Raman and Infrared Spectra of Cytochrome *c* Peroxidase–Carbon Monoxide Adducts in Alternative Conformational States†

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ABSTRACT: Resonance Raman (RR) spectra are reported for CO-bound cytochrome *c* peroxidase (CCP). At low pH, two forms are observed: form II, with ν_{Fe-C} = 530 cm^{-1} and δ_{FeCO} = 585 cm^{-1}, and form I, with ν_{Fe-C} = 495 cm^{-1} and no detectable δ_{FeCO}. They appear to have coincident ν_{CO} infrared bands, at 1922 cm^{-1}. These low-pH forms, similar to those observed for horseradish peroxidase (HRP), are attributed to tilted, H-bonded CO and perpendicular CO, respectively. The frequencies differ between the two proteins, a weaker H bond to CO being indicated for CCP. As with HRP, the equilibrium between forms I and II is shifted toward the latter at increasing CO concentrations, suggesting that secondary binding of CO perturbs the distal residues. At high pH [8.4, tris(hydroxymethyl)aminomethane buffer] the form II fraction converts to another form, II′, with ν_{FeC} = 503 cm^{-1}, δ_{FeCO} = 575 cm^{-1}, and ν_{CO} = 1948 cm^{-1}; a tilted, non-H-bonded geometry is suggested. If phosphate buffer is used, however, form II (H bonded) persists at pH 8.4. This result establishes a role for phosphate in stabilizing the H-bonded form of the enzyme; it is suggested that phosphate binds near the distal imidazole and substantially increases its pK_a. The conformational state is also influenced by aging. Fresh protein contains purely high spin Fe^{III} heme, as monitored by the high-frequency RR spectrum, and yields form II almost exclusively at elevated CO concentrations. Aged protein showed a substantial low-spin Fe^{III} component and a large fraction of form I, even at elevated CO concentration. In addition, the phosphate inhibition of the form II → form II′ conversion is lost upon aging. A model for CO binding is put forward, which involves alternative conformations of the distal residues. The equilibrium is influenced by the binding of protons, phosphate, and a second CO molecule and is sensitive to irreversible protein changes that occur upon aging.

The vibrational spectrum of the carbon monoxide adduct of heme proteins (Tsubaki et al., 1982) is proving to be a useful probe for interactions of the heme group with protein residues in the heme pocket. In the preceding paper in this issue (Evangelista-Kirkup et al., 1986a), alternative CO binding modes, one perpendicular to the heme plane and the other tilted and H bonded, have been characterized for horseradish peroxidase (HRP) with resonance Raman (RR) spectroscopy and infrared (IR) spectroscopy. The equilibrium between these forms was found to be dependent on pH and also, unexpectedly, on the CO pressure. It was suggested that a second CO binds in the vicinity of the heme group and perturbs the distal residues.

The observed vibrational frequencies were interpreted, with the help of model compounds, on the basis of H-bonding interactions on both the proximal and distal sides of the heme by using the crystal structure (Poulos & Kraut, 1980; Finzel et al. 1984; Poulos & Finzel, 1984) of the analogous enzyme cytochrome *c* peroxidase (CCP) as a guide. We now present RR data on the CO adduct of CCP itself, which show a pattern similar to that observed for HRP. There are significant differences, however, which imply that the disposition of the heme-linked residues is not the same for the two proteins. The distal H bonding is found to be significantly weaker in CCP than in HRP, and at high pH a tilted, non-H-bonded bound CO is observed with vibrational frequencies very similar to those of the CO adducts of myoglobin and hemoglobin (Tsubaki et al., 1982). The relative population of tilted and perpendicular forms of bound CO is dependent on CO concentration, as in the case of HRP, but also on protein aging. Aged protein shows a decreasing propensity to form tilted FeCO, and it shows an increasing fraction of low-spin heme in the native Fe^{III} state. Finally, it is shown that for fresh protein the titration of the distal group responsible for H bonding to the bound CO is strongly inhibited by phosphate, present in the usual buffer; this alteration implies an important role for phosphate in influencing the pK_a of this group, which is presumed to be the distal histidine. The binding of phosphate is also demonstrated by changes in the RR spectrum of the Fe^{III} state, which imply destabilization of the Fe-bound water molecule. However, the phosphate effect is quickly lost upon aging of the protein, suggesting dependence on a conformational equilibrium that is easily perturbed.

Experimental Procedures

Sample Preparation. Cytochrome *c* peroxidase was isolated from fresh commercial baker's yeast (Fleischman's) by following a modification of literature procedures (Yonetani & Ray, 1965; Nelson et al., 1977). A significant increase in the quantity of peroxidase recovered from the crude extract was achieved by (i) substituting cross-linked DEAE-agarose (DEAE-Sepharose CL-6B, Pharmacia) for DEAE-cellulose, (ii) extracting the enzyme in 0.05 M sodium acetate, which allowed direct loading on the DEAE column, and (iii) concentrating the enzyme prior to gel filtration by using ultraf-

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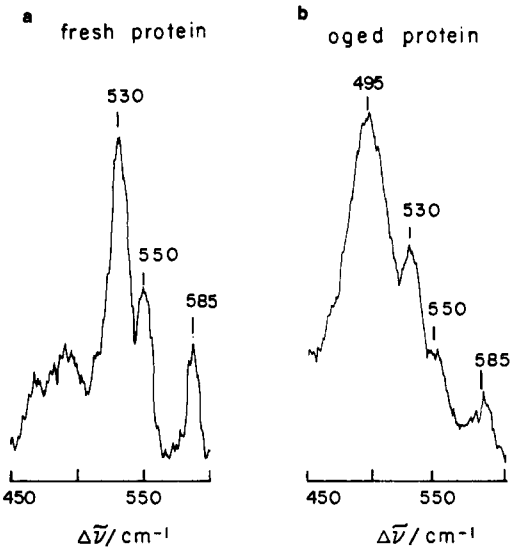

FIGURE 1: 413.1-nm-excited RR spectra near 500 cm^{-1} for CCP–CO at high CO concentration for (a) fresh (3.5 mM) and (b) aged (4 mM) protein (see Experimental Procedures) in phosphate buffer (10^{-1} M), pH 6. Experimental conditions: laser power, (a) ~5 or (b) ~7 mW at the sample; spectral slit width, 5 cm^{-1}; accumulation time, (a) 12 or (b) 15 s/0.5 cm^{-1}.

iltration instead of a second DEAE column. Enzymic activity during preparation was assayed at room temperature by the method of Yonetani and Ray (1965; Yonetani, 1976). The purity index A_{408}/A_{280} was ~1.5 after recrystallization.

The spectroscopic properties of the protein were found to depend on aging. In the subsequent discussion "fresh" protein refers to material that was in contact with ion-exchange resin for only 2 h and stored at 4 °C for less than 1 week. "Moderately aged" protein was stored at 4 °C for 1 month after isolation, then frozen (−10 °C), and stored for another 1 month. "Aged" protein had been in contact with DEAE-Sepharose for ~5 h and was stored at −10 °C for 1 year.

CCP crystals were dissolved in 0.1 M phosphate buffer at pH 7, and a minimal volume of sodium dithionite solution was introduced to prepare the reduced form. No significant pH change was detected in this solution. To prepare the alkaline form, the protein was dissolved in 0.01 M phosphate buffer, pH 7, to which an appropriate amount of 0.1 M phosphate buffer at pH 11 was added until the desired pH was obtained. The final phosphate concentration in these solutions was ~0.05 M. The CO adduct (high-CO form) was obtained by gently flowing CO from a gas cylinder over the surface of the reduced protein for 20 min. The low-CO form was prepared by carefully introducing small aliquots of CO from a 0.25-L gas flask (Cambridge Isotope Labs) to an evacuated NMR cell. Varying the concentration of the protein (Figure 2) and preparing the CO complex under identical conditions (flushing CO for 20 min) showed the same effects as changing the CO exposure.

Spectroscopy. Raman spectra were obtained with excitation from the 413.1-nm line of a Kr^+ laser (Spectra Physics 171) by using procedures described in the preceding paper (Evangelista-Kirkup et al., 1986). Infrared spectra were recorded at 25 °C with a Digilab FDS-20C FTIR[1] spectrophotometer, as previously reported (Evangelista-Kirkup et al., 1986). UV/visible spectra were collected before and after IR or Raman characterization to check sample integrity.

[1] FTIR, Fourier transform infrared; PPDME, protoporphyrin IX dimethyl ester; ImH, imidazole; Im^-, imidazolate; Hb, hemoglobin; Mb, myoglobin.

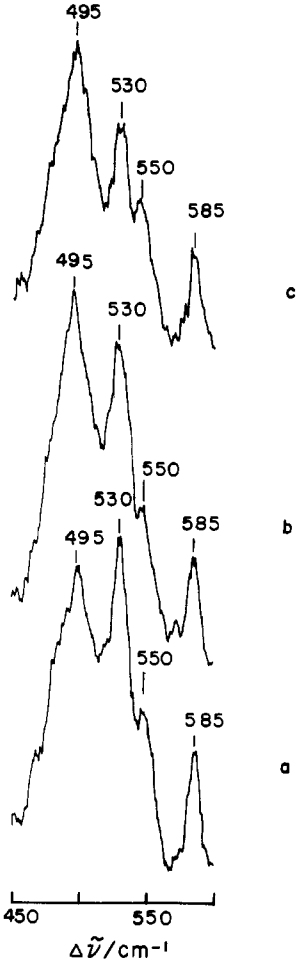

FIGURE 2: 413.1-nm-excited RR spectra near 500 cm^{-1} for CCP–CO (moderately aged protein) in phosphate buffer (0.1 M), pH 6, at different protein concentrations, (a) 10 (b) 7, (c) 3.5 mM, prepared with 20 min of CO flushing. Experimental conditions: laser power, ~7, mW at the sample; spectral slit width, 5 cm^{-1}; accumulation time, (a) 9 or (b and c) 15 s/0.5 cm^{-1}.

RESULTS

Figure 1 shows RR spectra, in the region of the Fe–C stretching and FeCO bending modes for the CO adduct of fresh and aged CCP preparations (see Experimental Procedures), exposed to 20 min of CO flushing. The aged sample shows a dominant 495-cm^{-1} band that is assigned to the ν_{FeCO} mode of perpendicularly bound CO, by analogy with the spectra of HRP–CO prepared with low CO exposure and/or high protein concentration (Evangelista-Kirkup et al., 1986). The fresh sample shows a dominant 530-cm^{-1} band and increased intensity for the 585-cm^{-1} band. By analogy with spectra of high-CO HRP–CO (Evangelista-Kirkup et al., 1986), these bands are assigned to tilted CO that is H bonded to a distal residue. H bonding is known to exist for CCP–CO from the 2-cm^{-1} downshift of the 1920-cm^{-1} infrared CO stretching band observed by Satterlee and Erman (1984) for CCP–CO prepared in D_2O. Our IR spectra showed a 1922-cm^{-1} band for both protein preparations, and we therefore presume that the ν_{CO} modes are accidentally coincident for the perpendicular and tilted FeCO forms. The frequency lies between those observed for perpendicular (1932 cm^{-1}) and tilted (1904 cm^{-1}) forms of HRP–CO (Barlow et al., 1976; Evangelista-Kirkup et al., 1986). In addition to the Fe–C stretching and FeCO bending modes, the RR spectra contain contributions from porphyrin modes at 495, 515, 550, and 585 cm^{-1} (Evangelista-Kirkup et al., 1985; Choi et al., 1982).

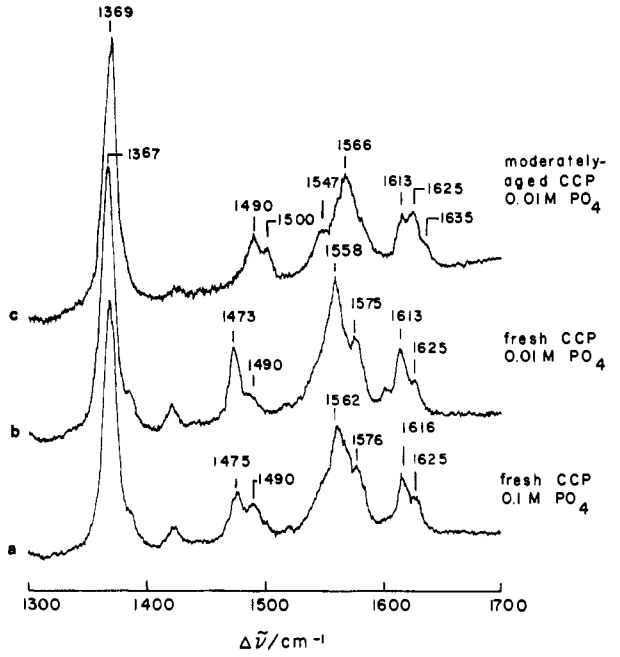

FIGURE 3: 413.1-nm-excited RR spectra in the high-frequency region for Fe^{III}–CCP in phosphate buffer, pH 7: (a) fresh protein, 0.1 M phosphate; (b) fresh protein, 0.01 M phosphate; (c) moderately aged protein, 0.01 M phosphate. Experimental conditions: laser power, ~30 mW at the sample; spectral slit width, 5 cm^{-1}; accumulation time, 3 s/0.5 cm^{-1}.

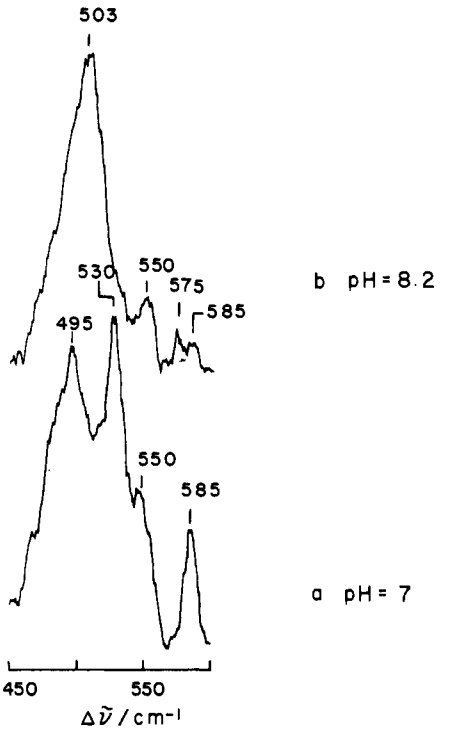

FIGURE 4: 413.1-nm-excited RR spectra near 500 cm^{-1} for CCP–CO (moderately aged protein) in phosphate buffer at (a) pH 7.5 (0.01 M phosphate) or (b) pH 8.2 (0.05 M phosphate). Experimental conditions: as in Figure 2, except accumulation time, (a) 6 or (b) 9 s/0.5 cm^{-1}.

Figure 2 shows RR spectra for moderately aged protein prepared at three different concentrations and flushed with CO for a constant interval, 20 min. As in the case of HRP, the bands assigned to tilted CO, at 530 and 585 cm^{-1}, increase at the expense of the perpendicular CO band at 495 cm^{-1} as the protein concentration decreases, an effect attributed to increasing CO concentration, due to decreased uptake of CO by the protein. Thus secondary binding of CO is implicated for CCP as well as HRP. A similar effect was observed for fresh protein; the 495-cm^{-1} band appears after very short CO exposures and is replaced with the 530-cm^{-1} band at longer (~10-min) flushing times. In the case of aged protein, however, the conversion to the tilted form was never complete, even for extended CO flushing times. Thus, storage of the protein produces a change that favors the perpendicular CO, perhaps by reducing the CO affinity of the secondary binding site.

Figure 3 compares RR spectra for fresh and moderately aged protein in the native Fe^{III} state, in the region of the high-frequency porphyrin skeletal modes. These modes are sensitive to the ligation and spin state of the Fe^{III} ion (Choi et al., 1982). CCP contains predominantly high-spin Fe^{III} heme (Yonetani, 1976), but the coordination number has been controversial. Five-coordination has been suggested on the basis of the RR spectrum (Sievers et al., 1979), but the crystal structure (Finzel et al., 1984) shows a distal water molecule, albeit at a somewhat long distance, 2.4 Å, from the Fe^{III} ion. It has now been shown via RR spectroscopy (Evangelista-Kirkup et al., 1985) that CCP contains a mixture of five- and six-coordinate high-spin Fe^{III} hemes, six-coordination becoming predominant at low temperatures. The 2.4-Å H_2O–Fe distance was interpreted as an average over bound (~2.0 Å) and unbound (~3.0 Å) H_2O molecules. This mixture of high-spin species can be seen in the RR spectrum of fresh CCP (Figure 3); the best indicator is the ν_3 band, appearing at 1490 and 1475 cm^{-1} for the five- and six-coordinate forms, respectively. The bottom spectrum of Figure 2 shows that increasing the concentration of the phosphate buffer from 0.01 to 0.1 M shifts the equilibrium toward five-coordination, as evidenced by the altered 1475 cm^{-1}/1490 cm^{-1} intensity ratio. We attribute this effect to destabilization of the Fe^{III}-bound H_2O by phosphate binding. The spectrum of moderately aged protein shows an additional ν_3 at 1500 cm^{-1}, a frequency characteristic of six-coordinate low-spin heme (Choi et al., 1982); other changes seen in the RR spectrum support this interpretation. This observation suggests that the same aging-induced alteration of the protein is responsible for a low-spin fraction in the native protein and a stable 495-cm^{-1} ν_{FeC} fraction of the CO adduct. We note that a CCP sample that had been left in contact with DEAE-agarose for ~12 h in the isolation procedure also showed a small low-spin component and that a variable fraction of low-spin hemes is detectable by electron paramagnetic resonance (EPR) for various CCP preparations (Yonetani, 1976). Thus the protein alteration in question may begin during isolation.

Figure 4 shows RR spectra for the CO adduct of moderately aged protein at increasing pH values. A new spectrum is observed at high pH, having a dominant band at 503 cm^{-1} and a subsidiary band at 575 cm^{-1}. This transition is concomitant with the replacement of the 1922-cm^{-1} IR band with one at 1948 cm^{-1}, as previously demonstrated by Yonetani and coworkers (Iizuka et al., 1985). The new set of frequencies is close to that of the CO adduct of hemoglobin, for which ν_{FeC}, δ_{FeCO}, and ν_{CO} have been found at 507, 576, and 1955 cm^{-1}, respectively, and attributed to CO bound in a tilted fashion (Tsubaki et al., 1982). Thus the high-pH form of CCP–CO contains predominantly a tilted but non-H-bonded FeCO unit. This situation is again in contrast to that of HRP, for which the high-pH form appears to be perpendicular FeCO, with the same vibrational frequencies as at low pH. There is, however, some intensity for the 1922-cm^{-1} IR band (Iizuka et al., 1985) under conditions where the 530-cm^{-1} RR band is absent, suggesting a fraction of perpendicular CCP–CO at high pH;

Table I: FeCO Frequencies (cm^{-1}) for Analogous Conformers in the CO Adducts of Heme Proteins

form	protein	ν_{FeC}	δ_{FeCO}	ν_{CO}	ref
I	CCP	495		1922	*a*
	HRP	490		1932	*b*
	Fe(PPDME)(Im^-)	490		1942	*b*
	Fe(PPDME)(ImH)	495		1960	*b*
II	CCP	530	585	1922	*a*
	HRP	537	587	1904	*b*
II′	CCP	503	575	1948	*a*
	Hb	507	578	1951	*c, d*
	Mb	512	577	1944	*c, e*

[a] This work. [b] Evangelista-Kirkup et al., 1985, 1986. [c] Tsubaki et al., 1968. [d] Alben et al., 1968. [e] Makinen et al., 1979.

the expected 495-cm^{-1} RR component for this species would have been obscured by the (rather broad) 503-cm^{-1} band of the dominant tilted species.

For the CO adduct of fresh protein, the low-pH spectrum containing the 530-cm^{-1} form exclusively (Figure 1) remained unaltered at high pH, provided that the pH adjustment was carried out with 0.1 M phosphate buffer. When, however, the protein was dissolved in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and the pH raised to 8.4, the 530-cm^{-1} form converted completely to the 503-cm^{-1} form. Evidently, phosphate stabilizes the 530-cm^{-1} form at high pH, presumably by raising the pK_a of the H-bonding residue(s). This effect, however, is very sensitive to aging. Thus, fresh protein that was stored at −10 °C for 1 week showed a mixture of 503- and 530-cm^{-1} forms in pH 8.4 phosphate buffer, although the low-pH spectrum showed the 530-cm^{-1} form exclusively, and no low-spin fraction was observed in the Fe^{III} RR spectrum. Phosphate binding appears to be lost before low-spin heme formation in the aging process.

Discussion

These observations on the CCP–CO Raman spectra make it clear that CCP and HRP share common CO binding properties, not seen in other heme proteins, but that they also show distinct differences. At low pH, both proteins show an equilibrium between two FeCO forms, I and II. Form I has CO bound in the normal fashion in the absence of distal constraints, with CO perpendicular to the heme plane. Form II has the CO tilted and H bonded to a distal residue. The vibrational frequencies, however, differ significantly in the two proteins, as summarized in Figure 5 and Table I. For II, ν_{Fe-C} is 7 cm^{-1} lower while ν_{CO} is 18 cm^{-1} higher in CCP than in HRP. In both proteins, II is known, from D_2O shifts of ν_{CO} (Smith et al., 1983; Satterlee & Erman, 1984), to involve distal H bonding to the bound CO. The unusually high ν_{FeC} and low ν_{CO} for HRP have been attributed (Evangelista-Kirkup et al., 1986) to enhanced Fe–CO back-bonding induced by strong H bonding to the O atom of the bound CO. The frequency differences therefore indicate that the H bonding is substantially weaker in CCP than in HRP.

For I, ν_{FeC} is 5 cm^{-1} higher while ν_{CO} is 10 cm^{-1} lower in CCP than HRP. Both ν_{CO} values are significantly lower than that observed in heme–CO model compounds (Evangelista-Kirkup et al., 1986; see Table I), even taking into consideration the effect of a strongly H-bonded proximal imidazole. For HRP the bound CO of I was considered by Smith et al. (1983) to be non-H-bonded, since the D_2O shift of ν_{CO}, ~1 cm^{-1}, was within the experimental error, in contrast to the 2.5-cm^{-1} shift for II. The situation for CCP is unclear since ν_{CO} appears to be coincident for I and II; the D_2O measurements of Satterlee et al. (1984) probably could not have determined whether both I and II were involved in the observed (2 ± 1-cm^{-1} shift. Even

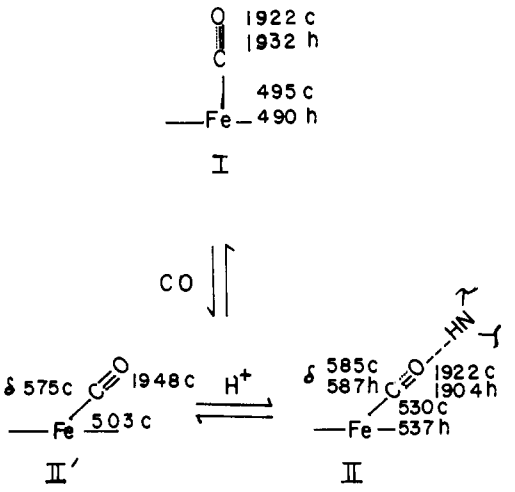

FIGURE 5: Schematic diagram for proposed binding modes for the CO adducts of CCP and HRP. The frequencies for ν_{CO} and ν_{FeC} are indicated (c = CCP, h = HRP) next to the CO bonds and those for δ_{FCO} next to the C atoms.

if H bonding is unimportant for I, it seems likely that polar interactions on the distal side of the heme group, e.g., with the positively charged arginine residue, Arg-48 (Poulos & Kraut, 1980), are responsible for the low values of ν_{CO}. Such interactions should have an effect on Fe–CO back-bonding similar to H bonding, lowering ν_{CO} and raising ν_{FeC}. They are apparently greater for CCP than HRP, producing the additional 10-cm^{-1} downshift of ν_{CO} and also the 5-cm^{-1} upshift in ν_{FeC}. Thus the distal interaction, polar interactions, and/or H bonding is greater in I, but less in II, for CCP than for HRP.

For both proteins, the H bond to the bound CO in II is titrated away at high pH, suggesting that the H-bonding residue is imidazole (Barlow et al., 1976; Iizuka et al., 1985). If this inference is correct, then the disposition of the imidazole must differ between the two proteins, to account for the difference in H-bond strengths. The crystal structure of CCP does show a distal histidine, His-52, although its orientation is suboptimal for H bonding to monatomic ligands bound to the heme Fe (Finzel et al., 1984). It is possible that the H-bond geometry is more favorable for HRP–CO than for CCP–CO. The titration appears to eliminate the tilted FeCO geometry in HRP, leaving perpendicular FeCO (form I) as the dominant form at high pH. The tilted form may be destabilized by an unfavorable interaction with the nearby unprotonated imidazole. CCP, in contrast, converts to a new form, II′, at high pH, whose vibrational signature, similar to those of HbCO and MbCO (Tsubaki et al., 1982; see Table I), suggests a tilted but non-H-bonded FeCO unit. Evidently the destabilizing interaction operative in HRP is attenuated in CCP, consistent with the weaker H bonding at low pH.

For both proteins, the equilibrium between I and II is shifted toward the latter with increasing CO concentration. This behavior is interpreted (Evangelista-Kirkup et al., 1986) in terms of a second binding site for CO, in the vicinity of the heme group, that alters the disposition of the distal residues in a manner favoring the H-bonded interaction with the distal imidazole. In the case of CCP, aging produces an irreversible change that favors I relative to II, possibly by lowering the affinity of the secondary CO binding site. This aging process also induces formation of an appreciable low-spin fraction in the Fe^{III} protein, indicating an alteration of the disposition of the distal residues. Most likely this alteration brings the distal imidazole into direct contact with the Fe^{III} ion, thus producing low-spin heme. The distal Fe–imidazole bond could still be

replaced by CO, following reduction, but in the altered conformation the displaced imidazole might not be able to form an H bond to the bound CO.

A remarkable effect is the stabilization of form II at high pH by phosphate, which we presume to bind in the distal region of the heme, thereby raising the pK_a of the distal imidazole. This effect has been observed only for CCP [the measurements on HRP–CO (Evangelista-Kirkup et al., 1986) were all carried out in 0.1 M phosphate and showed no evidence of form II at high pH] and only for fresh protein. Additional evidence for phosphate binding comes from the native protein RR spectrum (Figure 3), which shows an increase in the five-coordinate high-spin heme component, indicating that the bound phosphate destabilizes the Fe^{III}–OH_2 interaction. We infer that CCP exists in two conformations (in addition to a third conformation that develops in aged protein and is responsible for the low-spin heme component), one of which binds phosphate at a site where it increases the proton affinity of the distal imidazole and decreases the strength of the Fe^{III}–OH_2 interaction in the native protein.

What kind of conformational difference might account for this phosphate effect? An attractive possibility is suggested by the observation (Edwards et al., 1984) that Arg-48 occupies alternative postions in native CCP and its fluoride adduct. In the latter, Arg-48 swings into position (a ~2-Å movement) to H bond to the bound fluoride. Thus at least two conformations, A (native) and B (fluoride), are available to the protein, which differ principally in the disposition of Arg-48. As suggested previously (Evangelista-Kirkup et al., 1986), the secondary binding of CO in the distal region might displace the equilibrium toward conformation B, in which Arg-48 would offer steric hindrance to perpendicular FeCO, thereby stabilizing form II of the CO adduct. In conformation B, Arg-48 is also quite close to His-52. When the latter is protonated, a doubly positive charge site would be created, which might well have a high affinity for phosphate, the binding of which would have the effect of raising the His-52 pK_a. The crystal structure (Edwards et al., 1984) shows a water molecule (H_2O-596) H bonded to His-52, which could well be displaced by phosphate. Binding of phosphate at this site might weaken the interaction of His-52 with the heme-bound water molecule (H_2O-648) and stabilize five-coordinate heme.

The availability of a heme-linked phosphate binding site is relevant to the enzyme mechanism, since H bonding by both His-52 and Arg-48 has been implicated in the mechanism of peroxide heterolysis (Finzel et al., 1984; Poulos & Finzel, 1984), the first step in the enzymatic reaction. Control of this H bonding by phosphate, and perhaps by other anions, may therefore play a role in enzyme regulation. The loss of phosphate binding with protein storage and the stabilization of perpendicular relative to tilted and H-bonded CO indicate that the aging process leads to a stabilization of conformation A relative to conformation B. The nature of the protein modification responsible for this change is an important question for future studies.

Registry No. CCP, 9029-53-2; CO, 630-08-0; PO_4^{3-}, 14265-44-2.

References

Barlow, C. H., Ohlsson, P.-I., & Paul, K.-G. (1976) *Biochemistry 15*, 2225–2229.

Choi, S., Spiro, T. G., Langry, K. C., Smith, K. M., Budd, L. D., & LaMar, G. N. (1982) *J. Am. Chem. Soc. 104*, 443–445.

Edwards, S. L., Poulos, P. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 12984–12988.

Evangelista-Kirkup, R., Crisanti, M., Poulos, T. L., & Spiro, T. G. (1985) *FEBS Lett. 190*, 221–225.

Evangelista-Kirkup, R., Smulevich, G., & Spiro, T. G. (1986) *Biochemistry* (preceding paper in this issue).

Finzel, B. C., Poulos, T. L., & Kraut, J. (1984) *J. Biol. Chem. 259*, 13027–13036.

Iizuka, T., Makino, R., Ishimura, Y., & Yonetani, T. (1985) *J. Biol. Chem. 260(3)*, 1407–1412.

Nelson, C. E., Sitzman, E. V., Kang, C. H., & Margoliash, E. (1977) *Anal. Biochem. 83*, 622–631.

Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199–8205.

Poulos, T. L., & Finzel, B. C. (1984) in *Peptide and Protein Reviews* (Hearn, M. T. W., Ed.) Vol. 4, pp 115–171, Marcel Dekker, New York.

Satterlee, J. D., & Erman, J. E. (1984) *J. Am. Chem. Soc. 106*, 1139–1140.

Sievers, G., Osterlund, K., & Ellfolk, N. (1979) *Biochim. Biophys. Acta 581*, 1–14.

Smith, M. L., Ohlsson, P.-I., & Paul, K.-G. (1983) *FEBS Lett. 163*, 303–309.

Tsubaki, M., Srivastava, R. V., & Yu, N.-T. (1982) *Biochemistry 21*, 1132–1140.

Yonetani, T. (1976) *Enzymes (3rd Ed.) 13*, 345–361.

Yonetani, T., & Ray, G. S. (1965) *J. Biol. Chem. 240*, 4503–4508.