CO Recombination in Cytochrome *c* Peroxidase: Effect of the Local Heme Environment on CO Binding Explored through Site-Directed Mutagenesis†

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ABSTRACT: CO recombination to the cloned cytochrome *c* peroxidase [CCP(MI)] and mutants of CCP(MI) prepared by site-directed mutagenesis was examined as a function of pH by flash photolysis. The mutants examined included distal Arg 48 → Leu, Lys; proximal Asp 235 → Asn; and His 181 → Gly. At alkaline pH, ferrous CCP(MI) was converted to a hexacoordinate form by a cooperative two-proton ionization, apparent $pK_a = 8.0$. This change was observed in all of the mutants, although in the His 181 → Gly mutant, the conversion to the hexacoordinate form was the result of a single-proton ionization, implicating His 181 as one of the two residues deprotonated in this isomerization. The pH-dependent conversion of CO ferrous CCP(MI) from acidic to alkaline forms was also observed and was similar to that reported for cytochrome *c* peroxidase from bakers' yeast [Iizuka, T., Makino, R., Ishimura, Y., & Yonetani, T. (1985) *J. Biol. Chem. 260*, 1407–1412]. Photolysis of the acidic form of the CO complex of CCP(MI) produces a kinetic form of the ferrous enzyme (form A) which exhibits the slow rate of CO recombination ($l_1' \sim 10^3$ M^{-1} s^{-1}) characteristic of peroxidases, while photolysis of the alkaline form of the CO complex produces a second kinetic form (form B), which exhibits a much faster rate of recombination ($l_2' \sim 10^5$ M^{-1} s^{-1}). Kinetic forms analogous to forms A and B were observed in all of the mutants examined. A third kinetic form (form B*) with a bimolecular rate constant $l_3' \sim 10^6$ M^{-1} s^{-1} was also observed in the mutants at alkaline pH. Although the pH dependence for the conversion of form A to form B with increasing pH was altered by changes in the local heme environment, the rate of CO recombination by the respective forms was not dramatically altered in the mutants. Transient spectra of the reaction of CO with ferrous CCP(MI) after photolysis show that equilibrium between penta- and hexacoordinate ferrous enzyme is rapid relative to CO recombination. The presence of the internal sixth ligand has no discernible effect on the observed rate of recombination, however. The results presented indicate that in CCP(MI) the rate of ligand binding is determined primarily by isomerization of the protein from a closed conformation at acidic pH to an open conformation at alkaline pH and that polar effects of proximal Asp 235 and distal Arg 48 are of minor significance in the rate of CO recombination in both conformations.

The importance of protein structure in controlling heme reactivity is evidenced by the wide diversity of functions accomplished by heme proteins which contain protoheme as a prosthetic group. One aspect of heme reactivity that has received considerable attention is the influence of the surrounding protein environment on ligand binding rates. In the globins, kinetic studies of neutral ligand binding by flash photolysis on nanosecond and picosecond time scales have shown that the bimolecular recombination observed on the millisecond time scale is a complex process, involving at least two kinetically distinct concentration-independent steps at room temperature (Doster et al., 1982; Ansari et al., 1986; Gibson et al., 1986; Olson et al., 1987; Jongeward et al., 1988). Structural and spectroscopic characterization of some of the intermediates in this process has also been reported (Powers et al., 1987).

The characterization of ligand binding in peroxidases has received less attention. Although the catalytic cycle of these enzymes involves iron in the ferric and higher oxidation states, peroxidases reduced to the ferrous state under anaerobic conditions exhibit the reversible binding of small neutral ligands characteristic of pentacoordinate ferrous heme proteins (Keilin & Hartree, 1951). The observed bimolecular rate of ligand binding in peroxidases, however, is 100–1000-fold slower than the corresponding rate of recombination in other monomeric heme proteins, such as sperm whale myoglobin and leghemoglobin (Kertesz et al., 1965; Mims et al., 1983; Coletta et al., 1986). The crystal structure of yeast cytochrome *c* peroxidase (CCP)[1] (Poulos et al., 1980; Finzel et al., 1984) together with studies of model heme compounds [cf. Traylor et al. (1985), Traylor (1981), and Stanford et al. (1980)] permits several structural features which may influence the rate of ligand binding in peroxidases to be identified.

First, the distal heme pocket of CCP is more polar than the globins, due to the presence of an arginine residue (Arg 48) near the distal histidine (Poulos et al., 1980; Finzel et al., 1984). The guanidinium side chain of this residue creates a hydrogen-bonding network that extends from the heme pro-

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; CCP(MI), the cloned cytochrome *c* peroxidase.

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pionates to the ligand binding site (Finzel et al., 1984). The distal arginine is conserved among the peroxidases for which sequence data are available (Welinder, 1976; Welinder & Mazza, 1977; Mazza & Welinder, 1980; Tien & Tu, 1987). By comparing the recombination rates of CO and isonitriles in a variety of pentacoordinate heme proteins, Mims et al. (1983) concluded that the slower rate of CO recombination observed in ferrous peroxidases is due to the increased polarity of the distal heme pocket. The potential influence of distal polarity and steric effects on the rate of ligand binding are well-known through the study of model heme compounds [Traylor et al. (1985) and references therein].

A second structural feature of CCP that may influence the rate of ligand binding is a hydrogen-bonding interaction between the carboxylate side chain of an aspartic acid residue (Asp 235) and N_δ of the proximal imidazole ligand (His 175). This interaction is expected to increase the imidazolate character of the proximal base in CCP relative to the globins, since in the latter, the proximal imidazole forms a hydrogen bond with a backbone carbonyl. This view is supported by the higher Fe–imidazole stretch frequency in ferrous peroxidases relative to globins (Hashimoto et al., 1986; Smulevich et al., 1988a). Studies of model heme compounds have shown that deprotonation of the proximal imidazole ligand results in a dramatic decrease in the rate of CO recombination, leading to the proposal that the increased imidazolate character of the proximal base may be important factor in controlling the rate of ligand binding in peroxidases (Mincey & Traylor, 1979; Swartz et al., 1979, Stanford et al., 1980).

Finally, the extent of exposure of the bound heme to solvent is quite different in the globins and peroxidases. In myoglobin, the heme edge is directly exposed to the solvent, and the heme propionates bend back to form favorable ionic interactions with surface residues [cf. Takano (1977)]. In CCP, the heme is sequestered from bulk solvent, and although the propionate groups are completely extended, direct exposure to the solvent is not observed (Finzel et al., 1984). It is also interesting to note that although the heme edge is solvent accessible in myoglobin, no obvious path for ligand migration to the iron is evident (Case & Karplus, 1978), while in CCP, a solvent channel which is approximately 5 Å wide and 10 Å deep leads from the surface of the molecule to the iron (Poulos et al., 1980). Kinetic evidence that ligand entry into the protein occurs through this channel has been reported for peroxide and cyanide binding to ferric CCP (Hoth & Erman, 1984; Kang & Erman, 1982; Nicholls & Mochan, 1971). Differences between the accessibility of the heme iron center may therefore also influence the rate of ligand binding in peroxidases and globins.

Experimental support for the influence of protein structure on the overall rate of ligand binding has recently been reported. Studies of recombination on nanosecond and picosecond time scales suggest that the differences in the bimolecular rate of dioxygen and isonitrile binding in globins are due to differences in the rate of diffusion of ligand into the protein (Jongeward et al., 1988; Olson et al., 1987, Gibson et al., 1986). Moreover, Olson et al. (1988) have shown that the bimolecular rates of CO and dioxygen binding are affected similarly by changes in the distal environment of myoglobin and hemoglobin, indicating that steric effects influence the rate of binding of both ligands. Recent studies of CO recombination in ferrous horseradish peroxidase suggest that the entry of CO into ferrous HRP is severely restricted by a conformational barrier prior to entry of the ligand into the distal pocket (Doster et al., 1987).

CCP provides a convenient system for the study of the influence of enzyme conformation on ligand binding rates, since unlike other peroxidases that have been characterized (Kertesz et al., 1965; Coletta et al., 1986), a 50-fold increase in the rate of CO binding has been observed in CCP at alkaline pH (Iizuka et al., 1985). The observed increase in CO recombination rate is believed to be associated with a conformational change affecting the heme environment (Iizuka et al., 1985). A histidine residue (His 181) located in the vicinity of the heme propionates at the molecular surface has been shown to influence the stability of ferric CCP at alkaline pH (Miller et al., 1988), and deprotonation of this residue has been shown to make distal His 52 accessible to chemical derivatization (Bosshard et al., 1984). For this reason, the effect of His 181 on rates of CO recombination has also been examined here.

In the present work, the kinetics of CO recombination were examined as a function of pH in the cloned cytochrome *c* peroxidase, and in single-substitution mutants at positions 48, 235, and 181 described above. The results indicate that control of the rate of CO recombination is linked to the conformation of the protein and that the effect of a particular mutation is exerted primarily on the pH dependence of the conversion of ferrous CCP(MI) from the acidic (slow CO binding) to the alkaline (fast CO binding) form. In comparison to the change in rate of CO recombination due to the pH-dependent conformational change, the change in the rate of CO recombination observed when Arg 48 and Asp 235 are replaced with uncharged groups is minimal, suggesting that these features are not important in determining the bimolecular rate of CO recombination.

Materials and Methods

Materials. Chemicals employed in this study were of reagent grade or better. Carbon monoxide (99.9%) was from Matheson. Argon was passed through an in-line oxygen trap (Scientific Products) prior to use.

Preparation of CCP(MI) and Mutant Proteins. Expression of the cloned gene for cytochrome *c* peroxidase (Kaput et al., 1981) in *E. coli* has been described previously (Fishel et al., 1987). The mutations described in this study were introduced into the cloned gene, and the resulting enzymes were purified and converted to holoenzyme essentially as described by Fishel et al. (1987). Holoenzymes were twice crystallized by exhaustive dialysis against H_2O at 4 °C and stored as crystalline suspensions in H_2O at −70 °C until use.

Spectra of Ferrous CCP(MI) and Mutant Proteins. Enzyme stock solutions were freshly prepared at ~30 μM in 100 mM potassium phosphate buffer, pH 6.0, and were kept at 4 °C. Molar extinction coefficients for the Soret maximum of the ferric enzymes were determined in 0.1 M potassium phosphate, pH 6.0, at 23 °C by the pyridine hemochromogen method (Paul et al., 1953). For the CO ferrous derivatives, ϵ was determined frm the absorbance at the Soret maximum of the CO complex of a sample of known concentration, prepared by adding 20 μL of a ferric enzyme stock solution (deoxygenated under a stream of H_2O saturated Ar) to a sealed cuvette containing 2 mL of 0.1 M deoxygenated potassium phosphate buffer equilibrated with 1 atm of CO at the desired pH, in the presence of a slight excess of sodium dithionite. Spectra of the ferrous enzymes were determined as follows: a small aliquot of the enzyme stock solution was placed in the side arm of a sealed cuvette, and 2 mL of 0.1 M potassium phosphate buffer at the desired pH was placed in the optical chamber. The side arm was immediately immersed in an ice bath, while the optical chamber was main-

Table I: Spectral Data for Ferrous CCP and Mutant Proteins[a]

enzyme	coordination[b]	absorption maxima: Soret (nm)	Soret ϵ (mM^{-1} cm^{-1})	visible (nm)	5c/6c isosbestic points (nm)	pK_a[c]	n[c]
CCP[d]	5c	438	93	559		7.7	2.0
	6c	426	120	530, 562			
CCP(MI)	5c	438.9	103	559	433, 553, 569	8.0	2.2
	6c	426.7	138	530, 561			
Asn 235	5c	435.7	109	557	432, 552, 569	7.6	2.0
	6c	426.1	155	530, 561			
Leu 48	5c	438.9	104	559	433, 551, 566	7.2	1.8
	6c	425.2	124	531, 560			
Lys 48	5c	438.4	104	559	433, 552, 568	7.2	2.0
	6c	426.1	148	529, 560			
Gly 181	5c	436.1	103	559	433, 552, 570	7.3	0.9
	6c	426.3	140	530, 561			

[a] Spectra of protein solutions were recorded in 0.1 M phosphate at 23 °C, as described under Materials and Methods. [b] The spectra of penta- and hexacoordinate forms were determined under conditions where the respective forms were at least 98% of the total enzyme, as determined from the reported pK_a. [c] The reported values for the pK_a and n were determined from the x intercept and the slope in Hill plots of the change in absorbance at 580 nm versus pH for each protein. [d] Data from Conroy et al. (1978).

tained at ambient temperature. Argon (H_2O saturated) was bubbled through the buffer (and over the protein sample) for 30 min, at which time the cuvette was sealed. The sample and buffer were mixed, and the spectrum of the ferric enzyme was recorded. A slight excess of sodium dithionite (prepared in H_2O under anaerobic conditions) was introduced through an injection port sealed with a silicon septum, and the spectrum of the ferrous enzyme was recorded. Agitation of the samples was minimized during this procedure, particularly at alkaline pH, to avoid denaturation of the protein. Carbon monoxide was then passed over the sample for 10 min, and the spectrum of the CO complex was recorded. The pH of each sample was measured at the conclusion of each experiment. The protein concentration of each sample was calculated from the extinction coefficient of the CO complex at the Soret maximum, and ϵ at the Soret maximum of the ferrous enzyme was calculated by using this value. Direct comparison of spectra of the ferrous and the CO ferrous complex of each sample was used for determination of isosbestic points for reaction of CO with the ferrous enzyme.

Spectra were recorded at 23 °C on a Perkin-Elmer Lambda-3B dual-beam spectrophotometer equipped with a thermostated cuvette holder, and driven by a software package from Softways (Riverside, CA).

Kinetics of CO Recombination. Enzyme crystals were dissolved in 0.1 M potassium phosphate (pH 6.0) at 4 °C, brought to a final concentration of 0.2 mM, and 100 μL of this solution was deoxygenated under a gentle stream of argon for 30 min at 4 °C. Deoxygenated buffer (4 mL of 0.1 M potassium phosphate at the desired pH) was prepared in a 130-mL tonometer by four cycles of freeze–pump–thaw, followed by equilibration with argon (1 atm) at room temperature. The desired volume of carbon monoxide was added through an injection port sealed with a silicon septum. The deoxygenated enzyme solution was added through a cannula, followed immediately by the addition of a slight excess of sodium dithionite (prepared anaerobically, 45 mM in H_2O).

Samples with observed recombination rates less than 50 s^{-1} were photolyzed with an unfiltered Sunpak 611 photographic flash. Samples with observed recombination rates greater than 50 s^{-1} were photolyzed with a Rhodamine 560 dye laser. The monitoring apparatus employed has been described in detail elsewhere (Deardruff, 1985). The rate of CO recombination was determined by monitoring re-formation of the CO complex at the Soret maximum. At least five transients were averaged for each rate determination. Occasionally, recombination rates were also determined by monitoring disappearance of the ferrous enzyme at 438 nm. Similar recombination rates were obtained at both wavelengths. In all cases, the slower recombination rates (10^3–10^5 M^{-1} s^{-1}) were linear with CO concentration over a 10-fold range (10^{-4}–10^{-3} M), while the faster rates (10^6 M^{-1} s^{-1}) were linear over a 4-fold range [(1–4) × 10^{-4} M].

Monophasic recombination was analyzed by a linear least-squares fit program; biphasic processes were analyzed by the nonlinear least-squares programs EX2 developed by M. Lopez and NONLIN developed by one of us (M.F.F.). The agreement between the two nonlinear programs was within the error of the experiments. For biphasic fits, the relative percentage of recombination by each process was estimated as the ratio of the relative amplitude (ΔOD) of each process to the total amplitude determined from the curve fitting programs. The change in molar absorbance resulting from re-formation of the CO complex differs significantly between pentacoordinate ferrous enzyme and hexacoordinate ferrous enzyme, as well as between the acidic and alkaline CO complex (cf. Figure 5). To correct for this variation, the percentage of the total enzyme reacting by each process was determined by dividing the change in absorbance (amplitude) for each process by the change in molar absorbance ($\Delta\epsilon$) for each process at the wavelength of observation. In calculating $\Delta\epsilon$, form A and form B* were assumed to represent conversion of the pentacoordinate ferrous enzyme to the acidic and alkaline forms of the CO complex, respectively. Form B was assumed to be an equilibrium mixture of penta- and hexacoordinate forms at the pH of observation, according to the data in Table I. The change in molar absorbance for this form was calculated according to $\Delta\epsilon_B$ = (fraction 6c)($\Delta\epsilon_{6c}$) + (fraction 5c)($\Delta\epsilon_{5c}$), where $\Delta\epsilon_{6c}$ and $\Delta\epsilon_{5c}$ are the change in molar absorbance for conversion of pure 5c and 6c forms of the ferrous enzyme to the alkaline form of the CO complex at the wavelength of observation.

Transient Spectra of CO Recombination with Ferrous Peroxidase. CO ferrous enzyme samples were prepared at the desired pH in 0.1 M potassium phosphate buffer, essentially as described above. After photolysis of the sample with an unfiltered photographic flash, transient spectra were recorded on a Princeton EG&G Model 1215 optical multichannel analyzer at designated intervals over the course of the recombination reaction. For samples where k_{obs} for CO recombination was greater than 2 × 10^5 M^{-1} s^{-1}, the location of the isosbestic points for CO recombination and the changes in absorbance attributed to these processes were confirmed independently by monitoring the absorbance changes at several

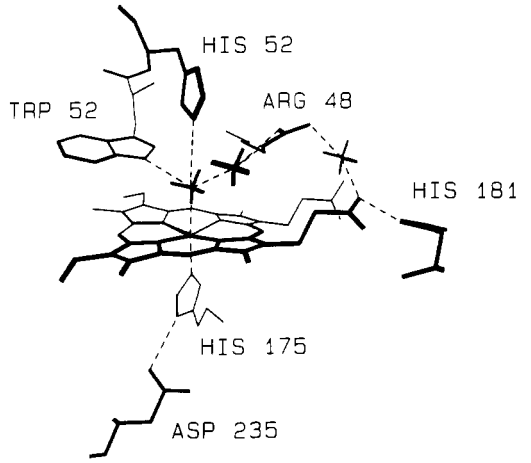


FIGURE 1: Structure of the heme binding pocket of ferric CCP(MI). Fixed solvent molecules are indicated by tetrahedra; hydrogen bonds are indicated by dashed lines.

wavelengths with the monochromatic detection system.

RESULTS

The gene for yeast CCP which has been expressed in *Escherichia coli* in this laboratory was cloned from *Saccharomyces cerevisiae* (Kaput et al., 1981). The protein expressed in *E. coli* differs from CCP isolated from bakers' yeast (for which the crystal structure has been reported; Poulos & Kraut, 1980; Finzel et al., 1984) in two ways. First, Thr 53 and Asp 152 in the bakers' yeast enzyme are replaced by Ile 53 and Gly 152 in the cloned enzyme. Second, expression of the cloned enzyme in bacteria involved addition of a methionine residue and an isoleucine residue at positions −2 and −1, respectively, of the N-terminus of the enzyme (Fishel et al., 1987). The enzyme produced in *E. coli* is therefore designated CCP(MI). The effect of these alterations on the structure of CCP is minor (Wang, 1988). Spectral and catalytic characteristics of CCP(MI) which have been examined show little difference from the bakers's yeast enzyme (Fishel et al., 1987).

The structure of the heme procket of the native ferric CCP(MI) is shown in Figure 1, and some important residues in the vicinity of the heme are indicated. The mutants of CCP(MI) examined here are single-residue substitutions at specific sites in the heme binding pocket. First, proximal Asp 235, which forms a hydrogen bond with N_δ of the proximal imidazole ligand (His 175), has been replaced with Asn in CCP(MI,N235). Second, the positively charged guanidinium side chain of distal Arg 48 has been replaced with the smaller primary amine side chain of Lys in CCP(MI,K48), or with an uncharged side chain (Leu) in CCP(MI,L48). Finally, His 181 has been replaced with glycine in CCP(MI,G181). Although His 181 is removed from direct contact with the distal face of the heme, it forms a hydrogen bond with the propionate of heme pyrrole IV. This propionate group also interacts with Arg 48 through mutual hydrogen bonds with a fixed water molecule (W348) (Figure 1), linking His 181 to the active site through the extended hydrogen-bonding network.

Spectra of Ferrous CCP(MI) and Mutant Proteins. Representative absorption spectra of ferrous CCP(MI) in the visible and Soret regions between pH 6.0 and pH 8.45 are shown in Figure 2. At pH 6.0 the spectrum is quite similar to that described for bakers' yeast CCP (Iizuka et al., 1985), with a Soret maximum at 438.9 nm, a single maximum in the visible region at 559 nm, and a shoulder at approximately 585 nm (Figure 2, Table I). These features are characteristic of pentacoordinate ferrous iron (Williams, 1951). As the pH is increased above 7.0, a blue shift in the Soret maximum is

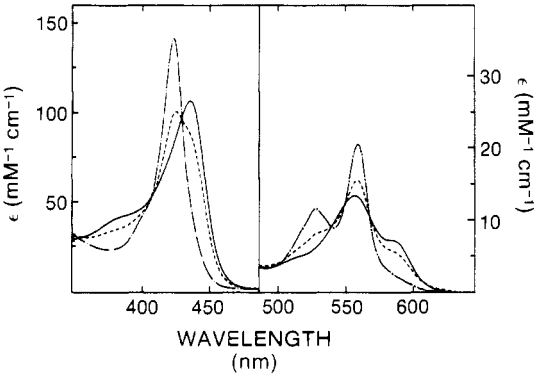


FIGURE 2: Dependence of visible and Soret region spectra of ferrous CCP(MI) on pH. The spectra were recorded in 0.1 M phosphate buffer, 23 °C, as described under Materials and Methods. Solid line, pH 6.0; dashed line, pH 7.8; broken line, pH 8.44.

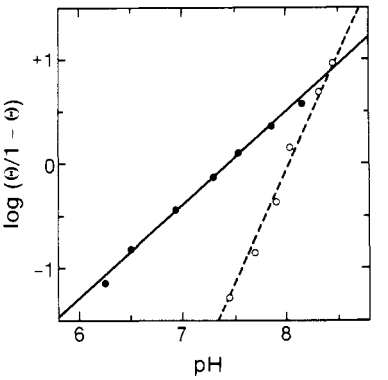


FIGURE 3: Hill plot of the conversion of ferrous CCP(MI) and ferrous CCP(MI,G181) from penta- to hexacoordinate forms. Theta (Θ) is the fraction of the enzyme in the alkaline form, monitored by the absorbance at 580 nm. Open circles = ferrous CCP(MI); solid circles = ferrous CCP(MI,G181).

observed, accompanied by the appearance of two maxima (α and β bands) in the visible region. The intensity of the α band (561 nm) is approximately twice that of the β (530 nm) band at pH 8.4. These changes are characteristic of the coordination of a basic sixth ligand to pentacoordinate ferrous heme (Williams, 1951). As noted by others, the spectral changes observed in both ferric and ferrous CCP from bakers' yeast at alkaline pH are complex, and although the major changes occur within a few seconds of pH adjustment, smaller changes are noted over a period of hours (Conroy et al., 1978; Gross & Erman, 1985; Dhaliwal & Erman, 1985). The spectra reported here were recorded immediately after exposure to alkaline conditions, and the experiments were complete within approximately 10 min. Long-term changes were not further investigated.

Hill plots of the change in absorbance at 580 nm with pH yielded a straight line, with a slope of approximately 2, consistent with the conversion of ferrous CCP(MI) from penta- to hexacoordinate forms by a cooperative two-proton ionization. An apparent pK_a = 8.0 (Figure 3) is indicated by the x intercept of the plot. An analogous change in coordination state of CCP from bakers' yeast resulting from a cooperative two-proton ionization has been described (Conroy et al., 1978), although a slightly lower apparent pK_a (=7.7) was reported. In addition to the transition from penta- to hexacoordinate CCP at high pH, Conroy et al. (1978) also described a single-proton ionization with an apparent pK_a = 5.7 which resulted in an increased molar absorbance of the Soret band with increasing pH. We have not detected an analogous effect in CCP(MI).

Table II: Bimolecular Rate Constants (l') for Recombination of Carbon Monoxide with Ferrous CCP(MI) and Mutant Proteins[a]

		CO rebinding				
enzyme	pH	l_3' ($\times 10^{-4}$ M^{-1} s^{-1})	l_2' ($\times 10^{-4}$ M^{-1} s^{-1})	l_1' ($\times 10^{-4}$ M^{-1} s^{-1})	% slow	λ_{max} (nm)
CCP(MI)	6.0			0.16 ± 0.02	100	423.4
	7.1			0.18 ± 0.03	100	423.4
	7.5		10 ± 1	0.20 ± 0.01	65	421.9
	7.8		8 ± 1[c]	0.49 ± 0.05	32	421.0
	8.3		15 ± 2[c]		0	420.4
Asn 235	6.0			0.56 ± 0.06	100	422.8
	7.1		15 ± 1	0.58 ± 0.02	71	421.4
	7.8	58 ± 10	5 ± 1[c]		53	420.4
Leu 48	5.0		13 ± 3	0.03 ± 0.01	74	422.6
	5.5		11 ± 1	0.09 ± 0.03	62	422.6
	6.0		8 ± 1	0.07 ± 0.01	62	422.1
	6.5		15 ± 1	0.08 ± 0.01	45	421.0
	7.1	140 ± 20	10 ± 2[c]		varies[b]	420.4
	7.8	130 ± 30	10 ± 2[c]		67	420.4
Lys 48	6.0		9 ± 3	0.8 ± 0.2	56	420.4
	7.1	140 ± 10	15 ± 3[c]		67	420.4
	7.8	110 ± 20	22 ± 4[c]		79	420.4
Gly 181	6.0		15 ± 3	1.0 ± 0.2	47	420.8
	7.1	140 ± 20	16 ± 4[c]		82	420.4
	7.8	140 ± 10	10 ± 1[c]		74	420.4

[a] Measurements were made by following the reformation of the enzyme–CO complex at the indicated Soret λ_{max} following flash photolysis of the complex. Samples were prepared in 0.1 M KPO_4 as described under Materials and Methods. [b] The percent slow rate decreased from 70% to 30% as the concentration of CO was raised from 1×10^{-4} to 7×10^{-4} M. [c] The presenceof the 6c form was observed in the transient spectra under these conditions.

The spectra of the CCP(MI) mutants were also examined in the Soret and visible regions. At pH 6.0, the spectra of the mutant proteins were characteristic of pentacoordinate ferrous peroxidase, similar to that described for CCP(MI). Small variations in the positions of the peaks were noted among the mutant proteins; these characteristics are summarized in Table I. The band at approximately 385 nm in CCP(MI) (Figure 2) was absent in the spectrum of CCP(MI,N235).

In each of the mutant proteins examined here, the pentacoordinate ferrous enzyme was converted to a hexacoordinate form when the pH was increased from pH 6.0 to pH 8.5. The spectra of the hexacoordinate forms were similar to that of parental CCP(MI), although, as with the pentacoordinate forms, small variations in the positions of the peaks in the visible and Soret regions were noted (Table I). The Soret band of hexacoordinate CCP(MI,L48) was significantly broader and weaker than those of the other proteins examined. The value for the apparent pK_a for conversion from penta- to hexacoordinate forms, determined from Hill plots of the change in absorbance at 580 nm with pH (not shown), is reported in Table I. In ferrous CCP(MI,N235), the apparent pK_a was 7.6, while in the distal ferrous CCP(MI,L48) and ferrous CCP(MI,K48), the apparent pK_a was lowered to approximately 7.2. In these mutants, Hill plots of the change in absorbance with pH had a slope of approximately 2, consistent with a cooperative two-proton ionization, as observed for ferrous CCP(MI).

Although the apparent pK_a for the conversion ferrous CCP(MI,G181) from pentacoordinate to hexacoordinate form was similar to that observed for the distal mutations, the Hill plot of the change in absorbance at 580 nm with pH for this mutant had a slope of approximately 1, indicating that the conversion from penta- to hexacoordinate forms results from a single-proton ionization (Figure 3). This observation implicates His 181 as one of the two residues that is titrated in the pH-dependent transition from penta- to hexacoordinated in ferrous CCP(MI).

Kinetics of CO Recombination of Ferrous CCP(MI). The kinetics of CO recombination of ferrous CCP(MI) were examined by photolysis of the CO complex under pseudo-first-order conditions between pH 6.0 and pH 8.4. The Soret maximum of CO ferrous CCP(MI) was located at 423.4 nm between pH 6.0 and pH 7.1. As the pH was increased above pH 7.1, a shift in the Soret maximum to shorter wavelengths was observed, until a value of 420.4 nm was reached at pH 8.3 (Table II). The position of the Soret maximum was not altered by further increases in pH. Iizuka et al. (1985) noted a similar blue shift in the Soret maximum of the CO complex of CCP isolated from bakers' yeast and found that this shift resulted from the reversible interconversion of acidic and alkaline forms of the CO complex by a two-proton cooperative ionization, apparent $pK_a = 7.6$. The shift in the Soret maximum in CO ferrous CCP(MI) exhibits similar characteristics (Miller, unpublished results) and is apparently directly analogous to that described for CCP. We have therefore adopted the designation *acidic* and *alkaline* for the 423.4- and 420.4-nm forms of the CO ferrous CCP(MI) complex, respectively.

The results of the kinetic experiments are summarized in Table II. Between pH 6.0 and pH 7.1, CO recombination occurs by a single exponential process. The observed bimolecular rate constant (l_1') for this process is similar to that observed for CO recombination in the acidic form of ferrous CCP from bakers' yeast (Iizuka et al., 1985) and to that observed for other ferrous plant peroxidases (Kertesz et al., 1965; Mims et al., 1983; Coletta et al., 1986). Above pH 7.1, CO recombination by a second, faster process was observed in addition to the l_1' process. The bimolecular rate constant (l_2') was approximately 50 times faster than l_1'. As the pH is increased, the percentage of recombination occurring by the faster process increase as well; at pH 8.3, recombination by the l_1' process was not detected.

To further characterize the fast and slow recombination processes, transient spectra of CO recombination with ferrous CCP(MI) were recorded; the results are presented in Figure 4. At pH 7.1, where recombination occurs by the l_1' process, the spectra indicate that ferrous CCP(MI) produced by photolysis is predominantly pentacoordinate (Figure 4A), and an isosbestic point was observed at 432 nm. At pH 7.8, the transient spectra indicate the presence of a significant amount of the hexacoordinate form of ferrous CCP(MI) immediately after photolysis. In the initial scans, where recombination by

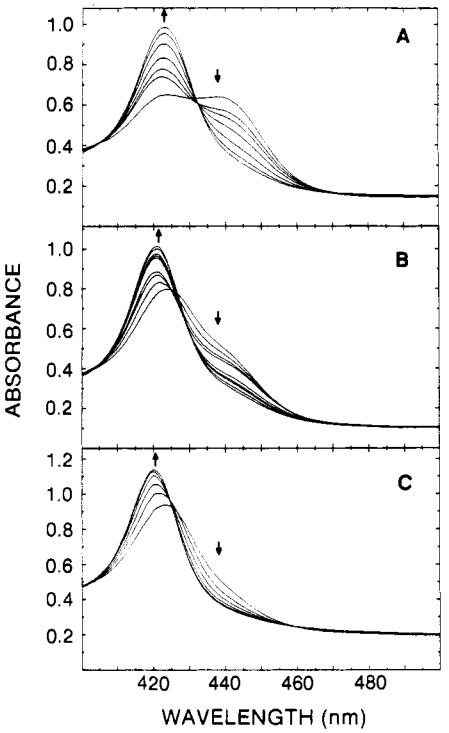


FIGURE 4: Transient absorption spectra of CO recombination with ferrous CCP(MI). Spectra of the samples were recorded after photolysis as described under Materials and Methods. Arrows indicate the direction of the change in absorbance with time. (A) pH 7.1, [CO] = 1×10^{-4} M, time sequence = 0.034, 1.0, 2.0, 4.0, 8.0, 15.0, 60.0 s. (B) pH 7.8, [CO] = 1×10^{-5} M, time sequence = 017, 0.425, 0.867, 1.275, 13.6, 20.4, 27.2, 108.8, 138.8 s. (C) pH 8.4, [CO] = 1×10^{-4} M, time sequence = 0.034, 0.085, 0.17, 0.34, 0.68, 1.36 s.

the l_2' process predominates, an isosbestic point was observed at 426 nm, while at later times, where recombination by the l_1' process predominates, an isosbestic point was observed at 431 nm (Figure 4B). At pH 8.3, where recombination occurs entirely by the l_2' process, the transient spectra indicate that the ferrous CCP(MI) produced by photolysis is primarily in the hexacoordinate form; an isosbestic point for the recombination reaction was observed at 425 nm (Figure 4C).

The location of the isosbestic points in the transient spectra can be rationalized by considering the pH dependence of the Soret region spectra of ferrous CCP(MI) and its CO complex. First, the pH-dependent change in the Soret region spectrum of ferrous CCP(MI) from completely pentacoordinate (λ_{max} = 438 nm) to completely hexacoordinate (λ_{max} = 426 nm) forms results in a blue shift in the isosbestic point for conversion to the CO complex as the percentage of the hexacoordinate species increases. This effect is shown by comparing the spectra of penta- and hexacoordinate ferrous CCP(MI) with the alkaline (420.4-nm) form of the CO complex (Figure 5A). Second, the isosbestic point for reaction of CO with ferrous CCP(MI) is also blue-shifted by the change in the CO ferrous CCP(MI) complex from the acidic (423.4-nm) to the alkaline (420.4-nm) form. The variation in the isosbestic point with pH for CO ferrous CCP(MI) and pentacoordinate ferrous CCP(MI) between pH 6.0 and pH 8.3 is shown in Figure 5B. For a given pH, the isosbestic point for each kinetic process therefore depends both on the percentage of the reacting population of ferrous CCP(MI) in penta- and hexacoordinate forms and on the percentage of the resulting CO complex in its acidic and alkaline forms. By comparison of the spectra of CO-free ferrous CCP(MI) and the CO complex for a given pH, the predicted isosbestic points shown in Figure 6A (solid line) were derived. Similarly, by comparison of the spectrum

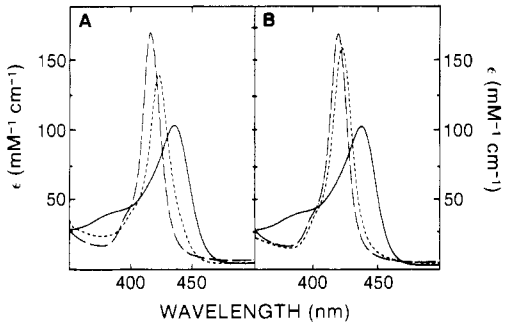


FIGURE 5: Dependence of isosbestic points for reaction of CO with ferrous CCP(MI) on pH. (A) The absorption spectra of pentacoordinate ferrous CCP(MI) (solid line) and hexacoordinate ferrous CCP(MI) (dashed line) are compared with the spectrum of the alkaline CO ferrous CCP(MI) complex (broken line). (B) The absorption spectra of the alkaline (broken line) and acidic (dashed line) forms of CO ferrous CCP(MI) are compared with pentacoordinate ferrous CCP(MI) (solid line). The spectra were recorded as described under Materials and Methods.

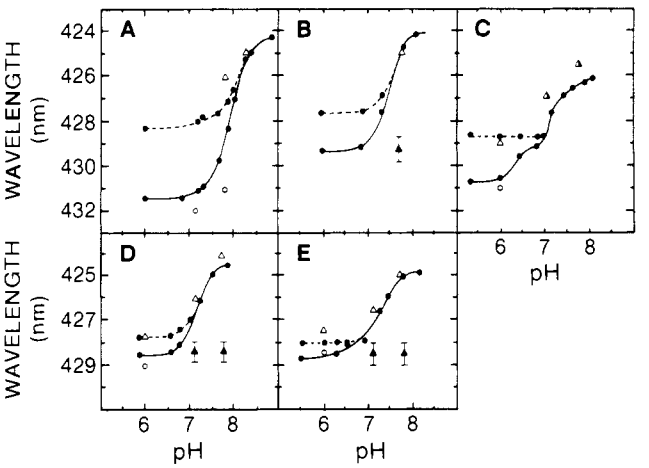


FIGURE 6: Dependence on pH of isosbestic points for reaction of CO with ferrous CCP(MI) and mutant proteins. Solid circles indicate the wavelength where the molar absorbance of CO-free ferrous enzyme and the subsequently formed CO complex were equal (solid lines) and the wavelength where the molar absorbance of CO-free ferrous enzyme and the alkaline form of the CO complex were equal (dashed lines). The lines have no theoretical significance. Open circles indicate the isosbestic point for reaction of form A with CO observed in the transient spectra. Open triangles indicate the isosbestic point for reaction of form B with CO in the transient spectra. Closed triangles indicate the isobestic point for reaction of form B* with CO, determined on the monochromatic detection system (see Materials and Methods). Panel A = CCP(MI); panel B = CCP(MI,N235); panel C = CCP(MI,L48); panel D = CCP(MI,K48); panel E = CCP(MI,G181).

of ferrous CCP(MI) from pH 6.0 to pH 8.4 with the alkaline (420.4-nm) form of the CO complex, the effect of the conversion of ferrous CCP(MI) from pentacoordinate to hexacoordinate forms on the isosbestic point for recombination can also be predicted (Figure 6A, dashed line).

By comparison of the isosbestic points observed in the transient spectra with those predicted above, the reaction of CO with ferrous CCP(MI) can be further described (Figure 6A). Recombination by the l_1' process, observed at pH 7.1 and 7.8, has an isosbestic point near 431–432 nm, consistent with reaction of CO with pentacoordinate ferrous CCP(MI) to produce the acidic (423.4-nm) form of the CO complex. In contrast, recombination by the l_2' process has isosbestic points at 426 nm at pH 7.8, and at 425 nm at pH 8.3, consistent with reaction of CO with a mixture of penta- and hexacoordinate ferrous CCP(MI) to produce the alkaline

(420.4-nm) form of the CO ferrous CCP(MI) complex (Figure 6A). The fact that this mixture of penta- and hexacoordinate forms yields a clean isosbestic point for CO recombination (Figure 4B,C) indicates that the equilibrium between the penta- and hexacoordinate forms reacting by the l_2' process is rapid relative to the rate of CO binding. These observations indicate that the coordination of the internal sixth ligand is both rapid and reversible in CCP(MI), in contrast to the slow, irreversible formation of the hexacoordinate species reported for bakers' yeast CCP (Iizuka et al., 1985).

Two forms of CO-free ferrous CCP(MI) can therefore be defined *based solely on the rate at which they bind CO.* The first, designated form A, binds CO according to the slow (l_1') rate, while the second, designated form B, binds CO at a rate (l_2') that is approximately 50-fold faster. The transient spectra recorded after photolysis of the CO complex suggest that form A is pentacoordinate and that form B is a mixture of penta- and hexacoordinate ferrous CCP(MI). The transient spectra further indicate that form A reacts with CO to produce the acidic (423.4-nm) form of the CO ferrous CCP(MI) complex, while form B reacts with CO to produce the alkaline (420.4-nm) form of the CO ferrous CCP(MI) complex. Accordingly, the increase in the relative amplitude of CO recombination by the l_2' kinetic process parallels the change in the Soret maximum of the CO complex from 423.4 to 420.4 nm (Table II). Form A and form B described here for ferrous CCP(MI) are directly analogous to the acidic and alkaline forms (respectively) of ferrous bakers's yeast CCP defined kinetically by Iizuka et al. (1985).

CO Recombination in CCP(MI) Mutants. Similar analyses were carried out in the subsequent characterization of CO recombination in the mutants of CCP(MI). In all of the mutants of CCP(MI) examined here, form A and form B are defined on the basis of kinetic criteria similar to those described above for ferrous CCP(MI). Form A has a bimolecular rate constant for CO recombination in the range from 10^3–10^4 M^{-1} s^{-1}, and form B has a bimolecular rate constant for CO recombination of (1–2) × 10^5 M^{-1} s^{-1}. As with CO ferrous CCP(MI), a pH-dependent change in the Soret maximum of the CO complex was noted in all of the mutants as the pH was increased. The interconversion between the two forms was reversible and showed isosbestic behavior in all cases (Miller, unpublished results), indicating the presence of two distinct forms of the CO ferrous enzyme complex. These two forms will be referred to as acidic and alkaline forms by analogy to the CO complex of ferrous CCP from bakers' yeast (Iizuka et al., 1985).

CO Recombination in Ferrous CCP(MI,N235). Photolysis experiments were carried out on CO ferrous CCP(MI,N235) at pH 6.0, 7.1, and 7.8. The position of the Soret maximum of CO ferrous CCP(MI,N235) varied from 422.8 nm at pH 6.0 to 420.4 nm at pH 7.8. Further changes in the Soret maximum below pH 6.0 and above pH 7.8 were not observed. At pH 6.0, ferrous CCP(MI,N235) is present entirely as form A, since CO recombination occurred by a single exponential process, with a bimolecular rate constant (l_1') 2–3-fold greater than that observed in CCP(MI) at this pH (Table II). At pH 7.1, a significant conversion of form A to form B was observed, since 32% of CO recombination occurred by a process with a bimolecular rate constant (l_2') similar to that observed for form B in ferrous CCP(MI) (Table II). At pH 7.8, form A is not present, since recombination by the l_1' process was not observed. Deprotonation of form A did not produce form B exclusively in ferrous CCP(MI,N235), however, since at pH 7.8 recombination by the l_2' process, and a third process, with a bimolecular rate constant (l_3') which was approximately 10-fold greater (Table II), was observed.

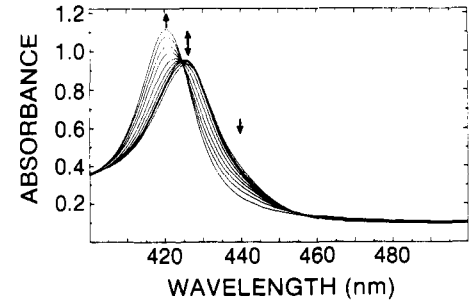


FIGURE 7: Transient spectra of CO recombination with ferrous CCP(MI,N235), pH 7.8. The experiments was performed as described under Materials and Methods. Arrows indicate the direction of the change in absorbance with time; the bivalent arrow indicates a region where the absorbance increases initially, followed by a decrease at later times. [CO] = 1 × 10^{-5} M, time sequence = 0.034, 0.051, 0.068, 0.119, 0.238, 0.476, 0.952, 1.7, 3.4, 14.96 s.

Transient spectra of CCP(MI,N235) were recorded at pH 7.8 to provide more information concerning CO recombination by the l_2' and l_3' processes. In the initial scans where the l_3' process predominates, a small increase in absorbance at 426 nm is observed, and an isosbestic point was located near 430 nm (Figure 7). Photolysis experiments with the monochromatic detection system showed that no change in transmittance due to the l_3' phase occurred at 430 nm, confirming the location of this isosbestic point. The increase in absorbance at 426 nm (cf. Figure 5A) and the location of the isosbestic point for recombination by the l_3' process (Figure 6B) indicate that this process results from reaction of CO with pentacoordinate ferrous CCP(MI,N235). At later times, where the l_2' process predominates, the transient spectra show that the absorbance at 426 nm decreases, and an isosbestic point is observed at 424.8 nm (Figure 7). The location of the isosbestic point (open triangle, Figure 6B) and the decrease in absorbance at 426 nm (Figure 5A) indicate that, in the l_2' process, CO is reacting with an equilibrium mixture of penta- and hexacoordinate forms at pH 7.8 (Figure 6B).

The data indicate the existence of a third kinetic form of the ferrous CCP(MI,N235) at pH 7.8. The bimolecular rate constant for this form is ~10-fold faster than that observed for form B and apparently results from the recombination of CO with a pentacoordinate form of the enzyme. The relative amplitudes of the l_2' and l_3' processes indicate that the two forms are present in nearly equal amounts at pH 7.8 (Table II). The spectrum of CO-free ferrous CCP(MI,N235) predicts that only ~20% of the enzyme exists in the pentacoordinate form at this pH, suggesting that equilibrium between penta- and hexacoordinate forms is not established in the third form. Reaction of CO with both forms apparently produces the same form of the alkaline CO complex, since no change in the spectrum of the resulting CO complex is detected. The third form, characterized by its rate of CO binding, is designated form B*, to indicate that reaction of this form with CO produces the alkaline form of the CO complex. The presence of form B* is not specific to ferrous CCP(MI,N235), however, since recombination by a process with a similar bimolecular rate constant, (0.5–1.5) × 10^6 M^{-1} s^{-1}, was observed in all of the mutants examined here (Table II and below).

Kinetics of CO Recombination in Ferrous CCP(MI,L48) and CCP(MI,K48). The results of flash photolysis experiments with ferrous CCP(MI,L48) are presented in Table II. The position of the Soret maximum of CO ferrous CCP(MI,L48) varied from 422.6 nm below pH 5.5 to 420.4 nm at pH 7.1.

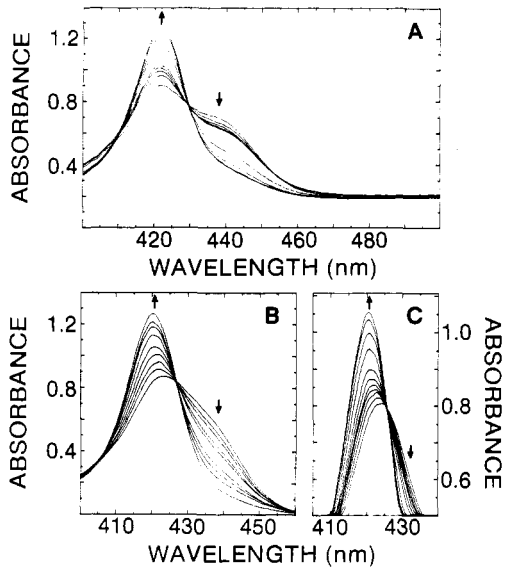


FIGURE 8: Transient spectra of ferrous CCP(MI,L48). Experiments were performed as described under Materials and Methods. Arrows indicate the direction of the change in absorbance with time. (A) pH 6.0, [CO] = 1×10^{-5} M, time sequence = 0.034, 0.051, 0.068, 0.085, 0.102, 30.0, 40.0, 50.0, 99.0 s. (B) pH 7.1, [CO] = 10^{-5} M, time sequence = 0.034, 0.051, 0.068, 0.102, 0.136, 0.281, 0.50, 0.80, 17.0 s. (C) pH 7.8, [CO] = 10^{-5} M, time sequence = 0.034, 0.051, 0.068, 0.085, 0.102, 0.136, 0.187, 0.391, 0.782, 1.7, 170.0 s.

The kinetics of CO recombination indicate that both form A and form B are present at pH 5.0, well below the pH where the CO ferrous CCP(MI,L48) complex is completely converted to the acidic (422.6-nm) form. The bimolecular rate constant (l_1') for form A was approximately 2-fold slower than that observed for CO recombination to form A of CCP(MI), and that for form B (l_2') was similar to that observed for recombination of CO with form B of CCP(MI). As the pH was increased, the relative proportion of form A decreased, until at pH 7.1, where the CO ferrous CCP(MI,L48) complex is entirely converted to the alkaline (420.4-nm) form, form A was no longer detected. At pH 7.1, forms B and B* were present, since recombination by processes with bimolecular rate constants (l_2' and l_3') analogous to those described for these forms was observed. Both processes were also observed at pH 7.8.

Transient spectra of CO recombination with ferrous CCP(MI,L48) are shown in Figure 8. At pH 6.0, the initial spectra show that pentacoordinate ferrous CCP(MI,L48) is produced by photolysis of the CO complex (Figure 8A). The isosbestic point for CO rebinding to ferrous CCP(MI,L48) was located at 429 nm in the initial spectra, where recombination was primarily due to the l_2' process, while at later times, where the l_1' process predominates, the isosbestic point was located at 431 nm. The observed isosbestic points for the l_2' and l_1' processes are in good agreement with those predicted for conversion of pentacoordinate ferrous CCP(MI,L48) to the alkaline (420.4-nm) and acidic (422.6-nm) CO ferrous CCP(MI,L48) complex, respectively (Figure 6C). At pH 7.1, the initial spectra show tht a mixture of penta- and hexacoordinate ferrous CCP(MI,L48) is produced by photolysis (Figure 8B). Despite the presence of two kinetically distinct forms at this pH, a constant isosbestic point (427 nm) was observed over the entire course of the reaction. Similar results were obtained at pH 7.8, where the initial spectra show that predominantly hexacoordinate ferrous CCP(MI,L48) is produced by photolysis, and a constant isosbestic point at 425.5 nm was observed (Figure 8C). The presence of a single isosbestic point for the l_2' and l_3' processes at pH 7.8 was confirmed by using the monochromatic detection system; at approximately 426 nm the change in transmittance corresponding to both l_3' and l_2' processes was eliminated.

The blue shift of the isosbestic point for recombination by forms B and B* with increasing pH paralleled that predicted for CO recombination with increasingly hexacoordinate ferrous CCP(MI,L48) (Figure 6C). Together with the observation of a single isosbestic point for the to processes, the data suggest that equilibrium between penta- and hexacoordinate ferrous CCP(MI,L48) is rapid relative to CO recombination in both form B and form B*.

The kinetic behavior of ferrous CCP(MI,L48) is clearly different from that observed for ferrous CCP(MI) and ferrous CCP(MI,N235) at acidic pH. Although the CO ferrous CCP(MI,L48) complex is entirely in the acidic (422.6-nm) form at pH 5.5, the kinetics of CO recombination indicate that a significant amount of form B is produced by photolysis even at pH 5.0. Moreover, although the percentage of the recombination attributable to form A decreased with increasing pH and was eliminated when the CO ferrous CCP(MI,L48) complex is converted entirely to the alkaline (420.4-nm) form, the relative amplitudes of forms A and B did not show the simple dependence on pH observed in ferrous CCP(MI). The conversion of the CO ferrous CCP(MI,L48) complex from acidic to alkaline forms has been found to correlate with a cooperative, two-proton ionization, however, with an apparent $pK_a = 6.2$ (Miller, unpublished results). Thus, unlike ferrous CCP(MI) and ferrous CCP(MI,N235), the disappearance of form A does not seem to directly parallel the conversion of the CO complex from acidic to alkaline forms.

Recombination in ferrous CCP(MI,L48) at alkaline pH was also anomalous, relative to the other mutants characterized here. First, although forms B and B* were observed at pH 7.1 and 7.8, the isosbestic point for CO recombination to the two forms was identical, indicating that form B* in ferrous CCP(MI,L48) represents an equilibrium mixture of penta- and hexacoordinate forms. Second, at pH 7.1, the percentage of the total enzyme present as form B* increased from 30% to 70% of the total as the concentration of CO was increased. This result may indicate that, at pH 7.1, form B* produced initially isomerizes to give form B at a rate that is sufficiently rapid to establish a competition between recombination and isomerization or that a slow isomerization occurs over the course of the experiment.

The results of flash photolysis experiments with ferrous CCP(MI,K48) are summarized in Table II. In CO ferrous CCP(MI,K48), no shift in the position of the Soret maximum is observed with pH. A change from acidic to alkaline forms is indicated by a small increase in the molar absorbance of the Soret band with increasing pH between pH 5.5 and pH 7.0, however (Miller, unpublished observation). At pH 6.0, ferrous CCP(MI,K48) is a mixture of forms A and B. Recombination by form A (l_1') was approximately 5-fold faster than that observed for recombination with form A of ferrous CCP(MI), while the rate of recombination with form B (l_2') was similar to that observed for CO recombination with form B in ferrous CCP(MI). At pH 7.1, form A was not detected, and recombination by form B and a process (l_3') corresponding to form B* was observed. Both form B and form B* were also detected at pH 7.8.

Transient spectra for CO recombination with ferrous CCP(MI,K48) are shown in Figure 9. At pH 6.0, the initial spectra indicate that pentacoordinate CCP(MI,K48) is produced by photolysis of the CO complex (Figure 9A). The

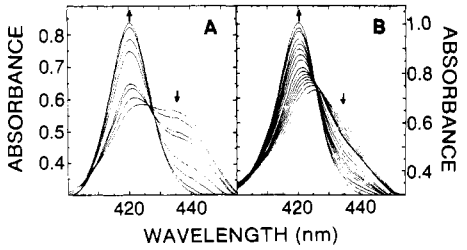


FIGURE 9: Transient spectra of ferrous CCP(MI,K48). Experiments were performed as described under Materials and Methods. Arrows indicate the direction of the change in absorbance with time. (A) pH 6.0, [CO] = 10^{-5} M, time sequence = 0.085, 0.153, 0.323, 0.425, 2.55, 5.95, 13.6, 28.9 s. (B) pH 7.1, [CO] = 10^{-5} M, time sequence = 0.034, 0.051, 0.068, 0.085, 0.102, 0.119, 0.170, 0.238, 0.34, 0.476, 0.51, 0.646, 0.85, 1.78 3.4, 13.6 s.

isosbestic point was located at 428 nm in the initial spectra, where recombination by form B predominates, and at 429 nm at later times, where recombination by form A predominates. The location of the isosbestic points suggests that reaction of pentacoordinate form A with CO (l_1') produces the acidic form of the CO complex, while reaction of pentacoordinate form B with CO (l_2') produces the alkaline CO complex (Figure 6D). At pH 7.1, photolysis of the CO complex produces a mixture of penta- and hexacoordinate ferrous CCP(MI,K48). In the initial scans, where recombination is predominantly by the l_3' process, an increase in absorbance at 426 nm was noted (Figure 9B). The location of the isosbestic point due to recombination by the l_3' process (form B*) could not be determined directly from the transient spectra, since recombination by this process was >75% complete before the second spectrum was recorded (0.051 s), even at low (2×10^{-5} M) CO concentrations. Experiments conducted with the monochromatic detection system showed that a change in transmittance due to the l_3' process was not detected between 428 and 429 nm, indicating that the isosbestic point for the l_3' process lies between these wavelengths. The location of the isosbestic point for recombination by this process was similar at pH 7.8. The increase in absorbance at 426 nm and the location of the isosbestic points suggest that form B* is pentacoordinate at pH 7.1 and 7.8. The results indicate that equilibrium between penta- and hexacoordinate form B* is established slowly relative to CO recombination.

In the later scans, where recombination is predominantly by form B (l_2'), an isosbestic point was observed at 426 nm at pH 7.1 (Figure 9B), and at 424 nm at pH 7.8. The shift in the isosbestic point for reaction of CO with form B as the pH is raised from pH 6.0 to pH 7.8 closely parallels that predicted by comparison of the spectra of ferrous CCP(MI,K48) and its CO complex over this pH range (Figure 6D). This indicates that equilibrium between penta- and hexacoordinate ferrous CCP(MI,K48) is rapid relative to CO binding in form B. The change in the ligation state of form B from pentacoordinate to hexacoordinate between pH 6.0 and pH 7.8 was essentially without effect on the bimolecular rate of recombination by this form (Table II).

Kinetics of CO Recombination in Ferrous CCP(MI,G181). The results of flash photolysis experiments with ferrous CCP(MI,G181) are summarized in Table II. The Soret maximum of CO ferrous CCP(MI,G181) was located at 420.8 nm at pH 5.5 and shifted to 420.4 nm at pH 7.1. This shift was accompanied by a small increase in molar absorptivity of the Soret band. Futher changes in this peak were not observed below pH 5.5 or above pH 7.1. At pH 6.0, ferrous CCP(MI,G181) is a mixture of forms A and B. The bimolecular rate constant (l_1') for recombination by form A was approximately 5-fold greater than that observed for recombination to form A of ferrous CCP(MI), while the bimolecular rate constant (l_2') for form B was similar to that observed in ferrous CCP(MI). At pH 7.1, form A was not detected. Two processes with bimolecular rate constants similar to those observed for recombination with forms B and B* of the other mutants were observed. Both processes were also observed at pH 7.8.

The transient spectra for CO recombination with ferrous CCP(MI,G181) were similar to those observed for ferrous CCP(MI,K48). At pH 6.0, an isosbestic point was observed at 427.5 nm in the initial spectra, where the l_2' process predominates, while in the later spectra, where the l_1' process predominates, an isosbestic point was observed at 428.5 nm. At pH 7.1, the initial transient spectra show that a mixture of penta- and hexacoordinate ferrous CCP(MI,G181) is produced by photolysis. In the region where the l_3' process predominates, an increase in absorbance at 426 nm was observed, and an isosbestic point was located between 428 and 429 nm by using the monochromatic detection system. In the later spectra, where the l_2' process predominates, an isosbestic point was observed at 426.5 nm. The isosbestic point for the l_3' process was located near 429 nm at pH 7.8, while that for the l_2' process was observed in the transient spectra at 425 nm. As shown in Figure 6E, the location of the isosbestic points for the l_2' process shows good correlation to those predicted by the spectra of ferrous CCP(MI,G181) and its CO complex between pH 6.0 and pH 7.8. The location of the isosbestic points for the l_3' process and the increase in absorbance at 426 nm observed during recombination by this process are consistent with reaction of CO with pentacoordinate ferrous CCP(MI,G181).

The results obtained with ferrous CCP(MI,G181) are essentially identical with those obtained for ferrous CCP(MI,K48). At pH 6.0, the kinetics of CO recombination indicate the presence of a mixture of forms A and B. When the pH was decreased to 5.3, no further shift in the position of the Soret maximum of the CO complex was noted and the kinetics of recombination remained biphasic (not shown), suggesting that form B is present below the pH at which conversion of the CO complex to the acidic form is complete. At pH 7.1, where conversion of the CO complex to the alkaline form is apparently complete, ferrous CCP(MI,G181) is converted entirely to forms B and B*. The equilibrium between penta- and hexacoordinate ferrous CCP(MI,G181) is therefore established rapidly relative to CO recombination in form B, while in form B* (predominantly pentacoordinate) this equilibrium established slowly.

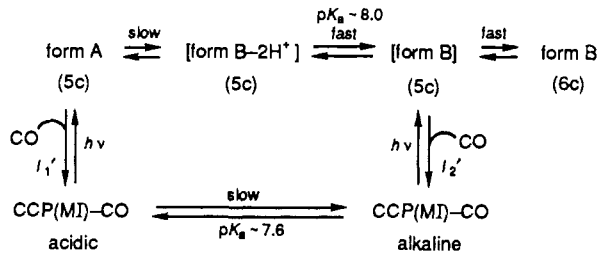
Discussion

CO Recombination in Ferrous CCP(MI). In the characterization of CO ferrous CCP from bakers' yeast, Iizuka et al. (1985) demonstrated the reversible interconversion of acidic and alkaline forms of the CO complex by a two-proton cooperative ionization, apparent $pK_a = 7.5$. The acidic form was characterized by a Soret maximum at 423.7 nm, and the C–O stretch frequency was observed at 1922 cm^{-1}, while in the alkaline form the Soret maximum is observed at 420 nm and the C–O stretch frequency at 1948 cm^{-1}. In the CO complex of ferrous CCP(MI), a shift in the Soret maximum from 423.4 nm at pH 7.1 to 420.4 nm at pH 8.4 is observed, and a shift in the infrared C–O stretch frequency in CO CCP(MI) from 1922 to 1948 cm^{-1} is observed between pH 7.0 and pH 8.4 (Smulevich et al., 1984). Thus, the acidic to alkaline transition in the CO complex of ferrous CCP(MI) is essentially identical with that observed in CCP from bakers' yeast.

Iizuka et al. (1985) reported that photolysis of the acidic and alkaline forms of CO CCP produced kinetically distinct forms of the ferrous enzyme; photolysis of the acidic CO complex produced a form of ferrous CCP with a slow bimolecular rate of CO recombination (1.1×10^3 M^{-1} s^{-1}), while photolysis of the alkaline form produced a form of ferrous CCP with a bimolecular rate constant that was approximately 30-fold faster (3.0×10^4 M^{-1} s^{-1}). Our results with ferrous CCP(MI) are consistent with these observations. Photolysis of the acidic and alkaline forms of CO ferrous CCP(MI) produces distinct forms of ferrous CCP(MI) which have characteristic rates of CO recombination. We have designated these kinetic forms of ferrous CCP(MI) as forms A and B, to distinguish them from the acidic (423.4-nm) and alkaline (420.4-nm) forms of the CO complex. The percentage of the enzyme present as the faster CO binding form B increases with increasing pH in parallel with the shift in the position of the Soret maximum of the CO complex from 423.4 to 420.4 nm (Table II). Between pH 7.1 and pH 8.3, where a mixture of forms A and B is produced by photolysis, the biphasic kinetics of recombination (Table II) and the distinct isosbestic points observed in the transient spectra (Figure 4) indicate that interconversion between forms A and B is slow relative to the rate of CO recombination. The transient spectra obtained with CCP(MI) (Figures 4 and 6A) show that recombination of CO with form A produces the acidic (423.4-nm) form of the CO complex and recombination with form B produces the alkaline (420.4 nm) form of the CO complex. The results are consistent with the conversion of form A to form B by a two-proton cooperative ionization, apparent pK_a = 7.6. The bimolecular rate constants for CO binding to forms A and B in CCP(MI) are consistently 2–3-fold larger than those reported for bakers' yeast CCP (Iizuka et al., 1985), probably as a result of slightly different experimental conditions.

In addition to the kinetically distinct forms of ferrous CCP(MI) observed in photolysis experiments, a pH-dependent conversion of ferrous CCP(MI) from the pentacoordinate form at pH 6.0 to a hexacoordinate form at alkaline pH was also noted in the visible and Soret region spectrum (Figure 2). The conversion of pentacoordinate to hexacoordinate ferrous CCP(MI) results from a cooperative two-proton ionization (apparent pK_a = 8.0). Similar results have also been described for ferrous CCP from bakers' yeast (Conroy et al., 1978), although a somewhat lower apparent pK_a (=7.7) was reported. The appearance of the hexacoordinate form at alkaline pH is presumed to result from coordination of a distal amino acid side chain (His 52) to the sixth coordination site of the iron.[2] The appearance of a hexacoordinate form in ferrous CCP(MI,L48) excludes Arg 48 as the sixth ligand (Table I), and the appearance of the hexacoordinate low-spin form has also been detected in a mutant where Trp 51 is changed to Phe (Smulevich et al., 1988a), eliminating Trp 51 as a possible ligand. Although a change in the proximal Fe–His stretching frequency was detected by resonance Raman spectroscopy in this pH range in ferrous bakers' yeast CCP and ferrous CCP(MI), a hexacoordinate form was not detected (Hashimoto et al., 1986; Smulevich et al., 1988a; Dasgupta et al., 1989). A recent reexamination of this discrepancy indicates that hexacoordinate ferrous CCP(MI) is converted to a photostable pentacoordinate form by the Raman laser under normal experimental conditions (G. Smulevich, personal communication). It is possible that the photostable state is related to the observed pentacoordinate form B*. The hexacoordinate form of ferrous CCP(MI,K48) was detected by resonance Raman spectroscopy, however (Smulevich et al., 1988a); the spectra were consistent with a bis(imidazole) heme, suggesting that the sixth ligand is His 52. The absorption spectrum of hexacoordinate ferrous CCP(MI) is also quite similar to that reported for ferrous cytochrome b_5 (Strittmatter & Velick, 1956), a protoporphyrin IX containing protein in which both fifth and sixth coordination sites are occupied by imidazole.

Scheme I



The change in the stoichiometry from a two-proton ionization to a one-proton ionization in ferrous CCP(MI,G181) identifies His 181 as one of the two residues that is deprotonated in the transition of ferrous CCP(MI) from pentacoordinate to hexacoordinate forms. The unusually high apparent pK_a (=8.0) of imidazole in this transition is most likely the result of the location of this residue within hyrogen-bonding distance of the carboxylate of Asp 37 and the propionate of heme pyrrole IV [Figure 1, Finzel et al. (1984)]. The importance of the interaction of His 181 with the heme propionate in the stability of pentacoordinate ferric CCP at alkaline pH is suggested by the reduced stability of the pentacoordinate form when either interaction is eliminated (Dowe & Erman, 1982; Miller et al., 1988).

The difference in apparent pK_a for the interconversion of forms A and B, and for the interconversion of penta- and hexacoordinate ferrous CCP(MI), may be explained either by the occurrence of two distinct transitions (net loss of four protons) or by a single transition (net loss of two protons), where the apparent pK_a is shifted from 8.0 to 7.6 by the presence of bound CO. The conversion of the CO complex from acidic to alkaline forms results from a two-proton ionization in CO ferrous CCP(MI) and a single-proton ionization in CO ferrous CCP(MI,G181) (Miller, unpublished results), indicating that deprotonation of His 181 is also involved in the conversion of the CO complex from acidic to alkaline forms. The observation that conversion of form A to form B parallels the conversion of the acidic form of the CO complex to the alkaline form in ferrous CCP(MI) favors the existence of a single transition, where the apparent pK_a of the transition is altered by the presence of bound CO. The difference in the apparent pK_a between the change in the coordination number of ligand-free ferrous CCP(MI) and that observed for conversion of form A to form B in photolysis experiments with CO ferrous CCP(MI) can be rationalized if the interconversion of form A and form B involves a pH-dependent conformational equilibrium, where protonation/deprotonation is fast, but the subsequent conformational equilibrium is achieved slowly, as shown in Scheme I, where the forms in brackets indicate nonequilibrium intermediates that result from photolysis. In this mechanism, conversion of pentacoordinate form A to hexacoordinate form B results from cooperative loss of two protons, and the apparent pK_a of the transition is shifted from

[2] Although the conversion of ferrous CCP(MI) to the hexacoordinate form by coordination of hydroxide, as has been demonstrated for ferric HRP (Sitter et al., 1988), cannot be rigorously excluded, we know of no precedent for a hydroxy sixth ligand in a ferrous heme with imidazole as proximal base.

8.0 to 7.6 by the presence of bound CO. A similar change in the apparent pK_a of a histidine residue due to CO binding has been described in deoxymyoglobin (Carver & Bradbury, 1984a,b). Between pH 7.1 and 8.5, pentacoordinate form B produced by photolysis will be protonated according to the apparent pK_a (=8.0) for penta- and hexacoordinate ferrous CCP(MI). The protonated form B subsequently isomerizes to give form A, while in deprotonated form B, the internal sixth ligand is coordinated to iron. If the isomerization of protonated form B to form A is slow and the coordination of the internal sixth ligand in deprotonated form B is fast, the percentage of total enzyme present as forms A and B will be distributed according to the apparent pK_a = 7.6, and the penta- and hexacoordinate forms observed in the transient spectra will be distributed according to the apparent pK_a = 8.0. This is consistent with the observations reported here. While the rate of isomerization of form B to form A has not been determined, pH jump experiments indicate that the conversion of form B–CO to form A–CO requires approximately 5 min at 23 °C (unpublished observation). This mechanism would also explain the absence of a spectral change in the CO-free ferrous protein with an apparent pK_a similar to that observed for the conversion of form A to form B.

The data therefore support the existence of a pH-dependent isomerization in ferrous CCP(MI) and its CO complex, resulting from the deprotonation of His 181 and a second, unidentified residue. The mechanism proposed above predicts that the conversion of form A to form B results from the same isomerization that results in the conversion of ferrous CCP(MI) from the pentacoordinate to hexacoordinate forms. The pentacoordinate form B detected in the kinetic experiments represents a nonequilibrium intermediate produced by photolysis of the CO complex. The percentage of the ferrous enzyme present as forms A and B after photolysis reflects the distribution of CO ferrous CCP(MI) between acidic and alkaline forms (apparent pK_a = 7.6), since isomerization of pentacoordinate form B to form A is slow relative to CO recombination.

The simplest explanation for the observed increase in CO recombination rates accompanying the isomerization is that deprotonation of the aforementioned residues results in a more open, or ligand-accessible, form of the protein. The observed coordination of a distal amino acid residue (presumed to be His 52, see above) indicates that a significant change in the distal environment of the heme accompanies this isomerization, since the N_ϵ of His 52 is 5.7 Å from the iron center in native CCP (Finzel et al., 1984). A significant change in the environment of the bound ligand is also indicated in the CO ferrous CCP(MI) complex by changes in the absorption spectrum, and by the shift in the infrared C–O stretch frequency (Iizuka et al., 1985; Smulevich et al., 1988b). Evidence that a more open conformation results from deprotonation of His 181 has been obtained from chemical modification studies of ferric CCP, where distal His 52 becomes accessible to derivatization with diethyl pyrocarbonate only when His 181 is deprotonated (Bosshard et al., 1984).

All of the mutants of CCP(MI) examined here showed a pH-dependent conversion of the CO-free ferrous enzymes from pentacoordinate to hexacoordinate form. In all the mutants except ferrous CCP(MI,G181), this conversion resulted from the cooperative loss of two protons (Table I) and is therefore presumed to be analogous to that observed in ferrous CCP(MI). The conversion of the CO complex from acidic to alkaline forms also results from a cooperative ionization of two protons, with the exception of CO ferrous CCP(MI,G181) (Miller, unpublished observations). Characteristic changes in infrared C–O stretch frequency coincident with conversion of the CO complex from the acidic to alkaline form have also been observed in all the mutants described here (Smulevich et al., 1986, 1988b; Smulevich, personal communication). The isomerization of the CO ferrous complex in the mutants is therefore also presumed to be analogous to that described for CO ferrous CCP(MI).

CO Recombination in Mutant Proteins—Form A and Form B. In each of the mutants examined, forms A and B were identified on the basis of the bimolecular rate of CO recombination. Recombination by form A for the mutants varied from 0.8×10^3 M^{-1} s^{-1} to 10×10^3 M^{-1} s^{-1}, and recombination by form B varied from 1×10^5 to 2×10^5 M^{-1} s^{-1} (Table II). For all of the mutants, the percentage of the total recombination by form A was decreased with increasing pH in the same range where conversion of the CO complex from the acidic to the alkaline form was observed, as determined by changes in the absorption spectra. The data from the transient spectra for each mutant suggest that reaction of CO with form A produces the acidic form of the CO complex while reaction with form B produces the alkaline form of the CO complex.

The replacement of proximal Asp 235 with asparagine results in a small change in the apparent pK_a for the conversion of form A to form B, since only form A was detected at pH 6.0, but the presence of form B was detected at pH 7.1. The Soret maximum of the CO complex is blue shifted at pH 7.1 relative to pH 6.0, indicating that the apparent pK_a for conversion of the CO complex from acidic to alkaline forms is also shifted downward relative to CCP(MI). A similar shift in the apparent pK_a for the conversion of the pentacoordinate to hexacoordinate form of CO-free ferrous CCP(MI,N235) was also observed (Table I). The effect on the apparent pK_a of the conversion of form A to form B may be due to a small structural adjustment along the loop leading from the proximal base (His 175), which interacts with Asp 235 directly, to the molecule surface at His 181; the largest structural changes in ferric CCP(MI,N235) have been noted between residues 180 and 191 (Wang, 1988).

A more dramatic effect on the conversion from form A to form B was observed in the mutants in which distal Arg 48 is replaced by leucine or lysine. Unlike CCP(MI), where conversion of form A to form B closely parallels the conversion of the CO complex from acidic to alkaline forms, CO recombination remained biphasic in ferrous CCP(MI,L48) at pH 5.0, where the conversion of the CO complex to the acidic form (as judged by the absorption spectrum in the Soret region) is complete. Thus, while protonation of His 181 and a second, undefined residue is sufficient to force the protein into a single closed conformation (form A) in CCP(MI), replacement of Arg 48 with leucine permits an equilibrium between form A and a more open form (form B) when both residues are protonated. The somewhat smaller change in the position of the Soret maximum [2.2 nm versus 3.0 nm in CCP(MI)] may reflect the presence of an alkaline form of the CO complex even when the enzyme is completely in the protonated form. Although the fraction of the enzyme that is present as form A decreases with increasing pH, and form A is not observed at pH 7.1, where the conversion of the CO complex to the alkaline fom is complete, the dependence of the relative amounts of forms A and B on pH is complex in ferrous CCP(MI,L48) (Table II). This is presumably due to the effect of other ionizing residues on the equilibrium between these forms.

Although the replacement of His 181 with glycine removes one of the two residues involved in the isomerization of ferrous CCP(MI) and its CO complex at alkaline pH, the effect of this mutation on the pH-dependent conversion of form A to form B in ferrous CCP(MI,G181) is similar to that observed in ferrous CCP(MI,L48) and ferrous CCP(MI,K48). Both form A and form B are detected in this mutant at pH 5.0, indicating that in ferrous CCP(MI,G181) a conformational equilibrium between closed and open forms of the enzyme exists when the enzyme is in the protonated form.

The similar effects of substitutions at Arg 48 and His 181 on the interconversion between forms A and B of CCP(MI) are most likely due to the interaction of Arg 48 and His 181 in CCP(MI) (Figure 1). Distal Arg 48 lies above heme pyrroles III and IV, where the guanidinium group forms hydrogen bonds with the water molecules in the active site, and interacts with the propionate of heme pyrrole IV through mutual hydrogen-bonding interactions with fixed water molecular 348. The hydrogen-bonding interaction between His 181 and the propionate of heme pyrrole IV therefore creates a hydrogen-bonding network that extends from His 181 through Arg 48 to the iron center. The replacement of either Arg 48 or His 181 would disrupt this network of hydrogen bonds, presumably permitting more flexibility of the protein in this region.

*CO Recombination in Mutant Proteins—Form B**. In CCP(MI), the conversion of form A to form B was complete at pH 8.4, resulting in monophasic recombination according to the expected rate for form B. In all of the mutants examined, however, a third kinetic form of the ferrous enzyme, designated from B*, was detected under conditions where conversion of form A to form B was expected to be complete. Data from the transient spectra for CO recombination with form B* suggest that it is primarily a pentacoordinate ferrous enzyme [with the exception of ferrous CCP(MI,L48), where it seems to be an equilibrium mixture of penta- and hexacoordinate forms] and is therefore distinct from form B, which is an equilibrium mixture of penta- and hexacoordinate enzyme in all cases (Figures 6C and 8). Due to the fast rate of CO recombination to this form, the CO-free spectrum of form B* has not been observed directly, and this evaluation should be considered preliminary at present. The simplest explanation of form B* is that it represents an even more opened conformation of the ferrous enzyme. The following possibilities can be ruled out at this time. (1) It is not an intermediate in the formation of form B, since the relative amplitudes of forms B and B* do not change with increasing CO concentration (with the exception of Leu 48, pH 7.1). (2) It is not a denatured form of the enzyme, since the Soret peak of the CO complex does not decrease with its appearance, and the spectra of the CO complexes are unaltered by its presence. (3) It is not the result of titration of an additional group on CCP(MI), since the percentage of recombination by form B* does not show a strong pH dependence.

Effect of the Local Heme Environment on CO Recombination in CCP(MI). One of the goals of this study was to determine the molecular basis for the slower rates of CO recombination characteristic of peroxidases (Iizuka et al., 1985; Kertesz et al., 1965) relative to the globins (Mims et al., 1983) by modification of the immediate heme environment. Although the effects of the mutations examined here are primarily exerted on the relative stability of forms A and B described for CCP and CCP(MI), it is useful to comment briefly on the rate of recombination by each form of the mutants described here.

The rate of CO recombination by form A in CCP(MI) is similar to that observed in other peroxidases (Iizuka et al., 1985; Kertesz et al., 1965; Mims et al., 1983). On this basis, it seems reasonable to presume that a common structural features(s) controls the rate of CO recombination in form A of CCP(MI) and the other peroxidases that have been characterized. By comparing CO recombination rates for form A of the mutants with CCP(MI), the effects of two important structural differences between myoglobin and peroxidases on the rate of CO recombination can be evaluated. First, the increased polarity in the distal heme pocket introduced by the invariant Arg 48 has been proposed to control ligand binding rates by peroxidases (Mims et al., 1983). This is clearly contradicted by the results obtained with ferrous CCP(MI,L48), since the slowest recombination rates were observed with this protein, where the distal Arg 48 is replaced with uncharged leucine (Table II). Second, the role of the increased imidazolate character of the proximal imidazole residue in determining the rate of ligand binding (Mincey & Traylor, 1979; Swartz et al., 1979; Stanford et al., 1980) is tested in ferrous CCP(MI,N235). Although the imidazolate character of the proximal base is reduced significantly in this mutant relative to ferrous CCP(MI) (Smulevich et al., 1988a), the recombination rate for form A in ferrous CCP(MI,N235) was only 3-fold higher than that observed in CCP(MI) (Table II). Studies with model heme compounds have shown that deprotonation of the proximal imidazole reduces the rate of CO recombination 100-fold, while a hydrogen bonding to the proximal imidazole reduces the rate of CO binding by approximately 3-fold (Stanford et al., 1980). The most straightforward interpretation of the small increase in the rate of CO binding in ferrous CCP(MI,N235) is that in ferrous CCP(MI) the proximal imidazole is strongly hydrogen bonded to Asp 235 but is not deprotonated by it. The deprotonation of the proximal imidazole suggested in the CO complex of ferrous CCP(MI) (Smulevich et al., 1988b) may therefore result from binding of CO. This situation may be analogous to the reported deprotonation of the proximal imidazole in ferric HRP when cyanide is bound, although this residue does not titrate in the absence of bound ligand (Thanabal et al., 1988).

Little variation was observed in the rate of CO recombination with forms B and B*. Thus, from the perspective of the approaching ligand, these forms must be essentially identical in the mutants examined here. Of particular interest is the observation that in all cases the rate of CO recombination in form B was unaffected by the presence of an internal ligand in the sixth coordination site. Since the presence of a sixth ligand is expected to reduce the rate of CO binding, it is clear that other effects are important. If the conformation and binding equilibria observed in CCP are represented as in Scheme I, CO recombination in CCP(MI) can be described as a situation where the removal of steric effects (conversion of form A to form B) results in an increase in the rate of binding despite the presence of a ligand in the sixth coordination site. Therefore, the increase in the rate of recombination due to the removal of a steric barrier more than compensates for the decreased availibility of the pentacordinate form.

One explanation for this observation can be described from data obtained from model heme compounds. Chelated mesoheme, in which a 1-imidazolylpropylamine moiety is attached through amide linkage to mesoheme, is pentacoordinate in benzene, due to coordination of the pendant imidazole to the iron. Dichelated mesoheme, where two 1-imidazolylpropyl-

amine groups are attached to mesoheme, exists as a mixture of penta- and hexacoordinate forms in benzene since both pendant imidazoles can coordinate to the iron (Geibel et al., 1975). The rate of CO binding by the hexacoordinate mesoheme is reduced 10-fold relative to the pentacoordinate chelated mesoheme in benzene (Geibel et al., 1975). When steric effects are imposed on pentacoordinate heme compounds by addition of a cyclophane strap across one face of the heme, the rate of CO binding in benzene is decreased by a factor of 10^3 compared to unhindered chelated mesoheme (Traylor et al., 1985). Clearly, removing the steric restraints imposed by the cyclophane structure on the pentacoordinate heme and replacing it with the hexacoordinate form seen in the dichelated mesoheme would cause an increase in the rate of CO binding. These observations stress the potential importance of steric effects on the rate of ligand binding in heme proteins.

Several observations indicate that the equilibrium between penta- and hexacoordinate form B is rapid: the rate of recombination is linearly dependent on CO concentration, independent of the coordination number of the ferrous enzyme; CO recombination to form B gives a constant isosbestic point in all cases (Figures 4 and 7–9); and in each case, the location of the isosbestic points for reaction of form B with CO is consistent with the predictions of the spectra of the unliganded ferrous form and the alkaline CO complex (Figure 6). Thus, in form B of CCP the rate of dissociation of the internal ligand is much faster than the rate of CO recombination in the concentration range examined here (k_{obs} = 10–100 s^{-1}). Although the predicted dissociation rate of the internal ligand is fast relative to those reported for dissociation of small ligands from ferrous heme proteins [cf. Mims et al. (1983)], the dissociation rate for imidazole from ferrous tetraphenyl and tetramesityl heme ranges from 10^3 s^{-1} for the bis(imidazole) compound to 10^8 s^{-1} for bis(1,2-dimethylimidazole) (White et al., 1979; Portella, 1987).

The recombination rate observed for form B is essentially constant over the range from completely pentacoordinate to completely hexacoordinate (Table II, Figure 6), indicating that the rates of recombination by hexacoordinate and pentacoordinate form B are equal. Recent studies of ligand binding in globins and model heme compounds (Jongeward et al., 1988; Olson et al., 1987; Ansari et al., 1986) suggest that, in these systems, the observed bimolecular rate constant for ligand binding is a function of a rapid equilibration of ligand into the heme binding site and the rate of Fe–ligand bond formation in the desolvated Fe–ligand pair:

$$k_{obs} = K_{eq}k_{BA}$$

where k_{BA} is the rate of Fe–CO bond formation and K_{eq} is the equilibrium constant for distribution of ligand into the heme binding site. If such a model is applicable to form B of CCP, the presence of an internal ligand in the sixth coordination site would be expected to reduce the rate of ligand binding by a factor equal to the dissociation constant for the internal ligand (K_D). One possible explanation for the similar rates of ligand binding in penta- and hexacoordinate form B is that the decreased rate of bond formation with CO due to competition with the internal sixth ligand for the sixth coordination site is fortuitously compensated by an equivalent increase in the rate of diffusion of CO into the protein (i.e., by increasing K_{eq}). For example, if the bimolecular rate constant for CO binding by pentacoordinate CCP is described by

$$k_{obs(5c)} = k_{BA}K_{5c}$$

and the bimolecular rate constant for hexacoordinate CCP is described by

$$k_{obs(6c)} = k_{BA}K_DK_{6c}$$

where K_{5c} and K_{6c} are the equilibrium constants for the distribution of CO into the heme pocket for the penta- and hexacoordinate forms, respectively, and K_D is the dissociation constant for the internal sixth ligand in the hexacoordinate form, then apparently, $k_{obs(6c)} = k_{obs(5c)}$ whenever $K_DK_{6c} = K_{5c}$. An alternative possibility, that the rate of ligand binding is limited by the initial rate of diffusion of the ligand into the protein, seems unlikely in view of the dependence of the observed bimolecular rate constant on the rate of bond formation noted in the globins and model heme compounds (Jongeward et al., 1988; Olson et al., 1988; Doster et al., 1987).

What Controls the Rate of CO Recombination in CCP(MI)? The results presented here describe a pH-dependent change in CCP(MI) from a slow binding conformation (form A) to a fast binding conformation (form B). Through characterization of several mutant enzymes, the rate of CO recombination by each conformation is shown to be relatively insensitive to changes in the polarity of residues at positions 235 and 48. The increased rate of ligand binding in form B can therefore be explained in terms of a conformational change in ferrous CCP at alkaline pH, which permits more rapid diffusion of the ligand into the heme binding pocket by removing a steric barrier. The role of steric effects on the bimolecular rate of rate of CO, isonitrile, and dioxygen binding is now well documented in the globins (Jongeward et al., 1988; Olson et al., 1988, 1986; Gibson et al., 1986). Moreover, the recent work of Doster et al. (1987) suggests that the entry of CO into the heme pocket in ferrous HRP requires a significant conformational change.

The existence of a proton-linked conformational change in CCP which alters the barrier to CO binding can also be reconciled with the observed differences in reactivity of ferric and ferrous peroxidases with small ligands. In ferric CCP and other peroxidases, the rate of reaction with peroxide is near the diffusion limit (Loo & Erman, 1975; Dunford & Stillman, 1976), suggesting that approach of peroxide to the iron is unhindered. In contrast, the reaction of ferrous CCP (Mims et al., 1983) and HRP (Phelps et al., 1974) with dioxygen is approximately 1000-fold slower than the reaction of the ferric enzyme with peroxide, despite the similar size of these ligands. Importantly, anion binding (Erman, 1974a,b; Dunford & Stillman, 1976) and the reduction of CCP and other heme peroxidases from ferric to ferrous form Conroy et al., 1978; Ricard et al., 1972) are accompanied by uptake of a proton. Thus, in ferric CCP, the proton that is bound along with anions may induce a conformational change which is important in the mechanism of reaction with peroxide, as suggested by Pratt (1986). The same change, induced by proton binding upon reduction of CCP from ferric to ferrous form may impose a barrier to the approach of neutral ligands. This would explain the much slower rate of ligand binding in the ferrous peroxidases.

A likely site for proton uptake in the reduction of CCP is the distal histidine (His 52). The protonation of His 52 in ferrous CCP is consistent with the conversion of the pentacoordinate to hexacoordinate transition from a one-proton ionization in ferric CCP (Gross & Erman, 1985) to a two-proton ionization in ferrous CCP, since coordination of His 52 to Fe would induce deprotonation of this residue. This residue has already been implicated as the proton acceptor in the reaction of CCP with HOOH (Poulos & Kraut, 1980), and protonation of the distal His has been shown to accompany cyanide binding in ferric HRP Thanabal et al., 1988). The difference in the apparent pK_a for His 52 between the ferric

and ferrous forms could result from Coulombic interactions accompanying the change in the Fe oxidation state from ferric to ferrous forms; such effects have been shown to alter the apparent pK_a of titratable residues near Fe by as much as 5 units in protein-free heme systems (Baldwin et al., 1981, 1984).

Recently, the importance of the distal (E7) His as a barrier to ligand binding in globins has been demonstrated (Olson et al., 1988; Morikis et a., 1989). Since N_ϵ of His 52 in CCP is located between the solvent channel and Fe in ferric CCP (Finzel et al., 1984), it is possible that protonation of this residue may increase the barrier to ligand binding by inducing a more extensive conformation change which alters the steric constraints of the access channel, by decreasing the mobility of His 52, or by the influence of increased polarity in the heme pocket, as suggested by Mims et al. (1983). Further kinetic and structural investigations will be required to evaluate these possibilities.

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Effects of the Bacteriophage T4 Gene 41 and Gene 32 Proteins on RNA Primer Synthesis: Coupling of Leading- and Lagging-Strand DNA Synthesis at a Replication Fork†

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ABSTRACT: We have demonstrated previously that the template sequences 5′-GTT-3′ and 5′-GCT-3′ serve as necessary and sufficient signals for the initiation of new DNA chains that start with pentaribonucleotide primers of sequence pppApCpNpNpN or pppGpCpNpNpN, respectively. Normally, the complete T4 primosome, consisting of the T4 gene 41 (DNA helicase) and gene 61 (primase) proteins, is required to produce RNA primers. However, a high concentration of the 61 protein alone can prime DNA chain starts from the GCT sites [Cha, T.-A., & Alberts, B. M. (1986) *J. Biol. Chem. 261*, 7001–7010]. We show here that the 61 protein can catalyze a single-stranded DNA template-dependent reaction in which the dimers pppApC and pppGpC are the major products and much longer oligomers of various lengths are minor ones. Further addition of the 41 protein is needed to form a primosome that catalyzes efficient synthesis of the physiologically relevant pentaribonucleotides that are responsible for the de novo DNA chain starts on the lagging strand of a replication fork. The helicase activity of the 41 protein is necessary and sufficient to ensure a high rate and processivity of DNA synthesis on the leading strand [Cha, T.-A., & Alberts, B. M. (1989) *J. Biol. Chem. 264*, 12220–12225]. Coupling an RNA primase to this helicase in the primosome therefore coordinates the leading- and lagging-strand DNA syntheses at a DNA replication fork. Our experiments reveal that the addition of the T4 helix-destabilizing protein (the gene 32 protein) is required to confine the synthesis of RNA primers to those sites where they are used to start an Okazaki fragment, causing many potential priming sites to be passed by the primosome without triggering primer synthesis.

An accurate and efficient DNA replication process has been reconstituted using purified components specified by bacteriophage T4 (Alberts et al., 1983; Nossal & Alberts, 1983; Alberts, 1984, 1987). A mixture of seven T4-encoded proteins—the products of T4 genes 43 (DNA polymerase), 45 and 44/62 (DNA polymerase accessory proteins), 41 (DNA helicase), 61 (primase), and 32 (helix-destabilizing protein)—catalyzes rapid DNA replication fork movement. These replication forks closely resemble those formed by the T4 replication apparatus in vivo, having a similar speed, geometry, and fidelity of DNA synthesis (Hibner & Alberts, 1980; Sinha et al., 1980).

At a DNA replication fork, the leading strand is synthesized continuously, and the lagging strand is synthesized discontinuously. Leading-strand DNA synthesis requires the concerted action of helix unwinding by the 41 protein and DNA synthesis by the DNA polymerase holoenzyme (the gene 43 DNA polymerase and its three accessory proteins (Cha & Alberts, 1989). Lagging-strand DNA synthesis, on the other hand, requires the periodic synthesis of RNA primers that are efficiently utilized by a lagging-strand DNA polymerase holoenzyme to initiate successive Okazaki fragments. Previous studies have shown that the 41 and 61 proteins form the primosome that is responsible for RNA primer synthesis (Liu & Alberts, 1980, 1981; Nossal, 1980) and that the major products synthesized are pentaribonucleotides both in vivo

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