Conversion of an Engineered Potassium-binding Site into a
Calcium-selective Site in Cytochrome c Peroxidase*

(Received for publication, June 16, 1999, and in revised form, September 21, 1999)

Christopher A. Bonagura‡, B. Bhaskar†, M. Sundaramoorthy§, and Thomas L. Poulos¶

From the Departments of Molecular Biology and Biochemistry and Physiology and Biophysics and the Program in
Macromolecular Structure, University of California, Irvine, California 92697-3900

The crystal structure showed Ca$^{2+}$ rather than K$^+$. Using the K$^+$-binding CCP mutant (CCPCA2) as a template protein, together with observations from structural modeling, mutants were designed that should bind Ca$^{2+}$ selectively. The crystal structure of the first generation mutant, CCPCA1, showed a smaller cation, perhaps Na$^+$, is bound instead of Ca$^{2+}$. This is probably because the full eight-ligand coordination sphere did not form owing to a local disordering of one of the essential cation ligands. Based on these observations, a second mutant, CCPCA2, was designed. The crystal structure showed Ca$^{2+}$ binding in the CCP cavity mutants where Trp191 is converted to Gly prefer to bind imidazolium cations in the pocket vacated by the missing Trp191 indole ring (15). In addition, electrostatic calculations (3, 14, 15) support the view that the Trp191 radical is cationic, although recent theoretical assessments (3, 14, 15) as well as the theoretical calculations (3, 16) show that the electrostatic environment of the protein surrounding Trp$^{191}$ is negative in CCP, which offers the possibility of stabilizing a cationic radical. Ascorbate peroxidase closely resembles the structure of CCP, including a homologue to Trp$^{191}$ (Trp$^{179}$ in ascorbate peroxidase). However, ascorbate peroxidase does not form a stable Trp$^{191}$-centered radical, but instead, a porphyrin-$\pi$-cation radical (18), as in other peroxidases. One hypothesis put forward to explain this difference is the electrostatic nature of CCP to more effectively stabilize the positive charge on the Trp$^{191}$ cationic radical. This view is supported by electrostatic calculations (3, 16). An obvious factor helping to alter the surrounding electrostatic environment near the ascorbate peroxidase Trp$^{179}$ residue is a K$^+$ metal cation bound ~8 Å from Trp$^{179}$ (Fig. 1). This cation site is conserved in all peroxidases for which structures are known, except CCP, where a water molecule occupies this position. We

* This work was supported by grants from the National Institutes of Health and the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ These authors contributed equally to this work.

§ Present address: Dept. of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160-6574.

¶ To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3900. Fax: 949-824-3280; E-mail: poulos@uci.edu.

The abbreviations used are: CCP, cytochrome c peroxidase; MES, 4-morpholineethanesulfonic acid.
have suggested that the positively charged cation in ascorbate peroxidase helps to prevent formation of a stable Trp_191 cationic radical owing to electrostatic destabilization (3, 18). This hypothesis has been tested by engineering the ascorbate peroxidase K^+ site into CCP. The resulting CCP mutant, designated CCPK2, binds K^+, has ~1% wild-type activity in the presence of K^+, and exhibits a significantly weakened EPR signal associated with the Trp_191 radical (3, 4). In addition, both enzyme activity and the Trp_191 EPR signal can be titrated with K^+, indicating that the binding of K^+ to the engineered site is responsible for loss of activity and destabilization of the Trp_191 radical.

Ascorbate peroxidase is the only member of the peroxidase family for which x-ray structures are available that binds K^+ rather than Ca^{2+}. Owing to our success in engineering the ascorbate peroxidase K^+ site into CCP, we next chose to convert this site to a Ca^{2+} site in CCP. One reason is the relative ease of using various chelating agents for removal of calcium, which should enable easier manipulation of the engineered cation site, which was not possible with the engineered K^+ site in CCP. This would potentially offer an easier means of controlling enzyme activity by providing a "molecular switch" that regulates the redox properties of Trp_191. In addition, because it was possible to convert CCP into a K^+-binding protein, we viewed CCP as an excellent system for probing in greater detail the general problem of designing metal-binding sites in proteins. The close structural homology among various peroxidases in the proximal cation-binding loop and the ability to readily obtain high resolution x-ray crystal structures afford an excellent opportunity to understand, in some detail, factors that can contribute to the successful design of a protein divalent metal-binding site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes and reagents for site-directed mutagenesis were purchased from Roche Molecular Biochemicals and New England Biolabs Inc. (Beverly, MA). Chromatography columns and media were purchased from Amersham Pharmacia Biotech. Horse heart cytochrome c, hydrogen peroxide (30%, w/v), EDTA, and EGT A were purchased from Sigma. 2-Methyl-2,4-pentanediol was purchased from Aldrich. All other chemicals were molecular biology grade or better and were purchased from Sigma or Fisher.

**Site-directed Mutagenesis**—Construction of CCPK2 has been previously reported (3, 4, 18). CCPK2 has the following mutations: A176T, G192T, A194N, T199D, and E201S, which alter the normal water coordination sphere. Site-directed mutagenesis was carried out by annealing this oligomer to the single-stranded uracylated CCPK2 template that was produced in Escherichia coli RZ1032 cells following the method of Kunkel et al. (19) as described previously (20, 21). The mutagenic oligomer was phosphorylated with calf intestine alkaline phosphatase; boiled with template at a ratio of 20 pmol oligomer to 1 pmol template; and annealed for 15 min each at 70 °C, 37 °C, and then at room temperature and finally on ice to add the reactants. Next, 1 mM ATP was added with T4 DNA polymerase and T4 DNA ligase in excess to the reaction mixture and incubated at room temperature for 3–4 h. The reaction mixture was then transformed by electroporation into JV30 cells, and the colonies grown were selected under ampicillin resistance. Transformation efficiency by this procedure was typically low, but the transformants were likely to be positive clones without spontaneous mutations. All mutations were sequenced at the DNA level (Promega thermal cycle sequencing) to ensure that the introduced mutations were only installed as predicted. The resulting mutant was called CCPCA1.

CCPCA1 was then changed to CCPCA2 using the same CCPK2 template and mutagenesis method described above with the following oligomer: GAA GGG CCA TGG GAC GCC ACT AAC AAT AAT TTC. The A194 residue on the CCPK2 template was installed to copy the corresponding ascorbate peroxidase cationic radical at position 194. Under “Discussion,” we will explain why there is the need to again alter this residue.

**Protein Expression and Purification**—The recombinant CCP mutants were expressed in E. coli BL21(DE3) cells under the influence of the T7 promoter with isopropyl-β-D-thiogalactopyranoside induction as described previously by Fishel et al. (22) and Choudhury et al. (21) using slightly modified conditions especially for the calcium-binding mutant CCPCA2. CCPCA1 was purified by column chromatography using the same conditions as used for wild-type CCP and CCPK2. CCPCA1 was stable in 50 mM potassium phosphate, pH 6.0. CCPCA1 was stored frozen at ~80 °C as microcrystalline precipitate after dialysis against water. CCPCA2 showed an obvious instability in potassium-containing buffers, and much of the protein tended to denature. Using calcium in phosphate buffers caused precipitation of calcium phosphate, which was detrimental to the stability of the mutant. As a result, CCPCA2 was purified in potassium phosphate buffer, pH 6.0; and after heme incorporation followed by DE52 anion-exchange chromatography, the protein was dialyzed against water followed by dialysis against water containing 100 μM calcium to stabilize the protein. Dialysis of CCPCA2 against distilled water did not result in crystals typically observed during CCP purification. Therefore, for long-term storage, CCPCA2 was dialyzed against 200 mM Tris/MES, pH 6.0, containing 50 μM calcium and stored as aliquots at ~80 °C. The CCP concentration was estimated spectrophotometrically using an extinction coefficient (ε) at 408 nm of 96 mM⁻¹ cm⁻¹.

**Steady-state Activity Assays**—For substrate titration kinetic assays, the steady-state oxidation of dithionite-reduced horse heart cytochrome c (ferrocytochrome c) was measured at 24 °C in a Cary 3E UV-visible spectrophotometer using Δε_550 = 19.6 mM⁻¹ cm⁻¹. The final reaction conditions consisted of 180 mM and 10 mM CCP and 30 μM ferrocyanochrome c in 5 mM Tris/MES, pH 6.0 (23). The initial linear region of the reaction slope was recorded and taken as least-squares fit using Cary Varian 101.00/06 software running on Windows 95. All data points reported in this paper reflect the average of at least three independent experiments averaged and plotted in Sigma Plot 4.0.

The following assay conditions were employed to measure steady-state activity as a function of cation concentration. CCPCA2 was added to 5 mM Tris/MES, pH 6.0, initially containing Ca^{2+} or K^+ ions or other ions at various concentrations, and then 180 and 40 μM ferrocyanochrome c were added in concert to begin the reaction. In the case of metal chelation experiments, the enzyme was preincubated at an end point...
concentration of 10 mM calcium in 5 mM Tris/MES, pH 6.0, before incubating with a metal chelator (EDTA or EGTA) at stoichiometric concentrations and then assaying for the remaining activity. Results are presented as the percentage activity remaining over the control in the absence of metal ions taken as 100%.

**Rapid Reaction Kinetics**—To evaluate the mutants for the efficiency of electron transfer rate, rapid reaction kinetics were performed at a single wavelength on a Hi-Tech Model SF-51 stopped-flow spectrophotometer equipped with a 1-cm path length. The output data were monitored and recorded on a Compaq PC for kinetic analysis using Hi-Tech IS-1 software suite version 1.0. Experimental conditions used to record Trp191 cation radical EPR spectroscopy were recorded on a Bruker ESP300 spectrometer equipped with an Air Products LTR3 liquid helium cryostat. Experimental conditions used to record Trp191 cation radical for crystallographic experiments were monitored and recorded on a Compaq PC equipped with Hi-Tech IS-1 software suite version 1.0. CCPCA2 compound I in various concentrations of Cu²⁺ or K⁺ was formed by the addition of an equal volume of 4.6 μM H₂O₂ and 4.0 μM CCPCA2 in 5 mM Tris/MES, pH 6.0. CCPCA2 compound I (2 μM) in one syringe was mixed with 2 μM horse heart cytochrome c in stoichiometric amounts. The oxidation of cytochrome c was monitored at 416 nm, an isosbestic point for the resting CCP ferric and oxyferryl compound I. Results are presented as the percentage of electron transfer rate over the control enzyme in the absence of metal ions taken as 100%.

**EPR Spectroscopy**—EPR spectra were recorded on a Bruker ESP300 spectrometer equipped with an Air Products LTR3 liquid helium cryostat. The remaining sample had wild-type CCP and mutants were as follows: microwave frequency, 9.475 GHz; microwave power, 0.5 milliwatts; modulation amplitude, 4.57 G; modulation frequency, 1000 kHz; field sweep rate, 11.92 G/s; time constant, 0.0256 ms; and receiver gain, 1 × 10⁴ (wild-type CCP) and 2.5 × 10⁵ (CCPCA2). The resting state sample had wild-type CCP and CCPCA2 at 300 μM in 5 mM Tris/MES, pH 6.0, in a total volume of 150 μl. Compound I was formed by the addition of 360 μM H₂O₂, and the samples were immediately frozen in quartz EPR tubes by submersion in liquid nitrogen over a period of 60–80 s. Spectra were recorded at 8 K. The data obtained were an average of five scans.

**Crystallization**—Because of the requirement to soak crystals in Ca²⁺ buffers, the usual use of potassium phosphate buffers could not be employed with CCPCA1 and CCPCA2. After several trials, crystals could be grown from 100 mM Trizma (Tris base), 50 mM acetic acid, and 50 mM MES, pH 6.0, or 50 mM Tris cacodylate, pH 5.6, using the usual 30% 2-methyl-2,4-pentanediol as the precipitant (24). CaCl₂ (10 and 2 mM) was included in the crystallization buffers for CCPCA1 and CCPCA2, respectively.

**X-ray Data Collection and Refinement Statistics**—Data for both CCPCA1 and CCPCA2 were obtained from one flash-frozen crystal each using an R-AXIS IV imaging plate and a Rigaku rotating anode x-ray source equipped with a Crystal Logic cryogenic N₂ delivery system. Initial image processing, indexing, and integration were performed with Denzo version 1.9.1, and the integrated data were scaled using ScalePack version 1.9.0 (25). The starting model for refinement was CCPK2. The models were refined with X-PLOR version 3.851 (26). Data collection statistics and final refinement parameters are given in Table I.

**RESULTS AND DISCUSSION**

**Crystal Structures**—Fig. 2 provides a sequence alignment of the proximal cation-binding loop in various peroxidases. Although the backbone polyepitope conformation of CCP and the proximal cation-binding loop of other peroxidases are nearly identical in this region, CCP lacks the side chain cation ligands that would bind the metal ion and hence binds water rather than a cation. CCPK2 is an engineered version of CCP that was designed to mimic the ascorbate peroxidase cation-binding site, and it does bind K⁺ (4). CCPCA1 was the first attempt to convert the K⁺ site in CCPK2 to a Ca²⁺-binding site. We reasoned that conversion of Thr¹⁹², a K⁺ ligand in CCPK2, to Asp would create a second negatively charged ligand, which, together with Asp¹⁹⁹, would provide a charge-neutralizing coordination environment of eight oxygens for Ca²⁺. Changing Thr¹⁹² to Asp in CCPK2 should install the bidentate bond at this side chain position that is observed in other peroxidase proximal cation loops such as the one found in lignin peroxidase (see Fig. 4) (27). This version of the CCP mutant was designated CCPCA1.

To see if Ca²⁺ binds to CCPCA1, the crystal structure was solved and refined to 1.9-Å resolution. An omit 2Fo — Fc electron density map of the wild-type protein and mutants was as follows: microwave frequency, 9.475 GHz; microwave power, 0.5 milliwatts; modulation amplitude, 4.57 G; modulation frequency, 1000 kHz; field sweep rate, 11.92 G/s; time constant, 0.0256 ms; and receiver gain, 1 × 10⁴ (wild-type CCP) and 2.5 × 10⁵ (CCPCA2). The resting state sample had wild-type CCP and CCPCA2 at 300 μM in 5 mM Tris/MES, pH 6.0, in a total volume of 150 μl. Compound I was formed by the addition of 360 μM H₂O₂, and the samples were immediately frozen in quartz EPR tubes by submersion in liquid nitrogen over a period of 60–80 s. Spectra were recorded at 8 K. The data obtained were an average of five scans.

**Engineering Specificity into a Protein Metal-binding Site**

### Table I

| Data collection and refinement statistics | CCPCA1 | CCPCA2 |
|-----------------------------------------|--------|--------|
| Total observations                      | 159,266| 155,672|
| No. unique reflections                  | 38,462 | 28,701 |
| Completeness (%)                        | 96.2   | 99.8   |
| Completeness in last shell (%)          | 76.3   | 99.0   |
| I0I in outer shell                      | 2.7    | 3.3    |
| Rmerge (%)                              | 0.062  | 0.082  |
| Unit cell dimensions (Å)                | 106.8, 75.8, 51.0 | 107.0, 75.5, 51.4 |
| Space group                             | P2₁2₁2₁ | P2₁2₁2₁ |
| Resolution for refinement (Å)           | 10 to 1.9 | 10 to 2.0 |
| No. reflections used for refinement     | 26,435 | 25,912 |
| Rfactor (%)                             | 22.1   | 19.2   |
| r.m.s. deviation of bonds (Å)           | 0.008  | 0.007  |
| r.m.s. deviation of bond angles         | 1.36°  | 1.32°  |

a Rmerge = Σ||Fo| - |Fc||/Σ|Fo|, R = Σ|Fo| - |Fc|/Σ|Fo|.

b Root mean square.

**Fig. 2**: Sequence alignments of the cation-binding loop in various peroxidases. Side chain ligands are in boldface. The remaining ligands are provided by peptide carbonyl oxygen atoms. WT, wild-type; APX, ascorbate peroxidase; LIP, lignin peroxidase.
tron density map around the cation-binding site is shown in Fig. 3. The omit $F_o - F_c$ map shows a 5σ peak at the position of the cation. This is far lower than would be expected for a Ca$^{2+}$ site since similar omit maps with other Ca$^{2+}$- or K$^+$-binding peroxidases we have studied often give peaks of 10σ or higher. This indicates that a smaller cation, most likely Na$^+$, is bound. The difference maps are flat when 0.55 K$^+$ (equivalent to Na$^+$) is modeled during refinement at the cation site in CCPCA1. In

Fig. 4. Stereoscopic models of the cation-binding sites in engineered CCP mutants, ascorbate peroxidase, and lignin peroxidase. APX, ascorbate peroxidase; LIP, lignin peroxidase.
addition, one of the anticipated ligands, Asn194, is pointing away from the cation (Fig. 4). The side chain density for Asn194 is not well defined, and there is a break in the electron density between Ala193 and Asn194 (Fig. 3). This failure of Asn194 to form a cation ligand and the local disordering most likely account for the failure of Ca$^{2+}$ to bind in CCPCA1. The ligand count in the pocket now drops to six oxygens, which is consistent with a Na$^+$ coordination sphere.

The reason why Asn194 does not coordinate the cation is most likely due to steric and electrostatic factors not considered in the original design of CCPCA1. If Asn194 were to rotate in toward the cation site, steric clashes would occur with Asp192, the newly installed cation ligand replacing Thr192. The new Asp192 could come within 1.3 Å from the normal position of the carbonyl group of Asn194 in the CCPK2 loop structure. We had anticipated sufficient adaptability of the site to relax such constraints, but apparently this assumption was incorrect. In addition, the hydrogen bond formed between Asn194 and Asp199 in CCPK2 (Fig. 4) was expected to remain in CCPCA1 and to provide a strong energetic incentive to correctly orient Asn194 toward the cation. Obviously, this hydrogen bond was not sufficient to maintain Asn194 in a coordination position. Since the only other Ca$^{2+}$-binding protein listed in Fig. 2, lignin peroxidase, has Thr at this position, we next replaced Asn194 with Thr and designated this mutant CCPCA2. We reasoned that removing the amide group from Asn194 would allow sufficient local relaxation to accommodate Asp192.

Fig. 3 shows the omit $2F_o - F_c$ maps for CCPCA2. Here, the $F_o - F_c$ map shows a 14σ peak at the cation site, compared with 5σ for CCPCA1. In addition, Thr194 now coordinates the cation, and the entire 192–199 section of polypeptide is well ordered as evidenced by strong continuous density in the omit $2F_o - F_c$ map. In addition, when the cation site is modeled as Ca$^{2+}$ (18 electrons), the temperature factor ($B$ factor) refined to a value of 14.4 Å$^2$, whereas in CCPCA1, the $B$ factor was 24 Å$^2$ even when the site was modeled with only 0.5 Ca$^{2+}$. Therefore, replacement of Asn194 in CCPCA1 with Thr enables the new mutant, CCPCA2, to bind calcium.

Another noticeable difference between CCPCA1 and CCPCA2 is the conformation of Asp192, a potential bidentate cation ligand. In CCPCA1, Asp192 cannot adopt the same conformation as in CCPCA2 owing to the presence of Asn194 in the cation coordination sphere (Fig. 4). Not too surprisingly, the conformation of Asp192 in CCPCA2 is very close to the corresponding Ca$^{2+}$ ligand, Asp194, in lignin peroxidase. The Cα–Cβ and Cβ–Cγ side chain torsion angles are 74° and 4°, respectively, in lignin peroxidase compared with 75° and 4° in CCPCA2 and −180° and −122° in CCPCA1. This enables both carboxylate oxygen atoms of Asp192 to ligate the calcium in CCPCA2 as in lignin peroxidase, whereas in CCPCA1, only one carboxylate oxygen atom is able to coordinate the cation while the other hydrogen-bonds to the peptide amide group of Ala193.

A comparison of the various peroxidase cation-binding sites is provided in Fig. 4.

**Enzyme Activity and EPR**—In our earlier work with CCPK2, we found that enzyme activity and the electron
transfer rate could be titrated down by the addition of K$^{+}$, which gave K_d = 10 mM (4). In addition, we found that the Trp191 EPR signal could also be titrated in a similar way with added K$^{+}$ ions. The close correlation between loss of EPR signal and loss of activity upon addition of K$^{+}$ indicated that electrostatic destabilization of the Trp 191 radical was responsible for the loss of enzyme activity. For our present purposes, activity and EPR can be utilized to see if, indeed, CCPCA2 now specifically binds Ca$^{2+}$.

For titrating enzyme activity, we again utilized both stopped-flow and steady-state kinetic assays. In the absence of added calcium, the activity of the CCPCA2 mutant is 28% that of wild-type CCP. In our previous study, we showed that this effect is due to a destabilization of the calcium loop including Trp191 (4). Despite this difference, a detailed kinetic analysis showed that CCPK2 behaves very much the same as wild-type CCP. As shown in Fig. 5A, Eadie-Hofstee plots for CCPCA2 give typical biphasic kinetics characteristic of wild-type CCP substrate turnover with very similar K_m values (4, 28). The two K_m values for wild-type CCP determined in our previous study, 3.6 and 29.8 mM (4), compare well with those for CCPCA2, 4.7 and 18.3 mM (Fig. 5A). In addition, the break point between the two kinetic phases is 12 mM ferrocytochrome c for CCPCA2 and 9 mM ferrocytochrome c for wild-type CCP (4). This indicates that the overall catalytic machinery of CCPCA2 remains intact even though activity can be decreased by the addition of Ca$^{2+}$. As shown in Fig. 5B, both K$^{+}$ and Ca$^{2+}$ addition led to a loss of activity, but Ca$^{2+}$ was much more effective with an apparent K_{d} of 27 mM. Just the opposite was found with CCPK2, the mutant designed to bind K$^{+}$. In this case, K$^{+}$ was more effective at decreasing activity than Ca$^{2+}$ (4). In the stopped-flow studies, CCP was treated with 1 eq of peroxide to give CCP compound I, followed by mixing with

The EPR signal in the absence of any added cations was considerably weaker than that of wild-type CCP, which correlated with the loss of activity. The experimental conditions are described under “Experimental Procedures.”

**Fig. 5.** Kinetic titration data of CCPCA2. A, Eadie-Hofstee plots for CCPCA2 for steady-state turnover of ferrocytochrome c. Assays were carried out in 5 mM Tris/MES, pH 6.0, with 10 nM enzyme and the following: left panel, 180 mM hydrogen peroxide and varying concentrations of ferrocytochrome c (cyt c) (the biphasic kinetics, the break point between the two kinetic phases, and apparent K_m values are similar to those of wild-type CCP); and right panel, 30 mM ferrocytochrome c and varying concentrations of hydrogen peroxide. B, titration of the steady-state activity with Ca$^{2+}$ and K$^{+}$. A steady-state assay was carried out on CCPCA2 at various concentrations of Ca$^{2+}$ and K$^{+}$. Assays were carried out in 5 mM Tris/MES, pH 6.0, 10 nM enzyme, 180 mM hydrogen peroxide, and 40 mM ferrocytochrome c at the indicated concentration of ions. The results are expressed as percent activity remaining over the control in the absence of metal ion taken as 100%. Inset, steady-state activity at various concentrations of Ca$^{2+}$. The Scatchard plot from this curve gave a K_D of 27 mM. C, titration of the single turnover electron transfer reaction from ferrocytochrome c to CCP compound I with Ca$^{2+}$ and K$^{+}$ by stopped-flow spectrophotometry. Reactions were carried out in 5 mM Tris/MES, pH 6.0, containing the desired concentration of Ca$^{2+}$ or K$^{+}$ with 2 mM CCPCA2 compound I mixed with 2 mM ferrocytochrome c in a stopped-flow spectrophotometer, and the rate of cytochrome c oxidation was measured at 416 nm. The results are expressed as percent of the electron transfer remaining over the control in the absence of metal ion taken as 100%.
Engineering Specificity into a Protein Metal-binding Site

reduced horse heart cytochrome c. Electron transfer from cytochrome c to CCP was monitored by following the oxidation of cytochrome c. As shown in Fig. 5C, the results are very similar to the steady-state assay results in that Ca2+ was much more effective at eliminating activity than K+. In both steady-state and rapid kinetic assays, other monovalent and divalent cations were ineffective in inactivating the enzyme. However, in steady-state assays, it was observed that inactivation of the enzyme by the added calcium could be partially reversed by the addition of the divalent metal chelator EDTA or EGTA (specific for Ca2+) in stoichiometric amounts (data not shown). This demonstrated that the reactivity of the Trp+ radical could be controlled by the addition of metal chelators.

In our earlier work on CCPK2, the EPR signal associated with the Trp193 radical was decreased more effectively by K+, and Ca2+ had very little effect (4). Fig. 6 shows the effects of both Ca2+ and K+ on the compound I EPR signal of CCPCA2. The EPR signal got progressively weaker as the CaCl2 concentration increased. Note, however, that 10 mM KCl caused only a modest decrease in signal. This again is the reverse of what was observed in the CCPK2 mutant designed to bind K+ (4). Taken together with the enzyme activity measurements, the EPR experiments demonstrate that CCPCA2 is, indeed, more selective for Ca2+ than K+.

CONCLUSIONS

This work demonstrates that it is possible to convert the engineered K+-binding site in CCP to a Ca2+-binding site. The crystal structure of CCPCA1 shows that CCPCA1 binds a smaller cation, possibly Na+, with only six protein cation ligands formed by the partially disordered cation-binding loop. In contrast, the crystal structure of CCPCA2 shows that the cation loop is well ordered, binds Ca2+, and has eight protein cation ligands. Both EPR and enzyme activity titration data illustrate that the engineered cation site in CCPCA2 now prefers Ca2+ over K+. In effect, we have systematically converted the natural CCP H2O site first to bind K+ in CCPK2, then Na+ in CCPCA1, and finally Ca2+ in CCPCA2.

Although it may have been possible to simply replace the entire cation-binding loop in CCP with the lignin peroxidase loop to achieve Ca2+ selectivity, the iterative process of first engineering in a K+ site and then Ca2+ has enabled a deeper appreciation for those structural factors that control protein metal site selectivity. The CCPCA1 structure demonstrated that providing a second Asp ligand was not sufficient to obtain Ca2+ selectivity. The engineered site bound to a smaller cation, possibly Na+. We had anticipated that ligands would adjust to accommodate Asn193 as a ligand, but instead, Asn193 oriented toward the molecular surface away from the cation. Replacing Asn193 with the corresponding residue in lignin peroxidase, Thr, “repaired” the site, enabling the full complement of eight ligands in the correct geometry to coordinate Ca2+. Perhaps even more interestingly, another ligand, Asp192, adopted quite different conformations in CCPCA1 and CCPCA2, with the CCPCA2 conformation more closely resembling that found in lignin peroxidase. This means that the nature of the ligand at position 194 strongly influences the conformation of the ligand at position 192. A priori, this would have been very difficult to model correctly since simple steric considerations indicated that the ligands should be able to accommodate Asp at position 192 without grossly affecting cation coordination or favored side chain torsion angles. Therefore, the somewhat more conservative iterative approach adopted here has provided some important insights into protein metal-binding site design.

Acknowledgment—We thank William Lanzalotta for invaluable technical expertise in EPR spectroscopic experiments.

REFERENCES

1. Stubbe, J., and van der Donk, W. A. (1998) Chem. Rev. 98, 705–762
2. Sundaramoorthy, M., Kishi, K., Gold, M. H., and Poulos, T. L. (1994) J. Biol. Chem. 269, 32759–32767
3. Bonagura, C. A., Sundaramoorthy, M., Pappas, H. S., Patterson, W. R., and Poulos, T. L. (1996) Biochemistry 35, 6107–6115
4. Bonagura, C. A., Sundaramoorthy, M., Bhaskar, B., and Poulos, T. L. (1999) Biochemistry 38, 5538–5545
5. Yeung, B. K., Wang, X., Sigman, J. A., Pettito, P. A., and Lu, Y. (1997) Chem. Biol. 4, 215–221
6. Wilecox, S. K., Putnam, C. D., Sastry, M., Blankenship, J., Chazin, W. J., McRee, D. E., and Goodin, D. B. (1998) Biochemistry 37, 16853–16862
7. Yonetani, T. (1976) in The Enzymes (Boyer, B., ed) Vol. 13, pp. 345–361, Academic Press, Inc., Orlando, FL
8. Sivaraja, M., Goodin, D. B., Smith, M., and Hoffman, B. M. (1989) Science 245, 738–740
9. Mauro, J. M., Fishe1, L. A., Hazzard, J. T., Meyer, T. E., Tollin, G., Cusanovich, M. A., and Kraut, J. (1987) Biochemistry 26, 6243–6256
10. Hahn, S., Green, L., Durham, B., and Millett, F. (1993) J. Am. Chem. Soc. 115, 3572–3577
11. Hahn, S., Miller, M. A., Geren, L., Kraut, J., Durham, B., and Millett, F. (1994) Biochemistry 33, 1463–1480
12. Pappa, H. S., Tajbaksh, S., Saunders, A. J., Pielak, G. J., and Poulos, T. L. (1996) Biochemistry 35, 4837–4845
13. Pelletier, H., and Kraut, J. (1992) Science 258, 1748–1755
14. Miller, M. A., Han, G. W., and Kraut, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11118–11122
15. Fitzgerald, M. M., Churchill, M. J., McRee, D. E., and Goodin, D. B. (1994) Biochemistry 33, 3807–3818
16. Jensen, G. M., Bunte, S. W., Warshel, A., and Goodin, D. B. (1998) J. Phys. Chem. B 102, 8221–8228
17. Menyhard, D. K., and Naray-Szabo, G. (1999) J. Phys. Chem. B 103, 227–233
18. Patterson, W. R., Poulos, T. L., and Goodin, D. B. (1995) Biochemistry 34, 4342–4345
19. Kunkel, T. A., Roberts, J. D., and Zokour, R. A. (1987) Methods Enzymol. 154, 367–382
20. Darwish, K., Li, H., and Poulos, T. L. (1991) Protein Eng. 4, 701–708
21. Choudhury, K., Sundaramoorthy, M., Hickman, A., Yonetani, T., Woebi, E., Dunn, M. F., and Poulos, T. L. (1994) J. Biol. Chem. 269, 20239–20249
22. Fishel, L. A., Villafranca, J. E., Mauro, J. M., and Kraut, J. (1987) Biochemistry 26, 351–360
23. Yonetani, T. (1986) Biochem. Prep. 11, 14–20
24. Edwards, S. L., and Poulos, T. L. (1990) J. Biol. Chem. 265, 2588–2595
25. Otwinowski, Z., and Minor, W. (1996) Methods Enzymol. 276, 307–326
26. Brunger, A. T. (1992) Yale University Press, New Haven, CT
27. Poulos, T. L., Edwards, S. L., Wariishi, H., and Gold, M. H. (1993) J. Biol. Chem. 268, 4429–4440
28. Kang, C. H., Ferguson-Miller, S., and Margoliash, E. (1977) J. Biol. Chem. 252, 919–926