. We have also identified a 2:1 ccp–cytochrome *c* complex. Higher molecular weight protein aggregates may be of catalytic significance.

Although the yield of 1:1 complex is slightly higher when ferrocytochrome *c* is used in the cross-linking reaction, the overall similarity of reaction products for the cross-linking reaction of ccp with both ferro- and ferricytochrome *c* suggests that both forms of cytochrome *c* interact with ccp in fundamentally similar ways. This result provides justification for the use of ferricytochrome *c* in studies designed to investigate the enzyme–substrate complex formed between ccp and cytochrome *c*.

Effect of Buffer and Ionic Strength on Yield of 1:1 Complex. Figure 2 shows that ccp and ferricytochrome *c* can be cross-linked in various salt solutions over a wide concentration range, whereas cross-linking conditions were limited to 10 and 200 mM sodium cacodylate–HCl, pH 6.0, in the study of Waldmeyer and Bosshard (1985). In this work, yields of 1:1 complex of approximately 35–40% were obtained under low ionic strength conditions in agreement with the earlier study. In contrast to these previous results, we find that cross-linking occurs with approximately equal efficiency in both cacodylate and phosphate buffers (pH 6.0) at low ionic strength. We also find, in contrast to the work of Waldmeyer and Bosshard (1985), significant complex formation at higher ionic strengths (100 mM for phosphate; 300 mM for cacodylate).

There is some evidence to support the idea that the yield of covalent complex under a given set of conditions is related to the ability of the two proteins to associate under those conditions (in the absence of EDC) as well as to the ability of the two proteins to interact in a catalytically productive way. For example, Kang et al. (1977) measured the effect of various anions on the V_{max} of ccp at pH 6.0 (the common cation was Tris). In the case of Tris–phosphate, pH 6.0 buffer, no enzyme activity was observed above 100 mM, whereas substantial activity was noted in 200 mM Tris–cacodylate, pH 6.0, buffer. This result can be correlated with the data in Figure 2. Very little covalent complex forms in 200 mM potassium phosphate, pH 6.0 (~5%), while a 25% yield of complex is obtained in 200 mM sodium cacodylate, pH 6.0. In addition, recent NMR studies[3] of the noncovalent interaction of ccp and cytochrome *c* in potassium nitrate–D_2O solutions indicate that the heme methyl resonance chemical shift changes indicative of complexation are still partially detectable in 100 mM KNO_3 (pH 6.7), whereas these changes are not seen at KNO_3 concentrations of 300 mM.

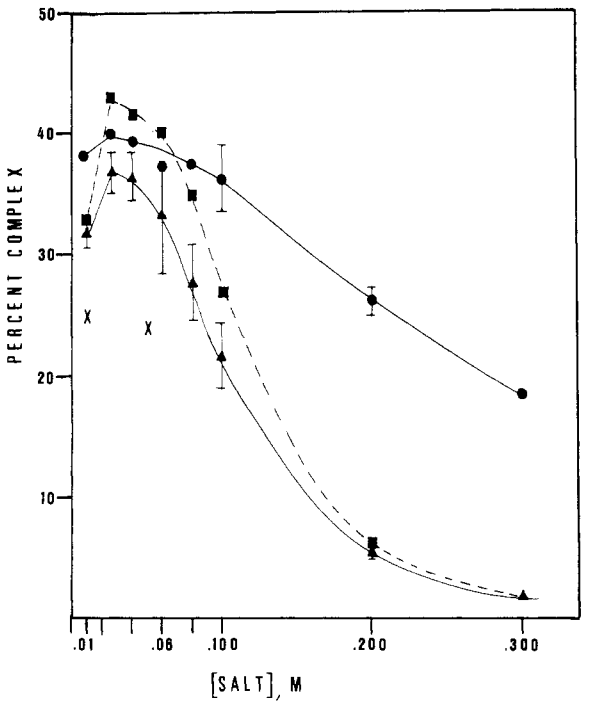

FIGURE 2: Percentage of 1:1 cross-linked complex formed in the presence of various salts relative to un-cross-linked ccp as estimated from densitometer traces of Coomassie Blue stained polyacrylamide gels: (□) potassium phosphate, pH 6.0, 0.01–0.3 M; (○) sodium cacodylate, pH 6.0, 0.01–0.3 M; (×) potassium phosphate, pH 7.0, 0.01–0.06 M; (△) potassium nitrate, pH ~7.0, 0.01–0.3 M. When cross-linking was done in neutral salts, the ratio of cyt *c* to ccp was 0.5 in order to keep the pH near neutrality. The cyt *c*:ccp ratio was 3.0 in the other experiments. Ferricytochrome *c* was used in all cross-linking experiments described here. The initial pH of the cross-linking reaction mixture in potassium nitrate (cytochrome *c*:ccp = 0.5) varied between 6.6 and 6.9 and increased 0.3–0.4 pH unit over the course of the incubation with EDC. Cross-linking was also carried out in sodium chloride, pH ~7.0. Results were very similar to those reported for potassium nitrate. These results are the average of two experiments. Error bars represent the variation in the two experiments, with the largest errors encountered for the neutral salts, probably as a result of no buffering capacity. Curves drawn through the points have no significance except to guide the viewer.

Evidence for EDC Modification of the 1:1 Complex at an Acidic Amino Acid Residue near 1-CH_3. The water-soluble carbodiimide EDC has been shown to be capable of modifying carboxylic acid groups (to *N*-acylurea) in the absence of nucleophiles that would be required to form the cross-linking amide bond (Timkovich, 1977). Accordingly, some of the 46 acidic amino acids (Asp and Glu) of ccp, especially those that occur on the surface of native ccp, are probably susceptible to EDC modification. Such modification is inferred from the fact that the ability of ccp to catalyze electron transfer from reduced cytochrome *c* to H_2O_2 was inhibited when the enzyme was pretreated with EDC (Waldmeyer et al., 1982). Here we present several pieces of evidence that demonstrate that EDC modification of the covalent cross-linked complex has occurred. Isoelectric focusing experiments (not shown) reveal that, whereas unreacted ccp can be focused into a tight band, the covalent complex, as well as ccp isolated from the reaction mixture, displays broad, diffuse bands. This suggests that the covalent complex isolated by our procedure contains a distribution of molecules that exhibit a range of isoelectric points due to EDC modification of the protein.

The proton NMR spectrum of hyperfine shift regions of the covalently cross-linked complex when both proteins are in the ferric oxidation state is shown in Figure 3A along with a portion of the ccp spectrum (Figure 3B) and the complete ferricytochrome *c* spectrum (Figure 3C), presented for comparison. Assignments designated for the covalent complex are made by comparison with the individual proteins and the noncovalent complex.

The most obvious NMR spectroscopic change observable for the covalent complex, relative to the free proteins, is the splitting of the resonance that corresponds in position to the assigned heme 1-CH_3 resonance of native ccp. In the spectrum of native ccp (Figure 3B), four heme methyl resonances (60–82 ppm) appear, whereas by comparison with the covalent complex spectrum (Figure 3A) the resonance corresponding to the heme 1-CH_3 group appears as two peaks. For several reasons, including the facts that similar splitting is not observed in the noncovalent complex NMR spectrum (Satterlee et al., 1987) and that the same 1-CH_3 resonance splitting is observed in the ccp monomer isolated from the reaction mixture (Figure 4), we conclude that the 1-CH_3 resonance splitting is due to EDC modification of an acidic amino acid (either to an *N*-acylurea or by the formation of an internal cross-link) that lies near the heme 1-CH_3 group in the ccp three-dimensional structure. A possible candidate for modification is Asp-146, which is in close proximity to heme 1-methyl while remaining exposed to the solvent as a surface amino acid (Poulos et al., 1980). This is shown in Figure 5. In this view, Asp-146 lies within 5 Å of the heme 1-CH_3. Modification, or partial modification, of this amino acid would, most likely, change the magnetic environment of the 1-CH_3. The remaining resonances are unaffected, indicating that the splitting is due to a highly localized effect and does not occur as a result of a more general conformational change.

Proton NMR Spectrum of the 1:1 Covalent Complex. Integration of the ccp methyl resonances and comparison with the integrated cytochrome *c* heme 8-CH_3 and 3-CH_3 resonances in the NMR spectrum of the covalently cross-linked complex show that the ccp:cytochrome *c* mole ratio is 1:1. Spectroscopic changes occurring upon complex formation include the rather large shifts in the cytochrome *c* heme 3-methyl and 8-methyl resonances (compared to uncomplexed cytochrome *c*) and the general line broadening that occurs for the cytochrome *c* resonances in the complex compared to cytochrome *c* alone (Table I). This line broadening has two possible origins. Upon covalent complex formation the effective molecular weight of cytochrome *c* nearly quadruples from approximately 12 kDa to over 46 kDa. Thus, the correlation time for motion of the heme group lengthens considerably as the overall rotational tumbling time of cytochrome *c* is changed to that of a molecule of nearly 4 times higher molecular weight. The effect of longer correlation times for proteins of this size is to cause line broadening via changes in the spin–spin (T_2) relaxation time of a given proton as well as to allow a second mechanism, Curie spin relaxation, to contribute to the resonance line width (Satterlee, 1986, 1987). The line broadening is much larger in the covalent complex than in the noncovalent complex due, in part, to the fact that in the noncovalent complex cytochrome *c* is rapidly exchanging between free and complexed states whereas in the covalent complex such exchange is impossible.

This inquiry was stimulated by the possibility of gaining experimental insight into the validity of the hypothetical ccp–cytochrome *c* molecular "docking" complex in solution. It is, perhaps, most instructive to consider these NMR results in light of this structure and results from the NMR study of the noncovalent complex. The computer modeling results and the analysis of the points of cross-linking in the covalent complex indicate that the primary sequence region 32–37 of

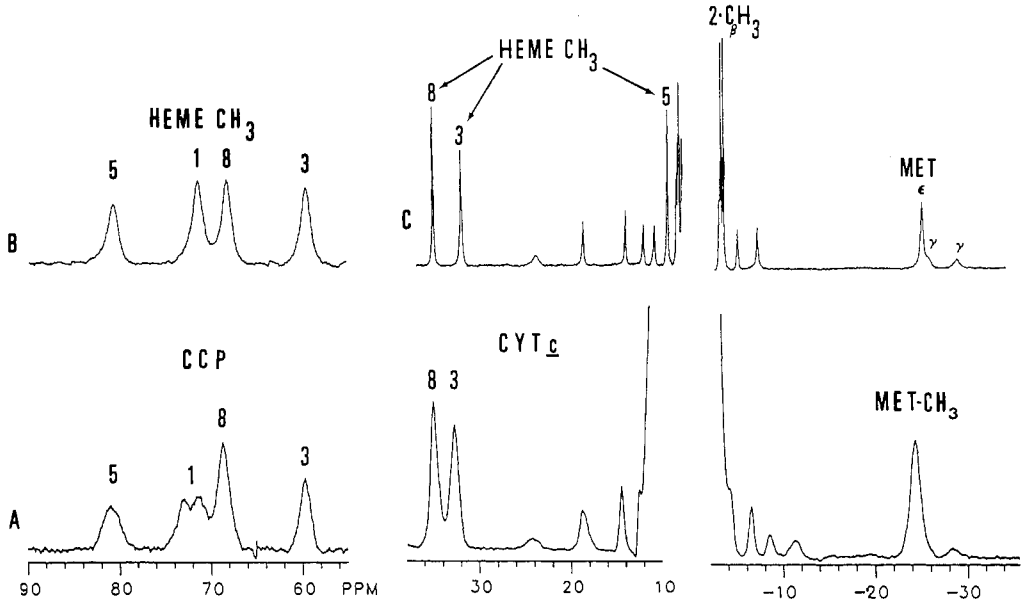

FIGURE 3: Proton NMR spectra of (A) the 1:1 ccp–cytochrome *c* covalently cross-linked complex, (B) the heme methyl region of native ccp, and (C) horse ferricytochrome *c*. Each spectrum was taken under the following conditions: pD 6.8 ± 0.2; temperature = 23 ± 1 °C; 10 mM KNO_3. The reference is the residual H^2HO peak, which was assigned a shift of 4.60 ppm. (Covalent complex shown here was cross-linked in 10 mM sodium cacodylate, pH 6.0.)

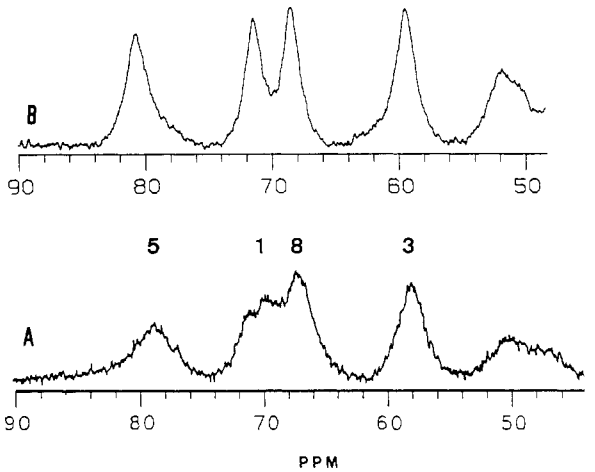

FIGURE 4: (A) Proton NMR spectra of the downfield heme methyl region of ccp that was isolated from the ccp–cytochrome *c* EDC cross-linking reaction. Experimental conditions: 10 mM KNO_3; pD 5.2; T = 30 ± 1 °C. (B) The same region of native ccp that has not been used in EDC cross-linking: 24 ± 1 °C; 10 mM KNO_3; pD 6.67. The difference in shift positions in the two spectra and in comparison to the spectrum in Figure 3 is due, in part, to the different pD and T values. Splitting of the 1-CH_3 resonance appears at all pD values between 5.2 and 7.2. Note the slightly different chemical shift scales in (A) and (B).

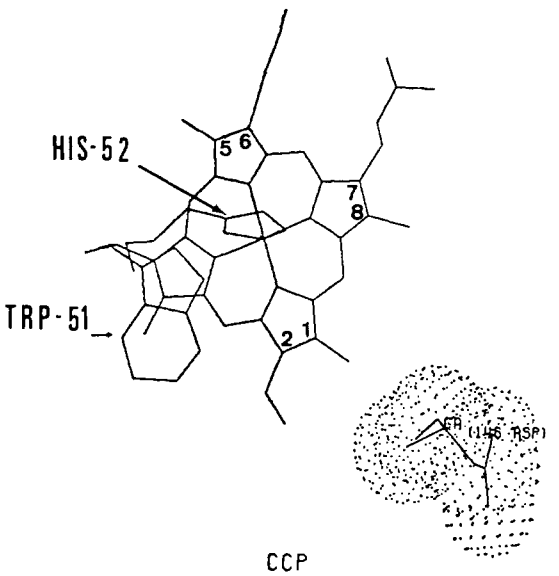

FIGURE 5: View of the ccp heme group from the distal side, perpendicular to the heme, showing the relative positions of the ccp heme 1-CH_3 and Asp-146 (which appears as a dotted van der Waals surface at the lower right). The distal amino acids Trp-51 and His-52 are also shown. This figure is constructed from coordinates in the National Protein Data Bank with the University of California, San Francisco, MIDAS program. The calculated Asp-146 C_α–1-CH_3 carbon distance is 4.85 Å.

ccp interacts with Lys-13 and Lys-86 on horse and tuna cytochromes *c* (Poulos & Kraut, 1980 Kraut, 1981; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985). In the three-dimensional structure of the hypothetical complex between ccp and tuna cytochrome *c*, the closest contact between the two proteins is made adjacent to the cytochrome's exposed heme edge at pyrrole II. Because of the overall similarity of the tuna and horse cytochrome *c* structures (Swanson et al., 1977), a virtually identical interaction should occur between horse cytochrome *c* and ccp. Our NMR results indicate this by revealing that it is the cytochrome *c* heme 3-methyl (located on pyrrole II) that experiences the largest shift upon complex formation in both the covalent and noncovalent complexes. Lys-13 is the next nearest neighbor to the heme 3-CH_3, which is itself partly exposed at the protein surface. Therefore, even minor structural rearrangement of Lys-13 upon complex formation with ccp would perturb the environment of the heme 3-CH_3. In addition, the chemical shifts of the 8- and 3-methyl groups of cytochrome *c* in the covalent complex are close to the corresponding shifts observed in the noncovalent complex of cytochrome *c* and ccp (Satterlee et al., 1987). This result indicates that the points of protein cross-linking determined by Waldmeyer and Bosshard (1985) are probably within the site at which the two proteins interact noncovalently. The heme methyl resonance shifts of ccp are essentially unaffected by either covalent or noncovalent complex formation. This is expected in view of the buried nature of the heme group in ccp (Finzel et al., 1984; Poulos et al., 1980), which, in contrast to the cytochrome *c* heme, is insulated from the interface contact region by the polypeptide. In summary, these NMR

Table I: Proton Shifts[a] and Line Widths[b] of Selected Hyperfine Resonances of the Covalent Cytochrome *c* Peroxidase–Cytochrome *c* (Horse) Complex in Comparison to Data for the Individual Proteins

	observed shift (ppm)			
resonance	covalent complex[c]	ccp[d]	EDC–ccp[e]	cytochrome *c*[f]
heme methyl				
5	80.8	80.7	78.8	
1	72.8		71.6	
1	71.1	71.5	69.7	
8	68.5	68.5	67.0	
3	59.5	59.5	57.8	
8′	34.9 (290)			35.2 (35)
3′	32.7 (300)			32.2 (50)
His-18	24.0			24.0
	18.6			18.8
	14.3			14.3
	12.4			12.4
	−2.59			
	−2.88			
	−4.40			−4.43
	−6.58			−6.60
4β-vinyl	−8.65	−8.42		
2β-vinyl	−11.1	−10.8		
Met-80 methyl	−24.5 (380)			−24.5 (110)
γ-methylene				−25.2
γ′-methylene	−28.6			−28.3

[a] Observed shifts are reported relative to internal H^2HO assigned a value of 4.60 ppm. [b] Line widths indicated in parentheses. [c] A 1.4 mM solution of the complex; pD 6.8; 23 ± 1 °C; 0.01 M KNO_3. [d] [ccp] = 0.003 M; 23 ± 1 °C; pD 6.8; 0.01 M KNO_3. [e] [ccp] = 0.001 M; 30 ± 1 °C; pD 5.2; 0.01 M KNO_3. Note different pD and *T* values. [f] [Cyt *c*] = 0.003 M; 23 ± 1 °C; 0.01 M KNO_3 plus 0.018 M ^2HCl; pD 6.5.

results are consistent with both the results of the previous cross-linking studies in the identification of the essential site of crosslinking and the Poulos–Kraut model for the structure of the complex.

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