CO Dissociation in Cytochrome *c* Peroxidase: Site-Directed Mutagenesis Shows That Distal Arg 48 Influences CO Dissociation Rates†

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Received April 18, 1990; Revised Manuscript Received July 23, 1990

ABSTRACT: To investigate the molecular basis for the 100-fold slower rate of CO dissociation in ferrous peroxidases relative to myoglobin, CO dissociation rates were measured as a function of pH in the cloned cytochrome *c* peroxidase from yeast [CCP(MI)] and in several mutants in the heme binding pocket prepared by site-directed mutagenesis. The mutants included Asp 235 → Asn; Arg 48 → Lys, Leu; and His 181 → Gly. Changes in the absorption spectrum with pH are consistent with conversion of the CO–ferrous CCP(MI) complex from acidic to alkaline forms by a two-proton cooperative ionization, with an apparent pK_a = 7.6, analogous to that described for CCP from bakers' yeast [Iizuka, T., Makino, R., Ishimura, Y., & Yonetani, T. (1985) *J. Biol. Chem. 260*, 1407–1412]. The rate of CO dissociation (k_{off}) was increased 11-fold (from 0.7×10^{-4} to 8.0×10^{-4} s^{-1}) by conversion of the acidic to the alkaline form. Analogous acidic and alkaline forms of the CO complex were also observed in the mutants of CCP(MI) examined here. In the acidic form, k_{off} was increased 5- and 20-fold when Arg 48 was replaced with Lys and Leu, respectively, while in the acidic form of mutants that possess Arg 48, k_{off} was similar to that observed in CCP(MI). Conversion of the CO complex from the acidic to alkaline form increased k_{off} in all the mutants, and the pH-dependent increase in k_{off} correlated with a two-proton cooperative ionization, except in the case of His 181 → Gly. In this mutant, pH-dependent increase in k_{off} correlated with a single-proton ionization, implicating His 181 as one of the two residues that is deprotonated in the conversion of CO–ferrous CCP(MI) from acidic to alkaline forms. Only a 2.5-fold variation was observed for k_{off} between the alkaline form of CCP(MI) and the Arg 48 → Leu mutant, suggesting that the influence of Arg 48 on the rate of CO dissociation is decreased in the alkaline form by a conformational change. Comparison of the relationship between log k_{off} and ν_{Fe-C} determined by resonance Raman spectroscopy [Smulevich, G., Mauro, J. M., Fishel, L. F., English, A. M., Kraut, J., & Spiro, T. G. (1988b) *Biochemistry 27*, 5486–5492] indicates that CO dissociation rates for the acidic form of CCP(MI) are independent of ν_{Fe-C} when Arg 48 is present and suggests that the 12-fold difference in k_{off} between the alkaline CO complex of CCP(MI) and myoglobin results from differences in the interaction of the heme with the proximal imidazole ligand.

Cytochrome *c* peroxidase (CCP)[1] from yeast *Saccharomyces cerevisiae* is a monomeric heme protein that catalyzes the peroxide-dependent oxidation of cytochrome *c*. Although the catalytic cycle of the enzyme involves iron in the ferric and higher oxidation states, when reduced to the ferrous state under anaerobic conditions, CCP exhibits reversible binding of small neutral ligands characteristic of ferrous heme proteins (Keilin & Hartree, 1951). Like other heme peroxidases that have been characterized (Kertesz et al., 1965; Wittenberg et al., 1967; Coletta et al., 1986), CO binding and dissociation rates in ferrous CCP are 100–1000-fold slower than that observed in the globins at neutral pH (Mims et al., 1983; Iizuka et al., 1985). Since protoporphyrin IX is the prosthetic group in both CCP and the globins, the differences in rates of ligand binding and dissociation can be ascribed to modification of the heme reactivity by the surrounding protein structure.

Recent structural and spectroscopic investigations have identified both proximal and distal features that distinguish peroxidases such as CCP and horseradish peroxidase (HRP) from the globins. In the peroxidases, a conserved arginine residue is present on the distal side of the heme (Finzel et al., 1984; Mazza & Welinder, 1980; Tien & Tu, 1987). In CCP, the distal arginine (Arg 48) increases the polarity of the distal side relative to the globins and participates in an extended hydrogen-bonding network with fixed water molecules and other residues in the active site. The imidazolate character of the proximal base is increased relative to the globins both in CCP (Smulevich et al., 1988a) and in HRP (La Mar & de Ropp, 1982; Teraoka et al., 1983). In CCP, this effect is

† This work was supported by NIH Grant HL13581 to T.G.T., NSF Grant DMB 85-11656-01 to J.K., and NATO Grant 86/0453 to G.S. M.A.M. was the recipient of a postdoctoral fellowship from Hemoglobin and Blood Protein Training Grant 5 T32 AM07233-11; J.M.M. was the recipient of NRSA Postdoctoral Fellowship PHS GM10292-02.

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[1] Abbreviations: CCP, cytochrome *c* peroxidase from bakers' yeast; CCP(MI), the cloned cytochrome *c* peroxidase; Fe^{II}(TPP), four-coordinate iron(II) *meso*-tetraphenylporphine; Fe^{II}(TpivPP), four-coordinate iron(II) picket-fence porphyrin, *meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-pivalamidophenyl)porphyrin; chelated mesoheme, mesoheme-*N*-[3-(1-imidazolyl)-propyl]amide; 1,2-Me_2Im, 1,2-dimethylimidazole; *N*-MeIm, 1-*N*-methylimidazole; Hb, hemoglobin; Mb, myoglobin; HRP, horseradish peroxidase; k_{off}, the first-order rate constant for CO dissociation.

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the result of a hydrogen-bonding interaction between the N^{δ} of the imidazole side chain of His 175 and the carboxylate oxygen of Asp 235 (Finzel et al., 1984; Smulevich et al., 1988a).

Extensive characterization of model heme compounds has shown that association and dissociation rates for a given ligand can be altered by modification of the proximal base and the distal environment of the heme. CO binding rates can be significantly decreased by increasing the steric hindrance on the distal side [cf. Traylor et al. (1985) and Collman et al. (1983a) and references cited therein] and by deprotonation of the proximal imidazole (Mincey & Traylor, 1979; Stanford et al., 1980). The rate of CO dissociation decreases with increasing proximal base strength for neutral ligands such as tetrahydrofuran, 1,2-dimethylimidazole, and 1-methylimidazole (White et al., 1979; Yu et al., 1983; Collman et al., 1983b) and increases with the exchange of thiolate for neutral imidazole (Chottard et al., 1984; Traylor et al., 1981a; Chang & Dolphin, 1976). Decreases in CO dissociation rates have also been reported to result from favorable dipole–dipole interactions between the bound CO and polar groups in the heme superstructure (Mispelter et al., 1983; Lavalette et al., 1984; Lexa et al., 1986a,b).

The recent cloning and expression of CCP in *Escherichia coli* (Kaput et al., 1982; Fishel et al., 1987) make it possible to induce specific alterations in the enzyme by site-directed mutagenesis. To evaluate the contribution of proximal and distal effects to the observed rates of CO binding and dissociation, single-residue substitutions have been introduced into the cloned CCP [designated CCP(MI)]. Previous work has shown that the rate of CO binding is relatively insensitive to changes which alter the polarity of the proximal and distal environment of the heme, while the rate of CO binding is increased approximately 50-fold by a pH-dependent change in the CO complex of the enzyme from acidic to alkaline form (Miller et al., 1989; Iizuka et al., 1985). These observations are consistent with recent evidence that the rate of CO binding in HRP is influenced by a steric barrier prior to the entry of CO into the heme pocket (Doster et al., 1987; Ascenzi et al., 1989).

To examine the effect of specific active-site residues on the rate of CO dissociation, the pH dependence of CO dissociation rates was examined in ferrous CCP(MI) and in the mutants Asp 235 → Asn; Arg 48 → Leu, Lys; and His 181 → Gly. The results presented here show that the slow rate of CO dissociation characteristic of the acidic form of CO–ferrous CCP(MI) is dependent upon the presence of distal Arg 48. Together with the recent resonance Raman characterization of the CO adducts of the mutants (Smulevich et al., 1988b), the data also suggest that the rate of CO dissociation in CCP is decreased relative to the globins through an effect of the proximal imidazole.

Materials and Methods

Materials. CO was from Matheson (99.9%). Argon was passed through an in-line oxygen trap (Scientific Products) prior to use. Other chemicals were of reagent grade or better.

Enzymes. The cloning and expression of the gene for yeast cytochrome *c* peroxidase in *E. coli*, as well as the detailed procedures for the introduction of single amino acid substitutions by site-directed mutagenesis, have been described (Fishel et al., 1987). The cloned CCP(MI) and the mutant apoenzymes examined here were isolated from 15–20-L cultures of *E. coli* and were converted to holoenzyme by previously described techniques (Fishel et al., 1987). The enzymes were twice crystallized by exhaustive dialysis against H_2O and were stored as crystalline suspensions in H_2O at −70 °C until use.

Absorption Spectra of the CO–Enzyme Complex. Crystals of the enzyme were dissolved in 0.1 M potassium phosphate (pH 6.0) to a final concentration of approximately 0.6 mM. A small aliquot (~20 μL) of concentrated enzyme solution was placed in the sidearm of a sealed cuvette, 2 mL of potassium phosphate (0.1 M at the desired pH) was placed in the cuvette chamber, and the sidearm of the cuvette was placed in an ice bath. Argon (H_2O saturated) was bubbled through the buffer solution and over the enzyme sample for 30 min, followed by admission of CO for 10 min. The cuvette was then sealed, the protein and buffer were mixed in the sidearm, and the sample was reduced by addition of a small excess of dithionite solution through an injection port. Spectra were recorded on a Perkin-Elmer Lambda 3b spectrophotometer with thermostated cuvette chambers at 23 °C. The pH of the sample was determined following the experiment. In some experiments, the pH of the sample was altered after the initial spectrum was recorded by introduction of a small aliquot (10–20 μL) of deoxygenated KOH or HCl through an injection port, with constant gentle mixing. The interconversion of acidic and alkaline forms was subsequently monitored. This technique was useful in confirming the location of isosbestic points between the acidic and alkaline forms.

Kinetics of CO Dissociation. CO dissociation rates were determined by the oxygen trap method of Wittenberg et al. (1967). Crystals of the enzyme were dissolved in 0.1 M potassium phosphate, pH 6.0, and brought to a final concentration of 0.4 mM, and 50 μL of the resulting solution was placed in a sealed serum vial at 4 °C. Argon (H_2O saturated) was passed over the sample for 30 min, followed by CO for 10 min. The sample was reduced by the addition of a slight excess of an anaerobically prepared dithionite solution (45 mM in H_2O) and was transferred to a gastight syringe. Reactions were initiated by injecting the sample into cuvette containing buffer (0.1 M potassium phosphate at the desired pH) equilibrated with atmospheric oxygen. To avoid photolysis of the CO complex by strong ambient light sources, the transfer was conducted in a darkened room. After an initial delay of 10 min to allow complete disproportionation of dithionite, spectra were recorded in the Soret region at varying time intervals; experiments were conducted for 2–6 half-lives. No increase in the rate of CO dissociation was observed when the sample was left in the incident beam of the spectrophotometer for the entire course of the experiment, due to the low energy of the incident beam. To obtain the end point of the reaction, the sample was placed near a standard fluorescent tube for 2 min, to completely photolyze the remaining CO complex. The pH of the sample was determined following the experiment. The disappearance of the CO complex was monitored at the wavelength where the maximum difference between initial and final scans was observed (see Figure 3 as an example). Rate constants for CO dissociation were obtained from linear least-squares analysis of plots of log $(A_0 - A_f)/(A_t - A_f)$ versus time over the course of the reaction, where A_0 is the initial absorbance, A_f is the absorbance at infinite time, and A_t is the absorbance at time t. In all cases, the rate of autoxidation of ferrous enzyme was at least 50-fold greater than the observed rate of CO dissociation, and no oxyferrous peroxidase intermediate was observed.

Results

Absorption Spectrum of the CO Derivative of Ferrous CCP(MI) as a Function of pH. The pH dependence of the absorption spectrum of CO–ferrous CCP(MI) in the visible

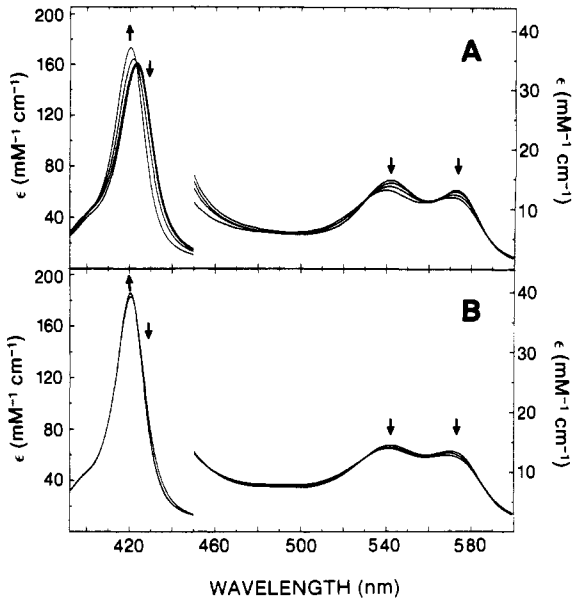

FIGURE 1: Dependence of the absorption spectrum of CO–ferrous CCP(MI) and CO–ferrous CCP(MI,K48) on pH. The spectra of enzyme solutions were recorded in potassium phosphate (0.1 M) at 23 °C as described under Materials and Methods. (A) CO–ferrous CCP(MI). Traces shown were recorded at pH 8.4, 7.8, 7.5, and 6.0. Direction of movement with increasing pH is indicated by the arrows. Isosbestic points were observed at 422.8, 529, 557, 565, and 584 nm. (B) CO–ferrous CCP(MI,K48). Traces were recorded at pH 5.5, 6.5, and 7.0. Direction of movement with increasing pH is indicated by the arrows. Isosbestic points were observed at 423, 529, and 584 nm.

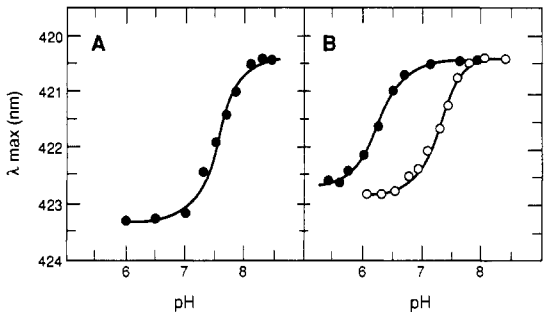

FIGURE 2: Shift in the position of the Soret maximum of the CO complex with pH. Soret maxima were determined from spectra of the respective enzyme solutions prepared in 0.1 M potassium phosphate at the indicated pH as described under Materials and Methods. (A) CO–ferrous CCP(MI). The solid lines represent the theoretical values for a cooperative, two-proton ionization, apparent pK_a = 7.6. (B) CO–ferrous CCP(MI,N235), open circles; CO–ferrous CCP(MI,L48), solid circles. The solid lines represent the theoretical values for a cooperative, two-proton ionization, apparent pK_a = 6.2 for CO–ferrous CCP(MI,L48) and apparent pK_a = 7.2 for CO–ferrous CCP(MI,-N235).

and Soret regions is shown in Figure 1. At pH 6.0, the Soret maximum was located at 423.4 nm ($\epsilon_{423.4}$ = 162 mM^{-1} cm^{-1}); α and β bands had maxima at 572 and 542 nm, respectively, in good agreement with the reported values for CO–ferrous CCP from bakers' yeast (Iizuka et al., 1985). Between pH 7.0 and 8.4, a blue shift in the Soret maximum to 420.4 nm was observed, accompanied by a small increase in molar absorptivity ($\epsilon_{420.4}$ = 170 mM^{-1} cm^{-1} at pH 8.4) and small changes in the visible region (Figure 1). The pH-dependent change in the absorption spectrum was reversible, and isosbestic points were observed (Figure 1). The change in the Soret maximum with pH fit well to the predicted titration curve for a cooperative, two-proton ionization, with an apparent pK_a = 7.6 (Figure 2A). The results indicate the existence

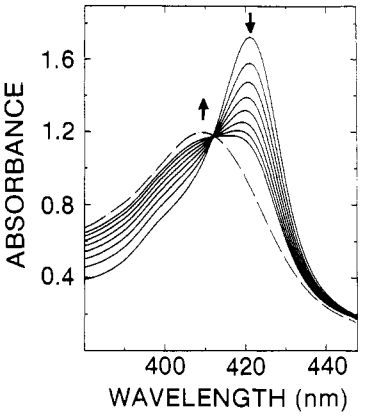

FIGURE 3: Conversion of CO–ferrous CCP(MI) to ferric CCP(MI) in the presence of oxygen. The experiment was conducted in 0.1 M potassium phosphate, pH 7.4, as described under Materials and Methods. Traces were recorded at 15-min intervals. The final trace (broken line) was recorded after the sample was exposed to light for several minutes. The direction of the change in absorbance with time is indicated by arrows.

of two distinct forms of the CO–ferrous CCP(MI) complex, which exist in an acid–base equilibrium involving cooperative ionization of two groups.

Distinct acidic and alkaline forms of CO–ferrous CCP from bakers' yeast were described by Iizuka et al. (1985); the conversion of the acidic to the alkaline form was monitored by a shift of the Soret maximum of the CO complex from 423.7 to 420.0 nm and a shift in the infrared C–O stretch frequency (ν_{C-O}) from 1922 to 1948 cm^{-1} at alkaline pH. The two forms were interconverted by a cooperative, two-proton ionization with an apparent pK_a = 7.5. In CCP(MI), the observed shift in the Soret maximum from 423.4 to 420.4 nm with increasing pH together with the shift in ν_{C-O} from 1922 to 1948 cm^{-1} (Smulevich et al., 1988a) indicates that the pH-dependent isomerization of CO–ferrous CCP(MI) is analogous to that observed in CCP from bakers' yeast. The effect of isomerization of the CO complex on the rate of CO binding is similar in both CCP and CCP(MI); the rate of CO recombination with ferrous CCP produced by photolysis of the acidic form of the CO complex is approximately 50-fold slower than that observed for recombination with the alkaline form (Iizuka et al., 1985; Miller et al., 1990). For the purposes of the present discussion, the distinct forms of the CO complex in CCP(MI) will be referred to as the acidic and alkaline forms, by analogy to the observations reported by Iizuka et al. (1985).

Kinetics of CO Dissociation in Ferrous CCP(MI). The CO dissociation rates for ferrous CCP(MI) were determined between pH 6.0 and 9.0. A typical experiment is presented in Figure 3; conversion from CO–ferrous CCP(MI) to ferric CCP(MI) is clearly shown. At each pH, a constant isosbestic point was observed in the Soret region at 411–414 nm during the course of the reaction, indicating that intermediates in the oxidation of the ferrous enzyme, such as the ligand-free ferrous enzyme and the ferrous–oxygen complex, do not accumulate at significant levels during the reaction. The rate of dissociation was determined by monitoring the conversion of CO–ferrous CCP(MI) to ferric CCP(MI) at the wavelength corresponding to the maximum difference between initial and final spectra (between 421 and 424 nm), as described under Materials and Methods. Variations in the isosbestic wavelength and the position of the maximum difference between CO–ferrous CCP(MI) and the ferric enzyme with pH were due to variability in the Soret region of both ferric (Smulevich et al., 1988b) and CO–ferrous CCP(MI) (Figure 1) with pH.

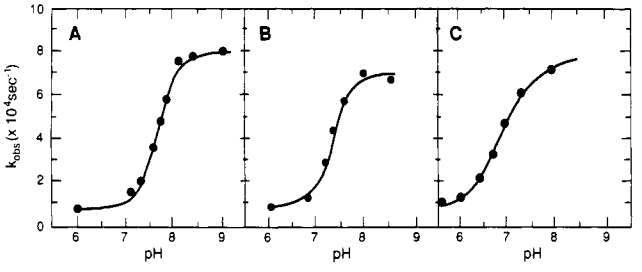

FIGURE 4: Dependence of CO dissociation rate (k_{obs}) on pH in CO–ferrous CCP(MI), CO–ferrous CCP(MI,N235), and CO–ferrous CCP(MI,G181). Rate constants were determined at the indicated pH as described under Materials and Methods. (A) CO–ferrous CCP(MI); (B) CO–ferrous CCP(MI,N235); (C) CO–ferrous CCP(MI,G181). The solid lines represent the fit of the data to the parameters reported in Table I, according to eqs 1–3, described under Results.

The reaction was characterized by a single-exponential process and gave linear plots of log $\Delta A_\infty/\Delta A_t$ versus time over the entire pH range examined here. At alkaline pH, a slow increase in the low-spin form of the ferric CCP from bakers' yeast has been reported (Dhaliwal & Erman, 1985). The constant isosbestic points observed for conversion of the CO complex to the ferric enzyme suggest that the spectrum of the ferric enzyme is not significantly altered by this process during the course of the reaction.

In CCP(MI), the CO dissociation rate increased from $k_{obs} = 0.7 \times 10^{-4}$ s^{-1} to $k_{obs} = 8.0 \times 10^{-4}$ s^{-1} as the pH was increased from 6.0 to 9.0. The increase in k_{obs} with increasing pH paralleled the blue shift in the Soret maximum of CO–ferrous CCP(MI), with a midpoint at approximately pH 7.7 (Figure 4A), suggesting that the rate of CO dissociation is increased by the conversion of the CO complex from acidic to alkaline form.

The dissociation of CO is a single-exponential process which is first order in the concentration of the CO complex, indicating that the equilibrium between acidic and alkaline forms is rapid relative to dissociation ($t_{1/2}$ = 160 and 14 min for acidic and alkaline forms, respectively, at 23 °C). Consistent with these observations, preliminary pH jump experiments show that interconversion of the acidic and alkaline CO complex requires approximately 5 min to complete at 23 °C ($t_{1/2} \sim$ 30–60 s).

A minimal mechanism for the pH dependence of CO dissociation in ferrous CCP(MI) is given by

$$\underset{\text{acidic}}{\text{CO–CCP(MI)}^{Fe(II)}:H_2} \overset{K_1}{\rightleftharpoons} \underset{\text{alkaline}}{\text{CO–CCP(MI)}^{Fe(II)}} + 2H^+$$

$$\downarrow k_a \qquad\qquad \downarrow k_b$$

$$\text{CCP(MI)}^{Fe(II)}:H_2 + \text{CO} \qquad \text{CCP(MI)}^{Fe(II)} + \text{CO}$$

The dependence of k_{obs} on pH can therefore be described by

$$k_{obs} = k_a \frac{[\text{CCP(MI)–CO}]_{acid}}{[\text{CCP(MI)–CO}]_T} + k_b \frac{[\text{CCP(MI)–CO}]_{alk}}{[\text{CCP(MI)–CO}]_T} \quad (1)$$

where k_a is the dissociation rate of the acidic form, k_b is the dissociation rate of the alkaline form, and $[\text{CCP(MI)–CO}]_{acid}/[\text{CCP(MI)–CO}]_T$ and $[\text{CCP(MI)–CO}]_{alk}/[\text{CCP(MI)–CO}]_T$ are the mole fractions of the CO complex in acidic and alkaline form, respectively. The equilibrium constant K_1 represents the product of the individual association constants for n ionizing groups. Assuming that conversion of the acidic to the alkaline form results from a cooperative ionization, the association constant (K_a) for each group is equal; for n ionizing groups, $K_1 = K_a^n$, and the concentrations of intermediate protonation states of the enzyme are assumed to be negligible. Under these conditions, the mole fractions of the acidic and alkaline forms at a given pH are given by eqs 2 and 3. The

$$\frac{[\text{CCP(MI)–CO}]_{acid}}{[\text{CCP(MI)–CO}]_T} = \frac{1}{(K_a/[H^+])^n + 1} \quad (2)$$

$$\frac{[\text{CCP(MI)–CO}]_{alk}}{[\text{CCP(MI)–CO}]_T} = \frac{1}{([H^+]/K_a)^n + 1} \quad (3)$$

values obtained for k_a, k_b, n, and apparent pK_a are reported in Table I; the fit of the data to the calculated values is shown in Figure 4A. The observed correlation of the increase in dissociation rate and the shift in the position of the Soret maximum with a two-proton ionization with similar apparent pK_a values strongly suggests that the increase in the rate of CO dissociation results from the conversion of the CO complex from the acidic to alkaline form.

Effect of the Local Heme Environment on CO Dissociation Rates. The influence of individual residues in the heme pocket of CCP(MI) on the rate of CO dissociation was further examined by characterization of mutants altered at specific residues around the heme binding pocket. The location of the altered residues is shown in Figure 5. The effect of each mutation on the conversion of the CO complex from acidic to alkaline form was monitored by observing pH-dependent changes of the Soret band, and CO dissociation rates were determined for acidic and alkaline forms of each mutant. In all of the mutants, the rate of oxidation of the ferrous enzyme was rapid relative to the rate of CO dissociation in the pH range examined (not shown). Consistent with this observation, a single isosbestic point in the region between 390 and 440 nm was observed for the entire course of the reaction over the pH range examined, indicating that, as with CCP(MI), the rate of oxidation of the ferrous enzymes is sufficiently rapid

Table I: Rate Constants for CO Dissociation from CCP(MI) and Mutant Proteins[a]

enzyme	k_a ($s^{-1} \times 10^4$)	k_b ($s^{-1} \times 10^4$)	n	pK_a
CCP(MI)	0.7 ± 0.1	8.0 ± 0.1	2	7.7
Asn 235	0.8 ± 0.1	7.0 ± 0.2	2	7.2
Gly 181	0.8 ± 0.1	7.9 ± 0.2	1	6.5
Lys 48	3.7 ± 0.2	6.4 ± 0.1	2	7.0
Leu 48	13.8 ± 0.5	20.0 ± 0.2	2	6.0

[a] The values presented represent rate constants derived from the results presented in Figures 4 and 6, fitted to eqs 1–3 under Results. k_a and k_b are the dissociation rate constants for the acidic and alkaline forms of the CO complex, respectively, and n is the number of protons ionized in the conversion of the CO complex from acidic to alkaline forms with the reported pK_a.

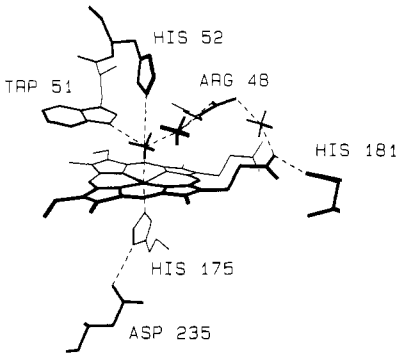

FIGURE 5: Structure of the heme binding pocket of ferric CCP(MI). Fixed solvent molecules are indicated by tetrahedra; hydrogen bonds are indicated by dashed lines. The data are from Wang et al. (1990).

to prevent the accumulation of intermediates. As with CO–ferrous CCP(MI), decay of the CO complex in the mutants was first order in concentration of the CO complex under all conditions and gave linear plots of log $\Delta A_\infty/\Delta A_t$ versus time.

Effect of the Asp 235 → Asn Mutant on CO Dissociation Rates. The effect of the interaction of Asp 235 with N^δ of the imidazole side chain of His 175 on CO dissociation rates was examined in CCP(MI,N235), where Asp 235 is replaced by Asn. The Soret maximum of CO–ferrous CCP(MI,N235) was located at 422.8 nm ($\epsilon_{422.8}$ = 167 mM^{-1}) at pH 6.0 and at 420.4 nm ($\epsilon_{420.4}$ = 178 mM^{-1}) at pH 7.8. Small changes in the visible region were also noted with increasing pH. As shown in Figure 2B, the pH dependence of the change in the position of the Soret maximum is consistent with a cooperative two-proton ionization with an apparent pK_a = 7.2, indicating that the conversion of the CO complex from acidic to alkaline form in this mutant is shifted downward relative to the parental CCP(MI) (apparent pK_a = 7.6). This conclusion is supported by the observation that at pH 7.0 two CO-dependent bands are observed in the infrared region, corresponding to the alkaline (ν_{C-O} = 1951 cm^{-1}) and acidic (ν_{C-O} = 1933 cm^{-1}) forms of the CO complex [Figure 6 in Smulevich et al. (1988b)].

The pH-dependent increase in CO dissociation rates in ferrous CCP(MI,N235) paralleled the blue shift in the Soret maximum, increasing from k_{obs} = 0.8 × 10^{-4} s^{-1} at pH 6.0 to k_{obs} = 7.0 × 10^{-4} s^{-1} at pH 7.8. When analyzed by eqs 1–3, the variation of k_{obs} with pH fit well to a cooperative two-proton ionization with an apparent pK_a = 7.2 (Figure 4B). Although the apparent pK_a for the interconversion of these forms in this mutant is shifted downward slightly relative to that of CO–ferrous CCP(MI), k_a and k_b are similar (Table I). The results demonstrate that the loss of imidazolate character of the proximal imidazole in ferrous CCP(MI,N235) (Smulevich et al., 1988a,b) does not significantly alter the rate of CO dissociation.

Effect of the His 181 → Gly Mutation on CO Dissociation Rates. Although His 181 is not located directly within the heme binding pocket, it interacts with the carboxylate of the heme pyrrole IV propionate and with residues in the heme pocket through extended hydrogen-bonding interactions (Figure 5). The effect of this residue on the conversion of the CO complex from the acidic to the alkaline form was examined in CCP(MI,G181), where His 181 is replaced by Gly. In CO–ferrous CCP(MI,G181), the Soret maximum at pH 5.5 was located at 420.8 nm ($\epsilon_{420.8}$ = 170 mM^{-1}) and shifted only slightly, to 420.4 nm ($\epsilon_{420.4}$ = 180 mM^{-1}), at pH 7.0. Since the shift in the Soret maximum with pH was small, the proton stoichiometry and apparent pK_a of the shift could not be accurately determined.

The rate of CO dissociation in CO–ferrous CCP(MI,G181) increased with increasing pH from k_{obs} = 0.8 × 10^{-4} s^{-1} at pH 5.5 to k_{obs} = 7.3 × 10^{-4} s^{-1} at pH 8.0 (Figure 4C). The relationship between k_{obs} and pH (Table I; Figure 4C) is consistent with a single-proton ionization, with an apparent pK_a = 6.5. The values for k_a and k_b obtained for the increase in CO dissociation rate with pH are similar to those obtained for CCP(MI). On this basis, we presume that the observed increase in CO dissociation rate is the result of the conversion of the CO complex from acidic to alkaline form. Further evidence favoring this assumption is provided by the observation that ν_{C-O} for the predominant form of the CO–ferrous CCP(MI,G181) complex is 1928 cm^{-1} at pH 6.0 and 1944 cm^{-1} at pH 7.0 (G. Smulevich, unpublished observation), suggesting a change in the environment of the CO ligand which is similar to that observed in ferrous CCP(MI) (Smulevich et al., 1988b). The involvement of a single-proton ionization in the conversion from acidic to alkaline form is in contrast to the results obtained for CO–ferrous CCP(MI) and the mutants reported here and implicates the imidazole of His 181 as one of the two residues that is deprotonated in this conversion. Evidence supporting the hypothesis that deprotonation of His 181 is accompanied by a significant conformational change in both ferric and ferrous CCP has been reported (Bosshard et al., 1984; Miller et al., 1988, 1990).

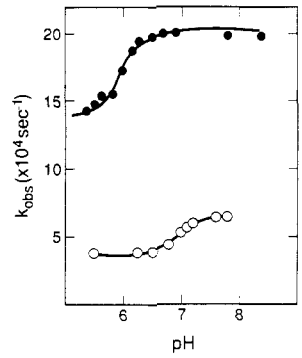

FIGURE 6: Dependence of CO dissociation rate (k_{obs}) on pH in mutants at distal Arg 48. Rate constants were determined at the indicated pH as described under Materials and Methods. CO–ferrous CCP(MI,L48), solid circles; CO–ferrous CCP(MI,K48), open circles. The solid lines indicate the fit of the data to the parameters reported in Table I, according to eqs 1–3, described under Results.

Although the pH-dependent changes in absorption spectra and CO dissociation rates are coincident in CO–ferrous CCP(MI) and CO–ferrous CCP(MI,N235), as described above, and in CO–ferrous CCP(MI,L48), described below, the pH dependence of the analogous changes in CO–ferrous CCP(MI,G181) is more complex. In this mutant, the pH-dependent changes in the Soret band occur as the pH is increased from 5.5 to 7.0, while the pH-dependent increase in k_{obs} is incomplete at pH 7.0. These observations suggest that for CO–ferrous CCP(MI,G181) the changes in the Soret and visible spectra are controlled by features that are distinct from those which cause the change in ν_{C-O} and the increase in CO dissociation rate. By the terminology employed in the other mutants we define the conversion from acidic to alkaline form in this mutant as the change that alters ν_{C-O} and results in an increased rate of CO dissociation.

Effect of Replacement of Distal Arg 48 on CO Dissociation. The effect of the polar distal Arg 48 of CCP on CO dissociation rates was examined by the characterization CO–ferrous CCP(MI,K48) and CO–ferrous CCP(MI,L48), in which Arg 48 is replaced with lysine and leucine, respectively. These two mutants were designed to permit separate evaluation of the role of charge and steric effects introduced by Arg 48 on CO dissociation rates.

At pH 5.0, the Soret maximum of CO–ferrous CCP(MI,K48) was located at 420.4 nm ($\epsilon_{420.4}$ = 184 mM^{-1}), the position characteristic of the alkaline CO complex of CO–ferrous CCP(MI). An increase in the molar absorptivity of the Soret maximum (to a final $\epsilon_{420.4}$ = 187 mM^{-1}) and a slight narrowing of this band were observed as the pH was increased from 5.5 to 7.0 (Figure 1B). The change in the shape and intensity of the Soret peak was reversible and gave constant isosbestic points, indicating the existence of two distinct forms of the CO complex (Figure 1B). The apparent pK_a for the intensity change of the Soret band could not be accurately determined since the change in molar absorptivity was small.

The rate of CO dissociation in CO–ferrous CCP(MI,K48) was constant between pH 5.5 and 6.5, and k_{obs} was approxi-

mately 5-fold greater than that observed in ferrous CCP(MI) at pH 6.0 (Figure 6). The rate of CO dissociation increased from $k_{obs} = 3.7 \times 10^{-4}$ s^{-1} at pH 6.5 to $k_{obs} = 6.4 \times 10^{-4}$ s^{-1} at pH 7.8. The fit of the data to eqs 1–3 suggests a two-proton cooperative ionization with an apparent pK_a = 7.0 (Figure 6). The values derived for k_a and k_b indicate that the CO dissociation rate of the acidic form is increased approximately 5-fold by the replacement of Arg 48 with lysine, while that of the alkaline form is affected to a lesser extent (Table I).

As noted above, the change in the absorption spectrum of CO–ferrous CCP(MI,K48) was complete at a lower pH than was the pH-dependent increase in CO dissociation rate and the pH-dependent change in ν_{C-O} observed previously (Smulevich et al., 1988b). The acidic and alkaline forms of CO–ferrous CCP(MI,K48) are defined here by the pH-dependent changes in the CO dissociation rate and ν_{C-O}.

In CO–ferrous CCP(MI,L48), the position of the Soret maximum was located at 422.6 nm ($\epsilon_{422.6} = 188$ mM^{-1}) at pH 5.5 and shifted to 420.4 nm ($\epsilon_{420.4} = 188$ mM^{-1}) at pH 6.8 The dependence of the position of the Soret maximum on pH (Figure 2B) is consistent with a cooperative two-proton ionization with an apparent pK_a = 6.2. As with the mutants described above, the observed changes in the Soret maximum were accompanied by small changes in the visible region and also exhibited isosbestic behavior. These observations suggest that the conversion of CO–ferrous CCP(MI,L48) from the acidic to the alkaline form is analogous to the conversion in CO–ferrous CCP(MI), although the apparent pK_a for the conversion is significantly lower. Moreover, the existence of distinct acidic and alkaline forms of CO–ferrous CCP(MI,-L48) is supported by the observed change in ν_{C-O} from 1941 cm^{-1} at pH 6.0 to 1950 cm^{-1} at pH 7.0 (Smulevich et al., 1988b).

The rate of CO dissociation for CO–ferrous CCP(MI,L48) increased from $k_{obs} = 13.8 \times 10^{-4}$ s^{-1} at pH 5.5 to $k_{obs} = 20.0 \times 10^{-4}$ s^{-1} at pH 7.0 (Figure 6). The increase in k_{obs} with pH is consistent with an increase in dissociation rate due to a cooperative two-proton ionization with an apparent pK_a = 6.0 (Table I). As with ferrous CCP(MI), the pH-dependent increase in k_{obs} parallels the blue shift of the Soret maximum of the CO complex and the increase in ν_{C-O} (Smulevich et al., 1988b), indicating that the observed increase in the CO dissociation rate results from the conversion of the CO complex from acidic to alkaline forms. The rate of CO dissociation from the acidic form of CO–ferrous CCP(MI,L48) (k_a) is increased approximately 20-fold relative to CCP(MI), while the rate of CO dissociation from the alkaline form (k_b) is increased only approximately 2.5-fold.

Discussion

Acidic–Alkaline Transition in CO–Ferrous CCP(MI) and Mutants. The results presented here, together with previous resonance Raman and infrared spectroscopic characterization of the CO complex of ferrous CCP(MI) (Smulevich et al., 1988b), demonstrate a pH-dependent isomerization of the CO complex between acidic and alkaline forms. The position of the Soret maximum as well as Fe–C (ν_{Fe-C}) and C–O (ν_{C-O}) stretching frequencies are altered by this isomerization. Similar results have been reported for CO–ferrous CCP from bakers' yeast (Iizuka et al., 1985; Smulevich et al., 1986). The rate of CO dissociation from the alkaline form of CO–ferrous CCP(MI) is approximately 10-fold greater than that observed in the acidic form. The data presented here also suggest that the change from the acidic to the alkaline form results from deprotonation of His 181 and a second residue that has not yet been identified.

The increase in the rate of CO dissociation with increasing pH is coincident with the conversion of the CO complex from the acidic to the alkaline form in each mutant, defined on the basis of pH-dependent changes in ν_{Fe-C} and ν_{C-O} (Smulevich et al., 1988b). Thus, the effect of mutations on the rate of CO dissociation can be evaluated for the acidic and alkaline forms of the CO complex. The results indicate that Arg 48 is important in determining the rate of CO dissociation in the acidic form of CO ferrous CCP(MI). When Arg 48 is replaced with Lys and Leu, the rate of CO dissociation for the acidic form is increased 5- and 20-fold, respectively. The rates of CO dissociation for the acidic forms of the Asp 235 → Asn and His 181 → Gly mutants are similar to that observed for the acidic form of CCP(MI), although these mutations lower the apparent pK_a [and in the case of CCP(MI,G181) alter the proton stoichiometry] for conversion of the CO complex from acidic to alkaline forms.

In the alkaline CO complex, the influence of Arg 48 on the rate of CO dissociation is apparently reduced. The rate of CO dissociation in the alkaline CO complex varied by only ~ 2.5-fold between CCP(MI) and CCP(MI,L48), indicating that the polarity of the residue at position 48 is less important in determining the rate of CO dissociation in the alkaline form of the CO complex than in the acidic form. Although the conversion of CCP(MI) from acidic to alkaline forms increases the rate of CO dissociation significantly, the dissociation rate for the alkaline form of CPP(MI) remains 12-fold slower than the rate of CO dissociation observed for myoglobin (Mims et al., 1983). Moreover, when Arg 48 is replaced with Leu, the rate of CO dissociation in the alkaline form is 5-fold slower than that observed in myoglobin. This indicates that the rate of CO dissociation in CCP is decreased relative to myoglobin by a second effect, which is not dependent on the interaction with distal Arg 48.

CO Dissociation Rate vs ν_{Fe-C} CO–Ferrous Peroxidases. It is possible to further clarify the influence of the local heme environment on the rate of CO dissociation in CCP(MI) by correlating the changes in the rate of CO dissociation among the alkaline and acidic forms of CCP(MI) and the mutants examined here with changes in the environment of the bound CO, as revealed by changes in ν_{Fe-C} and ν_{C-O} (Smulevich et al., 1988b). As a first step in elucidating the influence of the local heme environment on the rate of CO dissociation in CCP(MI), it is useful to examine the relationship between the strength of the Fe–C bond and the rate of CO dissociation (k_{off}). Uno et al. (1987) noted an inverse linear relationship between log k_{off} and ν_{Fe-C} for several heme proteins and concluded that the rate of CO dissociation is determined by Fe–C bond strength. When log k_{off} is plotted as function of ν_{Fe-C} for CCP(MI) and the mutants described here, together with a variety of heme proteins and model heme compounds, three distinct groupings are observed. First, the linear relationship between log k_{off} and ν_{Fe-C} noted by Uno et al. (1987) holds for cytochrome P-450, the globins, Fe^{II}(TPP) model heme compounds with the sterically hindered proximal base 1,2-dimethylimidazole (1,2-Me$_2$Im), and chelated mesoheme (points 1–12; Figure 7). Second, the alkaline CO complexes of ferrous CCP(MI) and the mutants, the acidic CO complexes of CCP(MI,K48) and CCP(MI,L48), and the model heme compound Fe(TpivPP) with the unhindered proximal base 1-methylimidazole (N-MeIm) (points 14–22, Figure 7) form a second line, which is parallel to the line that includes the globins. In these enzymes, k_{off} is approximately 10-fold slower than the globins for a given value of ν_{Fe-C}. Finally, the acidic forms of CCP(MI), HRP, and the mutants of CCP(MI) that

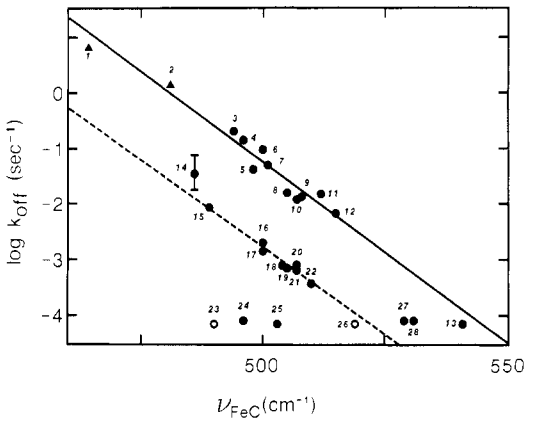

FIGURE 7: Experimental values for log k_{off} and ν_{Fe-C} in CO adducts of ferrous heme proteins and model heme compounds. (1) Cytochrome P-450; (2) cytochrome P-450+cam; (3) Fe^{II}(TPP)(1,2-Me_2Im); (4) Fe^{II}(TpivPP)(1,2-Me_2Im); (5) *Glycera dibranchiata* Hb; (6) *Chironomous thummi thummi* Hb; (7) chelated mesoheme; (8) leghemoglobin; (9) Hb (carp); (10) HbA (human); (11) Mb (sperm whale); (12) Mb (elephant); (13) HRP (acidic, form II). The solid line is described by the equation log k_{off} = −0.064 ν_{Fe-C} + 30.8; r^2 = −0.986. (14) The range shown is for Fe^{II} meso-, proto-, and diacetylporphyrins (*N*-MeIm), ν_{Fe-C} is for Fe^{II}(TPP) (*N*-MeIm); (15) Fe^{II}(TpivPP) (*N*-MeIm); (16) CCP(MI,L48), alkaline; (17) CCP(MI,L48), acidic; (18) CCP(MI,G181), alkaline; (19) CCP(MI,N235), alkaline; (20) CCP(MI), alkaline; (21) CCP(MI,K48), alkaline; (22) CCP(MI,K48) acidic. The dashed line is described by log k_{off} = −0.063ν_{Fe-C} + 28.7; r^2 = −0.984. (23) HRP-C, acidic, form I; (24) CCP(MI,G181), acidic, form I; (25) CCP(MI), acidic, form I; (26) HRP-C, acidic, form I; (27) CCP(MI,G181), acidic, form II; (28) CCP(MI,N235), acidic, form II. Data for points 1, 2, 5, 6, and 8–12 are from Mims et al. (1983), Li and Spiro (1988), and Uno et al. (1987) and references cited therein. Data for points 3, 4, and 15 are from Collman et al. (1983a–c); for point 7 from Traylor et al. (1979) and Mitchell et al. (1985); and for point 14 from Traylor et al. (1981) and Kerr et al. (1983). Data for points 13, 23, and 26 are from Evangelista-Kirkup et al. (1986), Uno et al. (1987), and Wittenberg et al. (1967); and for points 16–22, 24, 25, 27 and 28 are from Smulevich et al. (1988b) and the present work. Circles indicate imidazole or imidazole derivative as proximal base; triangles indicate thiolate as proximal base. The open symbols indicate conflicting values reported for acidic HRP-C (Evangelista-Kirkup et al., 1986; Uno et al., 1987).

are not altered at Arg 48 have similar rates of CO dissociation (~0.7 × 10^{-4} s^{-1}), although ν_{Fe-C} varies from 495 to 541 cm^{-1} (points 13, 23–28; Figure 7).

To interpret the different relationships between log k_{off} and ν_{Fe-C} for the three groupings shown in Figure 7, it is important to identify the interactions between the bound CO and the local heme environment that influence Fe–C bond strength. These can be defined by considering the variation in ν_{Fe-C} with respect to ν_{C-O} in relation to other heme proteins and model compounds (Li & Spiro, 1988). Since CO is a weak σ donor and a strong π acceptor, ν_{Fe-C} in heme–CO complexes is influenced by both changes in Fe $d_\pi \rightarrow CO_{\pi^*}$ backdonation and the σ donor strength of the proximal ligand. Backdonation results from the transfer of electrons from Fe d_π to the CO 2π antibonding orbital. The effect of increasing backdonation is therefore to strengthen the Fe–C bond and to weaken the C–O bond. The strength of σ bonding between Fe and CO is influenced by competition between CO and the proximal ligand for Fe d_{z^2} orbitals. Increasing the base strength of the proximal ligand weakens the σ bond with CO. The 5σ bond of CO is slightly antibonding (DeKock et al., 1971); therefore, increased σ donor strength is also expected to weaken the C–O bond.

For the heme proteins and model heme compounds that fall on the two lines in Figure 7 and have a neutral imidazole (or imidazole derivative) as the proximal ligand (points 3–22), an inverse linear relationship between ν_{Fe-C} and ν_{C-O} has been shown to exist (Li & Spiro, 1988; Smulevich et al., 1988b). This suggests that the increase in ν_{Fe-C} for points along the two lines is primarily the result of increased backdonation. Changes in backdonation have been shown to result from interaction between CO and polar groups in the local heme environment, changes in the cis electron donating characteristics of the heme substituents, and changes in the steric constraints on the bound CO (Paul & Rosen, 1984; Paul et al., 1985; Yu et al., 1983; Li & Spiro, 1988). It is important to note, however, that of these three influences on backdonation only changes in local polarity have been reported to influence the rate of CO dissociation (Lexa et al., 1986b; see below). Both model heme compounds (Traylor et al., 1981b) and heme proteins reconstituted with synthetic hemes (Iizuka et al., 1982) show little variation in the rate of CO dissociation with changes in pK_3 of the porphyrin. Moreover, although Yu et al. (1983) have shown that ν_{Fe-C} and ν_{C-O} are significantly altered as a result of steric hindrance to linear CO binding, no effect of steric hindrance on the rate of CO dissociation has been observed (Ward et al., 1981; Traylor et al., 1985).

These observations suggest that the increasing values of ν_{Fe-C} along the two lines in Figure 7 result primarily from the influence of polar residues in the distal heme pocket on backdonation. Dipole–dipole/hydrogen-bonding interactions between CO and favorably oriented polar groups are expected to increase backdonation by permitting greater electron transfer from Fe to CO (Paul & Rosen, 1984). The trend toward increasing ν_{Fe-C} and decreasing k_{off} for the points corresponding to model compounds and heme proteins with proximal imidazole ligands along the two lines in Figure 7 is consistent with a dominant influence of local polarity on ν_{Fe-C}. The lowest values of ν_{Fe-C} are observed in simple hemes with no superstructure (points 3 and 14, Figure 7), while the highest values of ν_{Fe-C} are observed in acidic forms of HRP and CCP(MI,N235), which have a distal Arg residue in the heme pocket (points 13, 27 and 28, Figure 7). Evidence that CO forms a hydrogen bond with a residue or bound water molecule in the active site of these enzymes has been reported (Smulevich et al., 1988b; Smith et al., 1983; Hayashi et al., 1976; Lee et al., 1988).

Influence of the Proximal Imidazole Ligand on the Rate of CO Dissociation. The slopes of the two lines shown in Figure 7 are essentially identical, suggesting that the effect of increased polarity on ν_{Fe-C} and the rate of CO dissociation is similar for points that fall along the two lines. The points corresponding to the globins fall along one line, while the points corresponding to the alkaline forms of CCP(MI) and the mutants fall along another. This suggests that the difference in the rate of CO dissociation between the globins and the alkaline peroxidases for a given value of ν_{Fe-C} results from an interaction that does not increase the strength of the Fe–C bond. A possible basis for the difference in the rate of CO dissociation for a given value of ν_{Fe-C} between the two lines in Figure 7 is suggested by the model compounds that fall on the respective lines. Fe^{II}(TPP) derivatives with the hindered proximal base 1,2-Me_2Im fall on the line including the globins (points 3 and 4, Figure 7), while a Fe^{II}(TPP) derivative with the unhindered base *N*-MeIm (point 15, Figure 7) falls on the line including the alkaline CO complexes of CCP(MI) and the mutants.

The substitution of 1,2-Me_2Im for *N*-MeIm as proximal ligand in Fe^{II}(TPP) hemes decreases the value of ν_{Fe-C} (compare points 4 and 15 in Figure 7), consistent with the predicted effect of decreased competition of the weaker 1,2-Me_2Im base for Fe d_{z^2} orbitals (Kerr et al., 1983). Despite the apparent

increase in Fe–C bond strength, this substitution also increases the rate of CO dissociation (White et al., 1979; Traylor, 1981; Collman et al., 1983b). The increased rate of CO dissociation when 1,2-Me_2Im is substituted for *N*-MeIm indicates that although the strength of the Fe–CO bond is increased, the free energy of the ground-state CO complex is increased relative to that of the transition state by the 1,2-Me_2Im proximal ligand. This result has been rationalized as a loss in π forward donation of electron density from imidazole to the iron atom, due to the approximately 0.1-Å increase in the Fe–Im bond length when 1,2-Me_2Im is substituted for *N*-MeIm (Jameson et al., 1980; Kerr et al., 1983). The fact that model compounds with *N*-MeIm as the proximal base fall on the line including the alkaline CO complexes of CCP(MI) and the mutants while model compounds with 1,2-Me_2Im as the proximal base fall on the line including the globins (Figure 7) may indicate that the rate of CO dissociation in the globins is increased relative to the alkaline peroxidases by a similar increase in the length of the Fe–Im bond as a result of "tension" on the proximal base imposed by the protein structure.

It is interesting to note that although k_{off} decreases with increasing proximal base strength in the series tetrahydrofuran < 1,2-Me_2Im < *N*-MeIm (Kerr et al., 1983), increasing the strength of the proximal base by substitution of thiolate for 1,2-Me_2Im increases the rate of CO dissociation (Chang & Dolphin, 1976; Traylor et al., 1981a; Chottard et al., 1984). The points corresponding to cytochrome P-450, which has a thiolate proximal ligand (points 1 and 2, Figure 7), fall along the same line as the globins, indicating that the increase in σ donor strength of the thiolate ligand relative to 1,2-Me_2Im is reflected by an increase in the rate of CO dissociation. Thus, the substitution of the stronger thiolate base for 1,2-Me_2Im does not provide the additional stabilization of the CO adduct observed when 1,2-Me_2Im is replaced by the unhindered *N*-MeIm ligand.

Influence of Arg 48 on the Rate of CO Dissociation in the Acidic CO Complexes of CCP(MI). The rate of CO dissociation in the acidic CO complex is strongly influenced by the identity of the residue at position 48, as shown by the 20-fold increase in the rate of CO dissociation for the acidic CO complex when Arg 48 is replaced by Leu in CCP(MI,L48). The increased rate of CO dissociation in CCP(MI,L48) does not result from a large structural change in the enzyme, since the structural changes in the ferric enzyme introduced by the Arg 48 → Leu mutation are small and confined to the immediate vicinity of the substitution (Wang, 1988). The influence of Arg 48 on the rate of CO dissociation is therefore most likely the result of the interaction between the charged guanidinium side chain of Arg 48 and the CO adduct. The potential influence of fixed polar groups on the rate of CO dissociation is suggested by characterization of the so-called "basket-handle" hemes (Lexa et al., 1984, 1986a,b; Lavalette et al., 1984).

The interaction between Arg 48 and CO in the acidic CO complex has been described by examining the changes in ν_{Fe-C} and ν_{C-O} accompanying the conversion of the alkaline CO complex to the acidic CO complex for CCP from bakers' yeast, CCP(MI), and mutants of CCP(MI) with respect to other heme proteins (Smulevich et al., 1986, 1988b; Dasgupta et al., 1989). Two conformations of the acidic CO complex have been described for CCP from bakers' yeast, designated form I ($\nu_{Fe-C} \sim 495$ cm^{-1}) and form II ($\nu_{Fe-C} \sim 530$ cm^{-1}). In form II, a significant tilt of the bound ligand is suggested by the observation of a moderately strong $\delta_{FeCO} = 580$ cm^{-1}. Two analogous forms have also been described for the CO complex of HRP at pH 7.0 (Evangelista-Kirkup et al., 1986; Uno et al., 1987).

Although the strength of the Fe–C bond is significantly different between form I and form II, the rate of CO dissociation is the same for both conformations. This is shown explicitly by the similar rates of CO dissociation observed for CCP(MI) and CCP(MI,N235), which exist entirely as form I and form II, respectively (Smulevich et al., 1988b). The rate of CO dissociation is also unaffected in the acidic CO complex of CCP(MI,G181), which exists as a equilibrium mixture of form I and form II (G. Smulevich, unpublished observation), implying that the rate of CO dissociation is similar for both conformations in this mutant as well. Therefore, the apparent independence of the rate of CO dissociation on the frequency of ν_{Fe-C} in the acidic CO complexes of the peroxidases (Figure 7) results from the equivalent rates of CO dissociation between the high ν_{Fe-C} (form II) and low ν_{Fe-C} (form I) conformations.[2]

The environment of the bound CO is quite different between these two conformations of the CO complex. In form II, the value of ν_{Fe-C} is increased and the value of ν_{C-O} is decreased relative to the alkaline CO complex, suggesting that a hydrogen bond is formed between CO and a distal residue or bound water molecule (Smulevich et al., 1988b). Evidence that a hydrogen bond is formed between CO and a bound water molecule located between Arg 48 and the oxygen atom of CO has been obtained from the crystal structure of the CO complex of CCP from bakers' yeast (Edwards & Poulos, 1990). Accordingly, points corresponding to form II [CCP(MI,N235) and CCP(MI,G181); points 27 and 28] fall near the line corresponding to the alkaline forms of CCP(MI) and the mutants in Figure 7; i.e., the rate of CO dissociation decreases as the strength of the Fe–C bond increases when the alkaline form of the complex (points 18 and 19, Figure 7) is converted to form II. In form I, the values of ν_{Fe-C} and ν_{C-O} are decreased relative to the alkaline CO complex. This is inconsistent with an increased interaction between CO and Arg 48 with respect to the alkaline CO complex. Apparently, the tilting of the Fe–CO unit observed in form II is required for formation of a hydrogen bond between CO and the water molecule located between CO and Arg 48. The observed decrease in both ν_{Fe-C} and ν_{C-O} in form I relative to the alkaline CO complex is the predicted result of deprotonation of the proximal imidazole (Evangelista-Kirkup et al., 1986) and is presumed to reflect transfer of the proton from N^{δ} of His 175 to Asp 235 (Smulevich et al., 1988b). The points corresponding to form I [CCP(MI) and CCP(MI,G181); points 24 and 25] fall below the line corresponding to the alkaline forms of CCP(MI) and the mutants; i.e., the rate is lower than that predicted by the frequency of ν_{Fe-C} in form I.

Since the decreased rate of CO dissociation in form I with respect to the alkaline form of the CO complex does not result from hydrogen bonding between Arg 48 and CO, an alternative possibility is that the decrease in the rate of CO dissociation results from the increased imidazolate character of the proximal ligand in form I relative to the alkaline CO complex. Deprotonation of the proximal imidazole ligand in simple heme compounds reduces the CO affinity (Mincey & Traylor, 1979) primarily by a decrease in the rate of CO binding (Stanford et al., 1980), however, and predicts little change in the rate of CO dissociation as a result of depro-

[2] On the basis of the similarity between the two conformations of the CO complex observed in CCP and HRP and the similar rates of CO dissociation in the two enzymes, the rates of CO dissociation in both forms of the CO complex of ferrous HRP were assumed to be equal in Figure 7, although this has not been explicitly demonstrated.

tonation of the proximal imidazole. Therefore, it is unlikely that the 10-fold decrease in k_{off} between the alkaline form of the CO complex and form I results from deprotonation of the imidazole ligand alone.

While a strong interaction between CO and Arg 48 is not required to produce the slow rate of CO dissociation observed in the acidic CO complex of CCP(MI), the data suggest that the rate of CO dissociation is influenced by the charge and orientation of the residue at position 48. In the acidic CO complex, the importance of the orientation of the positively charged residue at position 48 is indicated by the 5-fold increase in the rate of CO dissociation when Arg 48 is replaced with Lys (Table I). The difference between CCP(MI,K48) and CCP(MI) may result from anchoring of the guanidinium side chain of Arg 48 in a specific orientation through hydrogen bonds with fixed water molecules to the propionate of heme pyrrole IV and active site (Figure 5); the *n*-butylamino side chain of Lys in CCP(MI,K48) cannot be anchored in this fashion. In the alkaline CO complex, the increased rate of CO dissociation relative to the acidic form is accompanied by a decreased influence of Arg 48 on the rate of CO dissociation. This suggests that the orientation of Arg 48 with respect to the CO adduct is altered by a conformational change in the distal region of the enzyme. Evidence for a significant change in the distal heme environment of ligand-free ferrous and ferric CCP has been reported (Miller et al., 1990; Smulevich et al., 1988a; Yonetani & Anni, 1987; Iizuka et al., 1985; Bosshard et al., 1984).

Since the active-site structures of HRP and CCP are quite similar (Thanabal et al., 1988), similar local polarity effects may influence the rate of CO dissociation in HRP as well. It is interesting to note that the rate of CO dissociation is increased 30-fold, without effect on the rate of CO binding, in HRP reconstituted with protoheme dimethyl ester (Coletta et al., 1986). The increase in the rate of CO dissociation may indicate that the orientation of the distal Arg is perturbed as a result of the loss of an anchoring interaction with the heme propionates.

Since the decrease in the rate of CO dissociation in CCP(MI) does not correlate with increased imidazolate character of the proximal ligand, it seems likely that the rate of CO dissociation is influenced by interaction of the positively charged guanidinium side chain of Arg 48 with the heme. Extensive characterization of the basket-handle heme compounds has shown that fixed polar groups increase the affinity of tetracoordinate ferrous hemes for imidazole by promoting the transfer of electron density from the proximal ligand to the iron atom or to the porphyrin via the iron atom (Lexa et al., 1984, 1986a,b; Lavalette et al., 1984). The rate of CO dissociation in these compounds apparently decreases in parallel with increased affinity for the proximal imidazole ligand (Lexa et al., 1986b). The affinity of these model ferrous hemes for the proximal ligand and CO is dependent on the number of polar groups in the heme superstructure and their orientation with respect to the heme (Lavalette et al., 1984; Lexa et al., 1984, 1986a,b). Since the influence of Arg 48 on the rate of CO dissociation is similarly dependent on the charge and orientation of the guanidinium side chain, this residue may promote transfer of electron density from the proximal ligand to the iron atom or to the porphyrin. This would explain the partial or complete transfer of the proton from N^{δ} of His 175 to the carboxylate of Asp 235 in form I; in the absence of such an effect, the pK_a for imidazole is expected to be significantly higher than the pK_a for the carboxylate of Asp 235. The observation that the imidazole of His 175 is protonated as the pH is increased is also consistent with the prediction that the influence of Arg 48 is decreased in the alkaline CO complex.

The rate of CO dissociation in the acidic form of CCP(MI) is 100-fold slower than that observed in myoglobin despite a net decrease in ν_{Fe-C}, indicating that the strength of the Fe–C bond does not present an important kinetic barrier to CO dissociation in CCP(MI). Moreover, the rate of CO dissociation does not decrease when ν_{Fe-C} is increased by changes in the proximal ligand, changes in the electron-donating character of the porphyrin side chains, or steric effects (Ward et al., 1981; Iizuka et al., 1982; Collman et al., 1983b). This provides further evidence that the strength of the Fe–C bond does not have a strong influence on the rate of CO dissociation. The inverse linear relationship between ν_{Fe-C} and log k_{off} for the heme proteins and model heme compounds in Figure 7 must therefore be viewed as coincidental, in the sense that it does not reflect the influence of Fe–C bond strength on the rate of CO dissociation. The relationship apparently reflects similar effects of distal polar groups on CO dissociation and ν_{Fe-C} when CO is favorably oriented for interaction with the polar groups. Deviations from the linear relationship arise when (1) the orientation of CO prevents a strong interaction with distal polar groups, (2) the influence of the proximal base on the overall stability of the CO complex is not reflected by its σ donor strength alone (Kerr et al., 1983), (3) significant changes in the cis electron donating character of the heme are introduced by modifications of the porphyrin side chains (Iizuka et al., 1982), and (4) ν_{Fe-C} is altered by steric restrictions to linear binding of CO (Yu et al., 1983; Ward et al., 1981).

Separate Control of CO Binding and Dissociation Rates in CCP(MI). The present results indicate that the rate of CO dissociation in the acidic form of CCP(MI) is decreased relative to myoglobin by the introduction of an Arg residue into the distal heme pocket. While the increased polarity of the heme pocket can account for the slower rates of CO dissociation observed in CCP(MI) and HRP, the much slower rate of CO binding in ferrous CCP(MI) relative to the globins cannot be correlated with the influence of Arg 48. The slow rate of CO binding characteristic of the acidic peroxidases is not increased by substitution of Leu for Arg 48 in CCP(MI) (Miller et al., 1990). Moreover, although the pH-dependent increase in the rate of CO binding is coincident with the increase in the rate of CO dissociation in CCP(MI), the differential effect of pH on CO binding and dissociation rates in CCP(MI,K48) and CCP(MI,G181) suggests that different structural features control the rates of the two processes. Several conditions have also been described that increase the rate of CO dissociation in HRP without effect on the rate of CO binding (Coletta et al., 1986; Ascenzi et al., 1989). These results are consistent with the existence of a strong conformational barrier to CO binding in ferrous peroxidases (Miller et al., 1990; Ascenzi et al., 1989; Doster et al., 1987).

ACKNOWLEDGMENTS

We thank Dr. Jimin Wang for making this data available to us prior to publication and Drs. Douglas Taube and Jay Davies for helpful discussions.

Registry No. CO, 630-08-0; CCP, 9029-53-2; Arg, 74-79-3; His, 71-00-1; Asp, 56-84-8.

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Biochemical Properties of Site-Directed Mutants of Human Epidermal Growth Factor: Importance of Solvent-Exposed Hydrophobic Residues of the Amino-Terminal Domain in Receptor Binding†

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Received February 28, 1990; Revised Manuscript Received July 17, 1990

ABSTRACT: Eight analogues of human epidermal growth factor (hEGF) having specific amino acid substitutions in the β-sheet structure (residues 19-31) of the amino-terminal domain were generated by site-directed mutagenesis. Affinity of the epidermal growth factor (EGF) receptor for each of these mutant hEGF analogues was measured by both radioreceptor competition binding and receptor tyrosine kinase stimulation assays. The relative binding affinities obtained by these two methods were generally in agreement for each hEGF species. The results indicate that hydrophobic residues on the exposed surface of the β-sheet structure of the amino-terminal domain of hEGF have an important role in the formation of the active EGF-receptor complex. The substitution of hydrophobic amino acid residues, Val-19 → Gly, Met-21 → Thr, Ile-23 → Thr, and Leu-26 → Gly, resulted in decreased binding affinity, with the most severe reductions observed with the last two mutants. The mutations Ala-25 → Val and Lys-28 → Arg introduced amino acid residues resulting in slightly increased receptor binding affinity. Similar to previous results with acidic residues in this region [Engler, D. A., Matsunami, R. K., Campion, S. R., Stringer, C. D., Stevens, A., & Niyogi, S. K. (1988) *J. Biol. Chem. 263*, 12384-12390], removal of the positive charge in the Lys-28 → Leu substitution had almost no effect on binding affinity, indicating the lack of any absolute requirement for ionic interactions at this site. Substitution of Tyr-22, which resulted in decreased receptor binding affinity, provides further indication of the importance of aromatic residues in this region of the molecule, as found earlier with Tyr-29 (cf. reference above). These results provide direct evidence suggesting the importance of aliphatic and aromatic amino acid side chains, of the amino-terminal β-sheet of the EGF molecule, in receptor-ligand interactions.

Recent studies using site-directed mutagenesis have attempted to determine those amino acid residues of EGF[1] and the related transforming growth factor (TGF-α) which are directly involved in intermolecular interactions with the EGF receptor. For the most part, these studies have concentrated on altering amino acid residues in the carboxy-terminal domain (residues 32-53) of the growth factor peptide. The substitution of the highly conserved hydrophobic residue Leu-47 (Leu-48 in TGF-α) near the carboxy-terminal end of the peptide resulted in a marked reduction in the affinity of the receptor for hEGF (Engler et al., 1988; Dudgeon et al., 1989),[2] mEGF (Ray et al., 1988; Moy et al., 1989), and hTGF-α (Lazar et al., 1988). Other amino acids which have been examined by mutagenesis include the highly conserved Tyr-38, Arg-42, and Asp-47 residues of hTGF-α (Lazar et al., 1989; Defeo-Jones et al., 1988, 1989) as well as Tyr-37 and Arg-41 of hEGF (Engler et al., 1990). The results of those studies have demonstrated a nearly absolute requirement for Arg-41 (Arg-42

† Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract DE-AC05-840R21400 with Martin Marietta Energy Systems, Inc. The project described was supported by Grant CA 50735 from the National Cancer Institute, U.S. Public Health Service. S.R.C. was supported partly by National Cancer Institute Postdoctoral Training Grant CA 09336; R.K.M. and D.A.E. were supported by National Cancer Institute Predoctoral Training Grant CA 09104 to the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences.

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[1] Abbreviations: EGF, epidermal growth factor; hEGF, human epidermal growth factor; mEGF, mouse epidermal growth factor; TGF-α, transforming growth factor α; hTGF-α, human transforming growth factor α; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; 2D NMR, two-dimensional nuclear magnetic resonance spectroscopy; 1D NMR, one-dimensional nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography.

[2] R. K. Matsunami, G. T. Montelione, N. Greenfield, M. L. Yette, A. Stevens, S. K. Niyogi, Mutational Analysis of Leucine 47 in Human Epidermal Growth Factor (manuscript in preparation).

0006-2960/90/0429-9988$02.50/0