Subunit interactions change the heme active-site geometry in 
*p*-cresol methylhydroxylase

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ABSTRACT The enzyme *p*-cresol methylhydroxylase [4-cresol:(acceptor) oxidoreductase (methyl-hydroxylating), EC 1.17.99.1] contains two subunits: a cytochrome c (electron transfer) subunit (cytochrome *c*<sub>PCMH</sub>) and a flavin (catalytic) subunit. When these subunits are separated by isoelectric focusing, a stable cytochrome subunit is obtained. Significant differences are observed between the one-dimensional NMR spectra of oxidized cytochrome *c*<sub>PCMH</sub> and of oxidized *p*-cresol methylhydroxylase. Analysis of the two-dimensional nuclear Overhauser enhancement and exchange spectroscopy (NOESY) spectrum of reduced cytochrome *c*<sub>PCMH</sub> suggests that the axial ligand, Met-56, of the stable subunit reorients by a rotation about the Cy–S8 bond when cytochrome *c*<sub>PCMH</sub> binds to the flavin subunit. This reorientation must result in a change in bonding at the heme, which is reflected both in the paramagnetically shifted resonances and in the redox potential. *p*-Cresol methylhydroxylase thereby provides an interesting example of the coupling of subunit interactions to active-site structure and reactivity.

Interactions between individual subunits or domains are often believed to play a key role in modulating the structure and reaction of an active site (1–3). One classic example is offered by hemoglobin, where subunit contacts (in “relaxed” or “tense” states) are linked to active-site geometry and reactivity (3). Such inter-subunit communication is also likely to be important in multisite redox proteins. A well-characterized, two-site redox protein is *p*-cresol methylhydroxylase [PCMH; 4-cresol:(acceptor) oxidoreductase (methyl-hydroxylating), EC 1.17.99.1] (4–7). PCMH form A from *Pseudomonas putida* strain NCIB 9869 consists of two subunits, a flavin-containing catalytic subunit (M<sub>c</sub>, 48,600) and a smaller electron-transfer subunit (cytochrome *c*<sub>PCMH</sub>; M<sub>c</sub>, 8780) that contains a c-type heme. Although strongly bound, these subunits can be reversibly separated by isoelectric focusing (8). The independent cytochrome subunit is not denatured when separated but retains a compact, stable structure including the sensitive methionine charge-transfer band and upfield NMR shift. Thus, the intact enzyme can be functionally reconstituted from the isolated subunits (8). However, the isolated cytochrome PCMH subunit is functionally different from when it is incorporated into the holoenzyme. For example, dissociation shifts the heme redox potential by almost 400 mV; this shift is reversed upon reassembling the subunits. The structural bases for such functional changes that accompany subunit association have not, to our knowledge, been investigated.

A recent crystallographic study (7) has defined the structure of the intact, two-subunit PCMH (Fig. 1). The stability and low molecular weight of cytochrome *c*<sub>PCMH</sub> suggested that its solution structure could be determined by NMR methods with the aim of determining how subunit interactions may influence active-site structure and reactivity. As a first step in these studies, we have reported the assignments of some of the protons of the heme active site. These data show that an unusual active-site rearrangement occurs when cytochrome *c*<sub>PCMH</sub> binds to the flavin subunit to form the intact flavocytochrome. PCMH thus provides an example of a change in active-site geometry induced by assembling subunits into the fully active enzyme complex.

MATERIALS AND METHODS PCMH was isolated and purified as described (9, 10). Cytochrome *c*<sub>PCMH</sub> was obtained from PCMH by isoelectric focusing; the presence of a compact, highly folded structure is demonstrated by the hydrodynamic properties, which are those of a globular protein, not a random coil, and the highly characteristic CD of cytochrome PCMH, which shows substantial α-helix content, and, finally, by the reported NMR spectra reported (8). Because the cytochrome subunit constitutes only 16% of the mass of the intact flavocytochrome, it is difficult to isolate large amounts of cytochrome *c*<sub>PCMH</sub>. Thus, only a small amount (4 mg, 10<sup>−3</sup> M solution) of cytochrome *c*<sub>PCMH</sub> was available for these studies. Despite the use of relatively low concentrations, the spectral quality was sufficiently high to observe nuclear Overhauser enhancements (NOEs) between protons separated by up to 4 Å. NMR samples of PCMH and cytochrome *c*<sub>PCMH</sub> consisted of 1 mM protein and were prepared by exchanging the protein solution into 99.8% H<sub>2</sub>O/0.01 M P<sub>i</sub>, pH 7.0 (where pH includes a deuterium effect), in a miniaturized dialfiltration cell (Amicon 8MC) fitted with a YM30 or YM5 membrane. Adjustments to the pH were made with microliter aliquots of 3HCl and NaO<sub>D</sub>H with no correction being made for the deuteron isotope effect. Fully oxidized or reduced samples were obtained by treatment of the solution with a small excess of buffered K<sub>i</sub>Fe(CN)<sub>i</sub> (before dialfiltration) or Na<sub>i</sub>S<sub>i</sub>O<sub>i</sub> (after dialfiltration). After dialfiltration, 0.5 ml of protein solution was transferred to a NMR tube. As a precaution against atmospheric oxidation, reduced samples were transferred to a sealed NMR tube previously flushed with argon.

The crystallographic coordinates were derived from an electron density map of PCMH calculated at 3.0-Å resolution to which the known amino acid sequence of the cytochrome subunit was fitted. The protein phases were obtained by multiple isomorphous replacement and refined by cyclic averaging and solvent leveling. The model has not yet been

Abbreviations: PCMH, *p*-cresol methylhydroxylase; 1D, one-dimensional; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; NOE, nuclear Overhauser enhancement.

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RESULTS

Although PCMH is too large to give good quality two-dimensional NMR spectra, the small size of the cytochrome subunit of PCMH makes it suitable for analysis using two-dimensional NMR methods, particularly in the region of the heme. Both the 1D and NOE spectra confirm that cytochrome $c_{pc}$ retains a highly folded structure in solution. For example, the large chemical shift dispersion, particularly for the upfield ring current-shifted methyl groups, can only be obtained for a folded (native-like) structure. Particularly diagnostic in this regard is the upfield shifted methionine Met-50 $e$-CH$_3$ methyl resonance, which demonstrates that the conformationally sensitive Fe—S methionine bond is maintained in the isolated subunit. NOE methods have been used extensively to assign the individual resonances of heme active sites (14, 18). These methods are primarily applicable to diamagnetic, low-spin ferrous hemes (14, 18). Therefore, following the basic strategy of Williams et al. ("single proton" and "methyl proton" resonances can readily be distinguished by integrating the 1D spectrum) (14), the NOE connectivities (see Fig. 2) involving the heme meso protons of Fe(II) cytochrome $c_{pc}$ were examined.

The general approach to assignment follows, using the nomenclature outlined in Fig. 3: the $\alpha$ and $\beta$ meso protons should each give NOEs to two methyl groups. The NOEs involving heme methyl groups 3 and 5 will be of high intensity, whereas those involving the thioether methyl protons will probably be weaker and have an intensity that depends on the specific thioether stereochemistry. The $\gamma$ meso proton should not show a strong NOE to any methyl group because the closest methyl group [according to the crystal structure (7)] is $>5$ Å away; it should show NOEs to (at least) four different single proton resonances from the propionate $\alpha$ and $\beta$ protons centered around 2–3 ppm.


**Fig. 1.** Backbone structure of PCMH adapted from the coordinates provided by F. S. Mathews (5).

refined, since the amino acid sequence of the flavoprotein subunit is largely unknown.

The one-dimensional (1D) spectrum of oxidized PCMH was obtained on a Bruker AM600 spectrometer; the 1D and nuclear Overhauser and exchange spectroscopy (NOESY) (11) spectra of cytochrome $c_{pc}$ were obtained on a Bruker AM500 spectrometer. Both spectrometers are the property of the Oxford Centre for Molecular Sciences. HOHAHA spectra were recorded at 600 MHz on a Bruker AM600 spectrometer at 35°C, with a MLEV 17 mixing sequence (mixing time 36 ms) sandwiched between 2.5-ms trim pulses. Eight hundred and fifty $t_1$ increments of 2000 data points were collected with presaturation of the residual water resonance.

The NOESY (11) spectrum was recorded at 500 MHz on a Bruker AM500 spectrometer at 35°C with a mixing time of 125 ms. Seven hundred $t_1$ increments of 2000 data points were collected with presaturation of the residual water resonance. Two-dimensional spectra were recorded in the pure phase absorption mode using time-proportional phase incrementation (12). The reference phase of the spectrometer receiver was adjusted to yield a flat baseline in the $F_2$ dimension (13).

Both two-dimensional experiments were processed using Fourier transform NMR (Hare Research, Woodinville, WA) with a line-broadening parameter of $-15$ Hz and a Lorentz-to-Gaussian resolution enhancement parameter of 0.15 in the $t_2$ dimension. A 60°-shifted sine-squared bell was used in $t_1$ for the NOESY.

**Fig. 2.** Contour plots of the connectivities involving the meso protons from the NOESY spectra of the reduced cytochrome subunit of PCMH at pH* 7 and 37°C.

**Fig. 3.** Nomenclature for the active-site heme.
Finally, the δ meso proton should show two strong NOEs to heme methyl groups 1 and 8.

With these patterns in mind and using analogies with previous NMR studies of cytochromes, the heme meso proton resonances of cytochrome \( c_{\text{pc}} \) may be assigned. In the subsequent discussion, labels 1–4 of the meso protons are taken from Fig. 2. Meso proton 1 (at 10.18 ppm) does not show any NOE to the heme methyl resonances but does connect with four single proton resonances. This pattern clearly identifies the γ meso proton. Similarly, the δ meso proton may be assigned to meso proton resonance 2 at 9.48 ppm. This is the only resonance that shows NOEs to two heme methyl groups. These NOEs are of roughly equivalent intensity, as expected for the δ meso, which is equidistant from heme methyls 1 and 8. By elimination, the remaining resonances at 9.46 ppm and 9.15 ppm must be due to meso protons α and β. These resonances are differentiated by the observation that the resonance labeled 4 shows a unique NOE to the ε-methyl protons of the Met-50 axial ligand. Furthermore, Met-50 ε-CH₃ also shows a NOE to the heme methyl group, which is close to meso proton 4. Therefore, the α and β meso protons might, in principle, be distinguished by determining which proton more closely approaches Met-50 ε-CH₃. As the crystal structure of PCMH is known, we anticipated that this structural assignment would be straightforward. This assumption proved incorrect.

From the crystallographic coordinates (7) of the intact flavocytochrome, the expected NOEs to Met-50 ε-CH₃ can be clearly established. Only one strong NOE to any meso proton is predicted from the crystal structure, and this involves the γ meso proton. The crystal structure (7) further predicts that Met-50 ε-CH₃ will show no NOE to any other methyl group because all heme methyls lie >5 Å away. Clearly, both predictions are inconsistent with the observed NOEs in cytochrome \( c_{\text{pc}} \). The observed Met-50 ε-CH₃ NOE does not involve the γ meso proton but rather involves meso proton 4 (α or β) and its associated methyl group. We must conclude that cytochrome \( c_{\text{pc}} \) rearranges to a different orientation of Met-50 than that found in intact PCMH. The alternative possibility, that the position of Met-50 γ-CH₃ was incorrectly fitted to the electron density, could also be considered, but it is less likely, since the all-trans configuration of Met-50 is more consistent with the observed electron density.

The 1D NMR spectra of the Fe(III) forms of PCMH and cytochrome \( c_{\text{pc}} \) are shown in Fig. 4. Simply comparing the positions of the structure-sensitive paramagnetically shifted heme methyl resonances at a constant temperature of 32°C suggests that a change in heme electronic structure is caused when cytochrome \( c_{\text{pc}} \) binds to the flavin subunit to form intact PCMH. Although several factors might contribute to such a change, the large magnitude (up to 8 ppm) of the difference in chemical shifts suggests a possible shift in the position of the heme g-tensors, coincident with a change in Fe-ligand geometry. Such a geometric change would, indeed, occur if Met-50 rearranges as observed in Fe(II) cytochrome \( c_{\text{pc}} \) and is well preceded in the very different paramagnetic shifts observed for eukaryotic cytochrome c and cytochrome \( c_{551} \) (18), which also reflects a difference in the geometry of the axial Met—Fe bond. By analogy with cytochrome c, we attempted to confirm a geometry change using CD spectroscopy. There are, indeed, significant differences in the signs of the heme Cotton effects (particularly at ~430 nm) for PCMH when compared with the corresponding cytochrome PCMH subunit. However, the 690-nm region, which is conventionally assigned to a charge transfer, does not show a significant Cotton effect in either the holoenzyme or the isolated subunit, so that a direct confirmation of the NMR results by CD is not possible.

![Fig. 4. The 500-MHz \(^1\)H NMR spectrum of oxidized PCMH (a) and the cytochrome subunit of PCMH (b) showing the paramagnetically ring current-shifted heme methyl groups.](image-url)
DISCUSSION

Allosteric models of active-site regulation depend on the presumption that subunit (or domain) contacts modify active-site structure. Despite the prevalence of such models, there remain few clear examples of specific stereochemical changes in active-site structure that are coupled to interactions between subunits. The present data for the cytochrome c subunit of PCMH provide an interesting example of a specific structural change that accompanies assembly of the enzyme from its subunits.

Analysis of the NOESY spectrum of cytochrome c<sub>pc</sub> in conjunction with the crystal structure (7) of PCMH provides a simple suggestion for this structural change, as shown in Fig. 5A and B. A change in torsion angle about the C<sub>γ</sub>Sδ bond of Met-50 can swing the e-CH<sub>3</sub> group across the heme from a position near the γ meso proton [as observed in the holoenzyme crystal structure (7)] to a position near the α meso proton. This new orientation is nearly 180° from the crystallographic orientation and leads to close contacts of Met-50 e-CH<sub>3</sub> with the α meso proton and heme methyl 3, in agreement with the observed NOE pattern.

No simple change in torsion angle can result in a reorientation, which leads to close contact between Met-50 e-CH<sub>3</sub> and the β meso proton. This “least motion” analysis suggests meso proton resonance 4 should be assigned to the α meso proton. We, therefore, infer that a unique orientation occurs in cytochrome c<sub>pc</sub> (Fig. 5B), with a change to the crystallographic orientation when the subunits are assembled to form PCMH. Such a reorientation naturally predicts large changes in frequency for the paramagnetically shifted heme methyl resonances. Such differences between PCMH and its isolated cytochrome subunit are, indeed, observed.

This reorientation of Met-50 necessarily also reorients the ligand orbitals, which are available for back bonding to the iron atom. This change in bonding can have several effects. (i) The electron density within the ring will necessarily redistribute to accommodate the new bonding. This redistribution of electron density and associated spin density is responsible for the large differences in the paramagnetically shifted resonances between PCMH and cytochrome c<sub>pc</sub>. A strong analogy exists in the comparison of eukaryotic cytochrome c with cytochrome c<sub>551</sub> (18). The axial methionine ligand takes up different orientations in these two proteins, with concomitant large change in the pattern of paramagnetically shifted heme methyl resonances. Senn et al. (18) have argued that the function of these changes is to direct electron density toward the reactive heme edge to facilitate reaction with other redox proteins. A similar functional shift in electron density may accompany reorientation of the ligand that occurs when cytochrome c<sub>pc</sub> and the flavin subunit are assembled to form intact PCMH. A second related effect can be predicted. As pointed out by Moore et al. (19), an effect on the redox potential of the cytochrome subunit is expected. If methionine reorientation diminishes Fe-S back bonding, then a shift in redox potential favoring Fe(III) is expected. This result is, indeed, observed (20, 21); although cytochrome c<sub>pc</sub> in the intact flavocytochrome reduces at <i>E</i><sub>red</sub> = 254 mV (21) vs. normal hydrogen electrode, isolated cytochrome c<sub>pc</sub> is more difficult to reduce, with <i>E</i><sub>red</sub> = −179 mV (21) vs. normal hydrogen electrode. Protein reduction potentials depend on many interactions, including solvation, which may also change on forming the intact enzyme. Therefore, the magnitude of the shift to be ascribed to the bonding change is uncertain. It is certain, based on the earlier conformational characterization as well as the current NMR data that this shift reflects local bonding changes associated with subunit assembly and not simply global unfolding.

In summary, simple assembly of the enzyme from its subunits can lead to a directly observable change in active-site stereochemistry, with consequent effects on bonding and reactivity. It is not yet possible to say how the subunit interactions in PCMH lead to Met-50 reorientation, although it is noteworthy that the heme and Met-50, in particular, lie near the subunit interface as seen in the crystal structure (7). However, the reasonably high quality of the NOESY spectrum, even at low protein concentration, suggests the possibility that the structure of cytochrome c<sub>pc</sub> might be completely solved. Such a structure will permit a detailed analysis of the coupling of interface to active site.

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