

# Proton NMR Comparison of Noncovalent and Covalently Cross-Linked Complexes of Cytochrome *c* Peroxidase with Horse, Tuna, and Yeast Ferricytochromes *c*<sup>†</sup>

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**ABSTRACT:** Proton NMR spectroscopy at 500 and 361 MHz has been used to characterize the noncovalent or electrostatic complexes of yeast cytochrome *c* peroxidase (CcP) with horse, tuna, yeast isozyme-1, and yeast isozyme-2 ferricytochromes *c* and the covalently cross-linked complexes of cytochrome *c* peroxidase with horse and yeast isozyme-1 ferricytochromes *c*. Under the conditions employed in this work, the stoichiometry of the predominant complex formed in solution (which totaled >90% of complex formed) was found to be 1:1 in all cases. These studies have elucidated significant differences in the proton NMR absorption spectra and the one-dimensional nuclear Overhauser effect difference spectra of the complexes, depending on the specific species of ferricytochrome *c* incorporated. In particular, the results indicate that the noncovalent complexes formed between CcP and physiological redox partners (yeast isozyme-1 or yeast isozyme-2 ferricytochromes *c*) are distinctly different from the noncovalent complexes formed between CcP and ferricytochromes *c* from horse and tuna. Parallel chemical cross-linking studies carried out using mixtures of cytochrome *c* peroxidase with horse ferricytochrome *c*, and cytochrome *c* peroxidase with yeast isozyme-1 ferricytochrome *c* further emphasize such cytochrome *c*-dependent differences, with only the covalently cross-linked complex of physiological redox partners (cytochrome *c* peroxidase/yeast isozyme-1) displaying NMR spectra characteristic of a heterogeneous mixture of different 1:1 complexes. Finally, one-dimensional nuclear Overhauser effect experiments have proven valuable in selectively and efficiently probing the protein-protein interface in these complexes, including the environment around the cytochrome *c* heme 3-methyl group and Phe-82.

**E**lectron transfer between redox proteins enables energy to be directed in biological systems. Heme proteins such as *c*-type cytochromes are important electron transfer proteins, and there is presently a great deal of interest in complexes formed between cytochromes *c* and various partner metalloproteins (Kang et al., 1977, 1978; Poulos & Kraut, 1980a,b; Bechtold & Bosshard, 1985; Waldmeyer & Bosshard, 1985; Erman et al., 1987; Wendoloski et al., 1987; Moench et al., 1987; Satterlee et al., 1987a,b; Hazzard et al., 1987, 1988a,b; Liang et al., 1988; Northrup et al., 1988; Whitford et al., 1990; Burch et al., 1990; Bosshard et al., 1991; Zhou & Kostic, 1991). The role of protein structure in mediating the rate and specificity of electron transfer reactions between heme groups within protein complexes is an active area of research. Some factors that are considered to affect the physicochemical and kinetic properties of electron transfer complexes include (1) electrostatic and hydrophobic interactions that influence complex formation, (2) the overall dipole moments of the partner proteins, (3) the presence of certain aromatic groups in the electron transfer pathway, (4) the relative orientation of the heme groups in the complex, (5) the degree of exposure of the heme groups, (6) the distance between heme groups, (7) the

electromotive driving force, (8) the reorganization energy, and (9) the dynamics of the protein-protein interactions (McLendon, 1988; Everest et al., 1991).

An enzyme that has attracted interest in studies designed to sort out some of these factors is cytochrome *c* peroxidase (ferrocyclochrome *c*:hydrogen peroxide oxidoreductase; EC 1.11.1.5; CcP).<sup>1</sup> It is a 34-kDa, single-subunit native ferriheme enzyme from bakers' yeast that forms a reversible complex with various species of ferrocyclochrome *c* in the process of reducing hydrogen peroxide to water (Yonetani, 1976; Kang et al., 1977, 1978; Kraut, 1981; Poulos & Finzel, 1984; Bosshard et al., 1991). CcP and cytochromes *c* from the three species used in this work (horse, tuna, and yeast) are relatively small, water-soluble proteins that have been well characterized by X-ray diffraction methods so that structures are readily available (Poulos et al., 1980; Poulos & Finzel, 1984; Swanson et al., 1977; Takano & Dickerson, 1981; Louie et al., 1988; Louie & Brayer, 1990). In addition, both static and dynamic molecular modeling studies (Poulos & Kraut, 1980; Northrup et al., 1988) as well as extensive steady-state and transient kinetic studies (Kang et al., 1977; Kang & Erman, 1982; Erman et al., 1987; Hazzard et al., 1987, 1988a,b; Liang et al., 1988; Taylor-Conklin & McLendon, 1988) have been performed on CcP/cytochrome *c* complexes.

In this work we have undertaken studies of several CcP/ferricytochrome *c* complexes in which both physiological (yeast

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<sup>1</sup> Abbreviations: CcP, cytochrome *c* peroxidase; iso-1, yeast isozyme-1 cytochrome *c*; iso-2, yeast isozyme-2 cytochrome *c*; ferricyt, ferricytochrome; NOE, nuclear Overhauser effect; 1D, one dimensional; 2D, two dimensional; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

iso-1 and iso-2 ferricytochromes *c*) and nonphysiological (horse and tuna ferricytochromes *c*) redox partners for CcP have been used. The results presented here concern both covalently cross-linked (Moench et al., 1987) and noncovalent, or electrostatic, complexes (Satterlee et al., 1987a). They indicate significant species-specific differences in NMR spectra, chemical cross-linking products, and provide a preliminary qualitative assessment of complex dissociation dynamics. The combination of these data provides comparative information about factors 1, 3, and 9, described above, for the physiological and nonphysiological complexes.

This work relies primarily upon proton NMR spectroscopy, which has proven to be singularly sensitive in detecting complex formation between these proteins (Satterlee et al., 1987a,b; Moench et al., 1987). Also valuable is the fact that extensive proton assignments now exist for ferricytochromes *c* (Feng et al., 1989; Gao et al., 1990) which make possible structural interpretation of complex-induced NMR effects.

Whereas the kinetic studies of Kang et al. (1977) and Hazzard et al. (1987, 1988b) demonstrated that cytochrome *c* peroxidase is capable of kinetically discriminating between the mitochondrial cytochromes *c* from tuna, horse, and yeast and suggested that there may be differences in the orientation of the three cytochromes *c* within the noncovalent or electrostatic complex, our work reveals extensive proton NMR differences for complexes formed between CcP and physiological and nonphysiological redox partners. NMR results for the physiological redox pair indicate that a detailed kinetic analysis of complex dissociation dynamics will be possible in the future. The proton NMR and 1D NOE difference spectra obtained in this work have allowed us to investigate the effect of complex formation upon the solution-state structure of the various ferricytochromes *c* in the region of the protein near Phe-82. On the basis of studies of the complexes formed between wild-type and mutant cytochromes *c* with both CcP and cytochrome *c* oxidase, this highly conserved amino acid is believed to be situated at the protein-protein interface in the complexes and to be possibly involved in the formation of complexes or in the electron transfer process (Bosshard et al., 1991; Everest et al., 1991; Michel et al., 1989; Poulos & Kraut, 1980; Liang et al., 1988).

#### METHODS AND MATERIALS

**Noncovalent Complexes.** Horse (type VI), tuna (type XI), and yeast cytochromes *c* from *Saccharomyces cerevisiae* (types VIIIA and VIIIB) were purchased from Sigma Chemical Co. Yeast cytochrome *c* (type VIIIA, no longer commercially available) was found to be a mixture of isozyme-1 and isozyme-2, which were separated and purified as previously described (Satterlee et al., 1988; Moench & Satterlee, 1989). Yeast cytochrome *c* type VIIIB consisted solely of isozyme-1 with a minimal amount (~10%) of iso-1 dimer. All cytochromes *c* were oxidized with a stoichiometric amount of potassium ferricyanide (Fisher) in 0.05 M potassium phosphate (Fisher), pH 7.2. The proteins were then passed through small (1 × 1 cm) columns containing Dowex 1 × 8 resin (Bio-Rad) equilibrated in the same buffer, and the horse, tuna, and yeast iso-2 ferricytochromes *c* were extensively dialyzed against distilled, deionized water (Barnstead PCS). These ferricytochromes *c* were then lyophilized three times (twice in the presence of D<sub>2</sub>O). Yeast iso-1 ferricyt *c*, which has a tendency to autoreduce and to spontaneously dimerize during storage (Moench & Satterlee, 1989), was washed extensively with D<sub>2</sub>O using an ultrafiltration apparatus (Amicon) after elution from the Dowex column. Using an assay for free sulfhydryl groups (Habeeb, 1972; Zuniga & Nall, 1983), it was determined that

the yeast iso-1 cytochrome *c* was >90% monomer at the start of each NMR and cross-linking experiment. The cytochrome *c* peroxidase used in both the noncovalent and covalent complex experiments was isolated as previously reported (Erman & Vitello, 1980; Vitello et al., 1990). CcP samples for NMR studies were prepared by two methods. In the first method, crystals of the enzyme were dissolved in 0.1 M potassium phosphate, pH 7.0, and this solution was extensively dialyzed against distilled, deionized water. Before recrystallization occurred, the enzyme was lyophilized and then redissolved in D<sub>2</sub>O and lyophilized again. The second method involved dissolving the cytochrome *c* peroxidase crystals in 0.1 M potassium phosphate, pH 7.0, which was subsequently exchanged with D<sub>2</sub>O using an ultrafiltration apparatus as discussed above for yeast iso-1 cytochrome *c*.

NMR spectra were run at 500 MHz on a Varian Unity 500 (at 27 °C) and at 361 MHz (at 25 °C) on a GE360 spectrometer. All NMR samples were prepared with "100%" D<sub>2</sub>O (99.96%, MSD Isotopes or IsoTec) in the presence of 0.01 M KNO<sub>3</sub> (Mallinkrodt). Appropriate aliquots of 1 M KNO<sub>3</sub> in D<sub>2</sub>O were added to the protein solutions in D<sub>2</sub>O to make the solutions 0.01 M KNO<sub>3</sub>. Observed proton resonance shifts are reported relative to the residual water resonance assigned a value of 4.60 ppm. The pH, reported as pH', is the pH meter reading of D<sub>2</sub>O solutions uncorrected for the deuterium effect and was adjusted by the addition of small aliquots of DCl or NaOD (MSD Isotopes). Single-pulse and one-dimensional NOE measurements were conducted as previously described (Satterlee & Moench, 1987). One-dimensional NOE difference spectroscopy was favored over two-dimensional NOESY spectroscopy for the benefit of structural specificity and experimental efficiency not attainable by NOESY (Busse et al., 1990). The preirradiation pulse durations for NOE experiments were generally 100 ms, and decoupler power levels used for irradiation were 200 mW or less. The data are presented as difference spectra with the spectrum employing off-resonance irradiation subtracted from the corresponding spectrum employing on-resonance irradiation. Estimates of percent NOE of the Phe-82 phenyl ring proton resonances in the NMR spectra of the three ferricytochromes *c* were made as previously described (Moench et al., 1991).

**Covalent Complexes.** Horse and yeast iso-1 ferricyt *c* were chemically cross-linked to CcP in potassium phosphate buffers (10–300 mM) at pH 6.0, and analyses of the cross-linking reaction mixtures were carried out by SDS-PAGE as previously described (Laemmli, 1970; Moench et al., 1987). Isolation and purification of the covalent 1:1 complexes was carried out using a slight modification of our previous method (Moench et al., 1987), described below. The covalent complexes studied by proton NMR spectroscopy were cross-linked in 100 mM potassium phosphate, pH 6.0.

The sodium acetate-quenched cross-linking reaction mixture (50 μM CcP; 150 μM cytochrome *c* in a 175-mL volume) was concentrated by ultrafiltration to 20 mL and dialyzed extensively against 5 mM sodium cacodylate, pH 6.0. Analysis of the reaction mixtures were carried out by SDS-PAGE. The protein mixture was then loaded on to a small (1 × 10 cm) column of CM-Sepharose (Sigma) equilibrated with the same buffer and washed extensively with the buffer. Under these conditions, 70–90% of the uncomplexed CcP was eluted from the column. The remainder of the free CcP, as well as the majority of cross-linked CcP, was eluted from the column with 50 mM sodium cacodylate, pH 6.0, while 95–100% of the uncross-linked cytochrome *c* remained bound to the column. The covalent complex fraction was then concentrated to 5 mL

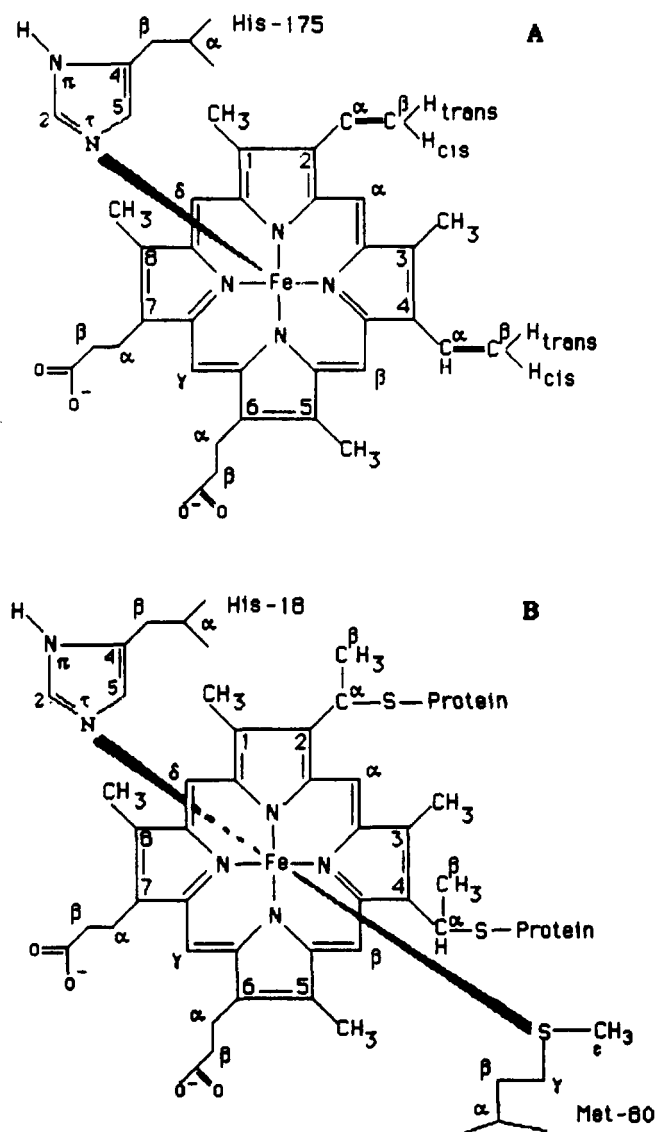


FIGURE 1: Coordination structure and labeling for heme and axial ligands of CcP (A) and cytochrome *c* (B).

via ultrafiltration and loaded on to a Sephacryl S-200 column (2.5 × 100 cm) equilibrated with 300 mM ammonium acetate, pH 5.0, and eluted at a rate of 10–20 mL/h with the same buffer. All fractions containing only 1:1 complex (as determined by UV-vis spectroscopy; Erman & Vitello, 1980) were pooled, and the sample was then exchanged into D<sub>2</sub>O/10 mM potassium nitrate solution by ultrafiltration. All 1:1 complex samples prepared in this way were determined by SDS-PAGE to be >95% pure with no free cytochrome *c* present. Final yields of the purified 1:1 complexes were approximately 50% of the total 1:1 complex initially formed in the cross-linking reaction mixture. Both yeast iso-1 and horse ferricytochromes *c* were also individually treated with EDC using the exact methods described above for the protein cross-linking reaction. The sodium acetate-quenched reaction mixtures of these ferricytochromes *c* were then directly exchanged into D<sub>2</sub>O/10 mM KNO<sub>3</sub> for NMR analysis.

The primary sequence numbering of yeast iso-1 that we have employed in this work is that which leads to the highest homology to horse cytochrome *c* (Senn et al., 1983). In this numbering scheme, the first five amino-terminal residues of yeast iso-1 are labeled -1 through -5, and the Gly that is actually residue 6 in the complete iso-1 sequence is labeled Gly-1 in order to correspond to Gly-1 in horse. Similarly, for

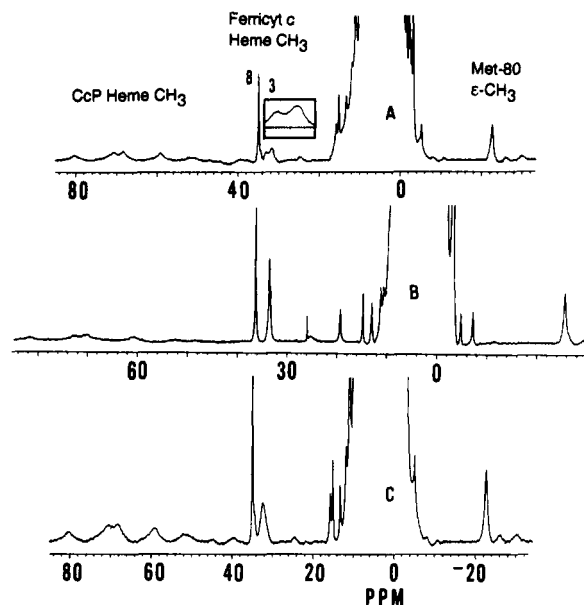


FIGURE 2: 500-MHz proton NMR spectra of noncovalent complexes of CcP (A) with yeast iso-1 ferricytochrome *c*, where [CcP] = 0.10 mM and [cyt *c*] = 0.26 mM; the box insert is an expansion of the heme 3-methyl resonances; (B) with horse iso-1 ferricytochrome *c*, where [CcP] = 0.12 mM and [cyt *c*] = 0.26 mM; (C) with yeast iso-1 ferricytochrome *c*, where [CcP] = 1.7 mM and [cyt *c*] = 4.1 mM. All samples were run in 10 mM KNO<sub>3</sub>/D<sub>2</sub>O solutions, 27 °C, pH 6.55. Panels A and C are plotted on identical horizontal scales; panel B has a different horizontal scale. The ferricytochrome *c* heme methyl resonances are aligned.

yeast iso-2 cytochrome *c* the amino-terminal sequence of residues are labeled -1 to -9 and the alignment with horse cytochrome *c* begins with Gly-10. This alignment allows us to refer, for instance, to the heme ligating histidine as His-18, uniformly without regard to species.

## RESULTS AND DISCUSSION

We have studied the following complexes of ferricytochromes *c* with CcP: (a) four different noncovalent complexes using horse, tuna, yeast iso-1, and yeast iso-2 ferricytochromes *c* as individual partners for CcP; (b) the covalently cross-linked (using EDC), isolated, and purified complex of CcP with horse ferricytochrome *c*; and (c) the covalently cross-linked (EDC), isolated, and purified complex of CcP with yeast iso-1 ferricytochrome *c*. The following presentation of results and discussion is organized into noncovalent and covalent complex sections.

### Noncovalent Complexes

**Complex-Induced Spectral Changes.** In all cases, formation of noncovalent complexes between CcP and partner cytochromes *c* at low ionic strength (10 mM KNO<sub>3</sub>) causes detectable changes in the proton NMR spectrum of each ferricytochrome *c*. As a reference for the ensuing discussion, we present the heme structures and labeling in Figure 1 and the 500-MHz proton NMR spectra of CcP/yeast iso-1 ferricytochrome *c* and CcP/horse ferricytochrome *c* noncovalent complexes along with selected resonance assignments in Figure 2. More complete complex-induced NMR shifts and assignments for all of the noncovalent complexes are presented in Table I. Figure 2 shows the highly shifted hyperfine resonances of CcP (a high-spin ferriheme protein) in the 40–80 ppm range (Satterlee et al., 1983) and the narrower, hyperfine-shifted resonances belonging to the ferricytochromes *c* (a low-spin ferriheme protein) in the 35 to 15 ppm and -5 to -30 ppm range (Satterlee, 1986). The resonances labeled in Figure

Table I: Changes in the Chemical Shifts<sup>a</sup> and Line Widths<sup>b</sup> of Selected Proton Resonances of the Heme and Neighboring Amino Acid Residues of Horse, Tuna, and Yeast Iso-1 and Iso-2 Ferricytochromes *c* upon noncovalent Binding to Cytochrome *c* Peroxidase in 1:1 Complexes

resonance <sup>c</sup>	chemical shift (ppm) <sup>d</sup> ferricytochrome <i>c</i>											
	horse			tuna			yeast iso-1			yeast iso-2		
	free	complexed	Δ(ppm)	free	complexed	Δ(ppm)	free	complexed	Δ(ppm)	free	complexed	Δ(ppm)
8-CH <sub>3</sub>	35.10 (32)	34.75 (103)	-0.35	35.09 (50)	34.86 (116)	-0.25	34.74 (38)	34.92 (128)	+0.18	34.20	34.48	+0.28
3-CH <sub>3</sub>	32.20 (40)	32.85 (116)	+0.65	32.33 (53)	32.97 (127)	+0.64	31.44 (36)	33.29 (118)	+1.85	30.81	32.61	+1.80
7α-CH	18.80	18.43	-0.37	19.62	19.24	-0.38	15.83	15.30	-0.63	17.14	16.54	-0.60
7α-CH							12.81	13.39	+0.58			
His-18β-CH	14.26	14.26	0	14.03	14.03	0	14.87	15.05	+0.18			
2β-CH <sub>3</sub>	-2.61	-2.56	+0.05	-2.52	-2.50	+0.02	-2.46	-2.36	+0.10			
Leu-68δ-CH <sub>3</sub>	-2.89	-2.83	+0.06	-2.98	-2.98	0	-3.42	-3.67	-0.25			
Pro-30δ-CH	-6.56	-6.47	+0.09	-5.93	-5.82	+0.11	-5.74	-5.25	+0.49			
Met-80ε-CH <sub>3</sub>	-24.44	-24.36	+0.08	-24.07	-23.94	+0.13	-23.35	-22.75	+0.60	-22.52	21.72	+0.80
Met-80γ-CH	-28.25	-28.22	+0.03	-28.19	-28.47	-0.28	-30.63	-29.94	+0.69			

<sup>a</sup>Proton shifts at 361 MHz are reference to HOD peak at 4.60 ppm. All samples are in 10 mM KNO<sub>3</sub>, pH' = 6.4, at 25 °C. Protein concentrations: ferricytochromes *c* alone = ~5–6 mM; ferricytochromes *c* in a complex = ~1.55 mM; CcP = 1.55–1.70 mM. <sup>b</sup>Line widths (Hz) of 3- and 8-CH<sub>3</sub> resonances are shown in parentheses. Line widths of complexed cytochromes *c* are calculated with 20-Hz line broadening. <sup>c</sup>Resonance assignments have been taken from a number of sources. (Keller & Wüthrich, 1978; Senn et al., 1983; Moore & Williams, 1984; Satterlee & Moench, 1987; Santos & Turner, 1987; Moench & Satterlee, 1989; Busse et al., 1990; Feng et al., 1989; Gao et al., 1990). <sup>d</sup>High frequency (downfield) shifts (Δ) are shown as positive in sign.

2 and Table I were assigned in previously published NMR studies of the four ferricytochromes *c* and for CcP (Keller & Wüthrich, 1978a,b; Satterlee et al., 1983; Williams et al., 1985a,b; Moore & Williams, 1984; Santos & Turner, 1987; Satterlee & Moench, 1987; Satterlee et al., 1988; Moench & Satterlee, 1989; Feng et al., 1989; Busse et al., 1990; Gao et al., 1990).

The data in Table I reveal that complex formation with CcP results in very similar complex-induced resonance shifts for horse and tuna ferricytochromes *c*. For horse and tuna ferricytochromes *c*, the largest complex-induced resonance shifts occur at the heme 8-CH<sub>3</sub> and 3-CH<sub>3</sub> resonances and at the heme 7α protons (Table I) (Satterlee et al., 1987a). For the remaining proton resonances listed in Table I and for several other resonances omitted from the table for brevity, the magnitude of complex-induced shifts for horse and tuna ferricytochromes *c* are either very small or zero.

In contrast to data for the horse and tuna ferricyt *c*:CcP complexes, Table I shows that noncovalent complex formation between CcP and both yeast iso-1 and iso-2 ferricytochromes *c* results in generally much larger and significantly more extensive complex-induced proton NMR shifts. These include the resolved, assigned resonances of amino acid protons that lie close to the heme or that are heme ligands, such as His-18, Met-80, Leu-68, and Pro-30, as well as the resonances of heme peripheral substituent groups. Similarly large shifts are found for the CcP/iso-2 noncovalent complex (Table I). The larger and more extensive shifts exhibited by both yeast iso-1 and iso-2 ferricytochromes *c* indicate that the physical and/or electronic structures of the iso-1 and iso-2 ferricytochromes *c* heme sites are more strongly linked to complex formation with CcP than are either the horse or tuna ferricytochromes *c*.

In several instances the direction of the complex-induced proton resonance shifts are different for the physiological redox complexes compared to the nonphysiological redox complexes. For example, when the heme 3-CH<sub>3</sub> and 8-CH<sub>3</sub> resonances in each complex of yeast iso-1 and iso-2 ferricytochromes *c* with CcP are compared, both shift in the same direction (to higher frequency), whereas for the horse and tuna complexes they shift closer together.

The respective movements of the heme methyl resonance pair of horse and tuna ferricytochromes *c* when complexed to CcP are precisely the kinds of shift changes induced in proton

NMR spectra of all three species of ferricytochromes *c* (horse, tuna, and yeast) as a result of increasing the solution ionic strength (Moench et al., 1991). Furthermore, the comparatively small magnitude of the complex-induced shifts demonstrated by the horse and tuna ferricyts *c* is remarkably similar to the magnitude of the changes caused by ionic strength variation (Moench et al., 1991). It therefore seems reasonable to conclude that a significant part of the horse and tuna ferricyt *c* complex-induced proton NMR shifts is attributable to CcP acting as a large polyelectrolyte. By comparison, the complex-induced heme methyl resonance shifts of yeast iso-1 and iso-2 ferricyts *c* are much larger in magnitude, in different directions, and, thus, are inconsistent with salt effects alone. So far no conditions of ionic strength, temperature, or pH have been found for which the complex-induced spectral changes for yeast iso-1 ferricyt *c* mimic those found for horse and tuna (unpublished data).

Resonance line broadening is also indicative of protein-protein association (Gupta & Yonetani, 1973; Eley & Moore, 1983; Satterlee et al., 1987a; Moench et al., 1987). Here, it is most readily illustrated by comparing Figure 3 panels A and E, which are typical of results observed for all of the noncovalent complexes formed at low salt. The broader lines (Table I) observed for the complexes are caused by the effective increase in rotational correlation time that results from the increased molecular mass of the complex.

**Complex Dissociation Dynamics.** The spectra presented in Figure 2 provide insights into the dynamics of cytochrome *c* exchange between CcP-bound and free (in solution) environments. This analysis focuses on the ferricytochrome *c* heme 3-methyl group resonance which occurs in the range 31–33 ppm, depending upon the species of cytochrome *c* and the extent of complex formation (Figures 2 and 3).

The proton spectrum shown in Figure 2A is that of a solution of CcP (0.10 mM) and yeast iso-1 ferricyt *c* (0.26 mM). The heme 3-methyl resonance is resolved into two resonances. The splitting is both temperature and concentration dependent and is most apparent at low temperatures and low protein concentrations. No similar splitting was observed for a mixture of CcP (0.12 mM) and horse ferricyt *c* (0.26 mM) under essentially identical conditions, as shown by Figure 2B, nor was heme 3-methyl resonance splitting resolvable in the CcP/iso-1 mixture at 16 times higher concentration of each protein (Figure 2C).

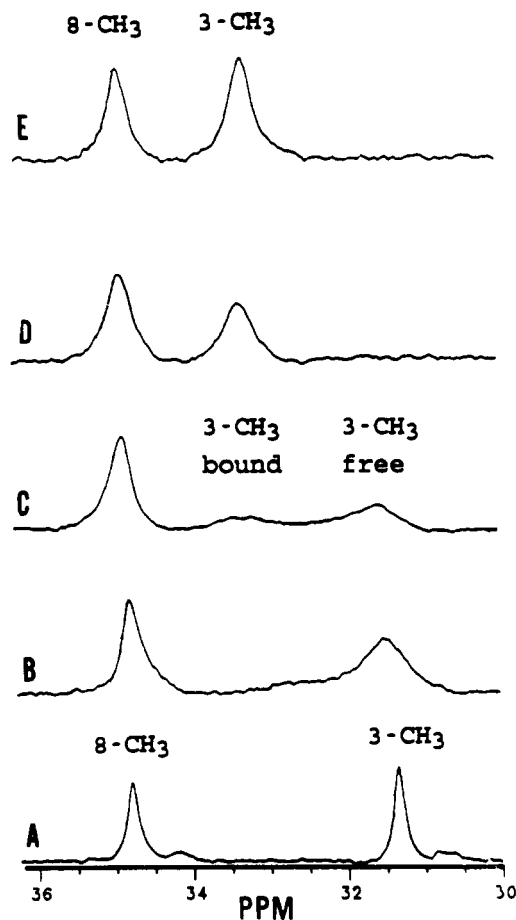


FIGURE 3: Expanded view of the heme methyl region of the 361-MHz proton spectrum of yeast iso-1 ferricytochrome *c* successively titrated with increasing amounts of CcP (10 mM KNO<sub>3</sub>, pH' 6.4, 25 °C). The ferricytochrome *c* concentrations were 0.3–0.6 mM. The concentration ratios, [CcP]/[cyt *c*], were (A) iso-1 ferricytochrome *c* alone, (B) 0.28, (C) 0.58, (D), 1.0, and (E) 1.5.

The "split-resonance" phenomenon (Figure 2A) is more easily seen in Figure 3, an expansion of the proton NMR spectrum around the iso-1 heme methyl resonances. This figure shows the effect of increasing relative amounts of CcP on the proton NMR spectrum of ferricyt *c*. Without CcP present in solution, the spectrum shows the free iso-1 ferricytochrome *c* heme 8-methyl (34.74 ppm) and 3-methyl (31.44 ppm) resonances (Figure 3A). Increasing the relative CcP concentration causes slight broadening and shifting of the heme 3-methyl resonance (Figure 3B) and then development of a second heme 3-CH<sub>3</sub> resonance at 33.29 ppm, corresponding to the CcP-bound form of iso-1 ferricyt *c* (Figure 3B,C). At the point where the CcP concentration is equal to or greater than the iso-1 concentration (Figure 3D,E), only the bound-form heme 3-methyl resonance of iso-1 is present (33.29 ppm).

The behavior of the heme 3-methyl resonance in the CcP/iso-1 noncovalent complex under conditions of low protein concentration (as in Figure 3) is characteristic of an exchange process occurring "slowly" on the NMR time scale (Pople et al., 1959). Under similar conditions, the simultaneous presence of individual free and CcP-bound heme 3-methyl proton resonances is not observed for the CcP/horse noncovalent complex; only a single peak is found (Figure 2B). Whereas in the CcP/iso-1 noncovalent complex distinct cytochrome *c* heme 3-methyl resonances are observed for free and CcP-bound forms of iso-1, in the CcP/horse noncovalent complex only a single-form cytochrome *c* heme 3-CH<sub>3</sub> resonance is observed, and its shift varies continuously during titration with

CcP (Satterlee et al., 1987a,b). This behavior exhibited by horse ferricyt *c* is characteristic of a process occurring "fast" on the NMR time scale, in this case yielding a single, averaged resonance for the heme 3-methyl environments in free and CcP-bound states (Pople et al., 1959).

One cannot conclude from these preliminary qualitative data that the dissociation rates are different for the two types of complexes, although that would be consistent with recent electron transfer results (Geren et al., 1991). Fortuitous observation of "slow" exchange in the CcP/iso-1 ferricyt *c* noncovalent complex derives from the larger complex-induced shift of the iso-1 ferricyt *c* heme 3-methyl group (668 Hz at 361 MHz) compared to the smaller complex-induced shift experienced by the horse ferricyt *c* heme 3-methyl group in the CcP complex (235 Hz at 361 MHz). These data emphasize the relative nature of the NMR exchange classifications "slow" and "fast", which are categorized relative to the frequency separations of identical resonances in the two (or more) environments (Pople et al., 1959). It is the uniquely large complex-induced shift of the iso-1 ferricytochrome *c* heme 3-methyl resonance (Table I) that provides the opportunity for potential elucidation of the rate law governing this exchange, as well as the kinetic constant.

Another aspect of the dynamic equilibrium association between CcP and yeast iso-1 ferricytochrome *c* is shown in Figure 2C. Figure 2C is a proton NMR spectrum taken when the *relative concentration ratio* of CcP and iso-1 is identical to that in Figure 2A but when the *absolute concentration* of each protein is 16 times larger than in the spectrum represented in Figure 2A. The situation represented by Figure 2C is one in which the CcP/iso-1 system demonstrates a spectrum that can be classified as "fast" exchange between free and CcP-bound forms. That is, the exchange rate has apparently increased to the point where individual heme 3-methyl resonances were not detected for the free and bound forms of iso-1 ferricytochrome *c*. Rather, an averaged heme 3-methyl resonance was observed under these higher concentration conditions. This piece of data illustrates the concentration-dependent exchange kinetics of noncovalent complex formation for the CcP/iso-1 noncovalent complex and suggests that a simple dissociation mechanism may not be consistent with the actual kinetic rate law. Our results under these specific conditions provide an estimate for the maximum iso-1 ferricyt *c* dissociation rate for the complex (668 s<sup>-1</sup>), although, in view of the previous discussion, we urge caution in interpreting this rate estimate until detailed kinetic studies, now in progress, are completed.

**Noncovalent Complex Stoichiometry.** Simultaneous detection of CcP-bound and free yeast iso-1 ferricyt *c* heme 3-methyl resonances allows straightforward determination of the stoichiometry for the predominant complex species in solution under the conditions of the NMR experiments. Figure 3 shows that at a [CcP]/[iso-1] ratio of 1.0 and higher all of the iso-1 ferricytochrome *c* is complexed, indicated by observed heme methyl resonance shifts of 34.92 and 33.29 ppm. At concentration ratios less than 1.0, where cytochrome *c* is in excess, uncomplexed iso-1 ferricytochrome *c* is clearly present, as indicated by the resonance corresponding to the heme 3-CH<sub>3</sub> of free iso-1 at 31.44 ppm and the smaller than maximal shift of the heme 8-methyl resonance at 34.83 ppm (Figure 3C). Titration results like these reveal that the stoichiometry of the predominant noncovalent CcP/yeast iso-1 ferricytochrome *c* complex is 1:1, within experimental error, consistent with the predominant product obtained by chemical cross-linking studies (vide infra) and with the previously established stoi-

Table II: The Effect of Noncovalent Binding to Cytochrome *c* Peroxidase on Selected Resonances in 1D NOE Difference Spectra Resulting from Heme 3-methyl Irradiation for Three Species of Ferricytochrome *c*

resonance <sup>b</sup>	chemical shift <sup>a</sup> (ppm)											
	horse			tuna			yeast iso-1			yeast iso-2		
	free	complexed	$\Delta$ (Hz)	free	complexed	$\Delta$ (Hz)	free	complexed	$\Delta$ (Hz)	free	complexed	$\Delta$ (Hz)
Phe-82 $\phi$ protons	5.88	5.96	29	6.01	6.08	25	5.97	6.27	108	6.00	6.29	104
4 $\beta$ -CH <sub>3</sub>	2.92	2.95	11	2.83	2.85	7	2.70	2.73	11	2.51	2.55	14

<sup>a</sup> Proton shifts at 361 MHz referenced to HOD peak at 4.60 ppm. Solution conditions: 10 mM KNO<sub>3</sub>, pH' 6.4, 25 °C. <sup>b</sup> Resonance assignments for free ferricytochromes *c* have been taken from a number of sources (Keller & Wüthrich, 1978; Satterlee & Moench, 1987; Moench & Satterlee, 1989; Feng et al., 1989; Busse et al., 1990; Gao et al., 1990). % NOE values of Phe-82  $\phi$  proton resonance relative to the heme 3-CH<sub>3</sub> resonance: 2–3% for free ferricytochromes *c*; 9–10% for complexed ferricytochromes *c*.

chiometry of the CcP/horse ferricytochrome *c* complex under similar conditions (Moench et al., 1987; Satterlee et al., 1987a).

Also, although these results confirm that the predominant species in these solutions are 1:1 complexes, we cannot rule out the presence of a small amount of 1:2 (CcP/iso-1) stoichiometry complexes on the basis of these data. As much as ~10% of a lower stoichiometry complex could go undetected in experiments such as those represented by Figure 3. Further, the absence of a free iso-1 ferricyt *c* heme 3-methyl resonance in solutions in which the relative concentration of CcP to iso-1 is  $\geq 1.0$  implies an equilibrium association constant at least as large as  $10^5$  M<sup>-1</sup>, consistent with the reported value of  $6 \times 10^6$  M<sup>-1</sup> (Erman & Vitello, 1980).

**Phe-82/Heme 3-Methyl Environment.** The heme 3-CH<sub>3</sub> substituent is situated near the exposed heme edge in all of the cytochromes *c*, and its resonance is the most perturbed by complexation. This area of the protein includes Phe-82 and forms part of the proposed cyt *c*/CcP interaction domain (Poulos & Kraut, 1980). There is conflicting evidence about a role for Phe-82 in these types of complexes. Contradictory evidence has been presented both for (Liang et al., 1988) and against (Everest et al., 1991) its participation in electron transfer with nonnative CcP. Phe-82 mutants of iso-1 displayed reduced activity with CcP (Pielak et al., 1985), and Phe-82 has been implicated in stabilizing complexes (Poulos & Kraut, 1980b; Wendoloski et al., 1987; Michel et al., 1989a,b; Bosshard et al., 1991). Regardless of the precise role of Phe-82, this region of cytochrome *c* is worthy of study since it involves the molecular docking site.

To probe the Phe-82/heme 3-CH<sub>3</sub> magnetic environment, we have used selective 1D NOE experiments. Previously, we demonstrated that proton resonances of Phe-82 in all four ferricytochromes *c* used in this study can be rapidly and selectively detected in 1D NOE experiments through connectivity to the irradiated heme 3-CH<sub>3</sub> resonance (Satterlee & Moench, 1987; Satterlee et al., 1988; Moench & Satterlee, 1989; Busse et al., 1990; Moench et al., 1991). For such selective information, we prefer 1D NOE methods, which we have found to be more efficient and specific than 2D methods (Busse et al., 1990) in situations like these. Figure 4 presents the results of proton homonuclear 1D NOE difference experiments, in which the on-resonance irradiation was selectively set at the heme 3-CH<sub>3</sub> resonance, for all four of the ferricytochromes *c*. Solid lines trace the spectrum for each of the free ferricytochromes *c*, and broken lines trace the spectrum for each of the CcP-complexed ferricytochromes *c*.

The most significant result is that complex formation with CcP induces shifts in the Phe-82 ring proton resonances for each of the ferricytochromes *c* (Figure 4; Table II). These complex-induced shifts are comparatively slight for the horse and tuna ferricytochromes *c* (~25 Hz), but they are over 4 times larger (~108 Hz) for the yeast ferricytochrome *c* isozymes. The larger shifts for iso-1 and iso-2 ferricytochromes

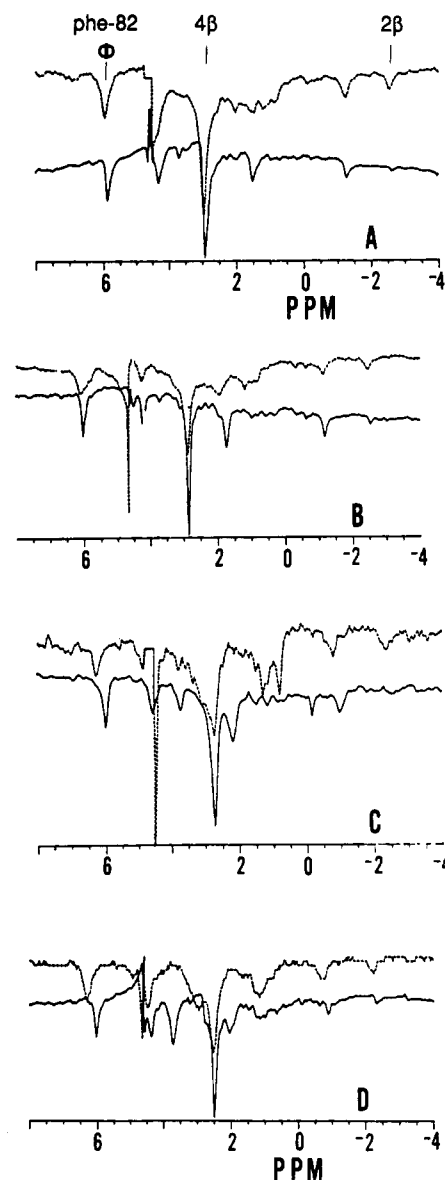


FIGURE 4: Selected region of the 361-MHz proton 1D NOE difference spectra obtained upon irradiation of the heme 3-methyl resonance for ferricytochromes *c* alone in solution (solid line) and in the presence of an equimolar amount of CcP (dashed line). (A) Horse ferricytochrome *c*. (B) Tuna ferricytochrome *c*. (C) Yeast iso-1 ferricytochrome *c*. (D) Yeast iso-2 ferricytochrome *c*. Cytochrome *c* concentrations were between 5 and 6 mM in samples without CcP, and the 1:1 mixtures were 1.55 mM in each protein; other solutions conditions were identical to those given in the legend to Figure 3.

*c* are in the direction (toward higher frequency) where the Phe-82 ring protons resonate in the reduced, diamagnetic forms of these cytochromes *c* (Senn et al., 1983; Gao et al., 1990). This type of shift is consistent with reorientation of Phe-82 away from the paramagnetic center and into the more dia-

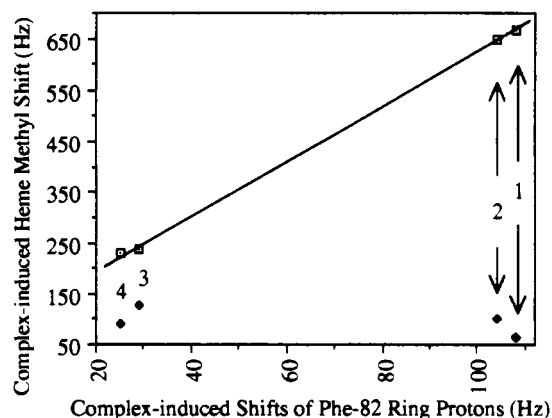


FIGURE 5: Magnitude of complex-induced shifts ( $|\delta_{\text{free}} - \delta_{\text{bound}}|$ ) for ferricytochrome *c* Phe-82 phenyl ring protons plotted against the magnitude of complex-induced shifts for the ferricytochrome's heme 3-methyl ( $\square$ ) and 8-methyl ( $\blacklozenge$ ) protons. Different 1:1 noncovalent complexes are designated by numbers: 1, CcP/yeast iso-1; 2, CcP/yeast iso-2; 3, CcP/horse; 4, CcP/tuna. The line drawn is from the linear least-squares regression analysis. The correlation coefficient for the 3-methyl data ( $\square$ ) is 1.00, while for the 8-methyl data ( $\blacklozenge$ ) it is 0.57.

magnetic environment of the protein-protein interface, in a manner similar to that proposed as part of the peroxidase mechanism (Poulos & Kraut, 1980a,b; Kraut, 1981; Finzel et al., 1984) and predicted to be possible in complexes of cytochromes *c* with other redox partner proteins (Wendoloski et al., 1988). The idea of conformational rearrangement of Phe-82 in complexes of cytochrome *c* and chemically modified cytochrome *c* derivatives has also been considered in discussions of other spectroscopic observations (Falk & Angstrom, 1983; McLendon, 1988; Hazzard et al., 1988; Michel et al., 1989a,b; Everest et al., 1991).

The relatively small Phe-82 shifts and the less extensive overall changes in the 1D NOE difference spectra exhibited by the horse and tuna cytochromes *c* complexed to CcP may be attributable primarily to electrostatic environment changes near the exposed edge of the ferricytochromes *c* because similar proton NMR spectral changes are caused by ionic strength variation of ferricytochrome *c* solutions (Moench et al., 1991). Similarly small shifts apparently occur for cytochrome *c* complexes with cytochrome *b*<sub>5</sub> (Eley & Moore, 1983; Burch et al., 1990; Whitford et al., 1990).

The larger magnitude and greater extent of the noncovalent complex-induced changes in the NOE difference spectra for the CcP complexes of yeast ferricytochromes *c* probably result from structural alterations in the heme environment that are comparatively less for CcP's nonphysiological redox partners. An apparent linear relationship exists between the magnitude of the complex-induced Phe-82 ring resonance shifts and the magnitude of the complex-induced heme 3-CH<sub>3</sub> resonance shifts (but not, for example, the 8-CH<sub>3</sub> shifts) for all of these ferricytochromes *c* (Figure 5), illustrating the intimate relationship of the magnetic environments of Phe-82 and the heme 3-methyl.

It is important to note that the CcP/ferricyt *c* NMR studies reported here have been performed under relatively low ionic strength conditions, on enzyme-product complexes of CcP and cytochrome *c*. While electron transfer rates in the horse and tuna CcP/cytochrome *c* complexes are relative high under low ionic strength conditions, the transfer of electrons in the CcP/yeast cytochrome *c* complexes is not as efficient (Hazzard et al., 1987, 1988a); so, to the extent that the ionic strength here differs from that of the yeast mitochondrial intermembrane space, the data presented here may not be characteristic

of the complex in vivo. A comprehensive study based upon ionic strength variations is currently underway.

### Covalent Complexes

**SDS-PAGE Analysis of EDC Cross-Linking.** In order to directly compare cross-linking results for physiological and nonphysiological CcP complexes, we have carried out identical cross-linking procedures in tandem on the CcP/yeast iso-1 ferricytochrome *c* pair and the CcP/horse ferricytochrome *c* pair. In view of other protein cross-linking work using EDC (Mauk & Mauk, 1989; Peery & Kostic, 1989; Zhou & Kostic, 1991; Matthews & Brittain, 1986), we have been careful to use appropriate controls so that valid comparisons can be made for cross-linking results with CcP and physiological and nonphysiological partners. The specific controls are described in subsequent sections. Results of the cross-linking experiments were analyzed by SDS-PAGE (not shown). In each reaction mixture, the major cross-linked product was the 1:1 complex. A comparison of the cross-linking products formed under a variety of buffer conditions indicated that several differences occur, depending on whether yeast iso-1 ferricyt *c* or horse ferricyt *c* was present in the reaction mixture. First, cross-linking under low ionic strength buffer conditions (buffer concentration = 10 mM) revealed that the yield of 1:1 CcP/yeast iso-1 ferricyt *c* covalent complex was lower (~10%) than the yield of the 1:1 CcP/horse ferricyt *c* complex (~40%). Second, at higher buffer concentrations (ranging up to 300 mM), the yield of 1:1 CcP/yeast iso-1 covalent complex was much higher (~20%) than the yield of the 1:1 CcP/horse ferricyt *c* covalent complex (<5%).

Lower yields for the 1:1 covalent CcP/yeast iso-1 complex relative to the CcP/horse complex formed under low ionic strength conditions may be due to the tighter binding suggested to occur in the former complex at low ionic strength (Kang et al., 1977; Das et al., 1988), which could inhibit EDC penetration of the interaction domain. Higher yields of 1:1 covalent complex for CcP/yeast iso-1 cytochrome *c* relative to CcP/horse cytochrome *c* under higher ionic strength conditions show that the interaction of the physiological redox partners is not as dominated by electrostatic effects as is the interaction of the nonphysiological redox pair and indicates that hydrophobic interactions play a substantial role in the binding energetics of the CcP/yeast iso-1 ferricytochrome *c* complex, as previously suggested (Das et al., 1988).

**NMR Comparison of Covalent Complexes.** Previous CcP/ferricyt *c* cross-linking work was carried out using EDC, in low ionic strength solutions of sodium cacodylate (buffer concentration = 10 mM, pH 6.0) with acetate ion quenching. Subsequent NMR comparisons of purified covalently cross-linked 1:1 complexes with horse cytochrome *c* were uniformly performed in low ionic strength solutions of potassium nitrate (10 mM, pH 6.4–6.9) (Moench et al., 1987; Satterlee et al., 1987a,b).

The purified 1:1 covalently cross-linked complexes prepared for the NMR studies presented here were also formed using EDC, and NMR spectra were also obtained on low ionic strength solutions of the purified complexes, but the cross-linking reaction was carried out in 100 mM potassium phosphate, pH 6.0. These buffer conditions were chosen as a result of steady-state and transient kinetic studies which revealed that the turnover numbers and rates of electron transfer were much higher in solutions of CcP and yeast iso-1 cytochrome *c* under high ionic strength conditions (Kang et al., 1977; Hazzard et al., 1988b).

Figures 6 and 7 show side-by-side comparisons of two portions of the proton NMR spectra of the isolated, purified



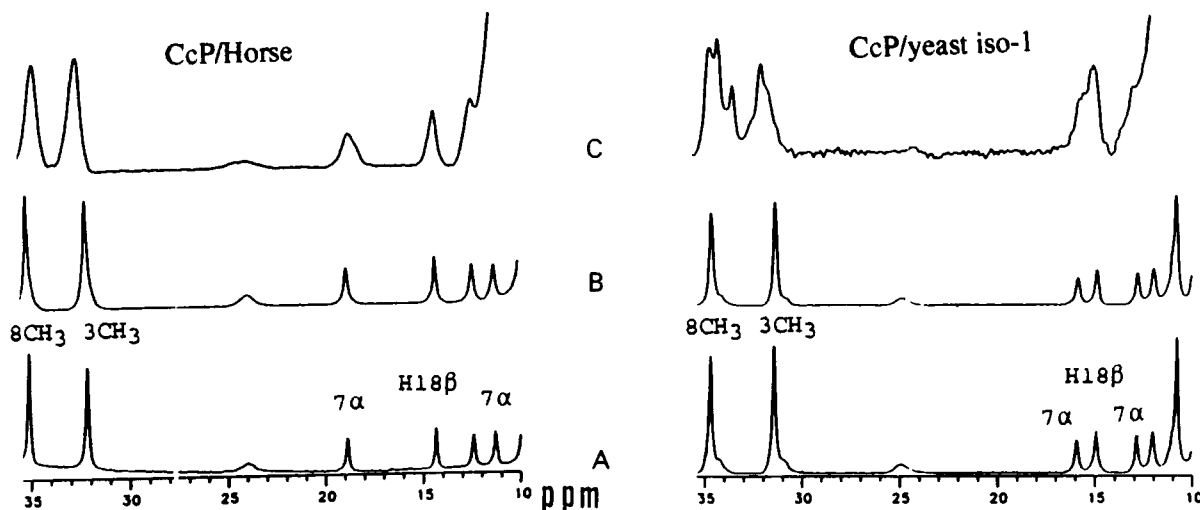


FIGURE 6: Downfield hyperfine shift region of the proton NMR spectra at 361 MHz of horse (left column of spectra) and yeast iso-1 (right column of spectra) ferricytochrome *c*. Spectral traces represent (A) unmodified, (B) modified with EDC, and (C) isolated 1:1 cyt *c*/CcP complex. Solution conditions: [free cyt *c*] = ~3 mM, pH' 6.3 (yeast), pH' 6.8 (horse); [cyt *c*/CcP complexes] ~2 mM, pH' 6.8 (horse) and ~1 mM, pH' 6.3 (yeast iso-1); all samples were in 10 mM KNO<sub>3</sub>, at 25 °C.

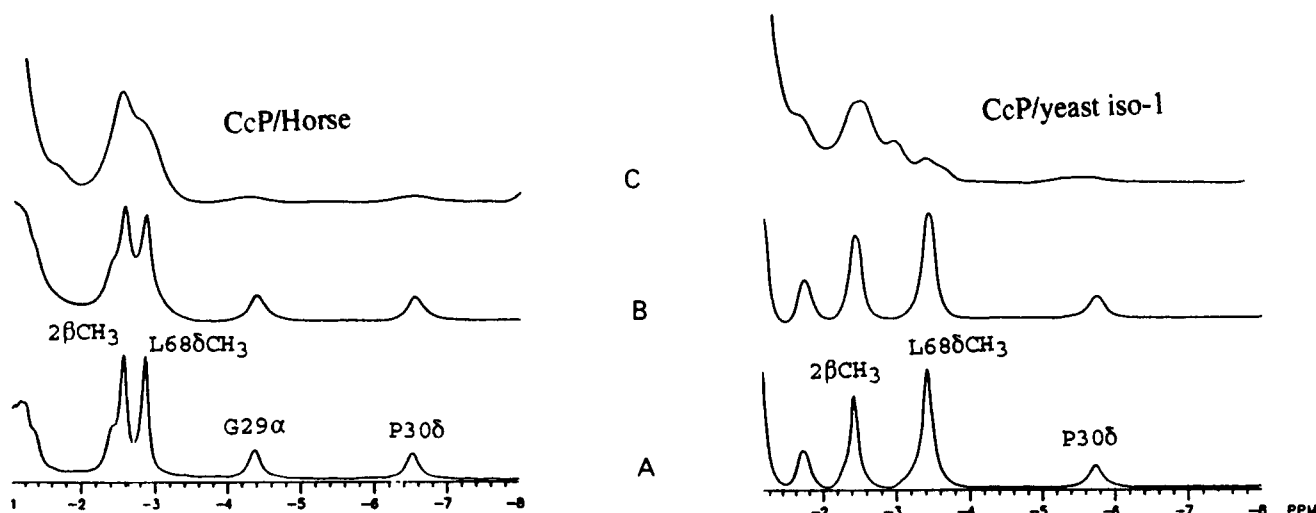


FIGURE 7: Near upfield hyperfine shift region of the proton NMR spectra at 361 MHz of horse (left column of spectra) and yeast iso-1 (right column of spectra) ferricytochrome *c*. Spectral traces represent (A) unmodified, (B) modified with EDC, and (C) isolated 1:1 cyt *c*/CcP complex. All conditions were identical to those described in the legend to Figure 5.

Table III: Chemical Shifts<sup>a</sup> and Line Widths<sup>b</sup> of Selected Hyperfine-Shifted Resonances from the 361-MHz Proton NMR Spectra of Horse and Yeast Iso-1 Ferricytochromes *c* Covalently Cross-Linked with Cytochrome *c* Peroxidase in 1:1 Complexes

resonance	Chemical Shift (ppm)					
	horse cyt <i>c</i>	EDC-modified horse cyt <i>c</i>	horse cyt <i>c</i> /CcP covalent complex	yeast iso-1 cyt <i>c</i>	EDC-modified yeast cyt <i>c</i> iso-1	yeast iso-1 cyt <i>c</i> /CcP covalent complex
8-CH <sub>3</sub>	35.12 (31)	35.18 (48)	34.83 (212)	34.74 (42)	34.72 (59)	34.82 ] <sup>c</sup> 34.45 ] 33.66 ]
3-CH <sub>3</sub>	32.17 (41)	32.20 (62)	32.64 (223)	31.43 (45)	31.38 (56)	32.76/32.19 ] <sup>c</sup> 31.35/31.80 ]
5-CH <sub>3</sub>	9.85	9.83		10.81	10.82	
7α-CH	18.85	18.86	18.73/18.27	15.90	15.90	15.79/15.06
7α-CH	11.26	11.27		12.86	12.85	13.71/13.15
His-18β-CH	14.32	14.32	14.29	14.90	14.90	15.09
2β-CH <sub>3</sub>	-2.63	-2.59	-2.57	-2.43	-2.44	-2.46
Leu-68δ-CH <sub>3</sub>	-2.92	-2.86	-2.87	-3.41	-3.42	-3.36/-2.58
Pro-30δ-CH	-6.58	-6.55	-6.50	-5.73	-5.75	-5.39
Met-80ε-CH <sub>3</sub>	-24.46	-24.42	-24.42	-23.32	-23.32	-23.03
His-18-C2H				-26.44	-26.45	-26.47
Met-80δ-CH	-28.25	-28.19	-28.48	-30.66	-30.61	-30.47

<sup>a</sup> Observed chemical shifts are reported relative to internal HOD assigned a value of 4.60 ppm. Solution conditions: 10 mM KNO<sub>3</sub>, pH' 6.3 (yeast samples), pH' 6.8 (horse samples), at 25 °C. Samples of horse and yeast cytochromes *c*, both unmodified and EDC modified, were present in 3–4 mM concentration. [Yeast:yeast covalent complex] = ~1 mM; [horse:yeast covalent complex] = ~2 mM. <sup>b</sup> Line widths (Hz) of 3- and 8-CH<sub>3</sub> resonances are shown in parentheses. Line widths of complexed cytochromes *c* are calculated with 20-Hz line broadening. <sup>c</sup> Brackets indicate that observed resonance cannot be unambiguously assigned.



1:1 complexes of CcP cross-linked to horse (left) and yeast iso-1 (right) ferricytochromes *c*. In both figures, trace A is the spectrum of each unmodified ferricytochrome *c* and trace B is the control spectrum that resulted from independently treating the individual ferricytochromes *c* with EDC under the cross-linking conditions. Both A and B serve as control spectra by which to judge trace C, which is the spectrum of each of the 1:1 covalent complexes. A summary of assignments and shifts is given in Table III.

Comparing the results tabulated in Tables I and III and the spectra shown in the left-hand column of both Figures 6 and 7 indicates that for the CcP/horse ferricytochrome *c* covalent complex the complex-induced shifts are quite similar in direction and magnitude to those found for the noncovalent CcP/horse cyt *c* complex (Satterlee et al., 1987a,b) and for the CcP/horse ferricytochrome *c* covalent complex that was cross-linked in the low ionic strength cacodylate buffer system (Moench et al., 1987).

Results shown in the right-hand columns of Figures 6 and 7, and Table III, for the CcP/iso-1 ferricytochrome *c* covalent complex are significantly different from both of the CcP/horse covalent complexes and from the CcP/iso-1 ferricyt *c* noncovalent complex (Table I; Figures 2 and 3). The principal difference is the large number of additional resonances for the purified 1:1 CcP/iso-1 ferricyt *c* covalent complex. For example, in the right-hand column of Figure 6, a number of "extra" hyperfine-shifted resonances occur in the heme methyl region (30–35 ppm) of the CcP/yeast iso-1 ferricyt *c* covalent complex (right column of Figure 6C) compared to the control spectra (Figure 6A,B). These occur as shoulders and additional resonances where only single resonances were found for the CcP/horse ferricyt *c* covalent complexes (left column of Figure 6C) and for the EDC treated individual cytochromes *c*.

We attempted to assess to what extent, if any, EDC and acetate caused nonspecific protein modification which might be the source of these NMR differences by performing a number of control experiments. These controls were (a) parallel cross-linking of CcP/horse ferricyt *c* and CcP/yeast iso-1 ferricyt *c* under identical conditions, with the NMR results presented in Figures 6C and 7C; (b) NMR analyses of both horse and yeast iso-1 ferricytochromes *c* that had been individually treated with EDC under the cross-linking conditions, with NMR results presented in Figures 6B and 7B; and (c) NMR analyses of uncross-linked ferricytochromes *c* isolated from each EDC reaction mixture (not shown). None of these controls displayed the extensive array of new or "extra" resonances that were exhibited by the CcP/yeast iso-1 ferricyt *c* covalent complex. In fact, a comparison of the spectra in Figures 6A and 7A, along with the tabulated shifts shown in Table III, revealed that the only detectable effect on NMR spectra of the individual EDC-treated, acetate-quenched ferricytochromes *c*, as well as for the ferricytochromes *c* isolated from the cross-linking reaction mixtures, was a slight resonance broadening of ~10–20 Hz. Thus, it is clear that the multiple resonances observed for the 1:1 CcP/iso-1 ferricyt *c* covalent complex must be due to a combination of EDC treatment and protein-protein association.

The multiple hyperfine-shifted resonances observed for the CcP/iso-1 ferricyt *c* 1:1 covalent complex indicate that a large degree of magnetic heterogeneity exists in the environments of specific yeast iso-1 ferricyt *c* proton groups in the complex. Such spectroscopic heterogeneity must be a manifestation of structural variations among the different types of 1:1 covalent complexes. This could be due to different orientations of bound

yeast iso-1 ferricyt *c* molecules at a single CcP binding site or to the presence of different iso-1 ferricytochrome *c* binding sites on CcP. The latter case is analogous to that proposed from Brownian dynamics modeling studies by Northrup et al. (1988) that identified a range of potential binding sites on the CcP surface. It is likely that the specific number of covalent cross-links is variable from one molecule of the complex to another. However, it is unlikely that the NMR spectral heterogeneity arises from such a phenomenon since such heterogeneity is not evident in the NMR spectrum of the CcP/horse ferricyt *c* 1:1 covalent complex.

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