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A Role of the Heme-7-Propionate Side Chain in Cytochrome P450cam as a Gate for Regulating the Access of Water Molecules to the Substrate-Binding Site

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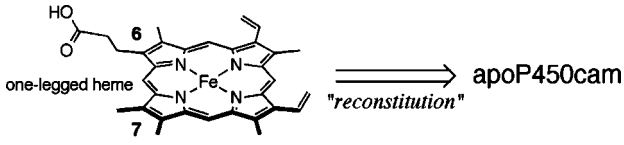
Cytochrome P450cam (P450cam) is a monooxygenase which catalyzes 5-*exo*-hydroxylation of *d*-camphor via reductive dioxygen activation using the thiolate-binding heme *b* as a prosthetic group.[1−4] A crystal structure analysis of P450cam indicates that the 6- and 7-propionate peripheral side chains form hydrogen bonding interactions with amino acid residues such as Arg, His, and/or Asp.[5,6] The heme propionate side chains have long been regarded simply as anchors for connecting the heme prosthetic group to the protein matrix.[7] We have recently reported that the heme-6-propionate side chain is essential for maintenance of the reactive P450cam protein, because removal of 6-propionate highly accelerates the conversion of P450cam into the inactive P420 species.[8] On the other hand, it was theoretically proposed that the 7-propionate−Arg299 salt-bridge would regulate the process of expelling the water cluster from substrate-binding site through a space transiently formed by a cleavage of the salt-bridge induced by a metastable rotamer of the Arg299 residue at the initial step of the catalytic cycle.[9,10] In addition, the QM/MM calculation study suggests that the carboxylate of the 7-propionate side chain partially contributes to tuning of the electron density of the activated heme species.[4,11] However, to the best of our knowledge, there is no experimental evidence that demonstrates a functional role of the 7-propionate side chain in the protein interior. We therefore prepared the reconstituted protein with an artificially created one-legged heme where the 7-propionate side chain is replaced by a methyl group (Scheme 1).[12,13] Here, we report the structure, physicochemical property, and reactivity of the reconstituted protein to evaluate the role of the 7-propionate side chain in the native protein.

Scheme 1. Insertion of One-Legged Heme into apoP450cam



The insertion of the one-legged heme into apoP450cam was carried out by the method described in the previous papers.[8,14] The reconstitution was confirmed by ESI-TOF mass spectrometry which showed a peak at 47104.7 (the calculated mass number is 47101.34). Purified reconstituted P450cam exhibits the ferrous CO-bound P450cam spectrum with the characteristic Soret band at 446 nm (Figure 1, inset), indicating that the thiolate of Cys357 is ligated to the heme iron of the one-legged heme as seen in the wild-type protein. In contrast, unexpectedly, the UV−vis spectrum of the reconstituted ferric P450cam (Figure 1, red spectrum) in the presence of 1 mM *d*-camphor is similar to that of the typical *d*-camphor-free low-spin ferric P450cam species with maxima at wavelengths of 417, 540, and 570 nm, whereas the wild-type ferric protein usually has a spectrum characteristic of the *d*-camphor-bound high-spin species with maxima at 391, 512, and 646 nm (Figure 1, blue spectrum) under the same conditions.[15] The content of the high-spin state of the reconstituted protein in the presence of 1 mM *d*-camphor and 100 mM KCl is estimated to be 26% on the basis of deconvolution of the Soret band region using the spectra of the camphor-free reconstituted protein and camphor-bound wild-type protein.

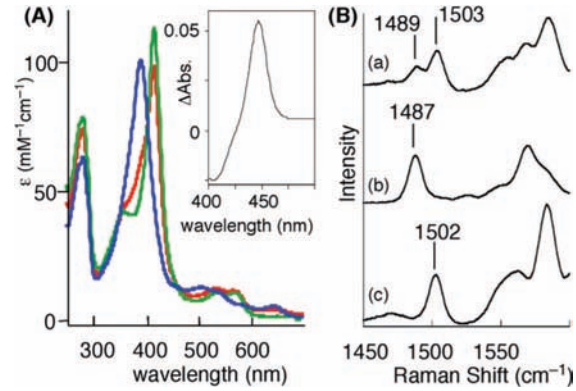


Figure 1. (A) UV−vis spectra of P450cams. The red and blue spectra represent the reconstituted and wild type ferric proteins, respectively, in 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl, and 1 mM *d*-camphor at 20 °C. The green spectrum represents the reconstituted protein under the same conditions except in the absence of *d*-camphor. The inset shows the difference spectrum of the CO-bound reconstituted protein. (B) Resonance Raman spectra of ferric P450cams in the 1450−1600 cm^{-1} region in 50 mM phosphate buffer, pH 7.4, containing 100 mM KCl, and 1 mM *d*-camphor (except trace c) at room temperature: (a) reconstituted P450cam spectrum, (b) wild type P450cam spectrum, and (c) *d*-camphor-free wild-type P450cam spectrum. The excitation wavelength was 413.1 nm with a laser power of 3 mW.

The high frequency resonance Raman spectrum of the ferric reconstituted P450cam also exhibits two evident υ_3 bands at 1503 and 1489 cm^{-1} which are derived from the low-spin and high-spin species, respectively.[16] From the UV−vis spectroscopic study, it is found that the *d*-camphor affinity for the substrate-binding site of the reconstituted protein is approximately 3 mM

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Table 1. Reactivities of Wild-Type and Reconstituted P450cams[a]

P450cam	k_{et} (s^{-1})[b]	K_d (μM) [c]	NADH oxidation[d] (μM/μM heme/min)	yield (%)[e]
wild type	53 ± 1.6	2.1 ± 0.25	$(1.35 \pm 0.05) \times 10^3$	100
reconstituted	0.54 ± 0.02	1.0 ± 0.15	27 ± 2	87 ± 2

[a] At 20 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 1 mM *d*-camphor. [b] Electron transfer rate constant from Pdx to ferric P450cam. [c] Affinity of Pdx for P450cam. [d] Rate of NADH oxidation by P450cam. [e] Yield of the product, 5-hydroxycamphor, based on the amount of oxidized NADH was determined by GC using benzyl alcohol as an internal standard.

at 20 °C, pH 7.4 in the presence of 100 mM KCl. From these results, removal of heme-7-propionate was found to dramatically decrease the *d*-camphor affinity by approximately 3 orders of magnitude, suggesting that the 7-propionate side chain plays a role in maintaining the high affinity of cytochrome P450cam for its substrate, *d*-camphor.

Although the affinity of the one-legged heme-reconstituted P450cam for reduced putidaredoxin (Pdx) is essentially the same as that of wild type P450cam, the process of electron transfer from reduced Pdx to ferric reconstituted P450cam is much slower (Table 1). In contrast, the electron transfer rate from reduced Pdx to oxygenated reconstituted P450cam was almost the same as that observed for the wild type protein (cf. Supporting Information).[17] As a result of this dramatically slowed first electron transfer in the reconstituted protein, the rate constant of the NADH-driven hydroxylation of *d*-camphor is only 8% of that determined for the wild-type protein. These findings are supported by the spectroscopic data that indicate the predominant presence of the less reactive low-spin species in the reconstituted protein. On the other hand, the product analysis suggests that the hydroxylation of *d*-camphor only occurs at the C-5 position as seen in the wild type protein. The reaction remains well coupled with oxidation of NADH as evidenced by the observation that $87 \pm 2\%$ of oxidized NADH was utilized to produce the product. These results indicate that the removal of the 7-propionate side chain dramatically decreases the *d*-camphor affinity, but exerts less of an influence on the other steps of the catalytic cycle of the monooxygenation reaction catalyzed by P450cam.

The X-ray structure of the reconstituted P450cam at a resolution of 1.8 Å reveals that the asymmetric one-legged heme is incorporated into the heme pocket in the same plane and in essentially the same conformation as the heme of the wild-type protein (cf. Supporting Information).[18] These observations suggest that the lack of the 7-propionate side chain does not exert an influence on the structure and electronic properties of the heme. This interpretation is in agreement with the spectroscopic data of P450cam.

The polypeptide Cα atoms of the wild type and reconstituted proteins are superimposable with a root-mean-square deviation of 0.507 Å. In the vicinity of the 7-propionate side chain, in the wild type protein there is a unique hydrogen bonding tetrad network comprising Arg299, 7-propionate, Asp297, and Gln322 (Figure 2A). Conformational differences are clearly observed for Asp297 and Gln322, which evidently deviate from their respective positions in the native structure, whereas, notably, the conformation of Arg299 is almost same as that of the wild-type protein (Figure 2B). The *d*-camphor substrate binds to the substrate-binding site with nearly the same conformation as that of the wild-type protein and forms a characteristic hydrogen bond with Tyr96 (Figure 2C). The C-5 atom is pointed toward the

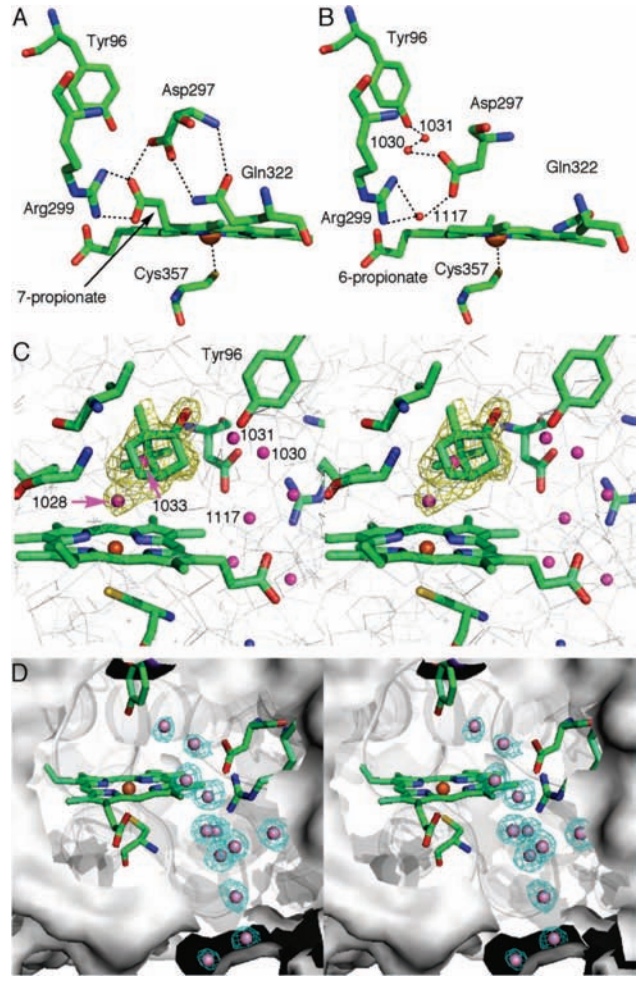

Figure 2. Crystal structure of P450cam showing the environment of heme groups in the presence of *d*-camphor. The characteristic water molecules in the crystals are also shown as spheres. Panels A and B represent the structures of the wild-type (2CPP) and reconstituted protein with the one-legged heme (2Z97). For clarity, *d*-camphor is omitted. Panels C and D are stereopair representations: (C) A view from a different angle focusing on the heme pocket with the yellow $F_o - F_c$ electron density map at 4.0σ based on the refined coordinates excluding *d*-camphor and water molecules. At least two water molecules, WAT1028 and 1033, were positioned into the $F_o - F_c$ electron density map and refined together with *d*-camphor and the protein. *d*-Camphor binds at the substrate-binding site with an occupancy of 0.25, whereas the coordinated WAT1028 and WAT1033 (see pink arrows) are located in the same area with an occupancy of 0.75. In addition, the map shows a small mass of unassignable density between WAT1028 and 1033 when contoured at 3σ. (D) The structure of the water array and the heme pocket. The cyan $2F_o - F_c$ map at 1.0σ is attached to each water molecule. For clarity, *d*-camphor and WAT1028, 1032 (data not shown) and 1033 are omitted. Occupancies and number designations of the representative water molecules are shown in Supporting Information.

heme iron, in an orientation consistent with the regiospecific *d*-camphor hydroxylation. However, the substrate binding site is not fully occupied with *d*-camphor. The $F_o - F_c$ map of the substrate-binding site clearly shows electron densities that fit to a camphor molecule and two water molecules with occupancies of 0.25, 0.75, and 0.75, respectively. This indicates coexistence of two substrate-binding site structures; the camphor-bound form and the camphor-free water-bound structure (their protein structures are crystallographically indistinguishable from each other). In the *d*-camphor-free structure, at least two distinct water molecules are located within the substrate-binding site;[19] WAT1028 is coordinated to the heme iron at a distance of 2.54

Å, while the other water molecule, WAT1033, is located within a hydrogen-bonding distance of WAT1028. The observed low occupancy of *d*-camphor is consistent with the low affinity observed by spectroscopic analysis.

Relative to the camphor-bound and camphor-free forms of the wild type protein, two additional water molecules (WAT1030 and 1031) are identified near Tyr96 and Asp297 of the reconstituted P450cam. Furthermore, a single water molecule (WAT1117) occupies the position of the 7-propionate carboxylate (Figure 2B). As a result, a unique array of water molecules extending from the Tyr96 residue to the outside of the protein is observed in the crystal structure (Figure 2D). This water array appears to prevent the exclusion of the inner water molecules from the substrate-binding site, thereby decreasing the *d*-camphor binding affinity of the reconstituted P450cam. Furthermore, in the reconstituted protein, it is noted that the Asp297 residue is remarkably flipped and its side chain Oγ1 and Oγ2 atoms are located within hydrogen bonding distance of WAT1030 and WAT1117. The hydrogen bonding interaction between the characteristic water molecules and the Asp297 residue disconnects the Gln322 residue from the Asp297 residue due to the cleavage of the O(Asp297)−HN(Gln322) interaction. This structural evidence suggests that the Asp297 and Gln322 residues are capable of undergoing a 7-propionate-associated conformational change in the protein interior.

It is known that the exclusion of water molecules from the substrate-binding site to bulk is the critical initial step of the P450cam catalytic cycle which yields the *d*-camphor-bound high spin active species. However, there is no clear pathway near the heme cavity for expulsion of water in the crystal structure of the wild type protein. In contrast, assuming that conformational changes of the tetrad occur to support the process of expulsion of water molecules upon binding of substrate, a simulation analysis suggests that a new space for expulsion of water molecules from the substrate-binding site is available near the tetrad.[20] Thus, we propose that the 7-propionate side chain functions not only as a water gate to repel water molecules from bulk to promote the high *d*-camphor affinity but also as a pivot in the hydrogen-bonding tetrad to regulate the water expulsion system at the initial step of P450cam catalytic cycle. Investigations of the water expulsion mechanism are now in progress. Another point of interest is the role of the 7-propionate side chain on the other reaction steps of the catalytic cycle of P450cam.[4,11]

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Supporting Information Available: Detailed experimental protocols, kinetic analysis of the protein reduction and X-ray crystallographic data and structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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