Converting Cytochrome b_5 into Cytochrome c-Like Protein

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Cytochrome b_5 (cyt b_5) is a well-studied b-type cytochrome with heme bound noncovalently.[1] Its heme-binding ability depends mostly on the strong axial ligation provided by residues His63 and His39 (Figure 1). Mutation studies on the axial ligands, aimed at creating new proteins with novel catalytic reactivity,[2] have been limited by the fact that such alteration would lead to a substantial decrease in the heme-binding stability.[3] Therefore, it would be of great interest to construct covalent linkages between heme and polypeptides so as to stabilize the heme prosthetic group, as found in *c*-type cytochromes,[4] in which heme is covalently attached to a polypeptide chain via two thioether bonds between the heme vinyl groups and the cysteine residues of a classic Cys-Xaa-Xaa-Cys-His (CXXCH) heme-binding peptide motif in the protein. By converting cyt b_5 into a cyt c-like protein, it will be much easier to fulfill the functional conversion of cyt b_5 mentioned above.

Based on the wealth of molecular information obtained from X-ray crystallography studies of cyt b_5 (PDB entry 1CYO),[5] we found out that residues Asn57 and Ser71 are close to the

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

  DOI: 10.1002/cbic.200500030

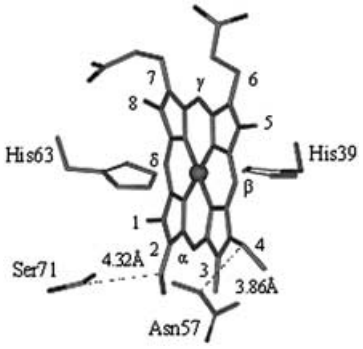

Figure 1. View of the heme-binding pocket of bovine liver microsomal cyt b_5. The residues close to the heme vinyl groups (Asn57 and Ser71) and the two axial residues (His39 and His63) are highlighted, as are the distances between the β carbons of Asn57 and Ser71 and the α carbons of the heme vinyl groups.

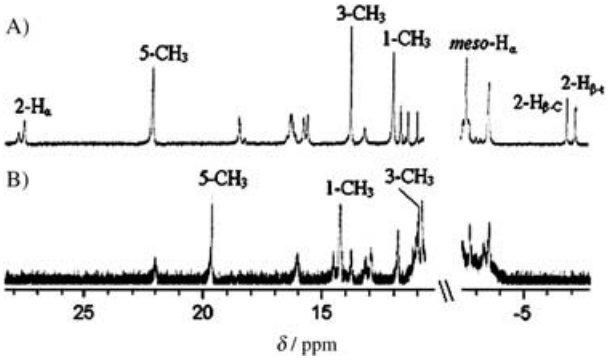

Figure 2. ^{1}H NMR spectra (600 MHz) of solutions of oxidized A) native cyt b_5 and B) *rb*$_5$ N57C/S71C in D_2O at 293 K; pD = 7.2 in 25 mM phosphate buffer. The protein concentrations were 2 mM. The most-shifted heme proton signals are labeled.

heme 4-vinyl and 2-vinyl groups with separations of 3.86 and 4.32 Å, respectively (Figure 1). These positions might be suitable for covalent-bond formation if the two residues were replaced with cysteine. Previous study has shown that in cyt b_5 a cysteine residue introduced at position 57 could form a thioether bond with the heme 4-vinyl group.[6] In order to further study the possibility of forming a covalent linkage between heme and the protein matrix, we replaced residues Ser71 and Asn57 in the native cyt b_5 with cysteine by site-directed mutagenesis.[7] Two major components with different colors (red and green) were obtained from the double-site mutation and were named *rb*$_5$ N57C/S71C (red) and *gb*$_5$ N57C/S71C (green).[8] The heme prosthetic group was shown to be covalently attached to the protein matrix in the double-site mutated cyt b_5, since the heme could not be extracted with acidified butan-2-one.[6]

The hemochrome spectrum of *rb*$_5$ N57C/S71C in pyridine exhibits an α-band at 550 nm, typical of a heme without double bonds conjugated to the porphyrin π-system, as in *c*-type cytochromes.[4] This demonstrates that two vinyl groups of heme in *rb*$_5$ N57C/S71C were saturated and that the heme group was attached through two covalent linkages, since one covalent attachment gives the α-band at 553 nm and when heme is free from covalent attachment the α-band is at 556 nm.[6] Furthermore, blue shifts in the visible spectra of *rb*$_5$ N57C/S71C in both oxidation states (ferric: 409 nm; ferrous: 416, 524, and 553 nm) were observed relative to those of native cyt b_5 (ferric: 413 nm; ferrous: 423, 527, and 556 nm). Ishida et al. have illustrated that these blue shifts of absorption bands agree with the differences between natural b- and c-type cytochromes and also with the mutational conversion of cytochrome b to c.[9]

The formation of covalent linkages in *rb*$_5$ N57C/S71C was also confirmed by its ^{1}H NMR spectrum (Figure 2). The resonances at 27.71, −7.19 and −6.86 ppm assigned to the heme 2-vinyl group in the spectrum of native ferric cyt b_5 were absent in the ferric *rb*$_5$ N57C/S71C, as in the case of ferric cyt b_5 reconstituted with deuterohemin or 2,4-dimethyl-deuterohemin.[10] At the same time, the different shifts of the heme methyl groups with respect to those of native protein reflect not only the different orientation of the two axial ligands,[11] but also the effects of covalent-bond formation at positions 2 and 4 of the heme.[12] Further evidence comes from the resonance of the heme *meso*-H_α, which shifted from −2.75 ppm in native cyt b_5 to 8.14 ppm in the double mutant. This shift is similar to those observed in many *c*-type cytochromes.[13]

Electrospray mass spectrometry provides further detailed information about the covalent linkages in *rb*$_5$ N57C/S71C. It reveals that the mass of *rb*$_5$ N57C/S71C is 11 269.8 ± 1 Da, instead of the expected mass of 11 254.8 Da (*rb*$_5$ N57C/S71C with covalently attached heme-thioether). The unexpected, additional mass of ~15 ± 1 Da implies that an extra oxygen atom was added in *rb*$_5$ N57C/S71C. This observation coincides well with the crystal structure reported recently for cytochrome *rC*$_{552}$ (PDB entry 1QYZ), which contains one extra oxygen atom since the 2-vinyl group of heme forms an unusual [heme-CO-CH_2-S-CH_2-C_α] linkage with cysteine residue.[14]

To identify the location of the additional oxygen atom (at the 4- or 2- vinyl group) in the double-site mutated cyt b_5, two single-site mutants, the cyt b_5 N57C and S71C variants, were prepared by the same procedure as used for the double-site mutants. The red components of each single-site mutant obtained, named *rb*$_5$ N57C and *rb*$_5$ S71C, respectively, were subjected to mass spectral analysis. The mass analysis of *rb*$_5$ N57C gave a value of 11 238.8 ± 1 Da, identical to *rb*$_5$ N57C with heme thioether covalently attached. The mass of *rb*$_5$ S71C with heme thioether covalently attached should be 11 266.8 Da, however the mass was determined to be 11 281.9 ± 1 Da, that is, ~15 ± 1 Da higher. These data suggest that the additional oxygen atom is at amino acid position 71 (heme 2-vinyl group) rather than at position 57 (heme 4-vinyl group) in the double-site mutated cyt b_5. This illustrates the two different cysteine–heme covalent linkages formed in *rb*$_5$ N57C/S71C: cysteine-57 forms the typical thioether linkage with the heme 4-vinyl group, while cysteine-71 forms an unusual [heme–CO–CH_2–S–CH_2–C_α] linkage with the heme 2-vinyl group.

Turning to the characterization of *gb*$_5$ N57C/S71C, in its visible spectra, the dramatically red-shifted Soret and α-, β-bands (ferric: 420 nm; ferrous: 434, 553 and 579 nm) indicate a

change of electron-density distribution in the porphyrin ring resulting from a structural disturbance.[6] In the pyridine hemochrome spectrum, there is an α-band at 581 nm identical to that of 2(4)-formyldeuterohemin;[14,15] this provides evidence for the presence of a formyl group in gb_5 N57C/S71C. Further evidence comes from the resonance Raman spectra of gb_5 N57C/S71C, which contain new, high-frequency bands at 1666 cm^{-1} for the oxidized and at 1643 cm^{-1} for the reduced sample, which are not present in the spectra of rb_5 N57C/S71C, corresponding to the characteristic resonances of a formyl stretch.[14]

Similarly, the mass spectrum of gb_5 N57C/S71C reveals that it also contains an additional oxygen atom (the measured mass is 11270.5 ± 1 Da). Actually, gb_5 N57C/S71C is the transformative product of rb_5 N57C/S71C, in which the oxidized heme 2-vinyl group has been converted to a formyl group, while the thioether linkage between cysteine-57 and the heme 4-vinyl group is maintained, as shown in the reported crystal structure of cytochrome p572 (PDB entry: 1ROQ).[14] It was found that heating can accelerate the conversion of rb_5 N57C/S71C to gb_5 N57C/S71C, as shown by the two additional peaks in the visible spectrum at 432 and 577 nm, which are characteristic of reduced gb_5 N57C/S71C (Figure 3). This behavior has also been observed in the conversion of cytochrome rC_{552} to cytochrome p572.[14]

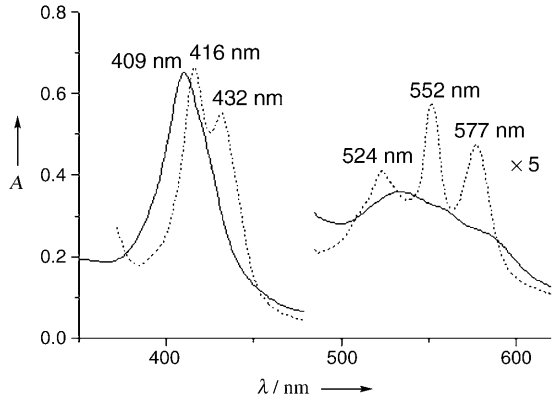

Figure 3. Effects of heating on the visible spectra of rb_5 N57C/S71C. Spectrum after heating the oxidized form of rb_5 N57C/S71C for 30 min at 70 °C in sodium phosphate buffer (50 mM, pH 7.0) and cooling to room temperature (——). An excess of sodium dithionite was subsequently added to obtain the reduced spectrum (-----) of the heated solution, which reflects a mixture of rb_5 N57C/S71C and gb_5 N57C/S71C.

It has been reported that unfolding induces peroxidase activity in c-type cytochromes,[16] this prompted us to evaluate the peroxidase activity of the cyt *c*-like cyt b_5 (rb_5 N57C/S71C) upon guanidine hydrochloride-induced unfolding.[17] The initial rates of product formation catalyzed by unfolded rb_5 N57C/S71C and unfolded wild-type cyt b_5 were found to be 3.5×10^5 M s^{-1} and 9.4×10^3 M s^{-1}, respectively. Meanwhile, control experiments showed initial rates of 1.0×10^3 M s^{-1} and 1.9×10^2 M s^{-1} for the folded native rb_5 N57C/S71C and cyt b_5, respectively. The considerably enhanced activity of unfolded rb_5 N57C/S71C could be attributed to an open crevice created at the heme upon unfolding by removal of the strong axial ligand and release of the rigid protein matrix, while maintaining the covalent heme–peptide attachment, as in the architecture of microperoxidases;[18] this would facilitate the formation of the catalytic oxo-ferryl intermediate and the access of the peroxide substrate to the heme iron center.[19] The story is not the same as for the wild-type cyt b_5; here the heme will dissociate from the protein matrix upon unfolding in the absence of covalent linkages.

In summary, we found that a cyt *c*-like cyt b_5 can be obtained by introducing a cysteine residue close to the heme vinyl groups without constructing the classic CXXCH heme-binding motif of cyt *c*.[20] The cysteine–heme covalent linkage could be either in the form of a thioether or [heme–CO–CH_2–S–CH_2–C_α] depending on their distance and spatial positioning. This study demonstrates that the classic heme-binding peptide motif "CXXCH" is not essential for the covalent attachment of heme to protein matrices in cytochromes. It will provide us with more information for understanding the in vivo and in vitro formation of a cyt c-like protein and for delineating the "structure–property–reactivity–function" relationship of hemoproteins. In addition, with heme covalently linked, the constructed cyt c-like cyt b_5 displays peroxidase activity in its unfolded form, transforming from an electron-transfer protein to a catalytic species. This study has also laid down a base for functional conversion of cyt b_5 by further structural modification based on the scaffold of cyt c-like cyt b_5.

Acknowledgements

It is a pleasure to acknowledge Professor A. G. Mauk of the University of British Columbia, Canada, for his kind gift of the cyt b_5 gene and discussion. This work was supported by the National Science Foundation of China.

Keywords: conversion · covalent linkage · cytochromes · enzymes · peroxidase activity

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[7] The cyt b_5 gene was amplified by polymerase chain reaction from pUC19 plasmid and subcloned into the *Nde*I/*Bam*HI-linearized pET-11c plasmid with BL21(DE3)pLysS as host cell. For the primers used, see the Supporting Information. The recombinant native cyt b_5 gene was first

mutated to the N57C variant and then further mutated to the N57C/S71C variant.
[8] They were separated on an ion-exchange column (Pharmacia, Mono Q) with NaCl as eluent under reducing conditions (5 mM sodium ascorbate),[6] the red component (rb_5 N57C/S71C) eluted from the column prior to the green component (gb_5 N57C/S71C).
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[17] Peroxidase activity was estimated with protein (1 μM) in sodium phosphate (2.0 mL, 100 mM) and guanidine hydrochloride (6 M) at pH 7.0 and 25 °C, with guaiacol (10 mM) as substrate. The reaction was initiated by the addition of hydrogen peroxide (final concentration 10 mM) to the starting mixtures and followed by monitoring the absorbance change of the product, tetraguaiacol, at 470 nm by using a molar absorbance coefficient of $\varepsilon = 26.6\ \mathrm{mM^{-1}cm^{-1}}$ (D. A. Baldwin, H. M. Marques, J. M. Pratt, *J. Inorg. Biochem.* **1987**, *30*, 203 – 217). The control experiments were carried out by following the same procedure, but without guanidine hydrochloride in the solution.
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Received: January 25, 2005

