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Effects of Temperature and Glycerol on the Resonance Raman Spectra of Cytochrome *c* Peroxidase and Selected Mutants†

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ABSTRACT: The high-frequency resonance Raman spectra of Fe^{III} yeast native cytochrome *c* peroxidase (CCP) and five of its mutants [CCP(MI), Phe-51, Leu-48, Lys-48, Asn-235, and Phe-191] were recorded in phosphate buffer, pH 7.0, and in glycerol/phosphate mixtures at 295 and 10 K. Glycerol induces heme coordination changes in some of the CCP mutants at room temperature. It apparently weakens the binding of the Fe atom to ligands in the distal heme cavity and drives the heme toward the 5-coordinate, high-spin state. At 10 K, native CCP and all the mutants (except Phe-51 which remains 6-coordinate, high-spin) show various distributions of spin and coordination states which differ from those observed at 295 K. Upon cooling in phosphate buffer, pH 7, and to a much lesser extent in 66% glycerol/phosphate, an internal strong-field ligand is coordinated to the Fe. A likely candidate is H_2O-595, which could become a strong-field ligand on H-bonding and/or proton transfer to H_2O-648, and/or the distal His-52. However, distal His-52 itself cannot be ruled out as the coordinating ligand considering that the Phe-51 mutant, which binds H_2O-595 at room temperature, does not show a large 6-coordinate, low-spin component at 10 K like the other mutants. These results clearly indicate that the Fe coordination in CCP and its mutants is sensitive to both temperature and solvent composition.

Changes in the electronic absorption and magnetic properties of hemoproteins versus temperature have been extensively reported [see Beetlestone and George (1964) and references cited therein and Iizuka and Kotani (1969a,b) and references cited therein]. Such changes were mainly ascribed to thermal mixtures of low- and high-spin states, rather than to changes in the axial ligation of the iron atom. However, recent reports (Schulz et al., 1984; Evangelista-Kirkup et al., 1985; Manthey et al., 1986; Andersson et al., 1987) have suggested that a number of hemoproteins may undergo freezing-induced conformational changes, which cause spin and coordination transitions due to the reversible binding of a sixth ligand to the iron atom. Glycerol addition to solutions of cytochrome *c* peroxidase (CCP)[1] (Yonetani & Anni, 1987) and HRP (Schulz et al., 1984) prevents the high-spin (hs) to low-spin (ls) transition observed on cooling these peroxidases. Since glycerol is known to stabilize the tertiary structure of proteins (Gekko & Timasheff, 1981), this suggests that a change in ligation occurs on freezing.

The effects of temperature on the absorption (Yonetani et al., 1966) and EPR spectra (Yonetani & Anni, 1987; Hori & Yonetani, 1985) and magnetic susceptibility of CCP (Iizuka et al., 1968) have been studied extensively by Yonetani and co-workers. On the basis of their results, they originally proposed that CCP was a pH-dependent mixture of acidic and alkaline forms between pH 4 and 8. They further concluded that *both* the acidic (pH 5) and alkaline (pH 7) forms possessed hs and ls states in thermal equilibrium; the alkaline form was ls below 173 K and became increasingly hs on heating, whereas the acidic form was hs below 173 K and showed an increasing ls component at high temperatures (Iizuka et al., 1968). Thus, while their visible absorption spectra were similar at room temperature, at 83 K the acidic and alkaline forms showed typical hs and ls spectra, respectively, and a pK_a of ~6 was estimated for the acidic–alkaline transition at 83 K.

Resonance Raman (RR) spectroscopy is a useful probe of heme structure since the Raman frequencies are sensitive to the coordination number as well as the spin state of the iron (Smulevich et al., 1988). Recently, Yonetani and co-workers reported the RR spectra of Fe^{III} CCP (Hashimoto et al., 1986). From these data, they concluded that CCP was a pH-dependent mixture of 6-coordinate (6-c) and 5-coordinate (5-c) hs species at room temperature. Since the 6-c form was dominant at pH 5, and the 5-c form at pH 7, these structures were assigned to the previously proposed acidic and alkaline forms of CCP, respectively, and a pK_a of 5.5 was obtained for the 6-c ⇌ 5-c transition at room temperature [see Figure 5

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; HRP, horseradish peroxidase; RR, resonance Raman; EPR, electron paramagnetic resonance; ls, low spin; hs, high spin; 5-c, 5-coordination; 6-c, 6-coordination.

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of Hashimoto et al. (1986)]. However, no ls component was observed in the Raman spectrum at pH 5 in contrast to the earlier report of a ls component for acidic CCP at room temperature (Iizuka et al., 1968).

A recent RR report by two of the present authors (Smulevich et al., 1986) revealed that CCP undergoes aging-induced changes. In particular, they observed that on extended storage CCP shows ls species, whereas the fresh (i.e., newly isolated) protein is purely hs. In a subsequent study, Yonetani and Anni (1987) examined this aging phenomenon in greater detail using absorption and EPR spectroscopy, and concluded that, contrary to their previous findings, at room temperature *fresh* CCP is purely 5-c,hs between pH 4 and 8. The enzyme converts to a 6-c,hs species upon aging due to the coordination of an endogenous weak-field ligand to the iron. Therefore, the proposed alkaline form of CCP is similar in coordination behavior to the fresh protein, whereas the acidic form arises from aging of the protein. In fact, both the 6-c,hs and 6-c,ls forms observed for acidic CCP at room temperature are due to aging since extended storage (at 240 or 77 K) further converts 6-c,hs CCP to 6-c,ls (Smulevich et al., 1986; Yonetani & Anni, 1987), as demonstrated by room temperature RR and absorption spectra. In conclusion, at room temperature, fresh CCP is purely 5-c,hs between pH 4 and 8, whereas the aged protein shows various amounts of 6-c,hs and ls components. *Above* pH 8, both fresh and aged CCP show a 6-c,ls form.

We have also reported the RR spectra of several CCP mutants (Smulevich et al., 1988) at room temperature. The mutants investigated included wild-type CCP cloned in *Escherichia coli*, CCP(MI), and those possessing additional mutations involving groups on both the distal (Trp-51 → Phe, Arg-48 → Leu and Lys) and proximal (Asp-235 → Asn, Trp-191 → Phe) sides of the heme. Our results show that like yeast native CCP (Yonetani & Anni, 1987), Fe^{III} CCP(MI) is mainly 5-c,hs from pH 5 to 8. However, a number of mutants show various amounts of 6-c,hs and ls forms.

Freezing-induced "artifacts" and their apparent prevention by high glycerol concentrations are of interest and concern in the use of low-temperature probes of CCP and other hemoproteins. For example, from EPR data, Yonetani and Anni (1987) report that at pH 7 fresh CCP possesses a significant ls component at 10 K; however, in the presence of 66% glycerol, fresh CCP remains 5-c,hs at 10 K; thus, the authors concluded that freezing induces the coordination of an internal sixth ligand to the iron and that glycerol prevents this freezing-induced "artifact". Hence, in order to clarify the influence of both freezing and solvent composition on the iron coordination of CCP, we have recorded the RR spectra of Fe^{III} CCP and its mutants at low temperature (10 K) both in the presence and in the absence of glycerol, and at room temperature (~295 K) in the presence of glycerol. The present study sheds new light on the effects of freezing and, also, of the presence of high glycerol concentrations on the spin and coordination states of the Fe^{III} atom in CCP.

Experimental Procedures

CCP was purified from bakers' yeast as previously reported (Smulevich et al., 1986; English et al., 1986) with the following modifications: ~500 mL of DEAE-Sepharose was added to the lysate supernatant following centrifugation and stirred for 1 h. The resin was collected under suction on a large Buchner funnel with a 15-cm coarse frit and washed with 1–2 L of 0.05 M acetate buffer, pH 5.0. The resin was then transferred to a 8 × 30 cm column, and CCP was eluted with 0.5 M acetate, pH 5.0. The CCP-containing band (~200 mL) was further purified by binding the protein to ~200 mL of DEAE in a 6-cm fritted-glass funnel and by washing the resin under suction with 0.2–0.25 M acetate buffer, pH 5.0, until the filtrate had an absorbance of <0.1 M at 260 nm. The CCP was again eluted with 0.5 M acetate, pH 5.0, and the resulting solution had a purity index (A_{408}/A_{280}) of >0.9. The solution was concentrated by using a YM 30 filter (Amicon) in a stirred cell and dialyzed at 4 °C for ~24 h versus 0.1 M phosphate, pH 7.0, to remove any acetate, which apparently binds to CCP (Yonetani & Anni, 1987), prior to crystallization. This procedure consistently gives purely 5-c,hs Fe^{III} CCP as determined by RR in 0.1 M phosphate, pH 6.0, using 413.1-nm excitation. On the other hand, if the purified protein is not dialyzed versus phosphate prior to crystallization, various amounts of the 6-c,hs form appear in the RR spectrum due to incomplete removal of bound acetate. In fact, this is clearly shown in our previous work [see Figure 3a,b of Smulevich et al. (1986)], where a shift to 5-c is reported on increasing the phosphate concentration from 0.01 to 0.1 M. We originally assigned this to a phosphate effect, but this effect is *not* observed if acetate is completely removed since CCP is purely 5-c. Furthermore, we observe no changes in purely 5-c,hs native CCP on storage at 77 K over a period of several months. On the other hand, if bound acetate is not removed, CCP is transformed to a ls form on aging. Because we observe no aging effects in 5-c,hs CCP, we refer to this form as "native" CCP rather than "fresh" CCP.

CCP(MI) and its mutants were isolated from *E. coli* as previously described (Fishel et al., 1987). Crystals of the proteins were dissolved in 0.1 M phosphate buffer, pH 7.0, and in glycerol/phosphate mixtures (v/v), to give a final protein concentration of ~0.3 mM. The buffer was prepared from monopotassium and dipotassium phosphate rather than the corresponding sodium salts to minimize the pH drop on freezing due to precipitation of Na_2HPO_4, which is less soluble than K_2HPO_4 (Orii & Morita, 1977). A closed-cycle He cryotip with automatic temperature control was used to obtain the low temperatures. The samples were introduced into the cavity of the cryostat cold finger and flash-frozen as previously described (Smulevich, 1985). Under these conditions, glassy samples were obtained for the glycerol/phosphate solutions, while the buffer solutions contained microcrystallites. This indicates that the samples possessed different aggregation states on freezing. The back-scattered light from a slowly rotating NMR tube (room temperature), or from the low-temperature cell, was collected and focused into a computer-controlled double monochromator (Jobin-Ivon HG-2S), equipped with a cooled photomultiplier (RCA C31034A) and photon counting electronics. The 413.1-nm line of a Kr^+ laser (Coherent Radiation Innova 90 K) was used as a radiation source. The room temperature absorption spectra were recorded with a Perkin-Elmer Lambda 5 spectrophotometer.

Results

295 K RR Spectra in Phosphate. The RR spectra of Fe^{III} CCP(MI), Leu-48, Lys-48, Phe-51, Asn-235, and Phe-191 at pH 7.0 are shown in Figure 1a. These spectra were previously published (Smulevich et al., 1988) but are shown here for ease of comparison. The coordination and spin state of the Fe atom can be determined from the position of the ν_3 band; as indicated in Figure 1, ν_3 bands at ~1480, 1492, and 1505 cm^{-1} correspond to 6-c,hs, 5-c,hs, and 6-c,ls hemes, respectively (Smulevich et al., 1988). Briefly, we concluded that CCP(MI) and Leu-48 are predominantly 5-c,hs but Lys-48 binds the Fe atom directly, giving rise to a 6-c,ls form. The Phe-51 mutant is 6-c,hs presumably because a water molecule (H_2O-595),

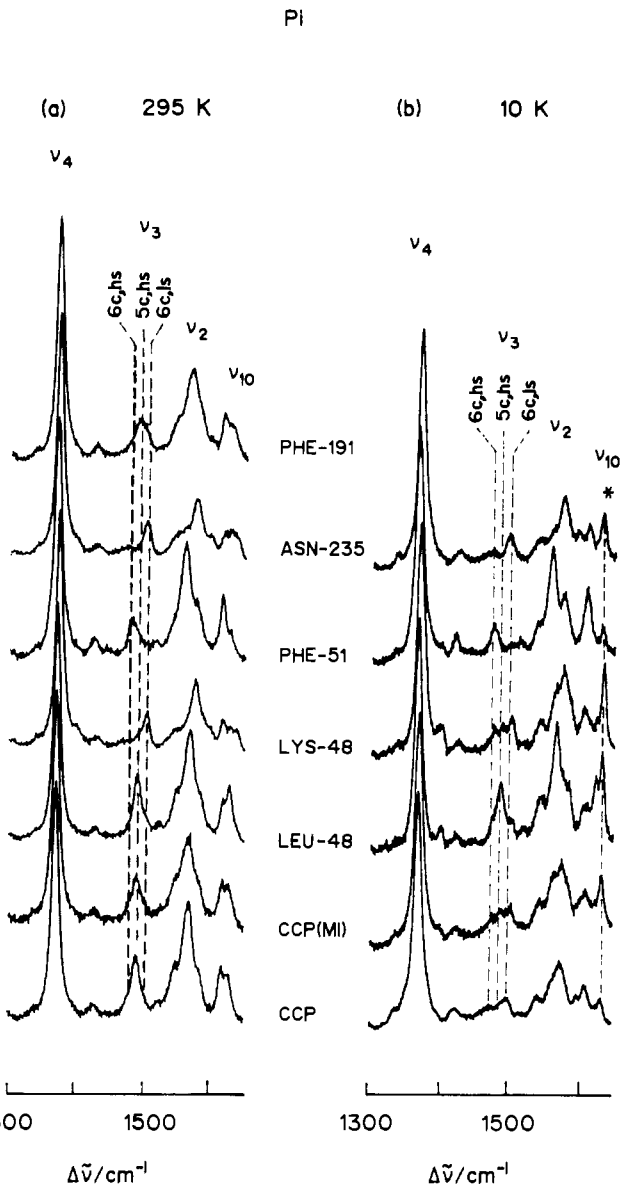

FIGURE 1: Resonance Raman spectra of Fe^{III} yeast native CCP and five CCP mutants (~0.3 M) in 0.1 M phosphate (P_i) buffer, pH 7.0, at (a) 295 K and (b) 10 K. Experimental conditions: 413.1-nm excitation, 50-mW laser power at the source, 5 cm^{-1} spectral slit width, and 1 s/0.5 cm^{-1} collection interval. The dashed lines that indicate the positions of ν_3 for 6-c,hs, 5-c,hs, and 6-c,ls hemes are at 1478, 1492, and 1505 cm^{-1}, respectively; the asterisk indicates a plasma line.

which is in close proximity to the heme, is bound to the Fe atom. Of the proximal mutants, Asn-235 is 6-c,ls, and Phe-191 is essentially 5-c,hs with minor 6-c components. The 295 K spectrum of yeast native CCP is also shown in Figure 1a; the ν_3 band at 1493 cm^{-1}, and the apparent lack of high- or low-frequency shoulders, clearly indicates that yeast Fe^{III} CCP is purely 5-c,hs under these conditions.

10 K RR Spectra in Phosphate. Figure 1b shows the RR spectra obtained on Soret band excitation for yeast native Fe^{III} CCP and its mutants in phosphate buffer at 10 K. Cooling gives rise to considerable changes in ν_3 for all the proteins except the Phe-51 mutant. In this mutant, the position of ν_3, which sharpens on cooling, is indicative of a mainly 6-c,hs heme at both 10 and 295 K, suggesting that the Fe coordination in this mutant is insensitive to temperature. Cooling the mutants which are essentially 5-c,hs at 295 K [i.e., wild-type CCP, CCP(MI), and Leu-48] gives rise to variable mixtures of all three Fe^{III} spin and coordination states, since their spectra show three peaks in the ν_3 region. Similarly, the 6-c,ls heme of Lys-48 gives rise to a mixture of all three states at 10 K, but the heme of Asn-235, which is also 6-c,ls at 295 K, remains mainly 6-c,ls at 10 K with a minor 6-c,hs component. In summary, it appears that the Fe coordination in mutants, which are either 5-c,hs or 6-c,ls, is sensitive to temperature, unlike the 6-c,hs state of Phe-51. We were unable to obtain the 10 K RR spectrum of Phe-191 because this mutant is not sufficiently soluble at low temperature in phosphate. Also, the phase separation on freezing reduces the optical quality of this sample.

295 K RR Spectra in Glycerol/Phosphate. Figure 2a shows the RR spectra on Soret excitation for the Fe^{III} forms of yeast native CCP and five mutants in 66% glycerol/phosphate at 295 K. The spectrum for CCP(MI) is omitted since it is identical with that given for yeast CCP. No change is observed in the ν_3 region of yeast CCP or Leu-48 on the addition of glycerol, indicating that these proteins remain 5-c. The effect of glycerol on the Phe-51 mutant is particularly noteworthy since the heme is converted from a mainly 6-c,hs species in the absence of glycerol (Figure 1a) to a predominantly 5-c,hs species in 66% glycerol (Figure 2a). Since the iron coordination of Phe-51 shows the greatest glycerol sensitivity, we investigated the effect of various glycerol concentrations on the 6-c $\rightleftharpoons$ 5-c equilibrium in this mutant. The results are summarized in Figure 3a–e, which shows the effect of increasing the percent glycerol on the ν_3 band of Phe-51. An examination of the spectra at 0% and 20% glycerol (Figure 3a,b) reveals that 20% glycerol is sufficient to shift the 6-c $\rightleftharpoons$ 5-c equilibrium toward the right and the addition of more glycerol shifts the equilibrium even further to the 5-c form. The spectra at 50% and 66% glycerol (Figure 3d,e) appear to be the same within the noise limit, which suggests that the full effect on the iron coordination occurs between 0% and 50% glycerol.

In 66% glycerol, the Lys-48 mutant shows two ν_3 bands at 1490 and 1502 cm^{-1}, corresponding to a 5-c,hs and a 6-c,ls heme, respectively. A similar trend is observed for Asn-235, where the ν_3 band shows a shift from 6-c,ls in buffer to a mixture of 6-c,hs and ls hemes in glycerol/buffer.

10 K RR Spectra in 66% Glycerol/Phosphate. Only minor spectral changes are observed on cooling the samples to 10 K in 66% glycerol (Figure 2b). Intensified low-frequency bands appear in the ν_3 region in the spectra of Asn-235 and Lys-48 (Figure 2b), which suggests enhanced 6-c,hs and 5-c,hs components, respectively, at 10 K. Low-frequency shoulders may also be present on ν_3 in the 10 K spectra of yeast native CCP and Phe-51, although the spectra are too noisy to confidently propose a 6-c,hs component for these proteins.

Soret Absorption Spectra in 66% Glycerol/Phosphate. The 295 K Soret absorption spectra of yeast native CCP, Phe-51, Lys-48, and Asn-235 in 66% glycerol/phosphate, pH 7, are presented in Figure 4. In 0.1 M phosphate in the absence of glycerol, the Soret maximum for 5-c,hs heme is ~408 nm with a strong shoulder at ~370 nm, as seen for yeast native CCP [see Figure 3 of Yonetani and Anni (1987)] and for CCP(MI) and Leu-48 [see Figure 14 of Smulevich et al. (1988)]. Conversion of hs heme from 5-c to 6-c is accompanied by intensification and narrowing of the Soret band, with a less distinct shoulder at 370 nm, and this is demonstrated by Phe-51 [see Figure 14 of Smulevich et al. (1988)]. The Soret maximum for 6-c,ls heme is red-shifted to ~412 nm as seen for Lys-48 [see Figure 14 of Smulevich et al. (1988)].

In 66% glycerol/phosphate, small wavelength shifts are observed compared to the phosphate only values. Nonetheless,

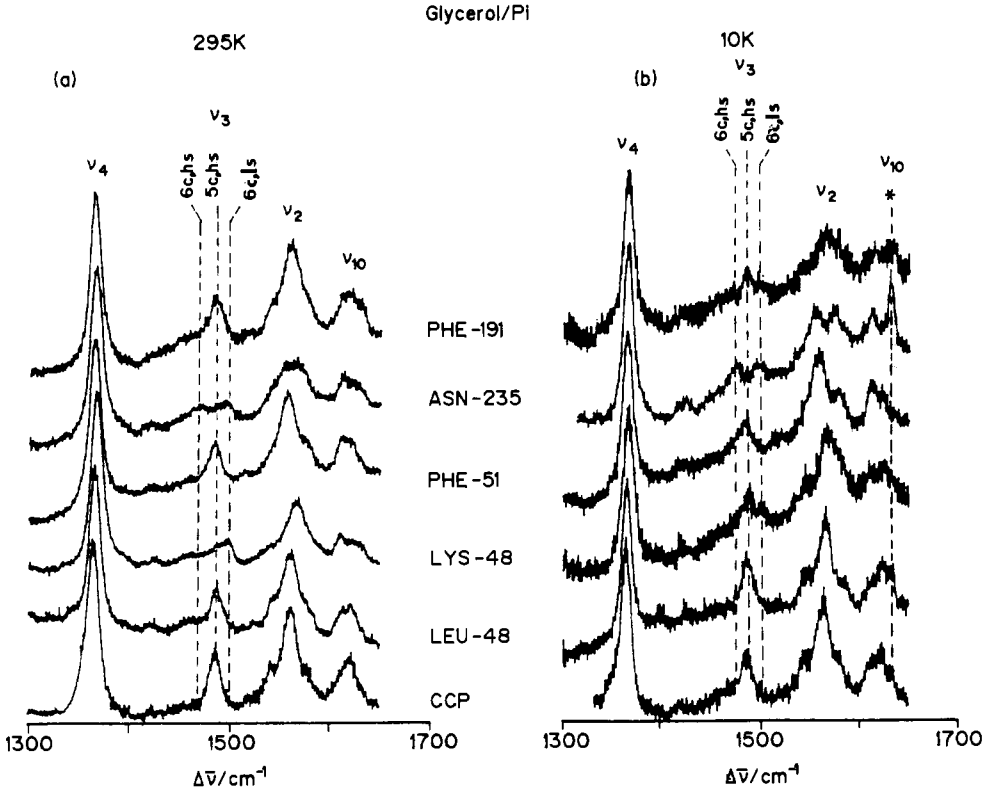

FIGURE 2: Resonance Raman spectra of Fe^{III} yeast native CCP and five CCP mutants (~0.3 mM) in 66% glycerol/0.1 M phosphate (P_i) buffer, pH 7.0, at (a) 295 K and (b) 10 K obtained with 413.1-nm excitation. The experimental conditions are given in the caption to Figure 1. The dashed lines that indicate the positions of ν_3 for 6-c,hs, 5-c,hs, and 6-c,ls hemes are at 1478, 1492, and 1501 cm^{-1}, respectively; the asterisk indicates a plasma line.

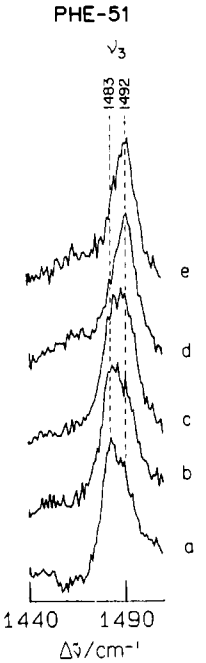

FIGURE 3: Resonance Raman spectra of the Fe^{III} Phe-51 mutant of CCP (~0.3 mM) in glycerol/0.1 M phosphate buffer, pH 7.0, at 295 K obtained with 413.1-nm excitation. The experimental conditions are given in the caption to Figure 1. The percent glycerol is as follows: (a) 0%, (b) 20%, (c) 33%, (d) 50%, and (e) 66%.

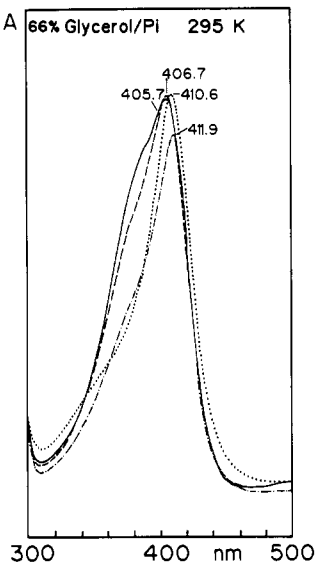

FIGURE 4: Soret absorption of Fe^{III} yeast native CCP and three CCP mutants in 66% glycerol/0.1 M phosphate (P_i) buffer, pH 7.0, at 295 K. (—) Yeast native CCP; (--) Phe-51; (···) Asn-235; (-·-) Lys-48.

the Soret bands show the same general features mentioned above and are readily interpretable as arising from variable contributions from 5-c,hs, 6-c,hs, and ls species. As can be seen from Figure 4, yeast native CCP [also CCP(MI) and Leu-48, spectra not shown] possesses a Soret maximum at 405.7 nm, with a prominent shoulder at 380 nm which is typical of 5-c,hs heme. In Phe-51 (and Phe-191, spectrum not shown), the Soret maximum shifts to 406.7 nm, and the shoulder decreases in intensity, indicating a 6-c,hs component in addition to 5-c,hs heme; in Lys-48, the maximum red-shifts to 411.9 nm, suggesting a 6-c,ls heme, but a 5-c,hs component is also present because a weak shoulder is visible at 380 nm. Asn-235 shows a maximum at 410.6 nm with no low-frequency shoulder, which is consistent with the presence of 6-c,hs and ls species only. Hence, the changes in the Soret spectra on the addition of 66% glycerol corroborate the RR data.

Table I summarizes the coordination and spin states of Fe^{III} in yeast native CCP and its mutants as determined from their

Table I: Fe^{III} Coordination and Spin-State Assignments for Yeast Native CCP and Its Mutants in Buffer (pH 7) and Glycerol/Buffer from Room and Low-Temperature Spectra[a]

	EPR		RR		Soret absorption	
protein	buffer	glycerol	buffer	glycerol	buffer	glycerol
CCP (yeast)						
RT			5hs[d]	5hs	5hs[c]	5hs
10 K	6hs m[b]	6hs vw[b]	6hs w	6hs vw ?		
	5hs m[b]	5hs m[b]	5hs w	5hs m		
	6ls m[b]		6ls m		6ls[e]	
CCP (aged)[h]						
RT			6hs w[f]			
			5hs m[f]			
9 K[f]/10 K	6hs m[b]	6hs vw[b]	6hs m[f]			
		5hs m[b]	5hs vw[f]			
	6ls vw[b]					
CCP(MI)						
RT			6hs vw[c]			
			5hs m[c]	5hs	5hs[c]	5hs
89 K[g]/10 K	6hs m[g]		6hs w			
	5hs m[g]		5hs m			
	6ls m[g]		6ls m			
Phe-51						
RT			6hs m[c]	6hs vw ?	6hs[c]	6hs w
			5hs w[c]	5hs m		5hs m
89 K[g]/10 K	6hs[g]		6hs	6hs w ?		
	6ls vw[g]					
Leu-48						
RT			5hs m[c]	5hs	5hs[c]	5hs
			6ls w[c]			
10 K			6hs vw			
			5hs m	5hs		
			6ls w			
Lys-48						
RT			5hs vw[c]	5hs w		5hs w
			6ls m[c]	6ls m	6ls[c]	6ls m
10 K			6hs w			
			5hs w	5hs m		
			6ls m	6ls w		
Asn-235						
RT				6hs m		6hs w
			6ls[c]	6ls m	6ls[c]	6ls m
10 K			6hs vw	6hs m		
			6ls m	6ls w		
Phe-191						
RT			6hs vw[c]	6hs vw ?	6hs w[c]	6hs w
			5hs m[c]	5hs m	5hs m[c]	5hs m
			6ls w[c]	6ls vw ?		
10 K				5hs		

[a] Abbreviations: RT, room temperature; 6hs, 6-coordinate, high-spin; 5hs, 5-coordinate, high-spin; 6ls, 6-coordinate, low-spin; medium (m), weak (w), and very weak (vw) refer to the relative intensities of the signals observed in the EPR and the Soret absorption spectra, and for the ν_3 bands in the Raman spectra; ? indicates that the Raman band is too close to the noise level to confidently assign this Fe^{III} state. The Raman frequencies of the ν_3 band range from 1477 to 1483 cm^{-1} for 6hs, from 1490 to 1494 cm^{-1} for 5hs, and from 1501 to 1507 cm^{-1} for 6ls. [b] Yonetani & Anni (1987). [c] Smulevich et al. (1988). [d] Hashimoto et al. (1986). [e] Yonetani et al. (1966). [f] Evangelista-Kirkup et al. (1985). [g] Fishel et al. (1987). [h] Aged yeast CCP (see Experimental Procedures).

10 K EPR spectra, 295 and 10 K RR spectra, and 295 K Soret absorption spectra.

Discussion

RR spectroscopy clearly indicates that yeast native CCP is purely 5-c,hs at room temperature (Figure 1a). This is consistent with its high-resolution X-ray structure (Poulos et al., 1980; Finzel et al., 1984), which shows a 5-c heme with a water molecule (H_2O-595) at 2.4 Å from the Fe atom (Figure 5).

The 10 K EPR spectrum of yeast native CCP in 0.1 M Mes, pH 7 (Yonetani & Anni, 1987; Anni & Yonetani, 1987), indicates the presence of 6-c,hs and ls states in addition to the 5-c,hs state. The observation of 6-c states was attributed to the reversible binding of an internal ligand following freezing-induced conformational changes in the protein. Unfortunately, it is not possible to measure the EPR spectrum of CCP at room temperature because of signal broadening (Yonetani & Schleyer, 1967); however, the RR spectra measured here, at 295 and 10 K, furnish results consistent with X-ray and EPR data, respectively (see Table I). A previous RR study (Evangelista-Kirkup et al., 1985) revealed a predominantly 6-c,hs heme for yeast CCP at 9 K in contrast to the largely 6-c,ls state observed in the present study. The presence of a predominantly 6-c,hs heme at 9 K indicates that the enzyme has converted to its aged form (Yonetani & Anni, 1987), which also shows a large 6-c,hs component at room temperature.

Freezing Effects. A comparison of the RR spectra of the CCP mutants at 295 and 10 K provides insight into the effects of freezing on the iron coordination. For example, yeast native CCP, CCP(MI), and Leu-48 possess a prominent ls component at 10 K which is not present at 295 K, where these proteins are mainly 5-c,hs; therefore, this ls component must result from the freezing-induced coordination of an internal strong-field ligand. The distal His-52 is a possible strong-field ligand as is H_2O-595. This water molecule is only 2.4 Å from the heme in yeast native CCP and is strongly H-bonded to a neighboring

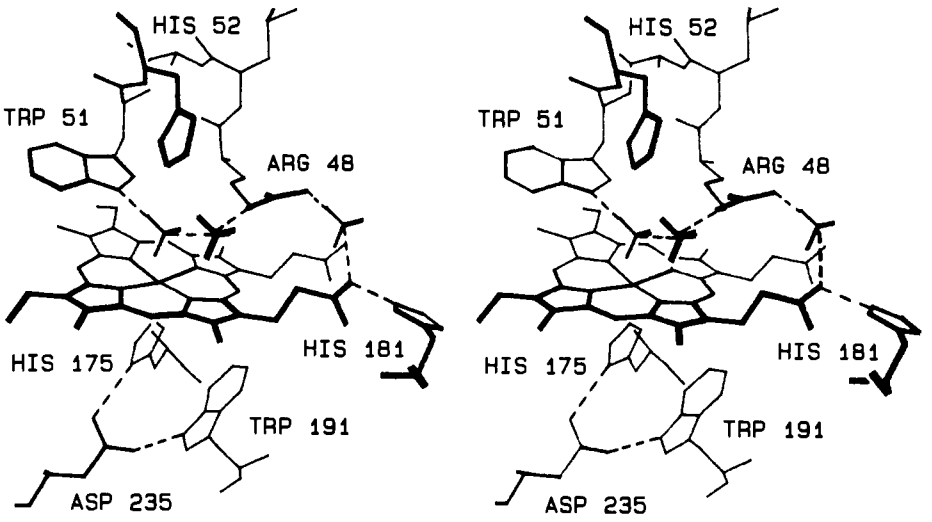

FIGURE 5: Heme crevice of Fe^{III} yeast native CCP from Finzel et al. (1984). Stereoscopic view showing amino acids which are important in the present study. Dashed lines indicate inferred H-bonds on the basis of distance criteria. Three fixed water molecules (from left to right, H_2O-595, H_2O-648, H_2O-348) are also shown.

water molecule (H_2O-648) and Trp-51 (Figure 5). Proton transfer from H_2O-595 to His-52 would allow the resulting OH^- group to drive the Fe^{III} atom ls. Close examination of Figure 1b reveals that the 6-c,hs and ls states are less populated at 10 K in Leu-48 than in yeast CCP and CCP(MI). This indicates that the side chain of Arg-48 in the distal cavity plays a role in driving the Fe atom 6-coordinate at 10 K. We proposed previously (Smulevich et al., 1988) that the side chain of Arg-48 can stabilize a heme-bound water or hydroxide, and the relatively low population of the 6-c states in the Leu-48 mutant is consistent with this proposition.

In the Phe-51 mutant, the 6-c,hs state at 295 and 10 K is most probably due to the binding of H_2O-595 to the Fe atom (Smulevich et al., 1988). Since the side chain of Trp-51 is a H-bond donor (Figure 5), which should *diminish* the "hydroxide" character of H_2O-595 in the other mutants relative to the Phe-51 mutant, the absence of a ls component in Phe-51 at 10 K is rather surprising. However, the less polar heme pocket on Trp-51 → Phe substitution may inhibit proton transfer from the distal water to His-52. Also, we cannot discount direct coordination of His-52 to the Fe atom in the ls mutants. It is relevant to note here that native CCP also goes from hs to ls under pressure, and the ΔV^0 associated with this spin transition is quite large (−30 mL mol^{-1}); thus, the pressure-induced changes may well involve displacement of H_2O-595 and coordination of His-52 directly to Fe^{III} in native CCP (Kornblatt et al., 1986). Direct binding of H_2O-595 to the iron could prevent its displacement by His-52 at low temperature in the Phe-51 mutant.

The spectra of the Lys-48 and Asn-235 mutants, which show predominantly 6-c,ls character at 295 K, more closely resemble those of yeast native CCP and CCP(MI) at 10 K (Figure 1b). The ls character of the Lys-48 mutant at room temperature is attributed to the coordination of the Lys side chain directly to the Fe atom, while the ls character of Asn-235 is attributed to the movement of the Fe atom into the porphyrin plane on breaking the proximal His-175 → Asp-235 H-bond (Figure 5), which permits the metal to interact more strongly with the distal H_2O-595 (Smulevich et al., 1988). The decrease in the relative intensity of the ls component at 10 K indicates that freezing *reduces* the interaction between the Fe atom and its sixth ligand in both of these mutants. However, the partial conversion of the ls component at 10 K in the Lys-48 mutant gives rise to a significant 5-c component (in addition to a 6-c,hs component), whereas the Asn-235 heme remains 6-c.

Glycerol Effects. Yonetani and Anni (1987) reported that the freezing-induced "artifact" in yeast native CCP (i.e., the hs → ls heme transition) can be effectively eliminated in the presence of glycerol. In fact, they observed using EPR that native CCP remains mainly 5-c,hs in a mixture of 66% glycerol/Mes (pH 7) at 10 K. The present study clearly shows that the CCP mutants, which are not purely 5-c,hs at 295 K, display a number of glycerol-induced "artifacts" even at room temperature (cf. Figures 1a and 2a). For example, in 66% glycerol, Phe-51 is converted from 6-c,hs to largely 5-c,hs, and Lys-48 shows a 5-c,hs component at the expense of the 6-c,ls component, indicating that glycerol reduces the interaction between Fe and the Lys-48 side chain in this mutant. Asn-235 follows a similar trend, since its heme goes from mainly 6-c,ls to a mixture of 6-c,hs and ls in glycerol/phosphate, binding of the sixth axial ligand being weakened. The absence of a 5-c state in Asn-235 in 66% glycerol is consistent with strong interaction between Fe and the distal H_2O-595 in this mutant (Smulevich et al., 1988), since the motion of the metal toward the distal cavity is not restricted by the proximal His-175 → Asp-235 H-bond (Figure 5), whereas the glycerol-induced shift to 5-c in the Lys-48 mutant further supports the proposed stabilization of a heme-bound water molecule by the side chain of Arg-48 (Smulevich et al., 1988).

In the presence of 66% glycerol, only minor differences are observed between the 295 and 10 K spectra. The 6-c,hs state seems to increase slightly in Phe-51, Asn-235, and perhaps to a lesser extent in yeast native CCP. This indicates that the effects of glycerol on the heme cavity at 295 K are partially reversed on freezing, except for the Lys-48 mutant, where the 5-c,hs state increases at the expense of the 6-c spin states. Thus, while the Lys side chain is bound directly to the Fe atom at 295 K, this interaction is weakened at low temperature both in the presence and in the absence of glycerol.

The reasons why glycerol reduces the interaction between the Fe atom and its distal ligand in the CCP mutants both at 295 and at 10 K are not clear at the present time. Glycerol is known to stabilize the tertiary structure of proteins due to their preferential hydration in glycerol/water mixtures (Gekko & Timasheff, 1981). This increased stabilization could give rise to small conformational changes that might decrease contact between the Fe atom and possible ligands in the distal cavity. However, the heme cavity in CCP is sufficiently large

to accommodate a molecule the size of glycerol (Poulos et al., 1980), which would displace distal water molecules or other nearby residues.

Freezing has the opposite effect on the 5-c mutants compared to glycerol since it tends to increase the interaction between the Fe atom and the distal cavity. However, those mutants which are mainly ls at 295 K (Lys-48, Asn-235) show less ls character at 10 K, due perhaps to reduced mobility of the Fe atom in Asn-235, and of the Lys side chain in Lys-48 at low temperature. Thus, freezing and glycerol induce similar changes in the Lys-48 and Asn-235 mutants, but the ls → hs heme transition is greater in glycerol at 295 K than in phosphate at 10 K (cf. Figures 1a, 1b, and 2a).

The effect of aging on yeast native CCP is similar to that observed on freezing. Both treatments increase the amounts of 6-c,hs and ls components observed, but from the Raman data, we cannot assume that the same conformational change is occurring on the distal side of heme. The effects of aging on the CCP mutants are not yet known.

In conclusion, the data on the mutant forms of CCP clearly indicate that glycerol alters the heme coordination at room temperature. This occurs even in 20% glycerol (Figure 3) and should be taken into consideration when investigating hemoproteins in the presence of antifreezing agents. For example, the crystals of native yeast CCP used for X-ray structure determination were grown from solutions containing 30% 2-methyl-2,4-pentanediol; hence, the heme coordination in crystals of the mutants may be susceptible to a solvent-induced "artifact" if grown from the same mother liquid.

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Registry No. CCP, 9029-53-2; Fe, 7439-89-6; heme, 14875-96-8; glycerol, 56-81-5.

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