OXYGENATED CYTOCHROME \( P-450_{\text{cam}} \): EVIDENCE AGAINST AXIAL HISTIDINE LIGATION OF IRON

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1. Introduction

Because of their unusual spectral and catalytic properties, the cytochrome \( P-450 \) enzymes have been intensely investigated \[1\]. A major objective has been to identify the non-porphyrin ligand(s) to the central iron in states 1–5 of the \( P-450 \) reaction cycle (see Scheme 1). Comparative physical properties of synthetic porphyrin complexes with various axial ligands, myoglobin complexes with axial histidine ligation, and \( P-450 \) reaction states 1, 2 and 5 have provided convincing evidence for cysteinate ligation in the latter \[2–9\]. Synthetic systems whose properties mimic those of reaction state 4 have not been prepared, making its axial ligand more difficult to ascertain. The identity of this ligand may be crucial for understanding the dioxygen activation step (Scheme 1, 4 \( \rightarrow \) 1).

Magnetic circular dichroism (MCD) spectroscopy has been particularly useful in studying the \( P-450 \) class of enzymes \[5,8,10–17\] because of its frequent ability to distinguish between porphyrin chromophores with different axial ligands. We have therefore obtained the MCD spectra of oxy-\( P-450_{\text{cam}} \) and of oxymyoglobin, the latter being a heme protein with known axial ligation by histidine. Substantial differences are seen, strongly suggesting the presence of an axial ligand in oxy-\( P-450_{\text{cam}} \) other than neutral histidine.

2. Methods

Cytochrome \( P-450_{\text{cam}} \) was isolated from the bacterium \( Pseudomonas putida \) \[18\] by a combination of the methods in \[19,20\]. A spectral purity ratio

\[
P-450(\text{Fe}^{3+}) \quad \text{substrate (CH)} \quad P-450(\text{Fe}^{3+}) \ldots (\text{CH})
\]

1 low spin 2 high spin

\[
e^{-}, 2H^{+} \rightarrow \text{C=OH, H}_{2}\text{O}
\]

\[
P-450(\text{Fe}^{2+})(\text{O}_2) \ldots (\text{CH}) \leftarrow P-450(\text{Fe}^{2+}) \ldots (\text{CH}) \rightarrow P-450(\text{Fe}^{2+})(\text{CO}) \ldots (\text{CH})
\]

4 low spin 3 high spin 5 low spin

Scheme 1
(A_391/A_280) of 1.42 was obtained. Camphor-bound oxy-P-450_cam was prepared as follows:

i. Deaeration.
ii. Reduction with Na_2S_2O_4.
iii. Cooling to -30°C.
iv. Exposure to oxygen.

Its absorption spectrum was nearly identical to [1,21,22]. Oxymyoglobin was prepared in a similar fashion with omission of the initial deaeration step. Its MCD spectrum is quite similar to [23]. MCD measurements were obtained as in [14]. The MCD spectrum of oxy-P-450_cam shown in fig.1 has been corrected for the presence of a small amount (ca. 15%) of ferric, camphor-bound P-450_cam by subtraction of its contribution from the observed spectrum. A similar problem (5–10% ferric contamination) was encountered [23] in the preparation of oxymyoglobin. We have not corrected our MCD spectrum of oxymyoglobin for the presence of ferric myoglobin. These methods have been detailed [24].

3. Results

The MCD spectra of oxy-P-450_cam and of oxymyoglobin are shown in fig.1. The spectrum of oxymyoglobin is composed of three derivative-shaped curves with crossover points at 577 nm, 549 nm and 418 nm and an additional broad negative trough at 353 nm. The spectrum of oxy-P-450_cam is noticeably more complicated with negative extrema at 587 nm, 561 nm, 427 nm and 360 nm; crossover points at 569 nm, 557 nm, 466 nm, 438 nm, 421 nm, 380 nm and 337 nm; and maxima at 533 nm, 446 nm, 407 nm and 327 nm.

4. Discussion

Oxy-P-450_cam has previously been studied by optical absorption [1,21,22] and Mössbauer [25,26] spectroscopy. These studies have clearly characterized it as an oxygenated ferrous hemeprotein. The identity of the axial ligand trans to dioxygen, however, is a matter of less certainty. This ligand may play an important role in modifying the reactivity of the iron in P-450 and giving rise to its unusual enzymic and spectral properties. The similarity in the Mössbauer and optical absorption properties of oxy-P-450_cam and oxyhemoglobin (and oxymyoglobin) makes it tempting to speculate that the axial ligand in P-450_cam is neutral histidine, as suggested [27]. There are, however, important differences which make this assignment less likely. The temperature dependence of the Mössbauer quadrupole splitting is much stronger for oxyhemoglobin than for oxy-P-450_cam [1,25,26]. Additionally [1,26], Mössbauer emission spectra of a series of oxygenated model heme complexes with various axial ligands trans to dioxygen [28] reveal that the technique is rather insensitive to the nature of the non-oxygen axial ligand bond.

The optical absorption spectra of oxy-P-450_cam and of oxymyoglobin, while similar in an overall
sense, also show important differences. The α and β bands of oxy-P-450_{cam} (ca. 550 nm) are shifted in location and intensity relative to oxyhemoglobin (and oxymyoglobin) and have merged together as in [21]. A corresponding difference is seen in reduced +CO P-450 and myoglobin [1,6,29]. An additional difference which has not been previously discussed, involves the near-ultraviolet δ band (ca. 350 nm). In oxymyoglobin this band is at 348 nm with a millimolar extinction coefficient of 26.2 [29], 20% of the value of 128 for the Soret (γ) band at 418 nm [29]. With oxy-P-450, the value for the δ band at 355 nm is 37, 60% of the Soret extinction coefficient of 62 at 418 nm [1,21,22,30]. Thus the ratio of Soret to δ band intensities changes as a result of enhancement of the δ band by 40% and diminution of the Soret band by 52%. Again, analogous differences are seen between reduced +CO P-450 and hemoglobin [29,31,32]. This intensity redistribution for reduced +CO P-450 has been discussed [32] in terms of the presence of a charge transfer (sulfur \( \rightarrow \) porphyrin) transition which mixes with the Soret transition, stealing some of its intensity and shifting it to the red. With oxy-P-450_{cam}, the intensity shift is experimentally observed but the wavelength shift is not. Calculations [32] for the reduced +CO chromophore show that a thiolate anion can give rise to the required sulfur to porphyrin charge transfer transition but that a coordinated thiol cannot.

A ferrous porphyrin dioxygen complex with an axial thiolate ligand has been reported [33] having an absorption spectrum substantially different from oxy-P-450_{cam}. This led them to conclude that oxy-P-450_{cam} does not have a thiolate ligand. The close similarity of their spectrum to those of thiol adducts of ferric P-450 [1,19,34] and of a bis-thiolate ferric porphyrin complex [35], however, likely means that their complex [33] is a ferric species resulting from oxidation by dioxygen.

Thus, the identity of the axial ligand trans to oxygen in oxy-P-450_{cam} is uncertain. The MCD spectra of oxy-P-450_{cam} and oxymyoglobin obtained here (fig.1) are strikingly dissimilar. The spectrum of oxymyoglobin consists of three derivative shaped curves at 577 nm, 549 nm and 418 nm which have been assigned as Faraday A terms [23]. The spectrum of oxy-P-450_{cam}, which cannot be properly assigned without examination of the effect of temperature, is considerably more complex and noticeably less intense than that of oxymyoglobin. In the 470–620 nm region, two symmetrically-shaped negative peaks and a complicated positive peak are seen. While the negative peak at 561 nm may be part of a weak Faraday A term, the other features most certainly are not. In the 320–470 nm Soret region, two symmetrical derivative-shaped curves of opposite sign (positive then negative as the wavelength decreases) to the single Soret feature seen in oxymyoglobin are found centered at 380 nm and 438 nm. Neither transition is centered at 418 nm, the absorption maximum of oxy-P-450_{cam}, as would be expected for a simple Faraday A term. An additional positive peak is seen centered at 337 nm. Because it is very unlikely that two identical chromophoric systems could have such different MCD spectra, it can at least be concluded that oxy-P-450_{cam} does not have an axial neutral histidine ligand.

The most reasonable remaining possibility is that oxy-P-450 has either an axial thiol or thiolate ligand. Coordination by imidazolate is also conceivable [36], but seems unlikely due to the rather weak acidity of neutral imidazole [37] even after coordination to iron [36,38]. The conclusion [27] that oxy-P-450_{cam} does have a neutral imidazole ligand trans to dioxygen was based on the spectral similarity between oxy-P-450_{cam} and oxymyoglobin (oxymyoglobin) and the differences seen between the spectra of oxy-P-450_{cam} and the oxygen complex [33]. As discussed above, these two interpretations are very likely incorrect.

In conclusion, we have obtained evidence from an MCD spectral examination of oxy-P-450_{cam} and oxymyoglobin against the presence of an axial neutral histidine ligand to iron trans to the dioxygen of oxy-P-450_{cam}. Arguments have also been presented to show that the data which to the conclusion [27] that neutral histidine is the ligand are incorrect. Determination of the true axial ligand awaits further experimentation including the preparation of a synthetic porphyrin model complex whose properties reproduce those of oxy-P-450_{cam}.

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References


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