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Yeast Cytochrome *c* Peroxidase: Mutagenesis and Expression in *Escherichia coli* Show Tryptophan-51 Is Not the Radical Site in Compound I†

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ABSTRACT: Using oligonucleotide-directed site-specific mutagenesis, we have constructed a system for the mutation and expression of yeast cytochrome *c* peroxidase (CCP, EC 1.11.1.5) in *Escherichia coli* and applied it to test the hypothesis that Trp-51 is the locus of the free radical observed in compound I of CCP [Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199–8205]. The system was created by substituting a CCP gene modified by site-directed mutagenesis, CCP(MI), for the *fol* gene in a vector previously used for mutagenesis and overexpression of dihydrofolate reductase. *E. coli* transformed with the resulting plasmid produced the CCP(MI) enzyme in large quantities, more than 15 mg/L of cell culture, of which 10% is holo- and 90% is apo-CCP(MI). The apoenzyme was easily converted to holoenzyme by the addition of bovine hemin. Purified CCP(MI) has the same catalytic activity and spectra as bakers' yeast CCP. A mutation has been made in CCP(MI), Trp-51 to Phe. The Phe-51 mutant protein CCP(MI,F51) is fully active, and the electron paramagnetic resonance (EPR) spectrum, at 89 K, of its oxidized intermediate, compound I, displays a strong sharp resonance at $g = 2.004$, which is very similar to the signal observed for compound I of both bakers' yeast CCP and CCP(MI). However, UV–visible and EPR spectroscopy revealed that the half-life of CCP(MI,F51) compound I at 23 °C is only 1.4% of that observed for the compound I forms of CCP(MI) or bakers' yeast CCP. Thus, Trp-51 is not necessary for the formation of the free radical observed in compound I but appears to exert a significant influence on its stability.

Yeast cytochrome *c* peroxidase (ferrocytochrome *c*:H_2O_2 oxidoreductase, EC 1.11.1.5) is a monomeric 294-residue enzyme (Kaput et al., 1982; Takio et al., 1980) that resides in the mitochondrial intermembrane space and contains a single noncovalently bound ferric protoporphyrin IX as a prosthetic group. It catalyzes the two-electron reduction of hydroperoxides by cytochrome *c*:

$$2H^+ + ROOH + 2\text{cyt } c(Fe^{2+}) \rightarrow 2\text{cyt } c(Fe^{3+}) + ROH + H_2O$$

The study of CCP[1] and its macromolecular redox partner, cytochrome *c*, affords insight into the enzymic mechanism of O–O bond cleavage, as well as the nature of macromolecular recognition and long-distance interprotein electron transfer. It is easily isolated and crystallized and has many similarities to more complex enzyme systems such as the membrane-bound cytochrome oxidases (Malmström, 1982; Hatefi, 1985). CCP is well suited for investigations of the relationship between the three-dimensional structure and the enzymic function since a large body of information already exists concerning its physicochemical properties (Yonetani, 1976), including the amino acid sequence (Takio et al., 1980) and the high-resolution X-ray crystallographic structure (Poulos et al., 1978, 1980; Finzel et al., 1984) of CCP from bakers' yeast.

The development of oligodeoxynucleotide-directed site-specific mutagenesis [reviewed by Smith (1985)] has made possible the selective replacement of specific amino acid residues of proteins by manipulation of the nucleotide sequences of their genes. The isolation (Goltz et al., 1982) and nucleotide sequence determination (Kaput et al., 1982) of the CCP gene from a standard strain of *Saccharomyces cerevisiae* opened the door to structure–function studies of CCP using directed

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[1] Abbreviations: CCP, cytochrome *c* peroxidase; DHFR, dihydrofolate reductase; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; ENDOR, electron nuclear double resonance; DEAE-cellulose, (diethylaminoethyl)cellulose; kb, kilobase(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; YT, 8 g of tryptone/5 g of yeast extract/5 g of NaCl per liter; LB, 10 g of tryptone/5 g of yeast extract/10 g of NaCl per liter.

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mutagenesis. Most importantly, the availability of precise geometrical information from the X-ray crystallographic structure permits the formulation of precise questions about the CCP molecule and allows the design of meaningful mutagenesis experiments.

Upon addition of a hydroperoxide, an intermediate is formed, termed compound I (Jordi & Erman, 1974), complex ES (Yonetani, 1966b), or compound ES (Coulson et al., 1971), which is two oxidizing equivalents above the resting (ferric) enzyme and is believed to contain both a ferryl heme iron [Fe^{IV}=O] and a free radical located on some unidentified protein group (Yonetani, 1976). Two principal hypotheses have been advanced regarding the nature and location of this radical, and a considerable body of evidence has been amassed for each. Yonetani et al. (1966c) proposed that an aromatic amino acid residue might be the free radical site, and on the basis of the X-ray crystallographic structure of bakers' yeast CCP, Poulos and Kraut (1980) suggested Trp-51, situated 3.3 Å above and nearly parallel to the heme plane, as the most likely possibility. Arguing from ENDOR spectroscopic data, on the other hand, Hoffman et al. (1979) have suggested that the radical is located either on a dimeric sulfur cation radical [$(R_2SSR_2)^+$] formed from two methionyl residues or on the sulfur atom of a nucleophilically stabilized methionyl residue (Hoffman et al., 1981).

In this paper we report on the design and construction of a system for the expression of yeast CCP in *Escherichia coli* and the generation, by oligodeoxynucleotide-directed mutagenesis, of site-specific mutations. We also describe the use of this system to investigate the possible role of Trp-51 as a free radical site in CCP compound I.

Experimental Procedures

Materials

Reagents. Restriction endonucleases, T4 DNA ligase, T4 kinase, and large fragment *E. coli* DNA polymerase I were purchased from New England Biolabs or Bethesda Research Laboratories (BRL) and used according to the suppliers' instructions. Dideoxynucleotides were obtained from BRL and deoxynucleotides from P-L Biochemicals. Ultrapure urea was purchased from Schwarz/Mann and acrylamide from BDH Chemicals. Trimethoprim, ampicillin, lysozyme, lyophilized horse heart cytochrome *c* (type VI), and bovine hemin (type I) were obtained from Sigma Chemical Co. Hydrogen peroxide (30%, AR grade) was purchased from Mallinckrodt, and Pentex bovine albumin (crystalline) was obtained from Miles Laboratories. All other reagents were of reagent grade. Bakers' yeast CCP, isolated from Red Star yeast, was donated by James Erman (Northern Illinois University).

Radioisotopes. [γ-^{32}P]Adenosine 5′-triphosphate (crude) was obtained from ICN. [^{35}S]Deoxyadenosine 5′-(α-thiotriphosphate) ([α-^{35}S]dATP) and [α-^{32}P]deoxycytidine 5′-triphosphate, tetra(triethylammonium) salt ([α-^{32}P]dCTP), were purchased from New England Nuclear.

Chromatography Supplies. Sephadex G-25 and G-75 were purchased from Pharmacia, Cellex-D (DEAE-cellulose) was purchased from Bio-Rad, and NACS-52 PREPAC columns were purchased from BRL.

Plasmids. YEp13CCP22 in *E. coli* RR1 was generously donated by James Kaput (University of Illinois, Urbana) and YEp13 in E. *coli* HB101 by David Stillman (University of California, San Diego).

Bacterial Strains and Bacteriophage. E. coli K12 JM103 was obtained from New England Biolabs, as was bacteriophage M13mp8 RF DNA (Messing & Vieira, 1982). *E. coli* CC2 (dam$^-$, F′) was generously provided by Charles Craik (University of California, San Francisco).

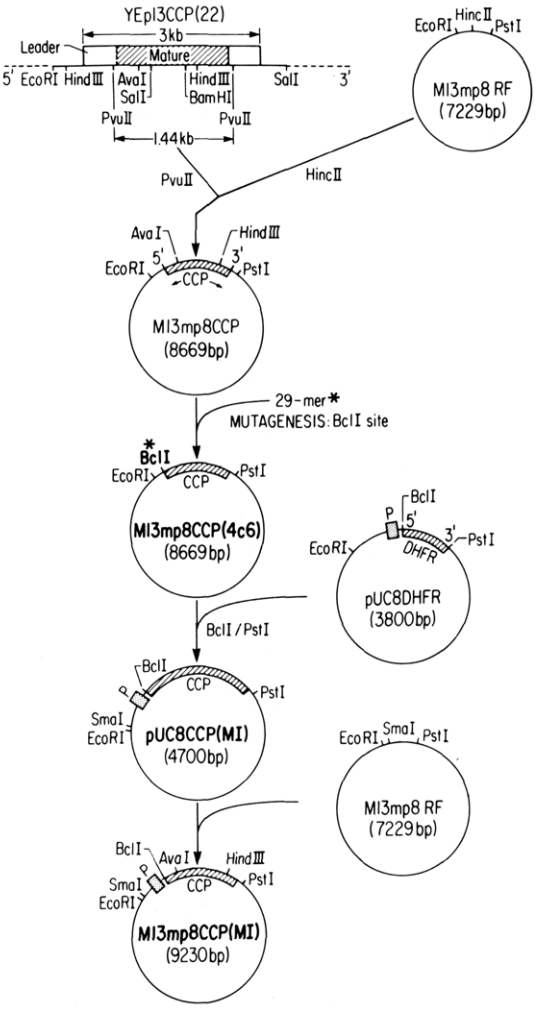

FIGURE 1: Construction of pUC8CCP(MI) and M13mp8CCP(MI). The restriction map of YEp13CCP(22) is as described in Goltz et al. (1982). Dotted lines indicate the remainder of the vector not shown in this figure. The 68 amino acid leader sequence of pre-CCP is denoted as "leader". The CCP inserts in M13mp8 and pUC8 have been drawn to scale in proportion to the total vector sizes. Due to size constraints, the pUC8 vectors have been drawn to the same size as those of M13mp8. (P) denotes the dihydrofolate reductase (DHFR) mutant promoter. Details of the construction of each vector are included in the text.

Methods

Construction and Mutagenesis of CCP Expression System. (A) Isolation of Mature CCP Gene from YEp13CCP22. YEp13CCP22, the plasmid YEp13 carrying an inserted *S. cerevisiae* gene encoding the enzyme cytochrome *c* peroxidase (Goltz et al., 1982; Kaput et al., 1982), was purified by standard methods and digested with the restriction endonuclease *Pvu*II to excise the CCP insert. The insert was identified by comparison with YEp13 also digested with *Pvu*II. This digestion resulted in removal of most of the CCP leader sequence, while leaving the remainder of the gene intact. Isolation and purification of the 1.44-kb fragment coding for mature CCP was accomplished by electrophoresis on a polyacrylamide gel, from which the band containing the single gene was excised and the DNA eluted (see Figure 1).

The purified mature CCP gene was then blunt-end ligated into *Hinc*II-cleaved bacteriophage M13mp8 RF DNA and the resulting vector used to transfect *E. coli* JM103. Transfected cells were plated onto a lawn of *E. coli* JM103 in the presence

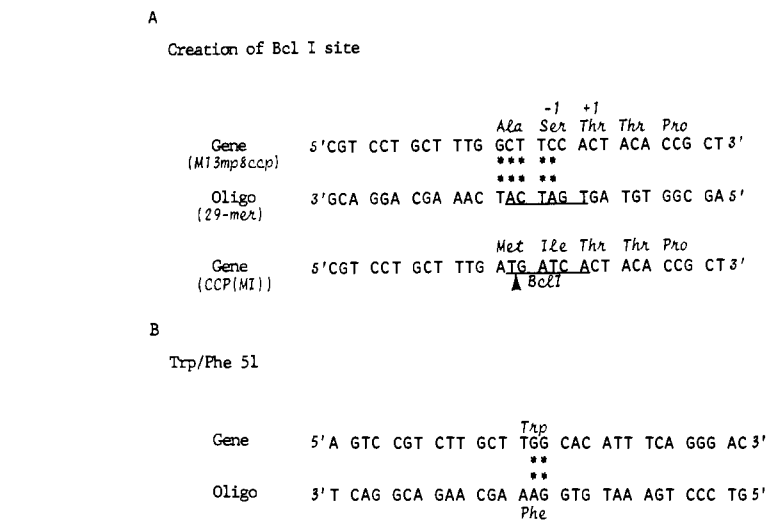

FIGURE 2: Mutagenic oligonucleotides and their complementary CCP gene sequences. (A) depicts the creation of the *Bcl*I site at the 5′ terminus of the mature yeast CCP gene. The numbering (−1/+1) indicates the demarcation line between the pre-CCP and mature CCP sequences, as reported by Kaput et al. (1982). Asterisks (*) denote mismatches between the gene and the oligonucleotide. The *Bcl*I restriction site is underlined, with a wedge (▲) marking the site of cleavage by *Bcl*I. (B) depicts the oligonucleotide used for the mutagenesis substituting Phe for Trp at position 51 of the CCP amino acid sequence.

of IPTG and X-Gal. Colorless plaques formed by cells infected with phage containing the *Pvu*II restriction fragment were removed for larger scale culture and analysis. Preparation of DNA from the plaques, followed by polyacrylamide gel electrophoresis of restriction endonuclease digests using *Eco*RI, *Pst*I, and *Bam*HI, allowed identification of those carrying the CCP insert. A single-stranded M13mp8CCP DNA template was then prepared (Sanger et al., 1980).

(*B*) *DNA Sequence Analysis.* DNA sequences were determined by the Sanger dideoxy method by using [α-^{32}P]dCTP (Sanger et al., 1977, 1980) or [α-^{35}S]dATP according to the modification of Biggin et al. (1983), with synthetic oligodeoxynucleotide primers complementary to regions of the CCP gene spaced 250–300 nucleotides apart.

(*C*) *Creation of a Met Start Codon and BclI Restriction Site at the 5′ Terminus of CCP.* This work was begun with a 29-mer oligodeoxynucleotide synthesized manually according to the phosphotriester method (Miyoshi et al., 1980), the sequence of which is shown in Figure 2A. Subsequent oligodeoxynucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. Synthetic oligodeoxynucleotides were radioactively labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP. The labeled products were separated from unincorporated label by chromatography on NACS-52 columns according to the manufacturer's instructions. Prior to its use in mutagenesis, the 29-mer was phosphorylated at the 5′ terminus with T4 polynucleotide kinase and ATP. It was then tested as a primer for dideoxy sequencing and found to give a uniquely interpretable pattern on a sequencing gel (Zoller & Smith, 1982; Villafranca et al., 1983).

Oligodeoxynucleotide-directed site-specific mutagenesis was conducted essentially as described by Zoller and Smith (1982). Following extension by *E. coli* DNA polymerase I (large fragment) and ligation by T4 DNA ligase, the entire extension–ligation mixture was used to transfect *E. coli* JM103 without further purification or processing. Screening for plaques containing mutant M13mp8CCP DNA was done by dot-blot hybridization with the mutagenic ^{32}P-labeled primer as a probe (Wallace et al., 1981; Zoller & Smith, 1982). Dissociation temperature differences were used to distinguish between wild-type and mutant phage. To obtain homogeneous mutants, positives at this stage were plaque-purified by infection of *E. coli* JM103 with mutant phage. Presumptive mutants were once again identified by dot-blot hybridization with the ^{32}P-labeled mutagenic oligonucleotide.

Use of the 29-mer resulted in the creation of a Met codon at the 5′ terminus of the mature *S. cerevisiae* CCP gene sequence, followed by an Ile codon, to form a *Bcl*I restriction site. The addition of the Met initiation codon was necessary to allow expression of the mature CCP gene followed by production of the mature CCP protein in an *E. coli* host. In yeast, mature CCP is obtained only after removal of the leader sequence by intramitochondrial proteolytic cleavage (Maccecchini et al., 1979; Kaput et al., 1982).

In order to generate DNA that can be cleaved by *Bcl*I, it is necessary to use a strain of *E. coli* deficient in adenine methylase. *E. coli* CC2 (dam$^-$, F′) was infected with the mutant M13mp8CCP phage isolated from *E. coli* JM103. DNA minipreps [RF DNA prepared from 5-mL cell cultures according to the method of Holmes and Quigley (1981)] of the M13mp8CCP DNA were digested with the restriction endonucleases *Bcl*I and *Pst*I or *Eco*RI and *Pst*I to confirm both the presence and the correct size of the CCP insert containing the *Bcl*I restriction site. Following determination of the nucleotide sequence of the CCP insert by using [α-^{35}S]dATP, a mutant designated M13mp8CCP(4c6) was selected for further manipulations.

(*D*) *Construction of pUC8CCP(MI) and M13mp8CCP(MI).* M13mp8CCP(4c6) RF DNA was digested with *Bcl*I and *Pst*I to separate the CCP(4c6) gene from the bacteriophage DNA. The gene encoding the enzyme dihydrofolate reductase (DHFR) was removed from its vector pUC8DHFR in the same manner. The plasmid pUC8DHFR contains, in addition to the DHFR gene, a mutant high-efficiency DHFR promoter (Smith & Calvo, 1982; Villafranca et al., 1983). The excised CCP gene was ligated into the *Bcl*I/*Pst*I-cleaved pUC8DHFR in place of the DHFR gene with T4 DNA ligase. The product of the coupling of the CCP(4c6) gene with the DHFR mutant promoter in pUC8 was designated pUC8CCP(MI).

The pUC8CCP(MI) ligation mixture was then used to transform the dam$^-$ *E. coli* strain SK383 (Zieg et al., 1978), according to methods described in Maniatis et al. (1982). Putative pUC8CCP(MI)-containing colonies were identified by replica plating on three types of agar plates—YT, YT/ampicillin (200 μg/mL), and minimal/trimethoprim (50 μg/mL). Since the pUC8 plasmid confers ampicillin resistance upon its host, growth on YT/ampicillin agar plates indicated successfully transformed colonies. Cells containing pUC8DHFR, and therefore expressing the gene for dihydrofolate reductase, showed resistance to trimethoprim. Cells containing pUC8CCP(MI) grew on YT and YT/ampicillin plates but were unable to survive in the presence of trimethoprim, while cells containing pUC8DHFR were able to survive on all three types of media. By comparisons of the YT/ampicillin and minimal/trimethoprim plates, it was possible to identify pUC8CCP(MI)-containing cells. These colonies were picked, and their plasmid DNA was isolated from DNA minipreps. Restriction digestions of the DNA samples confirmed the presence of the CCP insert in the pUC8 plasmid, as well as its size.

Finally, the entire CCP insert, including the promoter region, was excised from pUC8CCP(MI) with the restriction enzymes *Sma*I (or *Eco*RI) and *Pst*I and then ligated into

M13mp8 cleaved with the same enzymes. After propagation in *E. coli* JM103, the nucleotide sequence of the CCP insert and the promoter region of the resultant M13mp8CCP(MI) phage template was determined, in order to confirm that no unexpected mutations had been generated in either the CCP gene or the DHFR mutant promoter sequence.

(*E*) *Mutagenesis on M13mp8CCP(MI): Trp-51 → Phe.* A mutation was generated to replace Trp-51 with a Phe residue, by employing the same methods used for the creation of the *Bcl*I site. The sequence of the oligodeoxynucleotide primer used is shown in Figure 2B. In this case, the M13mp8CCP(MI) template already included the high-efficiency mutant DHFR promoter. Therefore, following mutagenesis, the new CCP insert was ligated directly into the plasmid pUC8, following restriction endonuclease digestion of both vectors with *Eco*RI and *Pst*I (see Figure 1). The ligation mixture containing pUC8CCP(MI,F51) was used to transform *E. coli* SK383, and the transformed cells were plated on L agar containing ampicillin (200 μg/mL).

Colonies were screened by colony hybridization with the ^{32}P-labeled mutagenic oligonucleotide as the primer according to the method of Grunstein and Hogness (1975), as described in Maniatis et al. (1982), and by substituting Whatman paper (541, ashless) for nitrocellulose paper (Gergen et al., 1979). Positives were cultured in LB/ampicillin (200 μg/mL) medium and small amounts of plasmid DNA isolated. Restriction digestions with *Eco*RI and *Pst*I were used to examine the CCP insert sizes. As with the CCP(MI) gene, the CCP(MI,F51) insert was removed from pUC8, ligated into the *Eco*RI/*Pst*I cloning site of M13mp8, and used to transfect *E. coli* JM103. Single-stranded M13mp8CCP(MI,F51) template was prepared and sequenced to confirm the integrity of the mutant CCP insert. The mutant CCP(MI,F51) was then expressed in *E. coli* SK383 as previously done for its parent CCP(MI).

CCP(MI) Protein Isolation and Purification. (*A*) *Cell Growth and Lysis.* In a typical preparation, 12 1-L cultures of *E. coli* SK383 transformed with pUC8CCP(MI) were grown to stationary phase in a shaker, at 37 °C, in LB medium containing 200 μg/mL ampicillin and 0.1% glucose. After centrifugation, the pelleted cells were lysed in 50 mM potassium phosphate, pH 6.0, containing lysozyme and EDTA. This was followed by a freeze/thaw step [CO_2(s)/2-propanol bath and thawing at 4 °C] and the addition of Brij 58 (Godson & Sinsheimer, 1967), DNase I, and RNase A. Sonication of the suspension and the pellet resulting from these procedures was found to improve the yield of CCP. After clarification by centrifugation for 35 min at 43540*g*, the supernatant was dialyzed 12–16 h against 50 mM potassium phosphate and 1 mM Na_2EDTA, pH 6.0, at 4 °C, to allow precipitation of several contaminating proteins to occur. The precipitate was removed by centrifugation at 43540*g* for 20 min prior to gel filtration chromatography. In later experiments, we found that sonication alone was also an efficient method of lysis.

(*B*) *Column Chromatography for CCP(MI) Purification.* The supernatant from the lysozyme/EDTA lysis (about 170 mL) was loaded onto a 5.0 × 180 cm Sephadex G-75 column and eluted with 50 mM potassium phosphate and 1 mM Na_2EDTA, pH 6.0. Fractions were analyzed by SDS-reducing PAGE on 13.8% gels stained with Coomassie Brilliant Blue R. Those containing large amounts of protein comigrating with bakers' yeast CCP were pooled.

(*C*) *Preparation of Holo-CCP(MI).* The following procedures were performed essentially as described by Yonetani (1967). A 5 mM stock solution of bovine hemin was prepared immediately before use by dissolving the hemin in a small volume of 0.1 N NaOH followed by dilution with 50 mM potassium phosphate, pH 7.5. Light was excluded from solutions during all procedures with the dissolved hemin. The pH of the pooled Sephadex G-75 fractions was adjusted to 7.5 by diafiltration with an Amicon concentrator (PM-10 membrane) at 4 °C. The hemin was added to the pooled CCP(MI)-containing fractions and the solution allowed to stand approximately 90 min at 4 °C. At least a 5-fold molar excess of hemin over the estimated CCP(MI) yield was used. The solution was then adjusted to pH 6.0 by addition of 1 M KH_2PO_4 followed by slow dilution with cold H_2O to a final concentration of about 50 mM and loaded onto a 2.5 × 8.5 cm Cellex-D (DEAE-cellulose) column equilibrated with 50 mM potassium phosphate, pH 6.0, following which the column was washed extensively with the same buffer. Bound CCP(MI) was then eluted with a linear 50–500 mM potassium phosphate gradient, pH 6.0. Alternatively, after the 50 mM wash, further washing with 0.1 M buffer until the CCP band spread down to the bottom of the column, followed by elution with 0.5 M potassium phosphate, pH 6.0, was found to be effective (Yonetani & Ray, 1965). Excess hemin remained irreversibly bound to the column. Column fractions were analyzed by SDS-reducing PAGE. The CCP(MI)-containing fractions were pooled, diafiltered, concentrated on an Amicon concentrator (PM-10 membrane) at 4 °C, and further purified by crystallization twice by exhaustive dialysis against H_2O (Yonetani et al., 1966a). The resulting needle crystals were collected by centrifugation and washed with ice-cold H_2O. Aliquots of the crystals suspended in H_2O were frozen in liquid nitrogen and stored at −80 °C. CCP(MI,F51) was also purified according to the above procedures.

Characterization of CCP(MI) and CCP(MI,F51) Proteins. (*A*) *Determination of Extinction Coefficients.* Total hematin content of electrophoretically pure enzyme samples was determined by the pyridine hemochromogen method with ϵ_{557} = 34.8 mM^{-1} cm^{-1} (Paul et al., 1953), and protein content was determined by the modified biuret assay of Yonetani (1961). SDS-reducing polyacrylamide gels were silver-stained according to the method of Morrissey (1981).

(*B*) *Cytochrome c Peroxidase Activity.* Enzyme activity was assayed at 23 °C in 0.02 M KH_2PO_4 adjusted to pH 6.0 with Tris base (Kang et al., 1977). Horse heart cytochrome *c*, reduced with dithionite, was separated from excess reducing agent as described in Yonetani (1966a), except that all operations were carried out under aerobic conditions. The final cytochrome *c* product was more than 98% reduced. H_2O_2 was standardized by titration with potassium permanganate (Kolthoff et al., 1957) and diluted with 0.01 M potassium phosphate, pH 7.0, containing 1 mM EDTA.

For CCP(MI), the apparent K_m for H_2O_2 was determined by holding the cytochrome *c* concentration constant at 35 μM while varying the H_2O_2 concentration from 2.5 to 80 μM. The apparent K_m for cytochrome *c* was determined by holding the H_2O_2 concentration constant at 75 μM while varying the cytochrome *c* concentration from 2.5 to 30 μM. In the case of CCP(MI,F51), the amounts of both substrates were varied simultaneously. The initial concentrations of cytochrome *c* were 2.5, 5.0, 10, and 15 μM, while those of H_2O_2 were 2.5, 5.0, 10, and 30 μM.

For activity assays, a stock solution of either enzyme was prepared in assay buffer containing 0.25 mg of bovine albumin/mL and stored on ice. Reactions were initiated by the addition of 5 μL of the enzyme stock solution to the assay cuvette. Concentrations of enzyme in the 1.0-mL reaction mixture were 2.5 × 10^{-10} M for CCP(MI) and 4.2 × 10^{-11} M

for CCP(MI,F51). A Perkin-Elmer Lambda 3A UV–visible spectrophotometer with attached 3600 data station was used for all assays.

(*C*) *EPR Spectroscopy.* EPR measurements of the resting and compound I forms of each enzyme were conducted by using an IBM ER 200D EPR spectrometer, with attached microwave frequency counter, NMR gaussmeter, and CS9000 computer. Analyses of first-derivative EPR spectra, including double integrations and area determinations, were carried out on the CS9000 computer with the IBM EPR applications package (version 2.0). The spectrometer was operated at X-band (9.4 GHz) with 100-kHz field modulation. The temperature of the double-rectangular (TE_{104} mode) cavity was maintained at 89 K with an IBM variable-temperature unit (ER4111 VT). Only one of the two cavities was used for these experiments. Samples of bakers' yeast CCP, CCP(MI), and CCP(MI,F51) were prepared as described by Yonetani et al. (1966c), on ice, at a final concentration of 0.74 mM. A 10% molar excess of H_2O_2 diluted in 0.1 M potassium phosphate, pH 6.0, immediately before use, or an equal volume of buffer alone, was added. The sample was then mixed gently and transferred to a quartz EPR tube (3-mm i.d.) and the tube immediately immersed in liquid nitrogen.

To compare bakers' yeast CCP, CCP(MI), and CCP(MI,F51) with regard to the number of spins per mole of each enzyme in the compound I state, the following spectrometer operating conditions were used: 54 mW microwave power, 2-G modulation amplitude, 6.32×10^4 receiver gain, and 500-G sweep width around the radical signal. Spectrometer settings used for other spectra are described in the caption for Figure 5.

A preliminary experiment examined the rate of decay of compound I of CCP(MI,F51) at 23 °C. A 0.75 mM solution of the enzyme was prepared at 23 °C in 0.1 M potassium phosphate, pH 6.0. A 10% molar excess of H_2O_2 was mixed with the CCP(MI,F51) and the mixture allowed to stand at 23 °C. At intervals over the next 6.5 min, aliquots were removed to EPR tubes and the tubes frozen immediately in liquid nitrogen. A Varian E-3 spectrometer was used for these measurements, operated at 9.15 GHz, with 100-kHz field modulation. Quartz sample tubes having an i.d. of 3 mm were used. The temperature was maintained at 77 K for all spectra.

Results

DNA Sequencing. DNA sequence analysis of M13mp8CCP(4c6), M13mp8CCP(MI), and M13mp8CCP(MI,F51) revealed no unexpected changes in either the CCP genes or the DHFR mutant promoter region in comparison with the published DNA sequences for CCP (Kaput et al., 1982) and DHFR (Smith & Calvo, 1980, 1982). In one instance, identification of several extraneous bands as artifacts of ^{35}S sequencing was obtained by resequencing the same regions with [α-^{32}P]dCTP. The ^{32}P gels did not contain the artifactual bands (data not shown).

Expression of CCP(MI) in E. coli SK383. Small-scale cell lysates of *E. coli* SK383 transformed with pUC8CCP(MI), when analyzed by SDS-reducing PAGE, revealed a prominent new protein band having the same mobility as bakers' yeast CCP (see Figure 3). Standard activity assays showed the presence of a significant amount of peroxide-dependent cytochrome *c* oxidation in the pUC8CCP(MI)-containing cell lysates, a result that was also observed with pUC8CCP(MI,F51)-transformed cells.

Determination of Apo-CCP(MI) Content in Crude Cell Lysates. Crude lysates of 1-L cultures of pUC8CCP(MI)/SK383 and pUC8/SK383, prepared by the lysozyme/EDTA lysis method described under Methods, were each divided into three fractions of equal volume. Fraction A was left undisturbed at 0 °C. Fractions B and C were adjusted to pH 7.5. Dissolved bovine hemin was then added to fraction B and an equivalent volume of 0.1 N NaOH/50 mM potassium phosphate, pH 7.5, to fraction C. The final pH values of solutions B and C were about 7.5. Fractions B and C were then adjusted to pH 6 by slow addition of 8.5% H_3PO_4 and all three fractions assayed for CCP activity. pUC8CCP(MI)/SK383 fractions A (untreated) and C (pH adjustments, but no hemin addition) had identical activities. Fraction B (pH adjustments with hemin addition) showed about a 9-fold increase in CCP activity. No CCP activity was observed in the pUC8/SK383 lysates that were treated identically (fractions A, B, and C). This experiment, performed in duplicate with identical results, demonstrated that *E. coli* SK383 transformed with pUC8CCP(MI) expresses approximately 90% apoenzyme.

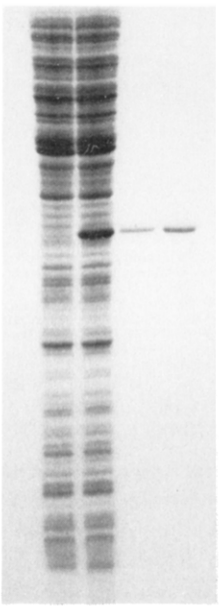

FIGURE 3: CCP(MI) protein production in *E. coli* SK383 visualized by Coomassie Brilliant Blue R staining of an SDS-reducing polyacrylamide gel. Production can be seen by comparison of the crude lysates of the *E. coli* containing the pUC8 vector with (lane B) and without (lane A) the CCP(MI) gene insert. Lanes C and D show CCP(MI) and bakers' yeast CCP, respectively, following final recrystallization by exhaustive dialysis against H_2O. The bottom of the gel is the anode end. Details of the protein purification are described in the text.

Of interest is the finding that the 10% holoenzyme produced in the *E. coli* contains ferric protoporphyrin IX, since the absorption spectrum of its reduced pyridine hemochromogen was identical with those of bovine hemin (ferric protoporphyrin IX) and bakers' yeast CCP (Yonetani & Ray, 1965). Therefore, all of the CCP(MI) expressed by the *E. coli* SK383 and later combined with bovine hemin contains the same prosthetic group—ferric protoporphyrin IX.

Characterization of CCP(MI). (*A*) *Purity and Extinction Coefficients.* The preparation was judged to be homogeneous by the observation of a single band when an SDS-reducing PAGE gel was overloaded and the silver-stained (data not shown). The ratio of absorbance at 408 nm to that at 280 nm (A_{408}:A_{280}) of a typical batch of pure CCP(MI) was 1.31 in 0.1 M potassium phosphate, pH 6.0, at 23 °C. Extinction coefficients determined on the basis of total hematin content, as measured by the pyridine hemochromogen method, were ϵ_{408} = 102 mM^{-1} cm^{-1} and ϵ_{280} = 77.9 mM^{-1} cm^{-1}, in good

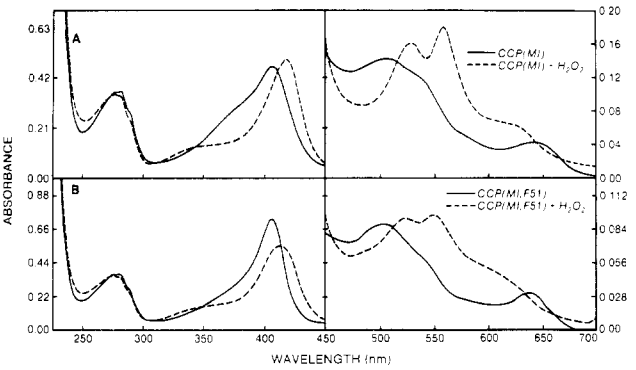

FIGURE 4: UV–visible absorption spectra of resting and compound I forms of (A) CCP(MI) and (B) CCP(MI,F51) in 0.1 M potassium phosphate, pH 6.0, at 23 °C.

agreement with results reported by previous workers with bakers' yeast CCP (Nelson et al., 1977; Yonetani, 1965). Comparison of the protein concentration, measured by the biuret reaction, with the heme content revealed the presence of 1 mol of ferric protoporphyrin IX per mole of CCP(MI). Thus, addition of bovine hemin to the CCP(MI) apoenzyme and subsequent purification yielded a pure product containing only holoenzyme.

(*B*) *UV and Visible Absorption Spectra.* The absorption spectra of CCP(MI) and its compound I, shown in Figure 4A, are indistinguishable from those obtained by Yonetani (1965). To obtain the spectrum of compound I, identical volumes of H_2O_2 were added to both the reference and the sample cuvettes. Compound I was observed to be very stable, having a half-life of several hours at 23 °C, in agreement with the reported stability of bakers' yeast CCP compound I (Yonetani et al., 1966b). On the basis of these criteria, expression of yeast CCP in *E. coli* followed by insertion of bovine hemin yields a normal enzyme.

(*C*) *Kinetic Parameters.* Values were determined from Lineweaver–Burk plots. Data were obtained in 0.02 M Tris–phosphate buffer, pH 6.0, at 23 °C. The results showed CCP(MI) has a k_{cat} of about 500 s^{-1}, an apparent K_m for horse heart cytochrome *c* of about 3.4 μM, and an apparent K_m for H_2O_2 of about 3.1 μM, all in close agreement with kinetic parameters obtained by other investigators for bakers' yeast CCP (Kang et al., 1977) and CCP derived from the same CCP gene we have used but expressed in yeast (Goodin et al., 1986).

(*D*) *EPR Spectroscopy.* Results obtained by EPR spectroscopy of CCP(MI) compound I (Figure 5D) revealed the presence of a strong free radical signal at $g = 2.004$, identical with that observed for bakers' yeast CCP compound I (Figure 5B) and of similar stability (Yonetani et al., 1966c). The EPR spectrum of resting CCP(MI) (Figure 5C) was also essentially identical with that of bakers' yeast CCP (Figure 5A).

To summarize, initial characterization of CCP(MI) and its compound I reveals no detectable differences from the bakers' yeast enzyme with respect to UV–visible spectra, catalytic parameters, or EPR spectra.

Characterization of CCP(MI,F51). (*A*) *Purity and Extinction Coefficients.* SDS-reducing PAGE, followed by silver staining of the gel, showed the preparation to be essentially homogeneous with only very minor amounts of contaminating proteins visible when the gel was overloaded (data not shown). CCP(MI,F51) exhibits the same mobility as CCP(MI). The A_{408}:A_{280} ratio of a preparation of CCP(MI,F51) was 1.97 when measured under the conditions described above for CCP(MI).

Extinction coefficients determined on the basis of total hematin and protein content were ϵ_{408} = 141 mM^{-1} cm^{-1} and

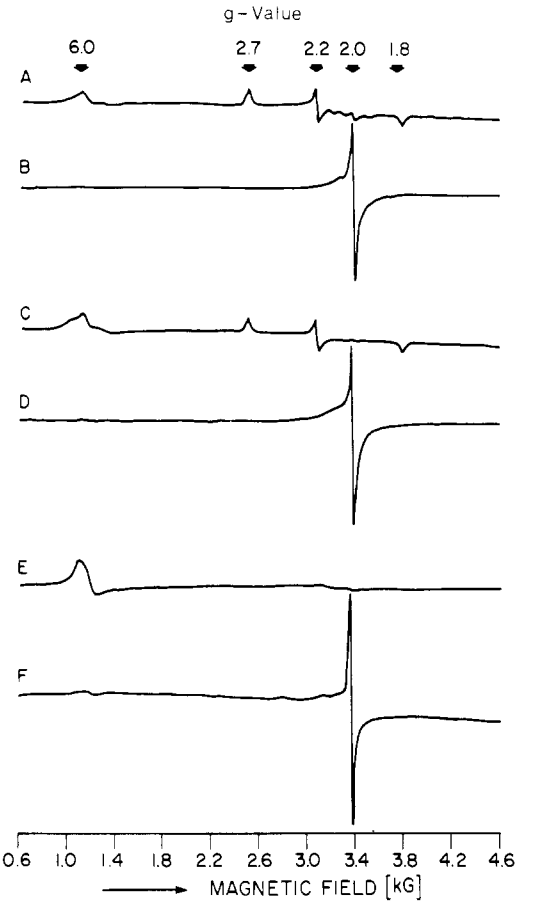

FIGURE 5: First-derivative EPR spectra of resting and compound I forms of bakers' yeast CCP, CCP(MI), and CCP(MI,F51) measured at 89 K: (A) bakers' yeast CCP; (B) bakers' yeast CCP + H_2O_2; (C) CCP(MI); (D) CCP(MI) + H_2O_2; (E) CCP(MI,F51); (F) CCP(MI,F51) + H_2O_2. All spectra were obtained under the following conditions: 0.74 mM final enzyme concentration, 54-mW (6-dB) microwave power, 9.4 GHz, 8-G modulation amplitude, and 7.96 × 10^4 receiver gain.

ϵ_{280} = 71.7 mM^{-1} cm^{-1}. Thus CCP(MI,F51) differs from the parent molecule CCP(MI) in two respects—the ϵ_{408} has increased and the ϵ_{280} has decreased.

(*B*) *UV and Visible Absorption Spectra.* The absorption spectra of CCP(MI,F51) and its compound I are shown in Figure 4B. The ultraviolet and visible spectra are very similar to those of CCP(MI) shown in Figure 4A, although there are some important differences.

The intensity of the Soret absorption of CCP(MI) compound I at 419 nm is greater than that of the resting CCP(MI) enzyme at 408 nm. Interestingly, the corresponding absorption for compound I of CCP(MI,F51) is lower than that of the parent CCP(MI,F51) enzyme at 408 nm, in addition to λ_{max} being located at a wavelength 3–5 nm shorter than that of CCP(MI) compound I.

The spectrum of compound I for CCP(MI,F51), shown in Figure 4B, rapidly decays back to that of the resting enzyme. This spectrum was only obtainable by scanning at the fastest scan speed available on our spectrophotometer, 480 nm/min. The peaks at 525 and 552 nm in Figure 4B were recorded about 20 s after the enzyme was mixed with a stoichiometric amount of H_2O_2. The peak at approximately 419 nm was observed by running a separate scan, and a similar decay rate was observed for this spectral feature. Decay of compound I back to the resting state appears to begin immediately after formation. Thus, we were not able to observe these peaks at their maximum intensity using a stoichiometric amount of

H_2O_2 at 23 °C. A nearly full recovery of the resting-state enzyme spectrum occurred in about 6 min. The half-life of CCP(MI,F51) compound I is reduced by a factor of about 70 from that of compound I of CCP(MI) or bakers' yeast CCP (Yonetani et al., 1966b). Thus, the stability of compound I has been drastically decreased by substituting Trp-51 with a Phe residue.

(*C*) *Kinetic Parameters.* Values derived from Lineweaver–Burk plots and corresponding secondary plots (Cleland, 1963) indicate that CCP(MI,F51) has a k_{cat} 2–4 times greater than that of CCP(MI), with similar increases in the apparent K_m values for H_2O_2 and horse heart cytochrome *c*. Further experiments are needed to determine these kinetic parameters accurately.

(*D*) *EPR Spectroscopy.* The EPR spectrum of CCP(MI,F51) compound I showed a strong free radical signal at $g = 2.004$, with a profile very similar to that observed for both bakers' yeast CCP and CCP(MI) (see Figure 5F). In order to minimize effects due to the observed rapid decay of compound I of the mutant enzyme discussed below, the sample used to obtain the EPR spectrum was prepared on ice and frozen in liquid nitrogen within 1 min. It is of interest to note that both wings of the radical signal are narrower than those of CCP(MI) and bakers' yeast CCP.

An experiment in which CCP(MI,F51) compound I was incubated at 23 °C revealed that the radical begins to decay immediately after its formation. By about 6 min after the enzyme was mixed with a 10% molar excess of H_2O_2, the appearance of the 77 K EPR spectrum was essentially that of the resting enzyme with only a small free radical signal still present (data not shown). No noticeable decay of the EPR spectrum of CCP(MI) compound I was observed over the same time period. Thus, the EPR results correlate well with the UV–visible results for the decay of CCP(MI,F51) compound I.

A significant difference is observed between the EPR spectrum of resting CCP(MI,F51) (Figure 5E) and those of resting bakers' yeast CCP (Figure 5A) and CCP(MI) (Figure 5C). CCP(MI,F51) showed predominantly high-spin iron, as indicated by a peak at about $g = 6.0$, with only a very small amount of low-spin iron, indicated by minor peaks at *g* values of about 2.7, 2.2, and 1.8. In contrast, the spectra of resting bakers' yeast CCP and CCP(MI) exhibited a mixture of spin states containing a much more prominent low-spin component.

In summary, substitution of Trp-51 by a Phe residue has not eliminated the free radical signal associated with the oxidized intermediate of the enzyme, compound I, but has caused a drastic decrease in its lifetime at 23 °C and has resulted in a shift in the population of spin states in the resting enzyme, from a mixture of high- and low-spin iron to predominantly high-spin iron, at 89 K.

Discussion

Expression of Yeast CCP in E. coli. This paper describes the design and construction of a system for site-directed mutagenesis and high-level production of yeast cytochrome *c* peroxidase (CCP) in *E. coli* and demonstrates its utility for studying structure–function questions concerning this enzyme. The system was created by substituting a modified CCP gene for the *fol* gene in a vector previously used for mutagenesis and overexpression of dihydrofolate reductase (Villafranca et al., 1983). *E. coli* as a host has two main advantages, in comparison with yeast (Goodin et al., 1986), for expression of CCP for use in biophysical studies. First, since there is no known CCP-like gene or gene product in *E. coli*, it was not necessary to inactivate or delete a host gene initially. Second, the level of production of plasmid-encoded CCP in *E. coli* is far higher than in yeast.

Both the yeast CCP gene and its protein product appear to be highly stable in their new host. In contrast to what was found in the expression of mutant dihydrofolate reductases (Villafranca et al., 1983), we observed no recombination between the CCP gene carried by the plasmid and *E. coli* host chromosomal genes. Additionally, the high-expression level of CCP protein caused no inhibition of growth in the *E. coli* even though the vector's promoter is unregulated.

In yeast, CCP is expressed as a pre-protein having a 68-residue leader sequence that presumably directs transfer from the site of synthesis in the cytoplasm and translocation into the mitochondrial intermembrane space. There, CCP resides as the mature protein lacking the leader sequence (Kaput et al., 1982; Maccecchini et al., 1979; Williams & Stewart, 1976).

In these experiments, we have constructed a new, mature CCP protein that has the leader sequence replaced by just two residues, Met-Ile. This new CCP is referred to herein as CCP(MI). Construction of the CCP(MI) gene involved replacement of codons specifying amino acid residues −2 and −1 of the leader sequence with codons specifying Met-Ile (see Figure 2) in order to create a *BclI* restriction site and a Met initiation codon. The CCP(MI) gene was then substituted for the *fol* gene at the *BclI* site of the plasmid pUC8DHFR in order to take advantage of its highly efficient mutant promoter.

It is important to note that CCP(MI) was expressed in *E. coli* as a mature protein, only slightly modified at the amino terminus by the addition of Met-Ile, not as a fusion protein. This modification is not expected to introduce any important alterations in molecular structure or functional properties since the crystal structure of bakers' yeast CCP reveals that the NH_2-terminal Thr residue is disordered (Finzel et al., 1984). It is therefore reasonable to assume that a high degree of flexibility at the amino terminus is an inherent property of the molecule which obtains not only in the crystal but also in the enzyme's normal physiological environment. As described under Results, our expectation that CCP(MI) is essentially identical in properties with bakers' yeast CCP has been confirmed by preliminary experiments.

Two obvious problems that might have interfered with a high level of expression of CCP(MI) in *E. coli* merit further discussion. One relates to codon preference and the other to the stability of the apoprotein. Codon preference in genes encoding highly expressed proteins is known to differ markedly between yeast and *E. coli* (Ikemura, 1982). Analysis of the nucleotide sequence of the CCP(MI) gene revealed that 40 of the 296 codons (294 + the amino-terminal Met-Ile) appear infrequently in *E. coli* genes. The rare codons specify Arg, Gly, Ile, Leu, Pro, and Thr. However, the presence of these codons did not appear to impair high-level expression; the final yield of more than 8 mg of pure crystalline enzyme/L of cell culture makes this system much more efficient than normal CCP production in yeast.

The high level of enzyme production in the *E. coli* system described here may be appreciated by noting that 12 L of pUC8CCP(MI)-transformed cell culture yielded a pellet of about 70 g wet weight from which more than 100 mg of crystalline enzyme was obtained (more than 8.3 mg/L of culture). In comparison, *S. cerevisiae* transformed with a plasmid carrying the entire pre-CCP gene (Goodin et al., 1986) was found to yield about 80 μg of purified enzyme/L of culture (before crystallization). Thus, expression of CCP in *E. coli* is more than 100 times as efficient as expression in the yeast/plasmid system. A further comparison may be made

with the yield of CCP from commercial pressed bakers' yeast; 2.27 kg of yeast gave about 35–73 mg of crystalline CCP (Poulos et al., 1978; Nelson et al., 1977). This high efficiency of CCP(MI) production from *E. coli* provides ample protein for kinetic, spectroscopic, and X-ray crystallographic studies from easily manageable volumes of cell culture.

Of particular interest was the finding that the CCP(MI) protein is expressed predominantly as the apoenzyme (about 90% apo and 10% holo), which is easily converted to active holoenzyme by addition of bovine hemin under suitable conditions. It should thus be possible to obtain large quantities of purified apo-CCP(MI) as well as the apo form of mutant CCP's generated by site-directed mutagenesis. The availability of these proteins may be helpful in studying the heme binding process in CCP. It is also noteworthy that the 10% holo-CCP(MI) produced by the *E. coli* contains ferric protoporphyrin IX, as evidenced by the spectrum of its reduced pyridine hemochromogen, which is identical with that of ferric protoporphyrin IX and with that obtained from bakers' yeast CCP (Yonetani & Ray, 1965). Therefore, all of the final holo-CCP(MI) product is identical in this respect.

Initial characterization of CCP(MI) has revealed no significant differences from the CCP expressed by Goodin et al. (1986) with regard to either catalytic activity or UV, visible, and EPR spectra. Therefore, none of the following appears to have altered these properties of CCP: (1) addition of two residues (Met-Ile) at the amino terminus of the mature protein, (2) expression in *E. coli*, or (3) addition of bovine hemin to convert ap into holoenzyme. Similarly, CCP(MI) shows no significant differences from bakers' yeast CCP. The latter contains two further internal sequence variations compared with CCP(MI), namely, Asp-152 instead of Gly and Thr-53 instead of Ile (Takio et al., 1980; Kaput et al., 1982; Finzel et al., 1984), which thus appear to have no effect on kinetic or spectroscopic properties.

Is Trp-51 the Free Radical Site in Compound I? A CCP(MI) molecule with a mutation at Trp-51 was designed in order to test the hypothesis that this residue is the locus of the free radical observed in compound I. The indole ring of Trp-51 is about 3.3 Å above and almost parallel to the heme plane on the distal side (Finzel et al., 1984). Substitution of a phenylalanine residue at this position should cause minimal structural perturbations while replacing the indole ring with a much less readily oxidizable benzene ring. Such a substitution is thus very likely to interfere with radical formation if Trp-51 is indeed the radical site, while leaving the rest of the enzymic mechanism intact. Additionally, there would be an analogy between the mutant CCP and catalase at the distal heme face (Murthy et al., 1981) where the side chain of Phe-160 is situated 3.5 Å above and parallel to the heme ring. Furthermore, sequence comparisons of CCP with the plant peroxidases (Takio et al., 1980; Poulos & Finzel, 1984) reveal that a phenylalanine residue in the plant peroxidases occupies a sequence position analogous to that of Trp-51 in CCP. In the two equivalent oxidized intermediates of catalase and the plant peroxidases, the presence of a less readily oxidized phenylalanine side chain presumably allows the heme to be preferentially oxidized, resulting in what is widely held to be a porphyrin π-cation radical (Dolphin et al., 1971; Dolphin & Felton, 1974; Yonetani, 1976; Roberts et al., 1981; Schulz et al., 1984).

Preliminary characterization of the mutant protein, CCP-(MI,F51), has shown that it is still an active peroxidase. The rate constant k_{cat} is increased by 2–4 times over that of CCP(MI), but the apparent K_m values for H_2O_2 and reduced horse heart cytochrome *c* are similarly increased.

The substitution of a phenylalanine for a tryptophan residue has caused an increase, by about 28%, in the molar extinction coefficient at 408 nm and a decrease, by about 8%, in the molar extinction coefficient at 280 nm. The underlying electronic reason for the increased ϵ_{408} value is not clear, but it must be a consequence of the difference between the way an indole ring and a phenyl ring interact with the heme. The decrease in ϵ_{280}, on the other hand, is obviously due to the lower intrinsic absorbance of phenylalanine as compared with tryptophan at 280 nm (Wetlaufer, 1962). It is not surprising that substitution of a benzene ring for an indole ring in contact with the heme has a significant effect on the kinetic and spectral properties of the enzyme.

EPR Spectrum of Mutant Compound I. An important result of this study is that the EPR spectrum of the peroxide-oxidized intermediate (compound I) of CCP(MI,F51) shows that the free radical type signal is still present. When recorded at 89 K, the EPR spectrum still displays a strong sharp resonance at $g = 2.004$. Moreover, the detailed profile of the latter (not shown) is similar to that observed for compound I of bakers' yeast CCP and of CCP(MI), with the interesting exception that the wings are much narrower. An analogous result was obtained by Goodin et al. (1986) when Met-172 was replaced by a serine residue. These spectra are quite different from that observed for compound I of horseradish peroxidase under comparable conditions (Morita & Mason, 1965; Aasa et al., 1975; Goodin et al., 1986).

Double integration of the first-derivative EPR curves for compound I of all three CCP variants—bakers' yeast CCP, CCP(MI), and CCP(MI,F51)—prepared and analyzed under the same conditions, showed their areas to be in the ratio 1:1:1 within 10% experimental error (data not shown). Thus, the compound I radicals of all three enzymes exhibit the same number of spins per mole of enzyme.

On the basis of single-crystal EPR measurements at 5 K, Hori and Yonetani (1985) have proposed the existence of at least two distinct types of free radical sites in compound I of bakers' yeast CCP. One gives a narrow EPR signal at $g = 2.004$ having hyperfine structure; the other is manifested as an axially symmetric signal, which is broadened to present the appearance of wings on the narrow signal when the temperature is raised to 77 K. Of great interest is our finding that these wings are obviously narrowed in the radical signal of the CCP(MI,F51) mutant compound I but they are not completely eliminated. However, as pointed out above, the area under the absorption curve, determined by integration, remains the same, and thus the area under the narrow signal must have increased. We conclude that Trp-51 is *not* the site responsible for the narrow signal at $g = 2.004$. Furthermore, Trp-51 is not solely responsible for the wing signals, since they were not completely eliminated by substitution with phenylalanine.

A second residue that has been implicated as the site of radical formation in compound I is Met-172 (Hoffman et al., 1981). When Goodin et al. (1986) replaced Met-172 with serine in the *S. cerevisiae* enzyme, they found that compound I of the mutant enzyme continued to display the characteristic narrow EPR signal at $g = 2$. However, the broad wings were significantly reduced in this case as well. Additionally, the activity of the mutant enzyme was unchanged.

In summary, different perturbations of the geometry of the heme binding pocket leave the narrow EPR signal intact but substantially reduce the size of the wings. These observations lead to two conclusions. The first is that neither Trp-51 nor Met-172 is the locus of the radical in compound I. The second

conclusion is that the EPR wings are a manifestation of some delicately balanced structural feature of the heme pocket. Moreover, whatever this feature may be, it is not essential for enzymic function since both mutant enzymes have undiminished activity.

Stability of Mutant Compound I. Of particular interest are the results of optical and EPR spectroscopy demonstrating that compound I of CCP(MI,F51) is much less stable than compound I of bakers' yeast CCP or CCP(MI). The observed half-life of less than 2 min at 23 °C instead of 2–4 h (Yonetani et al., 1966b) is similar to the corresponding value for HRP compound I (Chance, 1949a–c). Perhaps the decreased stability of the mutant compound I is also due to the heme pocket perturbation discussed above.

EPR Spectrum of Resting Mutant Enzyme. A further interesting result is that the EPR spectrum, at pH 6, of the mutant CCP(MI,F51) in the resting Fe^{3+} state is different from that of CCP(MI) or bakers' yeast CCP (see Figure 5E,C,A). The mutant shows predominantly high-spin iron, indicated by a strong signal at about $g = 6.0$, but little or no signal corresponding to low-spin iron at $g = 2.7$, 2.2, and 1.8. In contrast, the latter two resting enzymes show a mixture of high- and low-spin iron. In fact, the spectrum of resting CCP(MI,F51) at pH 6 closely resembles that of bakers' yeast CCP at pH 4.0–4.5 (Hori & Yonetani, 1985). One possibility, therefore, is that the mutation of Trp-51 to Phe has increased the pK of some group that affects the heme iron spin state. A likely candidate is the water molecule at the sixth coordination site of the iron atom, which is known to be hydrogen bonded to Trp-51 in bakers' yeast CCP (Finzel et al., 1984). A second possible interpretation is that the mutation has reduced the degree of occupancy by this water molecule.

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Registry No. CCP, 9029-53-2; L-Trp, 73-22-3; L-Phe, 63-91-2; H_2O_2, 7722-84-1; cytochrome *c*, 9007-43-6.

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Function of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase: A Model Study Using 6-Fluoro-5′-deoxypyridoxal- and 5′-Deoxypyridoxal-Reconstituted Enzymes†

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ABSTRACT: A new vitamin B_6 analogue, 6-fluoro-5′-deoxypyridoxal (6-FDPL), was synthesized and characterized. This analogue, as well as 6-fluoropyridoxal (6-FPAL), 6-fluoropyridoxal phosphate (6-FPLP), and 6-fluoropyridoxine, showed positive heteronuclear ^1H–^{19}F nuclear Overhauser effects between the 5′-protons and the 6-fluorine. Apophosphorylase reconstituted with 6-FDPL showed 1% of the activity of the native enzyme in the presence of phosphite. The kinetic pattern, apparent pH optimum of activity, and the activity–temperature dependency of the 6-FDPL–enzyme were virtually identical with those of phosphorylase reconstituted with the parent compound, 6-FPAL [Chang, Y. C., & Graves, D. J. (1985) *J. Biol. Chem. 260*, 2709–2714], except the K_m of phosphite toward the 6-FDPL–enzyme was 9 times higher than that with the 6-FPAL–enzyme and the 6-FDPL–enzyme showed a lower V_{max} value. Phosphorylase reconstituted with 5′-deoxypyridoxal (DPL) also showed activity in the presence of phosphite. The kinetics and the temperature–activity dependency of this reconstituted enzyme were investigated. ^{19}F nuclear magnetic resonance studies showed that the binding of glucose 1-phosphate to a 6-FDPL–enzyme–adenosine 5′-phosphate (AMP) complex shifted the ^{19}F signal 0.6 ppm upfield, whereas a 2.1 ppm change was observed when the 6-FPAL–enzyme–AMP formed a complex with glucose 1-phosphate [Chang, Y. C., Scott, R. D., & Graves, D. J. (1986) *Biochemistry 25*, 1932–1939]. Analysis of the activation parameters, activation enthalpy and activation entropy, of the reaction of glycogen degradation catalyzed by phosphorylase containing pyridoxal phosphate, 6-FDPL, pyridoxal, or DPL showed that modifications of the coenzyme molecule affected only the activation entropy, not the activation enthalpy. Results of this study indicate that the protein structure surrounding the coenzyme molecule, as well as the coenzyme configuration, is altered upon the binding of ligands. The 5′-OH group of the protein-bound coenzyme is a necessary factor for the completion of these conformational changes. A correct transformation of the protein structure, coordinated by the coenzyme molecule, is required for the high efficiency of catalysis.

Kinetic and equilibrium dialysis studies have shown that the activity of glycogen phosphorylase is determined by an equilibrium between at least two conformers, the active "R" and the inactive "T" conformers (Helmreich et al., 1967; Kastenschmidt et al., 1968). This equilibrium is affected by the binding of different ligands to the protein (Graves & Wang, 1972; Klein & Helmreich, 1980; Madsen & Withers, 1984). The reactivity of phosphorylase toward SH-modifying reagents (Avramovic-Zikic et al., 1970) as well as the electron spin resonance (ESR)[1] and fluorescence spectral studies of

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[1] Abbreviations: 6-FPAL, 6-fluoropyridoxal; 6-FPLP, 6-fluoropyridoxal phosphate; DPL, 5′-deoxypyridoxal; 6-FDPL, 6-fluoro-5′-deoxypyridoxal; NMR, nuclear magnetic resonance; AMP, adenosine 5′-phosphate; glucose-1-P, glucose 1-phosphate; EDTA, ethylenediaminetetraacetic acid; PLP, pyridoxal 5′-phosphate; TEAA, tetraethylammonium acetate; DTE, dithioerythritol; NOE, nuclear Overhauser effect(s); ESR, electron spin resonance; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Me_4Si, tetramethylsilane; Me_2SO, dimethyl sulfoxide.

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