Site-Directed Mutagenesis of Yeast Cytochrome *c* Peroxidase Shows Histidine 181 Is Not Required for Oxidation of Ferrocytochrome *c*†

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ABSTRACT: The long-distance electron transfer observed in the complex formed between ferrocytochrome *c* and compound I, the peroxide-oxidized form of cytochrome *c* peroxidase (CCP), has been proposed to occur through the participation of His 181 of CCP and Phe 87 of yeast iso-1 cytochrome *c* [Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 10322–10330]. We have examined the role of His 181 of CCP in this process through characterization of a mutant CCP in which His 181 has been replaced by glycine through site-directed mutagenesis. Data from single-crystal X-ray diffraction studies, as well as the visible spectra of the mutant CCP and its 2-equiv oxidation product, compound I, show that at pH 6.0 the protein is not dramatically altered by the His 181 → Gly mutation. The rate of peroxide-dependent oxidation of ferrocytochrome *c* by the mutant CCP is reduced only 2-fold relative to that of the parental CCP, under steady-state conditions. Transient kinetic measurements of the intracomplex electron transfer rate from ferrous cytochrome *c* to compound I indicate that the rate of electron transfer within the transiently formed complex at high ionic strength (μ = 114 mM, pH = 6) is also reduced by approximately 2-fold in the mutant CCP protein. The relatively minor effect of the loss of the imidazole side chain at position 181 on the kinetics of electron transfer in the CCP–cytochrome *c* complex precludes an obligatory participation of His 181 in electron transfer from ferrous cytochrome *c* to compound I. The stability and spectral properties of the mutant protein are significantly altered at pH >6.0, however, supporting a role for His 181 in the structural stability of CCP.

Much recent work has been dedicated to elucidating the mechanism of intermolecular electron transfer between proteins, both in physiological systems and in physiological redox complexes [cf. Marcus and Sutin (1985), Mayo et al. (1986), Hoffman and Ratner (1987), Kuki and Wolynes (1987), Cusanovich et al. (1988), and McLendon (1988)]. One well-characterized system for the study of this phenomenon is the electron transfer complex formed by yeast cytochrome *c* peroxidase (CCP)[1] and cytochrome *c*, its physiological redox partner. CCP catalyzes the peroxide-dependent oxidation of ferrous cytochrome *c* through one-electron transfers in 1:1 complexes (Leonard & Yonetani, 1974; Erman & Vitello, 1980). In the absence of cytochrome *c* or other electron donors, reaction of CCP with peroxide results in the formation of an unusually stable oxidized intermediate, compound I (or compound ES), which has an oxyferryl (Fe^{4+}) heme, and a protein-based radical(s) located at an undetermined residue(s) (Yonetani et al., 1966; Hoffman et al., 1979; Edwards et al., 1987; Hori & Yonetani, 1985; Goodin et al., 1986; Fishel et al., 1987). The stability of compound I ($t_{1/2}$ for spontaneous reduction is ~6.6 h at 25 °C, pH 6; Erman & Yonetani, 1975) has facilitated the development of techniques for direct measurement of the rate of electron transfer from ferrocytochrome *c* to compound I (Hazzard et al., 1987, 1988a,b,c).

A hypothetical structure for the CCP–cytochrome *c* complex has been proposed (Poulos & Kraut, 1980; Poulos & Finzel, 1984) on the basis of optimal pairing of complementary charged groups at the interface of CCP and tuna cytochrome *c*. Support for the existence of the favorable ionic interactions predicted by this model has been obtained from chemical modification and cross-linking studies (Bisson & Capaldi, 1981; Waldmeyer et al., 1982; Waldmeyer & Bosshard, 1985; Bechtold & Bosshard, 1985) and from NMR studies of the resulting cross-linked complex (Moench et al., 1987). The limitations inherent in this static view of the CCP–cytochrome *c* electron transfer complex are indicated, however, by recent work which suggests that more favorable orientations for electron transfer can be attained by weakening of the electrostatic interactions between cytochrome *c* and compound I through increased ionic strength or modification of charged residues at the molecular interface (Hazzard et al., 1988a,b,c). Moreover, Brownian dynamic computer simulations suggest that multiple orientations may occur during collisions between

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[1] Abbreviations: CCP, bakers' yeast cytochrome *c* peroxidase; CCP(MI), cytochrome *c* peroxidase expressed in *E. coli*; NMR, nuclear magnetic resonance spectroscopy; ZnCCP, zinc-substituted cytochrome *c* peroxidase; ^{3}ZnCCP, ZnCCP triplet state; $(ZnCCP)^+$, ZnCCP π-cation radical; BSA, bovine serum albumin; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; MES, 4-morpholineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TB medium, 10.8 g of tryptone, 21.6 g of yeast extract, 3.6 g of glycerol, 10 g of NaCl, 2.3 g of KH_2PO_4, and 12.5 g of K_2HPO_4 per liter of H_2O; $E_{m,7}$, midpoint potential at pH 7; 5-DRf, oxidized 5-deazariboflavin; 5-DRfH•, 5-deazariboflavin semiquinone; LfH_2, fully reduced lumiflavin; LfH•, lumiflavin semiquinone; CCP(IV,R•), peroxide-oxidized CCP, i.e., compound I.

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cytochrome *c* and CCP (Northrup et al., 1988).

The model for the CCP–cytochrome *c* complex also predicts that the hemes are parallel in the complex and separated by a distance of approximately 18 Å. The predicted separation between the hemes is supported by fluorescence energy transfer (Leonard & Yonetani, 1974; Kornblatt & English, 1986; Koloczek et al., 1987) and NMR (Gupta & Yonetani, 1973) studies. The rapid intermolecular electron transfer observed in this system therefore occurs despite the large separation between the heme edges. One explanation for this observation is the enhancement of the intermolecular electron transfer rate through the participation of aromatic residues in the intervening protein structure (Kuki & Wolynes, 1987; Closs & Miller, 1988; McLendon, 1988). Poulos and Kraut (1980) proposed the participation of the phylogenetically conserved Phe 87[2] of tuna cytochrome *c* and His 181 of CCP in electron transfer from ferrocytochrome *c* to CCP, as these two residues are approximately parallel with the hemes and form a bridge between the heme edges with separations no greater than 7 Å (Poulos & Finzel, 1984) in the hypothetical complex.

The role of Phe 87 of cytochrome *c* in electron transfer is unclear. Using site-directed mutagenesis, Pielak et al. (1985) found that substitution of Tyr, Ser, or Gly for Phe 87 of yeast iso-1 cytochrome *c* had only modest effects on the rate of peroxide-dependent oxidation of ferrocytochrome *c* by CCP under steady-state conditions, indicating that this residue is not an obligatory participant in electron transfer. Using ZnCCP, Liang et al. (1986, 1988) found that reduction of ferric cytochrome *c* by photogenerated ^{3}ZnCCP is also insensitive to the nature of the residue at this position of cytochrome *c*. In contrast, measurements of the intermolecular electron transfer rate (Liang et al., 1986) from ferrocytochrome *c* to the resulting cation radical, $(ZnCCP)^+$, have shown that the rate of electron transfer was reduced by 10000-fold when an aliphatic residue was present at position 87 (Liang et al., 1987, 1988). Thus, it appears that the residue at position 87 is highly influential in electron transfer from ferrous cytochrome *c* to $(ZnCCP)^+$. Recently, Liang et al. (1988) have proposed that the dramatic influence of this residue on thermal decay of the $(ZnCCP)^+$ is due to coupling of the heme π-electron systems by superexchange interactions (Miller & Beitz, 1981; McLendon, 1988) through Phe-87 of cytochrome *c* and His-181 of CCP.

Evaluating the role of His 181 of CCP in the electron transfer complex is therefore of considerable interest. While chemical modification studies (Bosshard et al., 1984) have suggested a role for His 181 in the oxidation of ferrocytochrome *c* by CCP, these studies did not attain an unambiguous modification of this residue. To directly evaluate the participation of this residue in electron transfer, we have taken advantage of the recent expression of CCP in *Escherichia coli* (Fishel et al., 1987) to create a mutant CCP in which His 181 is replaced by glycine. The results presented here show that the steady-state peroxide-dependent oxidation of ferrocytochrome *c* and the electron transfer rates from ferrocytochrome *c* to compound I are reduced by only 2-fold in the mutant protein, indicating that the imidazole side chain of His 181 is not required for electron transfer from ferrocytochrome *c* to compound I of CCP.

Materials and Methods

Materials. The oligonucleotide used in the mutagenesis was prepared on an Applied Biosystems 380A DNA synthesizer at the Agouron Institute, La Jolla, CA. Sources for other reagents used routinely in the preparation of mutant CCP(MI) proteins have been described elsewhere (Fishel et al., 1987). Cytochrome *c* (type VI from horse heart) and bovine serum albumin (fraction V) were obtained from Sigma. Hydrogen peroxide (Mallinckrodt) was periodically standardized with $KMnO_4$ (Koltoff & Belcher, 1957). Cytochrome *c* used in the flash photolysis experiments was further purified by ion exchange chromatography on CM-cellulose. Bakers' yeast CCP was isolated as described previously (Hazzard et al., 1987). 5-Deazariboflavin was the generous gift of Drs. William McIntire and Thomas Singer. The source of lumiflavin has been described elsewhere (Simondsen & Tollin, 1983).

Preparation of CCP(MI,G181). Expression of the cloned yeast CCP gene (Kaput et al., 1982) in *E. coli* has been described (Fishel et al., 1987). The mutation in the CCP coding sequence in M13mp8CCP(MI) was induced according to established procedures (Fishel et al., 1987), using a 22-base deoxyoligonucleotide primer with the sequence 5′-GTTCTTCAAGCCGGTCTTGCCC-3′. The underlined bases designate the change of the His codon (CAC) to a Gly codon (GGC) at position 181. The entire gene was then sequenced by the dideoxynucleotide method (Sanger et al., 1977; Biggin et al., 1983) to confirm the presence of the mutation and to ensure that no additional mutations were introduced during the mutagenesis procedure. The mutant gene was subsequently placed in a pUC8 expression vector by standard techniques (Fishel et al., 1987).

Proteins were prepared by aerobic culture of transformed *E. coli* SK383 in TB broth plus 300 μg/mL ampicillin for 27 h at 37 °C. Procedures for purification of the protein and heme insertion have been described elsewhere (Fishel et al., 1987). The proteins were twice crystallized from 0.025 M potassium phosphate (pH 6.0), by exhaustive dialysis against water at 4 °C, and stored as crystalline suspensions in H_2O at −70 °C until use.

X-ray Diffraction Studies of CCP(MI,G181). Crystals of CCP(MI,G181) were grown at 2 °C in 15-μL sitting drops containing 10 mg/mL protein in 50 mM potassium phosphate, pH 6.0, and 20% (v/v) 2-methyl-2,4-pentanediol by vapor diffusion against a reservoir of 30% (v/v) methylpentanediol and 50 mM K_3PO_4. A single crystal was induced in each drop by transfer of a partially dissolved seed crystal (Thaller et al., 1981). Single crystals were mounted in a glass capillary and fixed on the goniostat of the Mark II multiwire area detector diffractometer (Xuong et al., 1978). The crystal showed strong diffraction when placed in the incident X-ray beam. In contrast to crystals of the parent CCP(MI), however, the quality of the diffraction pattern deteriorated rapidly at room temperature. A low-temperature device was therefore employed to perform data collection at 3 °C. Under these conditions, the crystal was sufficiently stable to permit data collection for a period of 48 h.

The X-ray diffraction data were collected by continuous rotation of the crystal around the ω axis in increments of 0.1 °C, while a profile of counts per angular step was measured for each reflection.

Intensities were calculated by subtracting the estimated background from the reflection profiles which were then corrected for Lorentz and polarization effects. The multiple observations were merged together to produce a final data set of 13454 structure factors that scaled with an agreement of $R = 0.06$, where R is the unweighted residual based on intensities. This represents over 90% of the unique data to a

[2] The numbering for amino acids in tuna cytochrome *c* is based on a sequence alignment with yeast iso-1 cytochrome *c*.

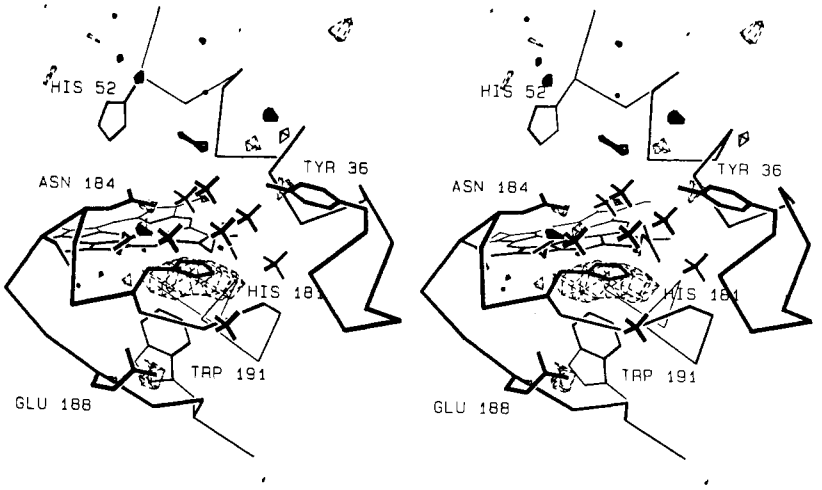

FIGURE 1: Difference Fourier map for CCP(MI,G181) minus CCP(MI) superimposed on a model of the parent CCP(MI) structure. Fixed water molecules are indicated by tetrahedra. The contour lines were drawn at $\pm 4.1\sigma$. Positive density is indicated by filled solid regions; negative density is indicated by regions enclosed by dashed lines.

resolution of 2.4 Å. The unit cell dimensions of the mutant crystal, 105.25 × 74.15 × 45.01 Å are within experimental error of the parent form, which has the same space group, $P2_12_12_1$.

Visible and EPR Spectroscopy. Visible spectra were recorded at 23 °C on a Perkin-Elmer λ 3B spectrophotometer equipped with a software package from Softways, Inc. (Riverside, CA). Extinction coefficients for the peroxidase enzymes were calculated from the total hematin concentration as determined by the pyridine hemochromagen method (Paul et al., 1953). To determine the rate of compound I decay, equimolar amounts of hydrogen peroxide and CCP(MI) or CCP(MI,G181) were mixed in 100 mM phosphate buffer, followed by incubation at 23 °C. Visible spectra were recorded at various time intervals, and the rate of compound I decay was determined by monitoring the oxidation of the oxyferryl center at 562 nm.

Samples were prepared for EPR spectroscopy as follows: 200 μL of a 1 mM solution of CCP(MI,G181) in 100 mM potassium phosphate (pH 6.0) was mixed with a 10% molar excess of hydrogen peroxide, added to a quartz (i.d. = 3 mm) sample tube, and immersed in liquid nitrogen. This procedure was completed within 1 min. EPR spectra were recorded at 77 K on a Varian E-3 spectrometer, operated at 9.15 GHz, with 100-kHz field modulation.

Steady-State Activity of CCP(MI) and CCP(MI,G181). The activity of CCP(MI) and CCP(MI,G181) was determined at 23 °C essentially according to the procedure of Yonetani (1965). To prepare stock solutions for enzyme assays, crystals of the enzyme were dissolved in 0.1 M potassium phosphate (pH 6.0) at approximately 10 mg/mL and were serially diluted in 0.1 M potassium phosphate, pH 6.0, containing 0.1 μg/mL BSA. Ferrous cytochrome *c* was prepared by the addition of a slight excess of dithionite to 200 μL of horse heart cytochrome *c* in water (400 mg/mL), and excess dithionite was removed by chromatography on a Sephadex G-25 column in 10 mM potassium phosphate–1 mM EDTA (pH 7.0).

The pH dependence of peroxidase activity was determined in a constant ionic strength buffer (μ = 50 mM) consisting of MES (25 mM), acetate (25 mM), and Tris (50 mM) (Ellis & Morrison, 1982) containing 30 μM ferrocytochrome *c* and 160 μM hydrogen peroxide in a final volume of 1 mL. Reactions were initiated by the addition of enzyme. The steady-state kinetic parameters were determined at 23 °C in 1 mL of 0.02 M potassium phosphate buffer adjusted to pH 6.0 with Tris (Kang et al., 1977). The apparent K_m for cytochrome *c* was determined by varying the concentration of ferrocytochrome *c* from 2 to 60 μM in the presence of 160 μM peroxide; the apparent K_m for peroxide was determined by varying the concentration of peroxide from 1 to 160 μM in the presence of 36 μM ferrocytochrome *c*.

Activities were calculated from the change in absorbance at 550 nm where ϵ_{550}(cytochrome $c_{Fe(II)}$) − ϵ_{550}(cytochrome $c_{Fe(III)}$) = 19.6 mM^{-1}. Apparent K_m and V_{max} values for each substrate were determined by the method of Eadie and Hofstee (1952).

Kinetics of Electron Transfer in CCP(MI) and CCP(MI,G181). Experimental procedures for the laser flash photolysis experiments have been described elsewhere (Hazzard et al., 1987, 1988a,b,c). The rate of compound I reduction by lumiflavin was measured in 5.4 mM phosphate buffer (μ = 8 mM, pH 6.0) containing 500 μM EDTA and 70 μM lumiflavin. The rate of compound I reduction by ferrous cytochrome *c* was measured as described (Hazzard et al., 1988a,c; Mauro et al., 1988), in 100 mM phosphate buffer (μ = 114 mM, pH 6.0) containing 500 μM EDTA, 90 μM 5-deazariboflavin, 30 μM ferric cytochrome *c*, and 9–70 μM compound I. The reduction of compound I is pseudo first order under these conditions, since the concentration of ferrous cytochrome *c* generated per laser flash is ≤0.1 μM (Simondsen & Tollin, 1983). A minimum of four transient decay curves, measured at the appropriate wavelength (see Results), were averaged for rate determinations at each compound I concentration. Pseudo-first-order rate constants (k_{obs}) were determined from plots of log Δ signal vs time. Such plots were linear for at least four half-lives. Second-order rate constants for the direct reduction of compound I by lumiflavin were obtained from plots of k_{obs} vs compound I concentration. For the reduction of compound I by ferrous cytochrome *c*, the rate constants were determined by nonlinear regression analysis of plots of k_{obs} vs compound I concentration according to the mechanism described in the text.

RESULTS

X-ray Diffraction Studies of the CCP(MI,G181) Protein. The difference Fourier for CCP(MI,G181) − CCP(MI) is shown in Figure 1, superimposed on the structure of CCP(MI) (Wang, 1988). The primary features of the difference map are located at the surface of the molecule in the vicinity of His 181. In addition to the large peak of negative density

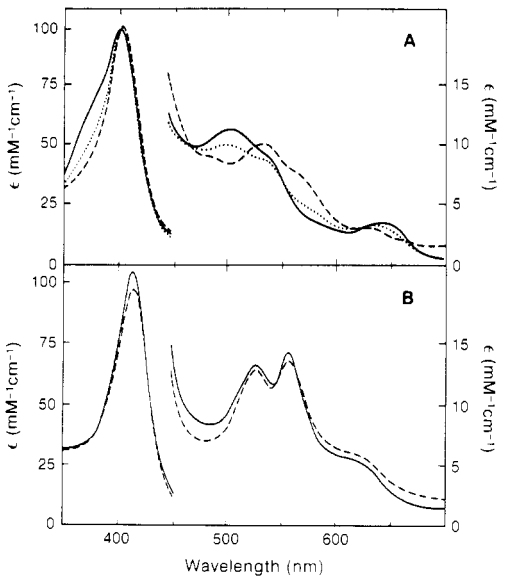

FIGURE 2: Absolute absorption spectra of CCP(MI,G181) and CCP(MI). (A) Absorption spectra of ferric CCP(MI) (solidus line) and CCP(MI,G181) (dotted line) at pH 6.0 and CCP(MI,G181) at pH 7.0 (dashed line). (B) Absorption spectra of CCP(MI) compound I (solidus line) and CCP(MI,G181) compound I (dashed line) at pH 6.0. All spectra were recorded at 23 °C in 100 mM phosphate buffer.

resulting from the loss of the His 181 side chain, small peaks of negative density are seen near Glu 188 and Asn 184, and a small peak of negative density is also seen near Tyr 36. These residues are not involved in the complementary surface interactions between CCP and cytochrome *c* in the hypothetical complex (Poulos & Finzel, 1984), and no evidence for their involvement has been obtained in chemical modification experiments (Bechtold & Bosshard, 1985; Waldmeyer & Bosshard, 1985).

On the basis of interatomic distance measurements made with the refined structure of CCP, His 181 is thought to form hydrogen bonds with Asp 37 and the propionate of heme pyrrole IV (Finzel et al., 1984). No features were observed in the immediate vicinity of either of these residues, however. The heme propionate of pyrrole IV also interacts through hydrogen bonds with Ser 185 and Asn 184 and with Arg 48 through water 348. Of these residues, only Asn 184 has a noticeable peak of negative density, perhaps resulting from the loss of the interaction with His 181.

Spectra of CCP(MI,G181). The visible absorption spectra of CCP(MI) and CCP(MI,G181) are shown in Figure 2A. The molar extinction at 408 nm for CCP(MI,G181) was identical with that of CCP(MI) (99.8 mM^{-1}), and the A_{408}/A_{280} ratio was 1.3. At pH 6.0, the CCP(MI,G181) mutant differs from CCP(MI) in the absence of the distinct shoulder at 390 nm which is characteristic of pentacoordinate high-spin iron (Yonetani & Anni, 1987) and in a small blue shift of the charge-transfer band from 645 to 632 nm. These characteristics suggest that CCP(MI,G181) exists predominantly in the hexacoordinate, high-spin form at pH 6.0, in contrast to the predominantly pentacoordinate high-spin form observed for CCP(MI). The intensity of the Soret band for CCP(MI,G181) is somewhat less than that reported by Yonetani and Anni (1987) for the hexacoordinate high-spin form of bakers' yeast CCP (120 mM^{-1} cm^{-1}).

Despite these observations, there are no features of the difference map which suggest a change in the coordination of the heme in the crystal structure (Figure 1). Similar results have been obtained for the Trp 51 → Phe mutation which also exists predominantly as a hexacoordinate high-spin form at pH 6.0 (Smulevich et al., 1988), with no obvious change in the coordination state of the crystal structure (Wang, 1988). This discrepancy is currently under investigation.

Although the visible spectrum of CCP(MI) was essentially unchanged from pH 6.0 to pH 8.0, CCP(MI,G181) underwent a transition from the hexacoordinate high-spin form at pH 6.0 to a spectrum characteristic of the alkaline (hexacoordinate low-spin) form of CCP(MI). This transition was characterized by a shift in the Soret peak from 408 to 413 nm, the shift of the β band from 512 to 534 nm, the shift of the α band from 536 to 564 nm, and the disappearance of the charge-transfer band at 632 nm (Figure 2A). The apparent $pK_a = 7.0$ for this transition, as determined from the observed increase in absorbance at 566 nm with increasing pH. The analogous transition is observed in CCP(MI) and bakers' yeast CCP at considerably higher pH ($pK_a = 8.6$; Dhaliwal & Erman, 1985; Gross & Erman, 1985).

The visible spectrum of compound I for CCP(MI,G181) formed by oxidation of the ferric enzyme with an equimolar amount of hydrogen peroxide was quite similar to that of CCP(MI) at pH 6.0 (Figure 2B). Compound I formed by CCP(MI,G181) was somewhat less stable at pH 6.0 than compound I of CCP(MI); the $t_{1/2}$ for decay of the oxyferryl center to the ferric form was 150 min in the mutant protein, compared to 450 min in CCP(MI). The stability of compound I in CCP(MI,G181) relative to CCP(MI) was reduced more dramatically at pH 7.0; $t_{1/2}$ for compound I decay was 5 min for CCP(MI,G181) compared to 320 min for CCP(MI).

When compound I of CCP(MI,G181) formed at pH 6.0 in 100 mM potassium phosphate was examined by EPR spectroscopy at 77 K, a radical signal similar to that of bakers' yeast CCP and CCP(MI) was observed (Fishel et al., 1987; Hori & Yonetani, 1985). The signal was centered at $g = 2.004$ and was accompanied by the broadened wings characteristic of CCP compound I (not shown).

Activity and Steady-State Kinetics of CCP(MI,G181). When the initial rates of ferrocytochrome *c* oxidation by CCP(MI) and CCP(MI,G181) were compared over the pH range from pH 5.0 to pH 7.0, both enzymes showed optimal activity between pH 5.3 and pH 5.5. Preincubation of CCP(MI,G181) at pH 7.0 for 30 min at 4 °C resulted in a 30% decrease in peroxidase activity. The activity of CCP(MI) was unchanged by similar treatment, indicating that CCP(MI,G181) is more labile than CCP(MI) under these conditions.

To further examine the effect of the His 181 → Gly mutation on steady-state activity with cytochrome *c*, kinetic parameters were determined for CCP(MI,G181) and CCP(MI) in Tris/phosphate buffer at pH 6.0. Linear Hofstee plots were obtained both for CCP(MI) and for CCP(MI,G181) for peroxide in the concentration range examined. The apparent K_m for peroxide was similar in CCP(MI,G181) and CCP(MI); values of 6.4 and 6.5 μM were obtained for the mutant and CCP(MI), respectively.

The initial rate of cytochrome *c* oxidation showed a hyperbolic dependence on cytochrome *c* concentration. Linear Hofstee plots were obtained over the concentration range from 2 to 60 μM ferrocytochrome *c*. The kinetic parameters derived from such plots showed the apparent K_m for ferrocytochrome *c* was increased approximately 4-fold in the CCP(MI,G181) mutant, from 2.5 to 9.5 μM. The value of V_{max} was reduced 2-fold for CCP(MI,G181) relative to CCP(MI); values of V_{max} = 560 μmol min^{-1} (μg of protein)$^{-1}$ (V_0/e = 319 s^{-1}) and V_{max} = 1100 μmol min^{-1} (μg of protein)$^{-1}$ (V_0/e = 625 s^{-1}) were obtained for CCP(MI,G181) and CCP(MI), respectively. Under similar conditions, Kang et al. (1977) have reported

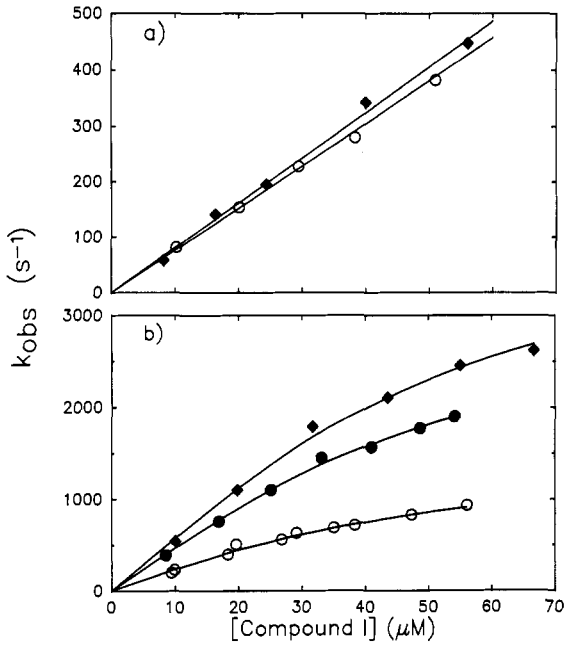

FIGURE 3: (a) Plots of k_{obs} for reduction of free compound I by fully reduced lumiflavin. (◆) CCP compound I from bakers' yeast, pH 6.0; (○) CCP(MI,G181) compound I, pH 6.0. All experiments were performed in K_3PO_4 (μ = 8 mM) containing 500 μM EDTA and 70 μM lumiflavin. (b) Plots of k_{obs} vs concentration for reduction of compound I in the presence of cytochrome *c*. Reduction of (◆) bakers' yeast compound I, (●) CCP(MI) compound I, and (○) CCP(MI,G181) compound I. For all reactions, the concentration of ferric cytochrome *c* was 30 μM in 100 mM phosphate buffer (μ = 114 mM) containing 500 μM EDTA and 90 μM 5-DRf. The solid lines represent best fits from a nonlinear regression analysis based on the mechanism described under Results.

biphasic Hofstee plots for bakers' yeast CCP. The values reported above for CCP(MI) and CCP(MI,G181) are within the range reported for the high-K_m phase by these authors for bakers' yeast CCP. The present results do not exclude the possibility that a low-K_m phase exists below the concentration range employed in this study. Alternatively, the absence of the low-K_m phase may represent the higher purity of the CCP(MI) and CCP(MI,G181) proteins employed in this study (based on the A_{408}/A_{280} ratio) or small structural differences between bakers' yeast CCP and CCP(MI).

Direct Reduction of Compound I by Reduced Free Flavins. The reduction of redox proteins by free flavin semiquinones and fully reduced flavins has been useful as a probe of the accessibility of the prosthetic group of a number of redox proteins (Tollin et al., 1986, 1987). Laser flash excitation of a free flavin in the presence of EDTA generates a flavin semiquinone species, which may either reduce a suitable electron acceptor or undergo a well-characterized disproportionation reaction ($k \simeq 10^9$ M^{-1} s^{-1}; Vaish & Tollin, 1971), resulting in the formation of fully reduced and fully oxidized flavin species. Previous studies (Hazzard et al., 1987) have shown that, in the absence of cytochrome *c*, compound I of bakers' yeast CCP is not reduced by photogenerated semiquinones of either lumiflavin ($E_{m,7}$ = −230 mV) or 5-deazariboflavin ($E_{m,m}$ = −630 mV) at pH 7.0, despite the high midpoint potential of compound I ($E_{m,7} \sim +1$ V).[3] This demonstrates that the reduction of compound I by $LfH^\bullet$ or $DRfH^\bullet$ is not rapid enough to compete with the respective

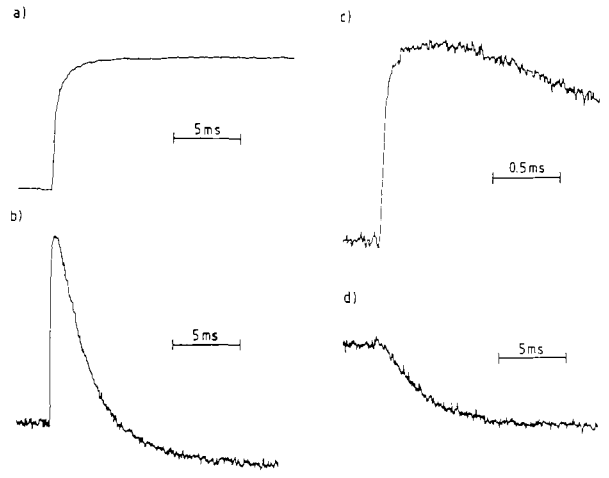

FIGURE 4: Transient decay curves for the reduction of CCP(MI,G181) compound I by photoreduced cytochrome *c*. For all traces, the concentration of ferric cytochrome *c* was 30 μM. (a) Transient signal observed at 550 nm in the absence of CCP(MI,G181) compound I; (b) transient signal observed at 550 nm following the addition of 20 μM CCP(MI,G181) compound I; (c) same as (B), on a 10-fold faster time scale; (d) same as (B), observed at 557 nm. For all plots, the reaction buffer contained 100 mM phosphate (μ = 114 mM), 500 μM EDTA, and 90 μM 5-DRf at pH 6.0.

semiquinone disproportionation reactions. However, direct reduction of compound I from bakers' yeast CCP and CCP(MI,G181) by fully reduced lumiflavin (LfH_2) is observed at pH 6.0. The pseudo-first-order rate constants for this reaction show a linear dependence on compound I concentration (Figure 3a); the second-order rate constants derived from these plots are 8.1 × 10^6 and 7.6 × 10^6 M^{-1} s^{-1} for bakers' yeast CCP and CCP(MI,G181), respectively.[4] These rate constants are the same within the error of the experiments (±10%), suggesting that the accessibility of the heme of compound I is not significantly altered by the His 181 → Gly mutation. In contrast, no measurable reduction of compound I by $DRfH_2$ was observed. This is not surprising, since oxidation of $DRfH_2$ is quite slow relative to that of other flavins (Edmondsen et al., 1972).

Reduction of Bakers' Yeast CCP, CCP(MI), and CCP(MI,G181) Compound I in the Presence of Cytochrome c. It has been shown previously that in the presence of ferric cytochrome *c*, CCP compound I is reduced indirectly by free flavin semiquinones (Hazzard et al., 1987). The kinetics of compound I reduction are consistent with the sequential reduction of cytochrome *c* by the semiquinone species, followed by electron transfer from ferrous cytochrome *c* to compound I (Hazzard et al., 1987). Moreover, it has been possible to determine the rate of intracomplex electron transfer from cytochrome *c* to CCP compound I at various ionic strengths and under conditions where electron transfer is the rate-limiting step in compound I reduction at pH 7.0 (Hazzard et al., 1987, 1988a).

To determine the effect of the His 181 → Gly mutation on the rate of electron transfer from ferrocytochrome *c* to compound I, similar experiments were performed at pH 6.0, under conditions of high ionic strength (μ = 114 mM). Representative kinetic traces for the flash-induced reduction of compound I of CCP(MI,G181) in the presence of cytochrome *c* are shown in Figure 4. The increase in absorbance at 550

[3] The value for $E_{m,7}$ for the Fe(IV)/Fe(III) couple has not been determined experimentally for CCP. The indicated value is assumed for the midpoint potential of the CCP Fe(IV)/Fe(III) couple calculated from the kinetics of CCP–compound II reduction by Fe(II) reductants at pH 5.26 (Purcell & Erman, 1976).

[4] Under the conditions of these experiments, we cannot distinguish between one-electron and two-electron reduction of compound I. The spectral changes observed are consistent with reduction of the Fe(IV) heme; however, there is no convenient means for evaluating the status of the protein-based free radical associated with compound I.

nm resulting from the reduction of horse heart cytochrome *c* in the absence of CCP(MI,G181) is shown in Figure 4a. The second-order rate constant for this process was 1×10^9 M^{-1} s^{-1} (data not shown), in good agreement with the value reported previously (Meyer et al., 1983). When the same experiment is performed in the presence of CCP(MI,G181) compound I, the rapid increase in absorbance at 550 nm is followed by a slower decay (Figure 4b,c). The trace in Figure 4d shows the decay in absorbance when the same reaction is monitored at an empirically determined isosbestic point for ferrous and ferric cytochrome *c* (557 nm). The decay in absorbance to a value below the original base line at 550 nm results from the simultaneous oxidation of cytochrome *c* and reduction of compound I (Hazzard et al., 1987), while the decay in absorbance at 557 nm results solely from the reduction of compound I. The rate constants determined at the two wavelengths were identical within the error of the experiments, indicating that the rates of cytochrome *c* oxidation and compound I reduction are also identical.

By analogous experiments, k_{obs} for oxidation of cytochrome *c* (and simultaneous reduction of compound I) was determined at 550 nm for bakers' yeast CCP, CCP(MI), and CCP(MI,G181) at varying compound I concentrations. The results are presented in Figure 3b. For the three enzymes examined, k_{obs} shows a saturation-type dependence on compound I concentration, with k_{obs} reaching a limiting value at high compound I concentrations. Similar observations have been reported elsewhere for horse, yeast iso-1, and yeast iso-2 cytochrome *c* oxidation at pH 7, at high ionic strength (μ = 260 mM) (Hazzard et al., 1988a,b,c). The simplest interpretation of the data presented in Figures 3 and 4 is the following mechanism:[5]

$$\text{5-DRfH}^{\bullet} + \text{Cyt } c(\text{III}) \xrightarrow{k_1} \text{5-DRf} + \text{Cyt } c(\text{II}) \quad (1)$$

$$\text{Cyt } c(\text{II}) + \text{CCP(IV,R}^{\bullet}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Cyt } c(\text{II})\text{–CCP(IV,R}^{\bullet}) \quad (2)$$

$$\text{Cyt } c(\text{II})\text{–CCP(IV,R}^{\bullet}) \xrightarrow{k_3} \text{Cyt } c(\text{III})\text{–CCP(III,R}^{\bullet}) \quad (3)$$

where Cyt *c*(II)–CCP(IV,R$^{\bullet}$) represents a transiently formed electron transfer complex. The data in Figure 4 (a and b) show that reduction of cytochrome *c* by 5-DRfH$^{\bullet}$ (k_1) is much faster than the subsequent reduction of compound I. Because of the large redox potential difference, it is assumed that the reverse reaction of eq 3 can be neglected. As described previously (Jung & Tollin, 1981; Simondsen et al., 1982) when the steady-state approximation is applied to this mechanism, the following relationship can be written for k_{obs}:

$$k_{obs} = \frac{k_2 k_3[\text{CCP(IV,R}^{\bullet})]}{k_2[\text{CCP(IV,R}^{\bullet})] + k_{-2} + k_3} \quad (4)$$

If one assumes that the reaction represents a rapid equilibrium formation of the electron transfer complex followed by electron transfer (i.e., $k_3 \ll k_2[\text{CCP(IV,R}^{\bullet})] + k_{-2}$), then saturation behavior will be observed, and under these conditions eq 4 reduces to

$$k_{obs} = \frac{k_2 k_3[\text{CCP(IV,R}^{\bullet})]}{k_2[\text{CCP(IV,R}^{\bullet})] + k_{-2}} \quad (5)$$

The data for k_{obs} as a function of compound I concentration were computer modeled by treating the mechanism as two consecutive first-order reactions (since all reactions were conducted under pseudo-first-order conditions) and programming an exact solution for the rate equations (Frost & Pearson, 1961). A constraint that any possible solution was ⩾90% monophasic was imposed on the data fitting process. The program used a steepest descent procedure to obtain a minimum least-squares error in fitting the experimental data. The derived values for k_3, K_2, and k_2 derived from this procedure are reported in Table I. Values for K_2 ($=k_2/k_{-2}$) and k_3 could be determined directly, whereas only a minimum value could be specified for k_2. Any value larger than the reported k_2 gave identical fits, within experimental error, when K_2 was held constant.

Table I: Kinetic Parameters for the Reduction of Bakers' Yeast CCP, CCP(MI), and CCP(MI,G181) Compound I by Ferrous Cytochrome *c* from Horse Heart[a]

protein	k_2 ($\times 10^{-7}$ M^{-1} s^{-1})	K_2 ($\times 10^{-4}$ M^{-1})	k_3 (s^{-1})
native	9.6	3.7	4500
CCP(MI)	8.0	3.4	3450
CCP(MI,G181)	6.7	2.3	1850

[a] Rate constants were obtained from nonlinear least-squares fits for the data shown in Figure 4B, according to the mechanism and procedures described under Results.

The effect of the His 181 → Gly mutation on the intracomplex electron transfer rate is relatively small, as k_3 for compound I of CCP(MI,G181) was 41% that of bakers' yeast CCP and 53% that of the parental CCP(MI). The similarity observed in the apparent rate constants (k_1) and the equilibrium constants (K_2) for the association of compound I of CCP, CCP(MI), and CCP(MI,G181) and ferrocytochrome *c* suggest only a small effect of the His 181 → Gly mutation on the formation of the electron transfer complex. The values obtained for K_2 are similar to the reported values for equilibrium binding of ferric CCP and ferric cytochrome *c* at pH 6.0 (Erman & Vitello, 1980).

Discussion

The diffraction studies, spectra of CCP(MI,G181) and compound I, and the kinetics of compound I reduction by LfH_2 at pH 6.0 all indicate that the structure of CCP(MI,G181) is not significantly altered by the loss of the imidazole side chain of His 181 at pH 6.0. Substitution of glycine for histidine at position 181 in CCP(MI) reduces the steady-state activity of the enzyme toward ferrous cytochrome *c* by 2-fold relative to that of the parental CCP(MI). A somewhat larger (7–10-fold) decrease in the steady-state activity of CCP from bakers' yeast was reported following photooxidation of His 181 with Rose Bengal (Bosshard et al., 1984). In the latter case, however, the photooxidation of His 181 was accompanied by destruction of two tryptophan residues, which may account for this discrepancy.

The transient-state kinetics of compound I reduction by cytochrome *c* show that k_{obs} for this reaction has a hyperbolic dependence on compound I concentration. This observation is consistent with a mechanism in which the rate of reduction is limited at low compound I concentrations by the concentration of the transient compound I–ferrocytochrome *c* complex

[5] We acknowledge the possible contribution of competitive binding of ferric cytochrome *c* with compound I to the overall mechanism

$$\text{Cyt } c(\text{III}) + \text{CCP(IV,R}^{\bullet}) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Cyt } c(\text{III})\text{–CCP(IV,R}^{\bullet})$$

The equilibrium constant for this reaction is given by $K_i = k_i/k_{-i}$. Summers and Erman (1988) have found that $K_i = 1 \times 10^6$ M^{-1} at μ = 10 mM, pH 7.5; essentially the same value was determined for ferrous cytochrome *c* (reaction 2). Assuming that these two constants also have similar values at μ = 114 mM ($\sim 3 \times 10^4$ M^{-1}), the percentage of free compound I which is present prior to the laser flash is calculated to be 61%, 71%, and 76% of the total compound I concentration at 10, 30, and 50 μM, respectively. Since the predicted effect of competitive binding of ferrous cytochrome *c* is therefore small, it has not been included in the computer modeling.

and at high compound I concentrations by the rate of electron transfer from ferrocytochrome *c* to compound I within the complex. By this mechanism, the limiting value at high compound I concentration represents a measure of the intermolecular electron transfer rate within the transiently formed electron transfer complex. Analysis of the data according to this mechanism shows that the rate of electron transfer in the transient complex for CCP(MI,G181) is decreased by 2-fold relative to that of the parental CCP(MI). The equilibrium constants derived for association of ferrous cytochrome *c* and compound I were similar for bakers' yeast CCP, CCP(MI), and CCP(MI,G181); thus, the 2-fold decrease in the electron transfer rate may reflect a slightly different orientation of the two proteins within the complex. The observed effect of the His 181 → Gly mutation on the electron transfer rate is therefore measurable, but small; rate differences of this magnitude have been observed in complexes formed between bakers' yeast CCP and cytochromes *c* from various organisms (Hazzard et al., 1987). In contrast, a much more dramatic reduction (at least 1000-fold) in the rate of electron transfer within the complex is observed when a nearby residue, Trp 191 of CCP(MI), is replaced by phenylalanine (Mauro et al., 1988), a substitution which also has relatively minor effects on the structure in this region.

The steady-state and transient kinetic results exclude His 181 as an obligatory participant in electron transfer in the CCP–cytochrome *c* complex. Thus, although the identity of the residue at position 87 of yeast iso-1 cytochrome *c* is quite influential in determining the rate of electron transfer from ferrocytochrome *c* to $(ZnCCP)^+$ (Liang et al., 1987, 1988), the proposed enhancement of electron transfer rates due to superexchange reactions through His 181 of CCP (Poulos & Kraut, 1980; Liang et al., 1988) does not appear to be operant in the physiological CCP–ferrocytochrome *c* complex. It is possible that the proposed rate enhancements occur through the participation of a residue(s) in CCP which has not yet been identified. Alternatively, the dramatic rate effects observed for substitutions at position 87 of yeast iso-1 cytochrome *c* may be due to an effect which is peculiar to the ZnCCP system, since the effect of nonaromatic substitutions on the rate of peroxide-dependent oxidation of ferrocytochrome *c* by native CCP is relatively minor under steady-state conditions (Pielak et al., 1985). A direct comparison of the respective CCP and cytochrome *c* mutants by the techniques described here and those employing ZnCCP would be useful in resolving this issue. It is important to note that we cannot, at present, exclude the possibility that removal of the imidazole side chain at position 181 results in electron transfer by an alternate, kinetically equivalent pathway.

The results presented here also provide direct evidence for the importance of His 181 in the structural stability of CCP at alkaline pH. The location of this residue in close proximity to the propionate of heme pyrrole IV and the carboxylate side chain of Asp 37 is thought to elevate the pK_a of the imidazole side chain (Poulos & Kraut, 1980), and deprotonation of the imidazole is thought to be involved in the alkaline denaturation of CCP (Bosshard et al., 1984). The results presented here are consistent with this hypothesis. As the pH is increased from 6.0 to 7.0, the appearance of the alkaline form of the enzyme is noted in CCP(MI,G181), accompanied by a significant increase in the rate of compound I decay and loss of peroxidase activity. The decrease of the pK_a for the transition from the acidic to alkaline form observed in CCP(MI,G181) suggests that the formation of the unstable hexacoordinate low-spin form of the enzyme is suppressed by the presence of the protonated imidazole side chain of His 181 in CCP(MI). This conclusion is supported by the observation that a similar reduction in the pK_a of the transition from the acidic to alkaline form of CCP is obtained in the dimethyl ester derivative of CCP (Dowe & Erman, 1982). Experiments to further characterize the transition of CCP from acidic to alkaline form are currently in progress.

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Registry No. CCP, 9029-53-2; His, 71-00-1; Gly, 56-40-6; cytochrome *c*, 9007-43-6.

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