Design and Creation of a Cu(II)-Binding Site in Cytochrome *c* Peroxidase that Mimics the Cu_B-heme Center in Terminal Oxidases

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Cu_B-heme centers are at the heart of the superfamily of terminal respiratory oxidases that include both cytochrome *c* oxidases (C*c*O) and quinol oxidases.[1] They catalyze the reduction of O_2 to H_2O, which is coupled to the generation of a proton gradient through the mitochondrial or cytoplasmic membrane. The proton motive force is then utilized for a variety of essential biological functions such as ATP synthesis. Understanding the structure and function of this important class of metal-binding centers has been the focus of intense biochemical,[2] spectroscopic,[3] X-ray crystallographic,[4] and biomimetic modeling studies.[5] For example, spectroscopic studies have shown that the Cu_B-heme center contains a spin-coupled Cu(II)−Fe(III) site, probably bridged by an unknown ligand.[3a,6] The successful synthesis of several biomimetic Cu(II)−Fe(III)porphyrin compounds containing bridging Cl^-, F^-, O^{2-}, or OH^- has helped elucidate the role of bridging ligands in the structure of the center.[5,7] However, recent X-ray structures of C*c*O failed to reveal any bridging ligand. Moreover, the mechanism of O_2 reduction remains to be fully understood.[8]

Design and construction of a Cu_B-heme center in proteins is an attractive approach toward the elucidation of the structure and function of this center. Like the syntheses of inorganic model compounds,[5] this minimalist approach can serve as a touchstone whereby the knowledge from the studies of native enzymes can be tested. Since the model system is free of other metal-binding sites in the oxidases, it can simplify many structural and functional characterizations. On the other hand, unlike inorganic model compounds, the designed protein utilizes the same type of ligands as in the native enzymes and can be studied under the same physiological conditions. An intriguing challenge is the construction of a stable and rigid Cu(II)-heme center without adding exogenous bridging ligands to test what ligand, if any, is necessary for the spectroscopic features of the center.

Cytochrome *c* peroxidase (C*c*P),[9] a heme peroxidase from yeast mitochondria, is a good system for the design and construction of the Cu_B-heme center. It has been used successfully as a template protein to create either a K^+ [10] or a Mn^{2+}-binding site[11] next to the heme center to elucidate the structure and function of ascorbate peroxidase and manganese peroxidase, respectively. However, while the creation of K^+- and Mn^{2+}-binding sites relies on the close structural homology of the template protein and target proteins, comparison of C*c*P and C*c*O showed no sequence or structural homology. Therefore, the design and construction of the Cu_B center next to the heme center in C*c*P poses a new challenge in our ongoing effort to engineer metal-binding sites in proteins.[11a,12] Here the design, construction, and spectroscopic characterization of the Cu_B-heme center in C*c*P is described.

An overlay of the Cu_B-heme active site of the bovine C*c*O with the active site of C*c*P is shown in Figure 1a. Different orientations of the C*c*P active site were sampled until an appropriate match of residues in C*c*P to the histidine ligands of the Cu_B site were found.[13] As shown, the relative positions of Arg48 and Trp51 correspond very closely to the position of two of the Cu(II) ligands in C*c*O (His290 and His291). Three residues in the active site of C*c*P, His52, Ser81, and Leu144 were available as candidates for the third ligand to Cu(II). The position of His52 was too different from the corresponding third ligand His240 in C*c*O and therefore was eliminated as a candidate by a His52Ala mutation. The histidines at positions 81 and 144 required rotations of the side chains toward Cu(II). We chose to characterize the Ser81His mutant first on the basis of its overall position relative to Arg48His and Trp51His. The active site of C*c*P(Arg48His, Trp51His, His52Ala, Ser81His, called Cu_BC*c*P thereafter) after minimization is compared to the Cu_B site of C*c*O in Figure 1b. The energy

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(13) Molecular modeling of the copper site in C*c*P was performed on a Silicon Graphics Irix2 computer utilizing Quanta97 (Molecular Simulations Inc.). The protein structure was minimized using the Steepest Descents method in CHARMm for 1000 steps.

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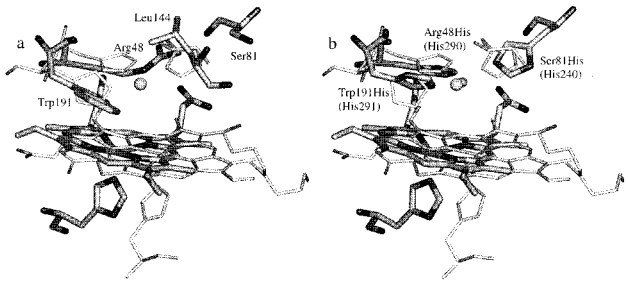

Figure 1. (a) Overlay of the Cu_B site in bovine C*c*O (thin lines) with the active site of WTC*c*P (thick lines); (b) overlay of Cu_B site of bovine C*c*O (thin lines) with active site of C*c*P with the Cu_BC*c*P (R48H, W51H, H52A, S81H) after minimization.

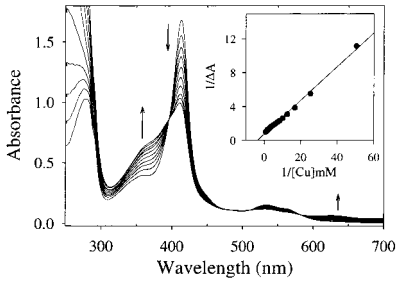

Figure 2. (a) UV−Vis absorption spectra of Cu_BC*c*P with the addition of increasing amounts of Cu^{2+}. The insert shows the double reciprocal plot of absorption change between 370 and 413 nm (ΔA) with respect to copper concentration. Binding data were calculated by titration of the protein (14 μM) in 10 mM malonate pH 7.

parameters for WTC*c*P and the mutant were closely matched (see supplemental information), indicating that the choice of mutations was appropriate.

When isolated,[14] the heme-incorporated, Cu(II)-free Cu_BC*c*P displayed a Soret band at 412 nm, and Q-bands at 534 and 563 nm, typical of a low-spin heme with either bis-His or His/hydroxide ligands (Figure 2). Upon addition of Cu(II) ions, the low-spin signals decreased with a concomitant increase of high-spin signals at 409, 525, and 624 nm, indicating that binding of Cu(II) caused the heme Fe(III) spin state change. The Cu_B-heme center in C*c*O is known to be in a high-spin state.

Isosbestic points at 396, 513, and 565 nm observed in the Cu(II) titration suggest a direct transition between two species. The addition of EDTA resulted in a spectrum identical to that before Cu(II) titration, indicating that the Cu(II) binding is reversible. An identical spectrum was also obtained after incubating the protein with Cu(II) and purifying though a gel filtration column (see supplemental information), indicating no heme loss in the process. The double-reciprocal plot, shown as an inset of Figure 2, indicates a dissociation constant of 250 μM. The Hill plot (see supplemental information) suggests one copper binding site per protein. Cu(II) binding to WTC*c*P was also observed (see supplemental information), although much more Cu(II) had to be added to see the spectral changes because the dissociation constant is 2.2 mM. Interestingly, addition of Zn(II) to the heme-incorporated Cu(II)-free Cu_BC*c*P resulted in similar spectral changes (see supplemental information). The Zn(II), being diamagnetic, serves as a control for the probe of the spin coupling between Cu(II)-heme Fe(III) (vide infra).

EPR studies were performed under two different conditions. Low microwave power (1 mW) and high temperature (20 K) are known to highlight low-spin heme signals, while higher power (10 mW) and lower temperature (4 K) tend to favor the observation of high-spin heme signals because the low-spin signals are saturated under the latter conditions. The EPR spectrum of Cu_BC*c*P at 1 mW power and 20 K (Figure 3a) showed mainly low-spin signals with $g = 3.08$, 2.27, and 1.44, consistent with the UV−vis study. The high-spin signals at $g = 6.00$ and 2.02 account for less than 6% of total spins based on an EPR spectral simulation and are due to freezing effect, as observed in other heme proteins.[9]

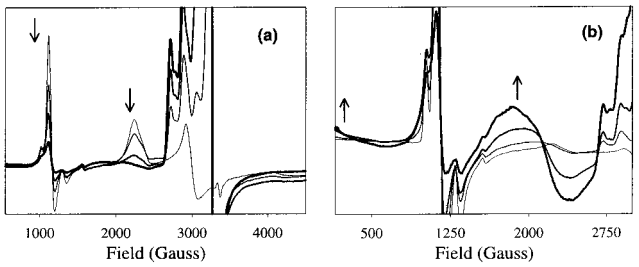

Figure 3. X-band EPR spectra of Cu_BC*c*P with increasing amounts of Cu^{2+} at (a) 20 K and 1 mW microwave power to highlight the low-spin heme signals; and (b) 4 K and 10 mW microwave power to highlight the high-spin heme signals. The final volume and concentration of the each EPR sample was 250 μL and 1 mM in 50 mM PIPES, pH 7.

More importantly, the addition of Cu(II) to Cu_BC*c*P caused a decrease in the intensity of *both* the high-spin and the low-spin signals. These results strongly suggest that the heme Fe(III) is antiferromagnetically coupled to the added Cu(II), just as they are in the Cu_B-heme center in all the terminal oxidases.

The above trend is also observed when EPR spectra are collected at 10 mW power and 4 K to highlight the high-spin signals (Figure 3b). Remarkably, broad signals around $g' = 12$ and $g' = 3$ increased with each addition of Cu(II). These signals have been observed in the slow form of bovine C*c*O and cytochrome *bo* oxidase and were attributed to exchange coupling between Cu_B and the heme iron.[3b,f,6,15] As control experiments (see supplemental information) Zn(II), which is diamagnetic, was added to Cu_BC*c*P and resulted in no decrease of heme EPR signals. The same observation applies to Cu(II) addition to WTC*c*P.

In summary, Cu_BC*c*P represents the first protein model of Cu_B-heme center and is the only model that shows spin coupling between Cu(II) and heme Fe(III) without added exogenous ligands. Since many proposed reaction intermediates in Cu_B-heme center have their peroxidase counterparts, engineering this center in C*c*P will allow us to provide insight into the reaction mechanism through a comparison of the two systems.

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Supporting Information Available: A table of energy parameters for WTC*c*P and Cu_BC*c*P after minimization, Hill plot of Cu(II) titration into Cu_BC*c*P, UV/vis spectra of Cu_BC*c*P before and after the addition and removal of Cu(II), UV/vis and EPR spectra of Zn(II) addition to Cu_BC*c*P, and Cu(II) addition to WTC*c*P. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) The Cu_BC*c*P protein was constructed, expressed, and purified as described previously (ref 11a,c). The proteins were characterized by polyacrylamide gel electrophoresis and electrospray mass spectrometry. The measured molecular weight of the mutant proteins corresponds to the calculated molecular weights within experimental error.

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