

Amino Acid Substitutions at Tryptophan-51 of Cytochrome *c* Peroxidase: Effects on Coordination, Species Preference for Cytochrome *c*, and Electron Transfer[†]

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ABSTRACT: Amino acid replacements of an aromatic residue, Trp-51, which is in contact with the heme of yeast cytochrome *c* peroxidase have a number of significant effects on the kinetics and coordination state of the enzyme. Six mutants at this site (W51F, W51M, W51T, W51C, W51A, and W51G) were examined. Optical and EPR spectra show that each of these mutations introduces a shift from the 5-coordinate to 6-coordinate form, and slightly increases the asymmetry of the heme ligand field. Conversion from a 6-coordinate high-spin form at pH 5 to a 6-coordinate low-spin form at pH 7 is observed for several of the variants (W51F, W51T, and W51A), while W51G and W51C appear as predominantly low-spin species between pH 5 and 7. Addition of 50% glycerol prevents the facile conversion to the low-spin conformation for W51F, W51T, and W51A, and only W51F can be stabilized in a 5-coordinate configuration by glycerol. For the oxidation of cytochrome *c* by H₂O₂, three of the variants (W51F, W51M, and W51T) exhibit values of $k_{\text{cat}}(\text{app})$ that are greater than for the wild-type enzyme, while the other mutations give decreased rates of enzyme turnover. Unlike the wild-type enzyme, which functions more efficiently with cytochrome *c* from yeast than with the horse heart protein, the mutant W51F does not show a preference for substrate from its native organism. The three mutants which exhibit increased values of $k_{\text{cat}}(\text{app})$ show a pH optimum at 6.8 compared with that of 5.25 for the wild-type enzyme when measured with horse heart cytochrome *c*. This shift in pH optimum is not observed with yeast cytochrome *c*. Construction of single and multiple mutations at Trp-51, Ile-53, and Gly-152 shows that these kinetic properties are not due to natural amino acid variations observed at these sites. Pre-steady-state kinetics show that the bimolecular rate constant for the fast phase of the reaction of the enzyme with H₂O₂ is only slightly decreased from $3.03 (0.09) \times 10^7$ to $2.2 (0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for W51F and to $1.5 (0.1) \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for W51A. The slow phase of the reaction (4.9 s^{-1}) which contributes approximately 30% to the amplitude of the change for the wild-type enzyme is not observed for W51F or W51A. The kinetic parameters indicate that the rate-limiting step of the reaction under these conditions is that of electron transfer from cytochrome *c* and that this process is made more rapid in the case of the hyperactive mutants. The increased rate of electron transfer may be a result of a change in the free energy of the oxidized enzyme (compound ES) brought about by amino acid or solvent contact with the heme. The effects of pH and substrate dependence observed for these mutant enzymes are consistent with proposals that the complex between horse heart cytochrome *c* and CCP may undergo small fluctuations in geometry and that the complex with yeast cytochrome *c* (iso-1) is much more specific and efficient.

Cytochrome *c* peroxidase (CCP)¹ (EC 1.11.1.5) is a yeast mitochondrial enzyme that catalyzes the oxidation of yeast cytochrome *c* (cyt *c*) by H₂O₂ (Yonetani, 1976; Poulos & Finzel, 1984). The enzyme is a soluble 34-kDa protein and contains a single ferric protoporphyrin IX in its native state. In the accepted mechanism for the function of this enzyme, H₂O₂ binds to the unoccupied axial coordination position of the heme and oxidizes the ferric enzyme by 2 equiv to give the oxidized intermediate, compound ES (Yonetani, 1976). This species is analogous to the compound I intermediate of other peroxidases in that it contains an oxyferryl center (Fe⁴⁺=O) [see Dawson (1988) and references cited therein].

However, instead of carrying the second oxidation equivalent on the porphyrin as is done by horseradish peroxidase compound I (Dolphin & Felton, 1974), the second equivalent of compound ES resides as a stable and reversibly oxidized form of Trp-191, at present the only known example of a tryptophan radical in biology (Sivaraja et al., 1989). Compound ES oxidizes two molecules of reduced cyt *c* in stepwise fashion to regenerate the native state of the enzyme (Yonetani, 1976). Thus, the mechanism involves a complex interplay of a number of chemical events including heterolytic cleavage of the peroxy

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¹ Abbreviations: CCP, cytochrome *c* peroxidase; CCP(MKT), cytochrome *c* peroxidase produced by recombinant expression in *E. coli* and containing Met-Lys-Thr on the N-terminus; cyt *c*, cytochrome *c*; EDTA, ethylenediaminetetraacetic acid; ES, peroxide complex of cytochrome *c* peroxidase; HRP, horseradish peroxidase; IPTG, isopropyl β -thiogalactopyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MPD, 2-methyl-2,4-pentanediol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; W51F, Trp-51 replaced by Phe.

bond, protein-protein recognition and binding, intermolecular electron transfer, and intramolecular oxidation-reduction of the tryptophan radical.

The factors controlling the kinetics of enzyme turnover under steady-state conditions have been well studied but remain incompletely understood. CCP, a yeast enzyme, functions most efficiently with cyt *c* derived from *Saccharomyces cerevisiae* (yeast), although cyt *c* from other species can be used as substrate (Kang et al., 1977). Oxidation of yeast cyt *c* at the optimum concentration of 100 mM phosphate is approximately 2-fold faster than that measured for horse heart cyt *c* at 20 mM phosphate. The different conditions of ionic strength optima presumably reflect differences in the nature of the complex formed between CCP and cyt *c* from various species (Kang et al., 1977). Previous kinetic models for enzyme turnover have included both single (Kang & Erman, 1982) and multiple (Kang et al., 1977, 1978) sites on CCP for binding of cyt *c* to explain the departure, under some conditions, from Michaelis-Menten behavior.

In a number of studies, the interaction of CCP and cyt *c* has been interpreted in terms of a geometrically well-defined electron-transfer complex originally proposed by Poulos and Kraut (Poulos & Kraut, 1980; Poulos & Finzel, 1984). The proposed complex, constructed by model building from the individual crystal structures of CCP and tuna cyt *c*, has served as the archetype for the interaction of electron-transfer proteins. Specific ionic interactions between spatially complementary charged residues were proposed to give the complex a defined geometry containing parallel hemes at a distance of 16.5 Å. Chemical modification studies have provided support for binding of cyt *c* at the proposed site of CCP (Kang et al., 1978; Waldmeyer et al., 1982; Bosshard et al., 1984; Waldmeyer & Bosshard, 1985). However, recent results have indicated that the interaction is considerably more complex. Brownian molecular dynamics calculations have suggested that formation of the initial CCP-cyt *c* complex is followed by a significant reorientation or diffusion of cyt *c* on the surface of CCP (Northrup et al., 1988). This would lead to a heterogeneity of geometries which could be visited during the lifetime of a single encounter of the two proteins. Crystallographic investigation of a complex between CCP and horse heart cyt *c* at low ionic strength has unfortunately not yet provided a resolution of this issue due to disorder of the cyt *c* in the crystal (Poulos et al., 1987). Species-specific differences in the electron-transfer rates have also been observed in the preformed complexes of CCP and cyt *c* from yeast and horse heart (Ho et al., 1985; Hazzard et al., 1988). These studies indicate that different types of cyt *c* result in the formation of complexes that differ in electron-transfer probability. Mutagenesis of yeast cyt *c* near the proposed site of binding to CCP has shown dramatic effects on electron-transfer rate constants (Liang et al., 1987, 1988). In addition, evidence has been reported indicating the existence of a conformationally controlled gate for electron transfer in the bound complex (Hazzard et al., 1987). As the electron-transfer efficiency is expected to be quite sensitive to the geometry of the heme centers, the dynamics of such a distribution of complexes may be essential for attainment of the optimized geometry under some conditions, and may limit enzyme turnover under others.

It has been noted previously (Goodin et al., 1987; Fishel et al., 1987; Smulevich et al., 1988; Wang et al., 1990) that alteration of Trp-51 has significant effects on the coordination properties and function of the enzyme. Trp-51 (distinct from the Trp-191 radical site) forms part of the distal active-site

cavity near the open coordination position of the heme iron, where it contacts the heme and forms a hydrogen bond with one of the water molecules that is displaced on binding H₂O₂ (Finzel et al., 1984). Other peroxidases, such as horseradish peroxidase, appear to have Phe in place of this Trp (Welinder, 1985). The CCP mutant W51F, with Phe in place of Trp-51, has been shown to be more active than the wild-type enzyme in reducing horse heart cyt *c* (Goodin et al., 1987), and this mutant has a compound ES intermediate that decays more rapidly than that of the wild-type (Fishel et al., 1987). Resonance Raman spectroscopy has shown that this mutation converts the enzyme from a 5-coordinate form to a 6-coordinate high-spin conformation that can be reversed by glycerol or 2-methyl-2,4-pentanediol (Smulevich et al., 1988, 1989, 1990). A Fourier difference map of the crystal structure of this mutant has been reported, and the effects of the substitution on the structure of the distal active-site pocket are small (Wang et al., 1990). Thus, despite detailed information on the structural consequences of the W51F mutant, it is not clear what specific role this residue plays in the function of CCP other than the fact that the rate-limiting step in enzyme turnover must have been altered by this change. In this paper, the functional properties and heme coordination of CCP expressed in *Escherichia coli* and six mutants containing substitutions at position 51 are examined. Steady-state and pre-steady-state kinetic studies using horse and yeast cyt *c* as substrates are reported. We show that all of the mutants have dramatically altered kinetics and that W51F has lost the species preference of wild-type CCP for yeast cyt *c*. These results are discussed in terms of alterations in the axial heme coordination and the effects of heme environment on the driving force for electron transfer.

MATERIALS AND METHODS

CCP Expression and Purification from *E. coli*. An expression vector was constructed which produced large quantities of the CCP apoprotein in *E. coli*. The 68 amino acid presequence (Kaput et al., 1982) was removed from the yeast CCP gene contained on the plasmid pEMBLCCP (Goodin et al., 1986) by oligonucleotide site-directed mutagenesis on single-stranded template DNA containing uracil as previously described (Goodin et al., 1987). This introduced an *Eco*RI site immediately followed by an artificial initiation codon at the +1 mature protein position. The gene for the mature protein, contained on a 2.1-kb *Eco*RI fragment, was introduced behind the lac promoter of the phagemid Δ¹³pEMBL8(+) (Luck et al., 1986). The *Pvu*II fragment of this plasmid, containing the promoter-CCP sequence, was joined with the *Pvu*II fragment of the phagemid pEMBL8(-) (Dente et al., 1983) containing the f₁ origin. The orientation of the packaged single strand was then verified by sequencing the packaged single-stranded DNA. In this construct, Δ¹³pEMBLCCP(-), the Shine-Dalgarno ribosome binding site of the lac promoter, was located 9 base pairs upstream of the CCP initiation codon, and the intervening sequence still contained the *Eco*RI site used for the construction. SDS-PAGE and Western blot analysis showed that this vector did not produce a significant amount of the protein in *E. coli* (JM101) grown in YT with or without added IPTG. The vector was further modified by mixed-oligonucleotide mutagenesis (Ner et al., 1988) to replace the sequence between the ribosome binding site and the translation start with the consensus sequence AGGAGGTATATTATG, and at the same time to randomize the second and third codons of the protein. The oligonucleotide used for this was 5'-TAACAATTTACACAGGAGGTATATTATGA,A/C,A/T,A/C,CGCTCGTTCATGTCGCC-

3', in which the boldface type represents sequence that was altered by the mutagenesis. Uracil-containing DNA was used for the mutagenesis, and random transformants of JM101 from the reaction were screened by SDS-PAGE for variants producing a protein of the expected electrophoretic mobility. A number of clones were identified and characterized by DNA sequence analysis. Considerable variation in the level of expressed protein was observed for several clones containing differing N-termini. One such variant, pLACCCP2-8, with the sequence **AGGAGTATATTATG**AAAACG and coding for Met-Lys-Thr (MKT) at the N-terminus, was identified as giving the highest level of expression. Protein expressed from this plasmid is thus designated CCP(MKT). Mutants at Trp-51 which were previously constructed in the vector pEMBLCCP (Goodin et al., 1987) were subcloned as the *Apal*/*Bam*HI fragment into the *E. coli* expression vector pLACCCPP2-8, and each gene was completely resequenced. The level of expressed protein from cells grown in YT+amp reaches a maximum approximately 24 h after saturation, and does not significantly decrease after this. The level of protein at this time is not dependent on the presence of up to 10 mM IPTG in the culture medium, even though the *E. coli* used (JM101 or HB2151) contained *lacI^q* for overexpression of the lac repressor. This is probably due to titration of the existing repressor by the high-copy plasmid.

As reported for a different expression system (Fishel et al., 1987), the enzyme was isolated from *E. coli* as the apoprotein which was purified and reconstituted as follows. A 2-L culture of *E. coli* HB2151 freshly transformed with pLACCCP2-8 was grown at 37 °C for 36 h in a medium containing per liter 10 g of bactotryptone, 8 g of yeast extract, 5 g of NaCl, 1 mL of glycerol, and 100 mg of ampicillin. Subsequent steps were performed at 4 °C. The cells were harvested by centrifugation at 6000g for 10 min, resuspended in 40 mL of buffer containing 200 mM potassium phosphate pH 7.5, 1 mM PMSF, and 1 mM EDTA, and lysed by passing twice through a French press at 16 000 psi. The lysate was diluted with 100 mL of cold H₂O and 300 mL of 50 mM potassium phosphate, pH 6.0, and centrifuged at 12000g for 15 min. The clear supernatant was loaded onto a DEAE-Sephadex CL-6B (3 × 5 cm) column equilibrated with 50 mM potassium phosphate, pH 6, and washed with the same buffer. After elution with 500 mM potassium phosphate, pH 6, the protein-containing fractions were diluted with an equal volume of cold H₂O and concentrated to approximately 4 mL by ultrafiltration (Amicon YM-10 membrane). The sample was centrifuged at 12000g for 2 min to remove insoluble material, loaded onto a Sephadex G-75 superfine column (3 × 60 cm), and eluted with 100 mM potassium phosphate, pH 6, and 1 mM EDTA. The apo-CCP eluted as the second peak as measured by the absorbance at 280 nm. A 2–3-fold molar excess of bovine hemin was dissolved in 5 mL of 100 mM NaOH and added to the apoenzyme. The pH was adjusted to 7.0 by dropwise addition of 100 mM NaOH. After 30 min on ice, the mixture was readjusted to pH 6 by addition of 1 M KH₂PO₄ and diluted with cold H₂O to give a conductivity of 5 mΩ⁻¹. This solution was loaded onto a small (2 × 3 cm) DEAE-Sephadex CL-6B column, washed with 50 mM potassium phosphate, pH 6, and eluted with 500 mM potassium phosphate, pH 6. The dark brown band was collected and concentrated by ultrafiltration as described above to a minimal volume (1–3 mL). The protein was recrystallized twice by dialysis against cold H₂O. Approximately 100 mg of recrystallized enzyme was obtained per liter of cell culture, which is substantially higher than previously reported methods

(Goodin et al., 1986; Fishel et al., 1987). The enzyme was stored as a crystal suspension in distilled H₂O at 77 K. CCP concentration was determined by using extinction coefficients determined for each mutant from the pyridine hemochromogen (Nicola et al., 1975).

Optical and EPR Spectra. UV/visible spectra were collected at 25 °C in the stated buffer using a Hewlett-Packard 8452A diode-array spectrophotometer. EPR spectra were collected in 3-mm quartz tubes at X-band on a Bruker ESP300 spectrometer equipped with an Air Products LTR-3 liquid helium cryostat. Temperature was measured with a GaAs diode calibrated to +0.1 K. Instrumental conditions are described in the figures.

Steady-State Kinetics. The steady-state rate of oxidation of cytochrome *c* by the enzyme was determined as previously described (Goodin et al., 1987). Cytochrome *c* was reduced with sodium dithionite and separated from the excess dithionite with a small Sephadex G-25 column. Kinetics were performed with >90% reduced cyt *c* at 25 μM using $\epsilon_{550} = 27.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{526} = 10.7 \text{ mM}^{-1} \text{ cm}^{-1}$ for horse heart cyt *c* and $\epsilon_{550} = 29.0 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{526} = 11.4 \text{ mM}^{-1} \text{ cm}^{-1}$ for yeast cyt *c* (Yonetani, 1965). The concentration of CCP used was approximately 300 pM and was adjusted to give a rate of change in absorbance (550 nm) of approximately $2 \times 10^{-3}/\text{s}$. Steady-state rate constants are reported in terms of enzyme (not cytochrome *c*) turnover. Reported values of the apparent k_{cat} [$k_{\text{cat}}(\text{app})$] were determined as a function of [H₂O₂] at a constant 25 μM cyt *c* concentration. This gives a rate that is approximately 90% of that at 60 μM cyt *c*. We note that it is difficult to saturate this rate, as Eadie-Hofstee plots are not linear, and true values of k_{cat} cannot be accurately obtained.

Stopped-Flow Kinetics. Transient kinetics for the reaction of CCP with H₂O₂ were measured at 25 °C on a Hi-Tech Scientific SHU stopped-flow spectrophotometer interfaced to a Hewlett Packard 9000 computer and using a 100-μs photomultiplier time constant. Absorption kinetics were made at 424 nm on 1 μM solutions of CCP in 100 mM potassium phosphate, pH 6.0.

Reagents and Chemicals. Horse heart cytochrome *c* type VI, *S. cerevisiae* cytochrome *c* type VIII-B, and bovine hemin type I were obtained from Sigma and used without further purification. Solutions of H₂O₂ were standardized by titration with KMnO₄ (Kolthoff & Belcher, 1957). All chemicals were reagent grade and used as received.

RESULTS

Effects of Trp-51 Mutations on Heme Coordination. Each of the Trp-51 variants examined changes the state of axial heme coordination. Most of the substitutions result in a change of the ferric high-spin heme from a 5-coordinate to a 6-coordinate form and cause an increased tendency for conversion to a low-spin species at neutral pH. These conclusions are drawn from the visible spectra of Figure 1 and from the high-spin EPR signals of Figure 2. The 5-coordinate form of CCP exhibits a charge-transfer transition at 645 nm which shifts to approximately 620 nm when coordinated by a sixth weak-field ligand such as fluoride or acetate (Yonetani & Anni, 1987). For the preparations of CCP(MKT) reported here, this charge-transfer band is consistently observed at 638 nm. The spectra of this material are fully independent of pH between 5 and 7 in phosphate, acetate, MES, or MOPS buffers and are not dependent on the presence of 50% glycerol (data not shown). Low-temperature EPR is a useful probe of the heme environment for ferric CCP because both the 5- and 6-coordinate high-spin species give rise to essentially axial

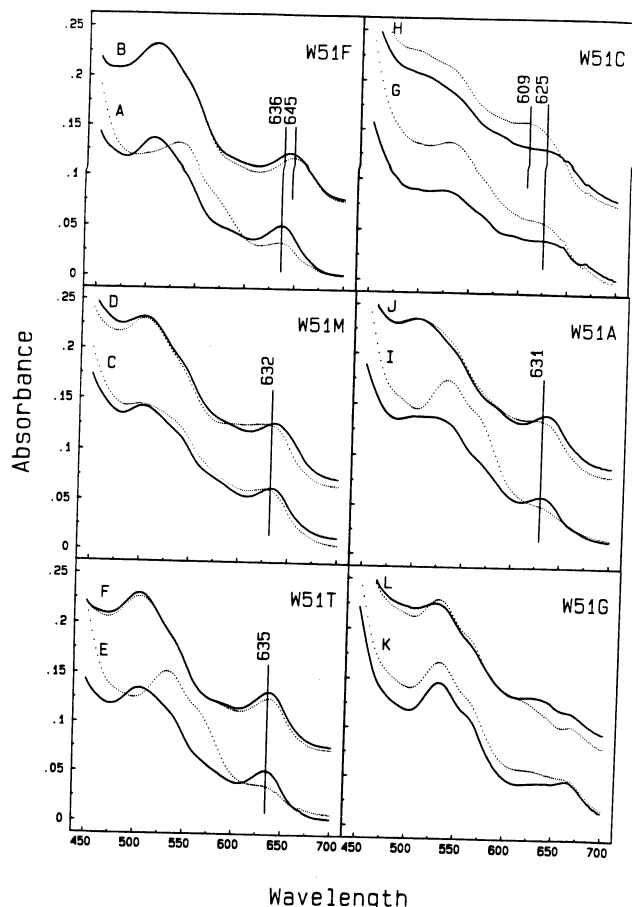


FIGURE 1: Visible spectra of Trp-51 mutants at 25 °C. Spectra (A, C, E, G, I, and K) were normalized to an absorbance of 1 at 280 nm and were collected on approximately 10 μ M protein solutions in 100 mM MES buffer, pH 5.0 (—), or 100 mM MOPS, pH 7.0 (---). Samples for the upper pair of spectra in each panel (B, D, F, H, J, and L) included 50% glycerol in the buffer.

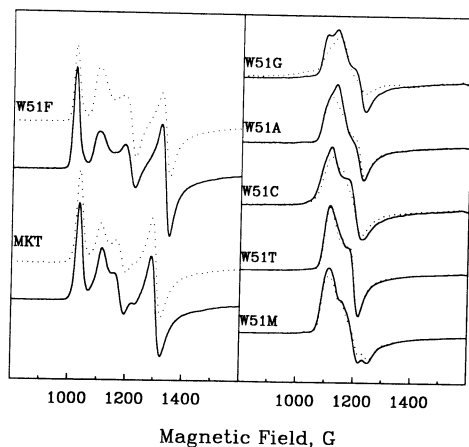


FIGURE 2: EPR spectra of Trp-51 mutants at 7 K. Spectra were collected in 3-mm OD quartz EPR tubes containing approximately 100–300 μ M CCP in 100 mM MES buffer, pH 5.0 (—), or 100 mM MOPS, pH 7.0 (---). All buffers contained 60% glycerol. Spectra were collected at 9.52-GHz microwave frequency using 2-mW microwave power and 5-G modulation amplitude at 100 KHz.

signals near $g = 6$ but differ in their degree of rhombic anisotropy (Goodin et al., 1987; Yonetani & Anni, 1987). The 5-coordinate species has rhombic components at $g_x = 6.60$ and $g_y = 5.23$, while the 6-coordinate form is more axially symmetric with $g_x = 6.13$ and $g_y = 5.81$. The EPR spectra at 7 K (Figure 2) show that CCP(MKT) is predominantly 5-coordinate (approximately 75%) from pH 5 to 7 and contains a small, relatively constant amount of the 6-coordinate species.

Table I: EPR Parameters Determined at 7 K for the High-Spin Ferric Signals of CCP Mutants at Trp-51^a

	rhombic signal			axial signal		
	g_x	g_y	E/D	g_x	g_y	E/D
CCP(MKT)	6.604	5.232	0.0286	6.128	5.814	0.0065
W51F	6.721	5.114	0.0335	6.212	5.645	0.0118
W51M				6.145	5.716	0.0089
W51T				6.145	5.716	0.0089
W51C				6.117	5.692	0.0094
W51A				6.212	5.622	0.0123
W51G				6.229	5.636	0.0124

^aSamples were in 100 mM MES, pH 5.0, and 60% glycerol.

Glycerol is included in the EPR sample buffers to prevent a freezing-induced transition to a low-spin species at high pH (Yonetani & Anni, 1987).

In contrast to these results for the wild-type enzyme, the EPR spectra of Figure 2 show that, with the exception of W51F, all of the variants at Trp-51 have axial rather than rhombic signals, indicating a fully 6-coordinate form. That these mutants also exist predominantly in a 6-coordinate form at room temperature is supported by the blue shift of the charge-transfer transitions from that of CCP(MKT) (Figure 1, A, C, E, and I) and by an increase in extinction coefficient of the Soret absorbance (data not shown). For example, the value of $\epsilon_{408} = 101.2 \text{ mM}^{-1} \text{ cm}^{-1}$ for CCP(MKT) is increased to 121.5, 117.0, 113.2, and 107.1 $\text{mM}^{-1} \text{ cm}^{-1}$ for W51F, W51M, W51T, and W51A, respectively. Three of these enzymes, W51F, W51T, and W51A, are partially converted to a low-spin species at 25 °C upon increasing the pH to 7 as is indicated by a weakening of the charge-transfer transitions at approximately 500 and 630 nm and by an increase in intensity of the α and β bands at 545 and 565 nm. In each case, this pH-dependent high- to low-spin conversion is prevented by the presence of 50% glycerol in the buffer (Figure 1, B, D, F, and J). As noted above, W51F is the only one of several mutants we examined that can be prepared in the 5-coordinate high-spin configuration. As observed earlier (Smulevich, 1989), this 5-coordinate species appears to be dependent on the presence of glycerol, because we observe that the axial high-spin EPR signal of the 6-coordinate species is replaced by the rhombic signal in buffers containing 50% glycerol (data not shown). This property of W51F is also observed at 25 °C in the visible spectra (Figure 1, A and B) in which the addition of glycerol induces a red shift in the charge-transfer band to 645 nm consistent with a 6- to 5-coordinate shift. Both W51C and W51G exhibit more profound changes in heme coordination that may result from a significant conformational perturbation of the distal heme environment. As shown in Figure 1 (G and K), these variants are largely in a low-spin state at both pH 5 and pH 7 which is not prevented by glycerol as observed above for the other mutants. It is of interest that these are the only mutations at W51F that gave protein that did not crystallize after purification. The apoprotein for the glycine mutant as isolated from *E. coli* was consistently green in color and exhibited absorption bands at 404 and 667 nm, very similar to those reported for the "verdochrome" oxidation product of HRP (Yamazaki et al., 1968). However, the chromophore could not be extracted by using the acid/2-butanone method (Yonetani, 1967), and probably represents a low occupancy, as the A_{404}/A_{280} ratio was only 0.12 and reconstitution of this material with hemin was successful. Finally, there is a small reduction in the symmetry of the crystal field for each of the Trp-51 mutants, as is shown in Table I by an increase in E/D for both the axial and rhombic EPR signals of the mutants with respect to the wild-type enzyme.

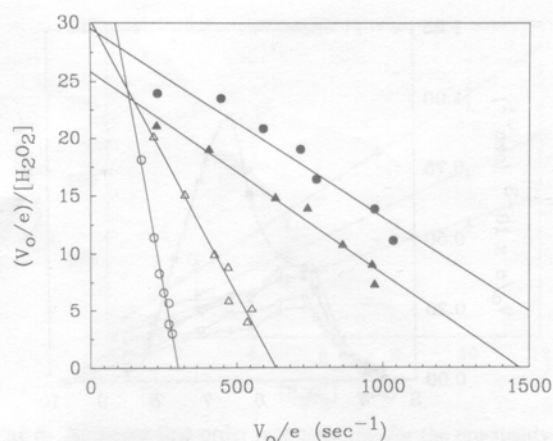


FIGURE 3: Eadie-Hofstee plot for the steady-state oxidation of reduced cytochrome *c* by CCP(MKT) and the mutant W51F. Kinetics were performed as described in the text at 25 °C in 20 mM Tris/phosphate, pH 6.5 (horse heart cyt *c*), or 100 mM potassium phosphate, pH 6.4 (yeast cyt *c*). These conditions of ionic strength have been shown to give optimum rates for the respective substrates (Kang et al., 1977). H_2O_2 concentrations ranged from approximately 9 to 133 μM . Kinetics were performed at 25 μM cyt *c*, and the CCP concentration was adjusted to between 25 and 250 pM to give cyt *c* oxidation rates of approximately $\Delta A_{550} = 0.002 \text{ s}^{-1}$. (○) CCP(MKT) oxidation of horse heart cyt *c*; (Δ) CCP(MKT) with *S. cerevisiae* (yeast) cyt *c*; (●) W51F with horse heart cyt *c*; (▲) W51F with yeast cyt *c*. The lines shown are linear least-squares fits to the data.

Stability of Peroxide Oxidized Forms. The replacement of Trp-51 with each of the residues described in this study results in a distinct effect on the stability of the compound I intermediate or on its reactivity toward H_2O_2 . W51F reacts with H_2O_2 to give an oxidized species containing the Trp-191 free radical (Goodin et al., 1987; Sivaraja et al., 1989) but which decays in the absence of reducing substrate at an enhanced rate (Fishel et al., 1987). We observe that W51F, W51M, W51T, and W51A each react with H_2O_2 at pH 6 to give a species with absorbance maxima that range on one extreme from 411, 528, and 552 nm for W51M to 418, 530, and 556 nm for W51F, consistent with an oxidized ferryl center. At 25 °C, these oxidized species decay exponentially with half-times of 1.8, 0.4, 0.8, and 1.0 min, respectively, considerably faster than observed for the wild-type enzyme (213 min). We observe only small changes in the UV/visible spectra of W51C or W51G upon stoichiometric addition of H_2O_2 . This indicates that these mutants, which are predominantly in a low-spin configuration, are relatively unreactive toward H_2O_2 .

Steady State Kinetic Properties of Trp-51 Mutants. Under steady-state conditions, the mutant W51F exhibits a higher turnover rate than the wild-type enzyme for cytochrome *c* from both yeast and horse heart. Data showing the initial turnover rate of CCP(MKT) and W51F as a function of H_2O_2 concentration are shown in Figure 3. Kinetic parameters are presented for both horse heart cytochrome *c* and *S. cerevisiae* (yeast) cytochrome *c*. Although the steady-state kinetics of CCP as a function of cyt *c* concentration have been shown to give complex, nonlinear Eadie-Hofstee plots (Kang et al., 1977; Kang & Erman, 1982), the data of Figure 3 with respect to H_2O_2 concentration exhibit linear behavior. Thus, well-defined values for $k_{\text{cat}}(\text{app})$ and K_m can be determined, and these values are presented in Table II. As previously reported, $k_{\text{cat}}(\text{app})$ for oxidation of horse heart cytochrome *c* is 6 times greater for the mutant W51F than for wild-type CCP(MKT) (Goodin et al., 1987; Fishel et al., 1987). In addition, the data of Table II show that when measured with yeast cytochrome *c*, W51F has a $k_{\text{cat}}(\text{app})$ that is about twice that for CCP-

Table II: Steady-State^a and Pre-Steady-State^b Rate Constants for CCP

	$k_{\text{cat}}(\text{app})$ (s^{-1})	K_m (μM)	$k_{\text{cat}}(\text{app})/K_m$ ($\mu\text{M}^{-1} \text{s}^{-1}$)	k_1^b ($\mu\text{M}^{-1} \text{s}^{-1}$)
Horse Heart Cytochrome <i>c</i>				
CCP(MKT)	298 (20)	7.1 (0.4)	42 (2)	30.3 (0.9)
W51F	1800 (200)	61 (7)	30 (2)	22 (1)
Yeast Cytochrome <i>c</i>				
CCP(MKT)	630 (60)	21 (2)	31 (2)	
W51F	1500 (100)	57 (4)	26 (1)	

^aKinetics at 25 °C were determined for the oxidation of the indicated cytochrome *c* as a function of $[\text{H}_2\text{O}_2]$. Horse heart cyt *c* kinetics were performed in 20 mM Tris/phosphate, pH 6.5. Yeast cyt *c* kinetics were in 100 mM potassium phosphate, pH 6.4. Parameters were obtained from a fit to the data of Figure 3, and errors correspond to standard deviations (in parentheses) in the fitted values. ^bSecond-order rate constant for the pre-steady-state oxidation of CCP by H_2O_2 at 25 °C in 100 mM potassium phosphate, pH 6. Values are included for comparison to those of $k_{\text{cat}}(\text{app})/K_m$.

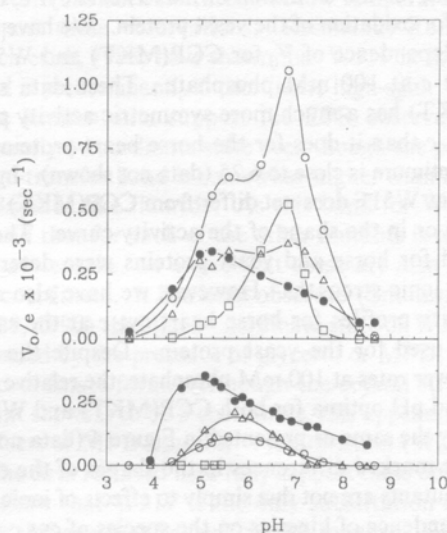


FIGURE 4: Kinetic profile as a function of pH for the steady-state oxidation of horse heart cyt *c* by CCP mutants at Trp-51 at 25 °C. Data were determined as in Figure 1 in 20 mM Tris/phosphate at 95 μM H_2O_2 , 23 μM cyt *c*, and 250–2500 pM CCP. Values of pH were measured for each reaction after kinetics. Curves are drawn only to aid interpretation of the data. Upper panel: (●) CCP(MKT); (○) W51F; (Δ) W51M; (□) W51T. Lower panel: (●) CCP(MKT); (○) W51C; (Δ) W51A; (□) W51G.

(MKT). It has been shown that yeast CCP oxidizes yeast cyt *c* more efficiently than horse heart cyt *c* (Ho et al., 1985; Hazzard et al., 1987, 1988; Kang et al., 1977). This is evident in Table II by the fact that $k_{\text{cat}}(\text{app})$ for CCP(MKT) measured with yeast cyt *c* is approximately 2-fold greater than that for horse heart cyt *c*, measured at their respective optimum ionic strengths. It is of interest that the mutant enzyme W51F does not show this preference for yeast cyt *c*. The data in Table II show that $k_{\text{cat}}(\text{app})$ for horse heart cyt *c* is at least as large as that for the yeast protein.

A number of substitutions at Trp-51 were examined to determine if the kinetic effects observed for W51F are due to specific interactions of tryptophan that are lost on mutation, or if the effects are peculiar to the placement of phenylalanine at this position. The pH dependence of the initial rate data for horse heart cyt *c* is presented for these mutants in Figure 4. In agreement with earlier data (Goodin et al., 1987), the wild-type enzyme gives an asymmetric pH profile with a maximum at pH 5.25 and a gradually declining kinetic rate as the pH is increased above this value. For the Trp-51 mutants, two distinct types of pH dependence are observed. Replacement of Trp-51 by Phe, Met, or Thr results in one type

of activity profile shown in Figure 4, in which the optimum rate for the mutant is shifted 1.5 units to pH 6.8 and $k_{\text{cat}}(\text{app})$ is greater at this pH than the wild-type enzyme. W51F clearly exhibits the most extreme rate enhancement. It and W51M show two peaks in activity, at pH 5.25 and 6.8. The second type of behavior is observed for W51C and W51A, as shown in Figure 4. These mutants are less active than wild-type enzyme and show a symmetric pH profile with optimum activity at pH 5.75, which is intermediate between that of wild-type enzyme and the hyperactive mutants of Figure 4. Finally, the mutant W51G showed no observable activity [$<1\%$ of that for CCP(MKT)] under these conditions (Figure 4). These data show that a range of functional properties can be produced by altering Trp-51 and that amino acids other than phenylalanine can to a variable degree produce the hyperactivity observed for W51F.

Although W51F and the wild-type enzyme exhibit different pH optima for the oxidation of horse heart cyt *c*, this is not the case for oxidation of the yeast protein. We have measured the pH dependence of V_0 for CCP(MKT) and W51F using yeast cyt *c* at 100 mM phosphate. These data show that CCP(MKT) has a much more symmetric activity profile for yeast cyt *c* than it does for the horse heart protein and that the pH optimum is close to 6.25 (data not shown). In addition, the mutant W51F does not differ from CCP(MKT) in the pH optimum or in the shape of the activity curve. The kinetics described for horse and yeast proteins were determined at different ionic strengths. However, we have also measured the activity profiles for horse heart cyt *c* at the same ionic strength used for the yeast protein. Despite the expected overall lower rates at 100 mM phosphate, the relative activities, shape, and pH optima for both CCP(MKT) and W51F were essentially the same as presented in Figure 4 (data not shown). Thus, the marked differences in the shapes of the curves for Trp-51 mutants are not due simply to effects of ionic strength. This dependence of kinetics on the species of cyt *c* used suggests a sensitivity to features of the CCP-cyt *c* interaction.

Insensitivity of Kinetics to Naturally Occurring Amino Acid Variation. It has been noted that there are two amino acid differences between the gene cloned from the laboratory strain of *S. cerevisiae* D273-10B, which contains Ile-53 and Gly-152 (Kaput et al., 1982), and the protein sequence of CCP obtained from commercial bakers' yeast, with Thr-53 and Asp-152 (Takio et al., 1980). The identity of the residues in the bakers' yeast enzyme has been confirmed by X-ray crystallography (Finzel et al., 1984). The residue at position 152 is on the surface of the molecule in a region that is remote from both the heme pocket and the proposed cyt *c* binding site of CCP. Thr-53, however, is part of the distal α -helix containing the active-site residues Arg-48, Trp-51, and His-52. Thr-53 forms a bifurcated hydrogen bond to the carbonyl oxygens of Ala-50 (3.2 Å) and Leu-49 (3.1 Å) and may thus provide an important stabilizing interaction for this helix. Such H-bonding interactions will be absent in the recombinant enzyme containing Ile-53. A difference Fourier map of a Thr-53 substitution in this enzyme shows minor shifts in the distal helix with a resulting movement of the active-site His-52 by 0.5 Å (Wang et al., 1990). It is thus of importance to determine whether the differences in functional properties observed in this study could be due to the combined effect of the Trp-51 mutation with the repositioning of the distal helix of CCP(MKT) containing Ile-53.

Although minor effects on the turnover rate are noted for alterations at position 53, the dramatically different behavior of W51F is unrelated to amino acid changes between the two

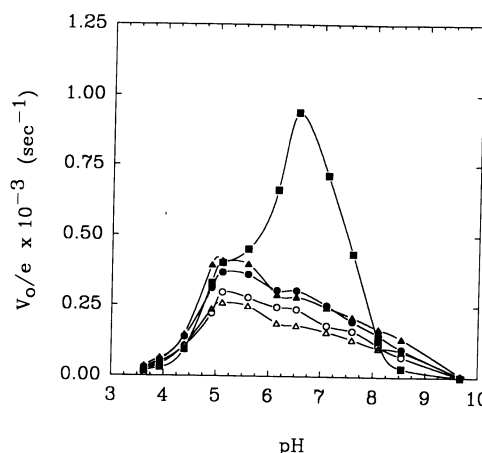


FIGURE 5: Kinetic profile for the oxidation of horse heart cyt *c* by variants of CCP representing differences observed in sequence between yeast strains. Steady-state kinetics were determined as described in Figure 4. Data are presented for (Δ) G152D, (\circ) CCP(MKT), (\blacktriangle) G152D, I53T, (\bullet) I53T, and (\blacksquare) W51F, I53T.

strains of yeast. To test for these effects, a number of mutant combinations were constructed in which both Gly-152 and Ile-53 were replaced individually and together for enzymes containing Trp-51. In addition, the double-mutant W51F,I53T was constructed. Each of these enzymes crystallizes normally, and no effects on the physical properties and optical spectra are evident (data not shown). The initial turnover number for oxidation of horse heart cyt *c* is shown as a function of pH for these enzyme variants in Figure 5. These results show that replacing Ile-53 with Thr slightly increases (approximately 20%) the initial rate but does not measurably affect the pH profile. In either of these backgrounds, the mutant G152D has no significant effect on the steady-state functional properties. Notably, regardless of the identity of the residue at position 53, each mutant containing Trp-51 exhibits the characteristics of the wild-type enzyme, with an asymmetric pH profile and a maximum rate at pH 5.25, while the variants containing Phe-51 show the characteristic rate enhancements and pH shifts discussed above. Thus, the effects presented for the W51F mutant are a property of the alteration at position 51 and not to more complicated secondary effects caused by differences in the two isozymes.

Pre-Steady-State Kinetics for the Formation of Compound ES. One might expect that mutations at Trp-51 would alter the rate of reaction of the enzyme with H_2O_2 . Trp-51 forms part of the distal active-site cavity in which the indole nitrogen makes a hydrogen bond with water-595 (Finzel et al., 1984). This water molecule is 2.4 Å from the 5-coordinate heme iron, slightly too far removed to be considered the sixth axial ligand. As shown above, mutations at Trp-51 alter the axial heme coordination state, and thus the rate of binding or oxidation of the ferric enzyme by H_2O_2 could be affected.

Stopped-flow kinetic measurements for the formation of compound ES were made for CCP(MKT), for the hyperactive W51F, and for W51A, which is less active than the native enzyme. Our results using CCP(MKT) agree well with those reported earlier for the yeast-derived enzyme. The second-order rate constant for this reaction at 25 °C has been reported to be $4.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Loo & Erman, 1975; Vitello et al., 1990a) and $3.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Nicholls & Mochan, 1971). More recent experiments at 2 °C have shown biphasic kinetics, with a fast component of $1.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ corresponding to the earlier results, and a much slower, peroxide-independent phase of 10^{-1} s^{-1} (Balny et al., 1987). Using CCP(MKT) at 25 °C, we observe the biphasic kinetics as noted by Balny et

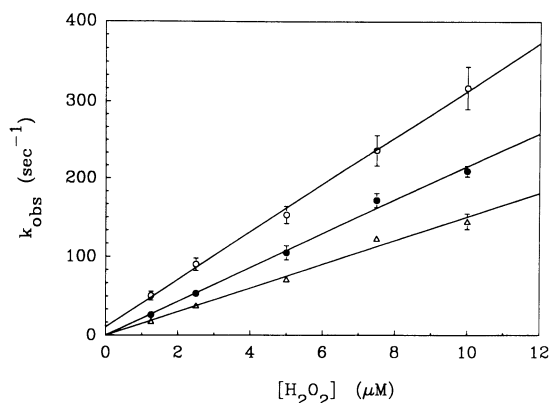


FIGURE 6: Apparent first-order rate constants for the pre-steady-state formation of compound ES. Reactions were carried out at 25 °C in 100 mM phosphate, pH 6, by measuring the increase in absorbance at 424 nm after mixing CCP at 1.0 μM final concentration with H₂O₂. The apparent first-order rate constants were determined by least-squares fit of the data to a single exponential and shown as a function of H₂O₂ concentration. Reactions performed for (○) CCP(MKT), (●) W51F, and (Δ) W51A.

al. (1987) with a fast component dependent on H₂O₂ concentration, and a slow, peroxide-independent phase. In good agreement with this work, the slow phase was observed with a first-order rate constant of 4.9 s⁻¹ and contributed approximately 30% to the amplitude of the total absorbance change. This slow kinetic phase was completely absent for both W51F and W51A mutant enzymes. The fast phase was analyzed by fitting the early data (*t* < 50 ms) to a single exponential with a variable offset. The pseudo-first-order rate constants for the fast phase are plotted as a function of [H₂O₂] for the three enzyme variants in Figure 6. From these data, we obtain a second-order rate constant for CCP(MKT), *k*_{1(fast)}, of 3.03 (0.09) × 10⁷ M⁻¹ s⁻¹, in excellent agreement with the work of Nicholls and Mochan (1971). The rate constants observed for W51F and W51A are 2.2 (0.1) × 10⁷ and 1.5 (0.1) × 10⁷ M⁻¹ s⁻¹, respectively. Thus, both Trp-51 mutations decrease the rate of compound ES formation, but not to a dramatic degree.

DISCUSSION

Previous studies have indicated that the pH dependence often observed in the position of the charge-transfer transition at 645 nm is due to an unknown variation in the protein related to storage or method of preparation (Yonetani & Anni, 1987; Vitello et al., 1990b). This variation results in the conversion to a 6-coordinate high-spin species at low pH that appears to slowly interconvert with the 5-coordinate native conformation. Although the nature of this modification was not understood at the time these studies were performed, several variations in the protein purification strategy were tried, and the one described here was developed to minimize the pH dependence of the enzyme. The preparation method used for these studies gives wild-type enzyme that is approximately 75% in the 5-coordinate conformation and that exhibits electronic spectra that are fully independent of pH between pH 5.5 and 7. We have recently determined that the modification of a specific methionine residue during culture or isolation can cause the above effects, and we will discuss these results in detail elsewhere (Goodin et al., unpublished results). In addition, we have found that the presence or absence of this modification does not significantly affect the steady-state or pre-steady-state kinetic parameters discussed in this work.

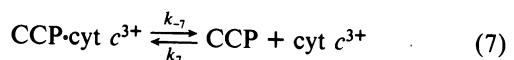
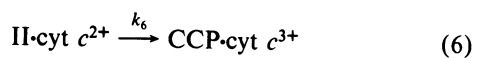
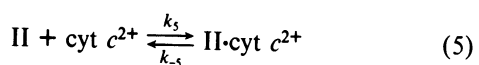
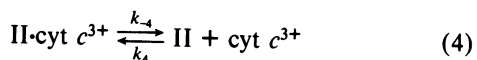
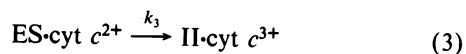
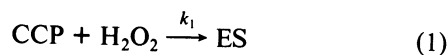
Several conclusions about the effects of Trp-51 substitutions on heme coordination can be drawn from examination of several different substitutions at the same site. The charge-

transfer bands observed for the mutants range from 631 to 636 nm. This is higher than typically observed for 6-coordinate CCP when fluoride or acetate serves as the sixth ligand, and suggests that the mutants are possibly mixtures of 5- and 6-coordinate forms. We have examined both W51F and W51A in the presence of fluoride and find that both bands are indeed at 620 nm. However, it is not known where the charge-transfer band lies in the fully H₂O-coordinated form of CCP because this form has not been well documented. In fact, for myoglobin-fluoride, this band is observed at 603 nm but shifts to 637 nm in aquometmyoglobin (Smith & Williams, 1968). Our data suggest some contribution from the 6-coordinate form, but the UV/vis spectra alone do not allow further conclusions. In the absence of glycerol, the EPR spectra of the mutants show no evidence for the 5-coordinate form, while in 60% glycerol, only W51F shows a significant population of this form. While it is possible that the coordination state has been perturbed at low temperature, other data support these observations. Resonance Raman data (Smulevich et al., 1988) have shown that W51F in the absence of glycerol is predominantly 6-coordinate high-spin. Thus, the resonance Raman data support the EPR observation of a predominantly 6-coordinate state even though the position of the charge-transfer band is somewhat higher than typical of charged ligands like fluoride. As this band for W51F is at 636 nm and that for each of the other mutants is at slightly shorter wavelengths, we argue that these are also predominantly 6-coordinate. It has been observed (Smulevich et al., 1988, 1989, 1990) that the 6-coordinate state of W51F could be suppressed by the presence of glycerol or MPD, resulting in the 5-coordinate form. Although the crystal structure of this mutant shows it to be 5-coordinate, this appears to be due to the effects of MPD used for crystallization (Wang et al., 1990). The data reported here fully support these observations but also show that W51F is the only substitution of the six studied at this site that behaves in this manner. Each of the other substitutions remains predominantly 6-coordinate in up to 50% glycerol. In some of these cases, glycerol prevents facile conversion to the low-spin configuration but does not recover a native 5-coordinate species as observed for W51F. This clearly underscores the delicate nature of the structure of the distal heme cavity of CCP. The role that agents such as glycerol play is not clear but could be related to stabilizing effects on tertiary structure (Smulevich et al., 1989) or partial dehydration of the distal substrate channel. It is likely that alterations other than W51F produce a perturbation that is larger than the small compensating tendency of glycerol to stabilize the native structure. Mutations at other sites, such as the proximal Asp-235, also have the effect of converting this enzyme to a 6-coordinate species (Smulevich et al., 1988; Wang et al., 1990), indicating further that many of the structural features of the active site serve to maintain a delicately poised 5-coordinate heme center.

We have observed a small difference in *E/D* for each of the mutants, indicating that replacement of Trp-51 results in a slightly more rhombic ligand field. It is possible that a small perturbation in the position of the nearby water molecule above the axial coordination position might lead to such a change in anisotropy. Similar effects have been noted in low-spin heme complexes depending on the orientation of the proximal histidine with respect to the pyrrole nitrogens (Quinn et al., 1987). However, this increased rhombicity is observed for both the 5- and 6-coordinate forms of W51F, regardless of the fact that the water position must be significantly different in the two forms. It is possible that a specific π-π interaction between

Trp-51 and the heme pyrrole contributes to the magnetic anisotropy. Just such an effect has been suggested to explain a rhombic perturbation in the NMR chemical shifts for the heme methyl resonances (Satterlee et al., 1983).

Mutationally altered enzymes often show impaired function because a minor structural perturbation at the active site could easily alter substrate binding, transition-state energetics, or positioning of chemically active functional groups. It is thus of some interest to understand the cause of the enhanced activity exhibited by the mutants described above. It may be, however, inaccurate to assume that nature has been outdone in this case, because enzymes are usually not driven under saturating conditions, and evolutionary pressure does not reward k_{cat} unless K_m also increases and remains less than the concentration of substrate *in vivo* (Fersht, 1977). The structural alterations introduced by these changes must necessarily have facilitated the rate-limiting step of the mechanism. It has not been completely clear what this rate-limiting step is, but a strong argument can be made that it is the rate of electron transfer within the CCP-cyt c^{2+} complex. A simple version of the accepted mechanism is presented in eq 1–7:



where ES represents the fully oxidized enzyme containing both the ferryl center and the Trp-191 free radical and II is the half-reduced species. A more complex version of this mechanism in which CCP is allowed to bind to cyt c^{2+} prior to oxidation by H_2O_2 has been analyzed (Kang & Erman, 1982). Such a consideration would not affect the present conclusions provided that the rates for reaction of H_2O_2 with the free and complexed enzyme are similar as has been reported (Kang & Erman, 1982). The complex rate equation resulting from the above mechanism has made meaningful interpretation of initial rate data vs [cyt c^{2+}] difficult. However, considerable simplification results for V_0/e as a function of $[\text{H}_2\text{O}_2]$ at constant saturating [cyt c^{2+}] with the assumption that [cyt c^{3+}] = 0. In this case, V_0/e reduces to a simple Michaelis–Menten form in which $1/k_{\text{cat}} = 1/k_3 + 1/k_6 + 1/k_{-4} + 1/k_{-7}$ and $k_{\text{cat}}/K_m = k_1$. Thus, the value of k_{cat} will be determined by the slowest unimolecular process on the forward reaction pathway. These are the two electron-transfer events, and the rate constant for dissociation of the CCP-cyt c^{3+} complex.

Our values of steady-state turnover are not inconsistent with measured rates of the electron-transfer reactions in eq 3 and 6. Values for k_3 and k_6 have been determined for complexes between compound ES or CCP(Fe^{4+}) and cyt c^{3+} by following the decay of the oxidized CCP after photochemical reduction of the bound cyt c^{3+} (Hazzard et al., 1987, 1988). At low ionic strength, the steady-state rate for enzyme turnover is significantly lower than at the optimum conditions for each type of cyt *c*. This is presumably due to the fact that enzyme

turnover is limited by complex dissociation at low ionic strength. By considering the steady-state rates determined at optimum ionic strength (20 mM Tris/phosphate, pH 6.5, for horse heart cyt *c* and 100 mM phosphate, pH 6.4, for yeast cyt *c*), we hope to suppress rate-limiting complex dissociation in order to see if the maximum turnover rate compares favorably with the known electron-transfer rate constants. The intrinsic electron-transfer rate constants have been reported under conditions similar to but not identical with those we used for optimal turnover. For horse heart cyt *c*, k_3 was reported as 730 s^{-1} in 3 mM phosphate, pH 7 (Hazzard et al., 1987), and 1460 s^{-1} for the yeast protein at 100 mM phosphate, pH 7 (Hazzard et al., 1988). It was also shown for horse heart cyt *c* that $k_6 = 0.62k_3$ (Hazzard et al., 1987). Thus, if product dissociation is not rate-limiting, k_{cat} should correspond to $k_3k_6/(k_3 + k_6)$ or $0.38k_3$. This gives an estimated k_{cat} limited by electron transfer of 277 s^{-1} for horse heart cyt *c* and 555 s^{-1} for the yeast protein, assuming a similar relationship between k_3 and k_6 . These values compare very well with the $k_{\text{cat}}(\text{app})$ values reported here of 298 and 630 s^{-1} , respectively. Considering that the values for $k_{\text{cat}}(\text{app})$ determined at $25 \mu\text{M}$ cyt *c* represent lower limits to the true value of k_{cat} , it is clear that if the cyt c^{3+} dissociation rates (eq 4 and 7) were much slower than the electron-transfer rates, then $k_{\text{cat}}(\text{app})$ would be smaller than observed. Thus, under these conditions, $k_{\text{cat}}(\text{app})$ for the wild-type protein appears to be limited not by dissociation of the protein–protein complex but by electron transfer. This indicates that substitution of phenylalanine for Trp-51 increases this limiting electron-transfer rate.

Pre-steady-state kinetics for compound ES formation were performed to examine the effect of the distal cavity mutants on the reactivity of the heme for H_2O_2 . This was particularly relevant because of the varied effects of the mutations on the heme coordination state. Our results provide two significant observations. The rate constant for the fast phase, representing the bimolecular reaction of ferric enzyme with H_2O_2 , has become slightly slower for both W51F and W51A. The reduced rate constants for these two mutants could be due to the fact that they are partially (in the case of W51F without glycerol) or predominantly (W51A) in a 6-coordinate conformation and must undergo ligand exchange before reacting with H_2O_2 . It is in fact somewhat surprising that the rate is not more seriously impaired considering the dramatic alteration in coordination state for these mutants. Alternatively, it is possible that Ala and Phe, which have smaller side-chain volumes than tryptophan, have increased the size of the “cage” around the peroxide binding cavity, leading to effects related to the probability of successful encounters of peroxide trapped within the channel. While it is possible that this reduced rate could account for the impaired turnover rate of W51A, it is significant that W51F nevertheless exhibits increased activity over wild-type enzyme. This observation is consistent with the above conclusion that the rate-limiting step is not compound ES formation but electron transfer. The second significant observation is that neither of the mutants exhibits the slow phase of the reaction. This slow phase has been interpreted as arising from the slow peroxide-independent conversion of the 6-coordinate to 5-coordinate form of the enzyme as the 5-coordinate form is depleted by the reaction. Our results show that this explanation cannot be complete, because both of the mutants are predominantly 6-coordinate, yet they react rapidly and exhibit no slow component. It is possible that the slow component represents some slow protein conformational shift from an inactive to active form and that the mutations have prevented this conformational equilibrium.

The results of the steady-state kinetic parameters support these conclusions. As discussed above, $k_{\text{cat}}(\text{app})/K_m$ with respect to $[\text{H}_2\text{O}_2]$ should be a measure of the bimolecular rate constant, k_1 , for the formation of ES, and the observed values of $k_{\text{cat}}(\text{app})/K_m$ agree well with the stopped-flow data. In addition, the mutation W51F slightly decreases the value of $k_{\text{cat}}(\text{app})/K_m$ and, as expected, this parameter is not sensitive to the type of cyt *c* used.

The above suggestion that W51F significantly alters the rate of electron transfer could have several implications concerning the role of Trp-51. It is possible that the alteration has affected the average orientation or distance between the heme centers of the CCP-cyt *c* complex to alter the electronic coupling of the two centers. This would have to arise from a long-range effect on the conformation or dynamics of CCP because Trp-51 is buried within the active-site cavity and not near to the areas on CCP implicated in cyt *c* binding. This change in geometry would also have to be such that the electron-transfer event is made more efficient. A similar change would have to occur for each of the several substitutions (W51F, W51M, and W51T) that show similar behavior, and the effect would have to operate for horse heart as well as yeast cyt *c*. Finally, the crystal structure of a mutant CCP containing the W51F mutation shows little evidence for such long-range structural perturbation. Thus, we consider long-range effects an unlikely explanation of the observed behavior. Instead, we propose that alteration of Trp-51, which makes direct van der Waals contact with the distal heme face, has a direct effect on the free energy of the ferryl heme center. Such an effect could arise from changes in the local dielectric constant, water occupation, or specific π interactions of the residue at position 51 with the heme. Another intriguing possibility was raised by Wang et al. (1990) in which the loss of the H-bond provided by the Trp-51 indole proton to the oxyferryl oxygen atom increases the energy stored in the ferryl center. Whatever the nature of the interaction, perturbing this site could result in a change in the energies of unoccupied acceptor states of the heme and result in an increase in driving force for the electron-transfer process. It is significant that the ES intermediate for each of the mutants decays more rapidly than that of the wild-type enzyme, although it is not clear from these data why W51A has reduced activity. These results support the notion of a more reactive ES complex for these mutants.

The above proposal can account for the fact that W51F shows no preference for yeast cyt *c* over the horse heart protein. There is significant evidence for intrinsic differences in the electron-transfer rates between different species of cyt *c* (Ho et al., 1985; Hazzard et al., 1987, 1988). These studies have concluded that slightly different geometries are achieved in the complexes of the respective proteins with CCP and that these differences result in differing electron-transfer probabilities. Horse cyt *c* requires low ionic strength for effective binding to CCP, whereas yeast cyt *c* will bind at higher ionic strength (Erman & Vitello, 1980; Kang et al., 1977). It is possible that CCP binds to horse heart cyt *c* at low ionic strength with more than one geometry as suggested by Northrup et al. (1988) but that these complexes are not of optimum geometry for electron transfer. In this case, overall enzyme turnover would be limited by the electron-transfer process, as discussed above. By increasing the driving force for electron transfer, several mutations at Trp-51 could all have the common effect of dramatically increasing the rate of the limiting step in the turnover of horse cyt *c*. Yeast cyt *c* at 100 mM phosphate may form a more well-defined, geometrically optimized complex. In this case, the enzyme may become

limited by cyt *c* dissociation or reaction with H_2O_2 , and increasing the driving force for electron transfer would have only a marginal effect. Thus, the cyt *c* species specificity could be lost because the energetically unstable compound ES for the hyperactive mutants no longer requires optimum complex geometry for efficient electron transfer.

The changes in the pH profiles for the mutant enzyme activities are complex and not fully understood. These include the observations that with horse cyt *c*, the wild-type enzyme functions optimally at pH 5.25, the hyperactive mutants at pH 6.75, and the hypoactive mutants at pH 5.8, while there is none of this shift observed with yeast cyt *c*. We cannot convincingly address the reasons for the lowered activity of W51C, W51A, and W51G. However, it is clear that these forms exhibit significantly perturbed heme coordination, and may thus be accompanied by conformational changes in the distal helix. These changes could affect any of the steps in the proposed mechanism. Several conclusions can be made about the hyperactive mutants, however. Accounting for the shift in optimum pH on the basis of direct changes in titratable groups near the active site is difficult. It is unreasonable to consider that a change in the $\text{p}K_a$ of the distal His-52, for example, is responsible for these effects. This is because such a change would most likely affect peroxide binding or heterolytic cleavage, and these steps do not determine the steady-state rate under these conditions. We suggest an alternative explanation consistent with our above conclusions. Following earlier proposals, it is likely that an encounter between CCP and horse heart cyt *c* results not in a single well-defined complex, but in several (Northrup et al., 1988). Each of these may have different electron-transfer efficiencies related to the varying complex geometry. The dissociation constants for these differing complexes may be a function of pH. Thus, horse cyt *c* bound to CCP at pH 5.25 may consist of a different distribution than at pH 6.75. Considering the results of Figure 4, the wild-type enzyme at pH 6.75 may be dominated by a poorly optimized, loosely bound cyt *c*, and mutants such as W51F, W51M, or W51T can thus dramatically influence the overall rate. At pH 5.25, however, the complexes may be more tightly bound, and may limit the increase in turnover rate for the mutants. Our results using the yeast protein are consistent with this as they would not show such behavior assuming that a more well-defined complex is formed. A more detailed examination of the thermodynamic parameters for CCP-cyt *c* binding will be necessary to test these proposals.

Substitutions of Trp-51 of CCP have a diverse range of effects on function, and none of the substitutions examined is unaffected. Most notably, at least three residues at this site result in enzyme that has greater than normal activity, indicating that the identity of this residue affects the rate-limiting step of the enzyme mechanism. We have shown that this residue does not significantly impair the rate of reaction of the enzyme with H_2O_2 . Instead, the results indicate that the rate-limiting electron-transfer step is altered by changes in the physical environment of the heme. Further investigation of the direct electron-transfer rates, reduction potentials, and electronic structure of the heme for these mutants will help to determine exactly how these interactions affect the heme.

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