OXYGENATED CYTOCHROME *P*-450$_{cam}$: EVIDENCE AGAINST AXIAL HISTIDINE LIGATION OF IRON

John H. DAWSON
Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, CA 91125

and

Stephen P. CRAMER
Department of Chemistry, Stanford University, Stanford, CA 94305 USA

Received 23 January 1978

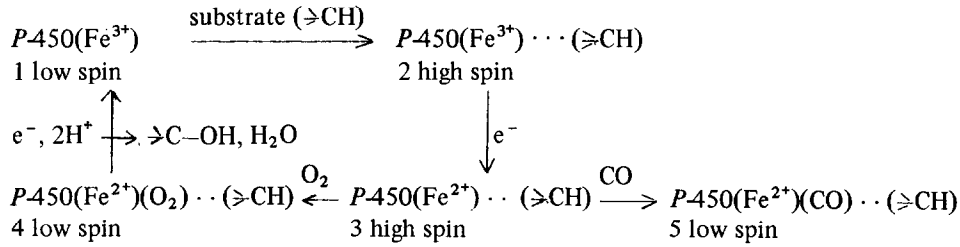
1. Introduction

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 → 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$_{cam}$ 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$_{cam}$ other than neutral histidine.

2. Methods

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



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.

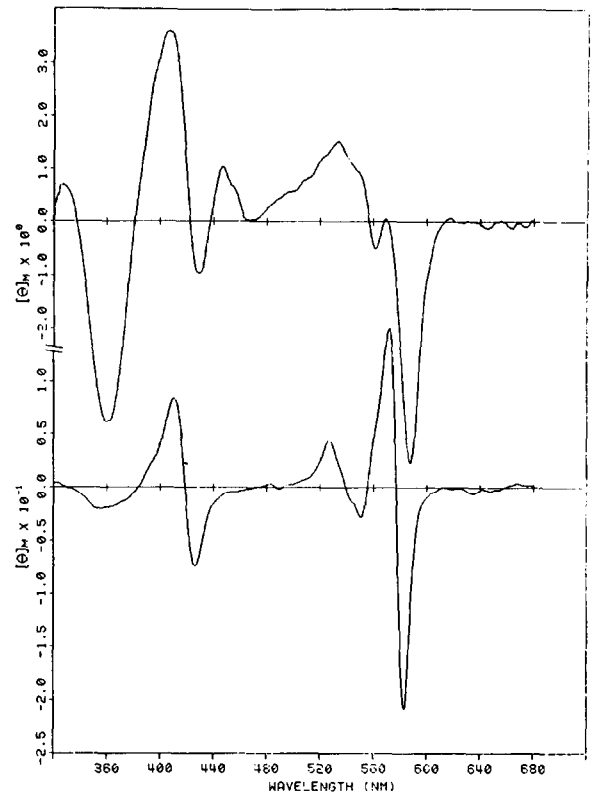


Fig.1. Magnetic circular dichroism spectra at −30°C of: (a) top, oxygenated ferrous cytochrome *P*-450$_{cam}$ in a 1:1 mixture of ethylene glycol and 50 mM potassium phosphate buffer (pH 7.4, 100 mM KCl, 1 mM D-camphor); (b) bottom, oxygenated ferrous myoglobin in a 1:1 mixture of ethylene glycol and 10 mM potassium phosphate buffer (pH 7.4). Please note that the scales are different for the two spectra.

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 $p^{\dagger} \rightarrow$ porphyrin $e_g(\pi)$) 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 oxyhemoglobin (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$_{cam}$.

Acknowledgments

This investigation was supported in part by an NIH fellowship (1 F32CAO5748–01) from the National Cancer Institute awarded to J.H.D. (1976–8). S.P.C.

was an IBM Fellow (1976–7). Additional support was provided by the NIH (GM20276, CA16748). We wish to thank E. Bunnenberg, C. Djerassi, K. Hodgson, and B. Hudson for their encouragement of this research; R. Records for technical assistance; R. Simoni and E. Shooter for use of laboratory facilities and technical advice; and E. Bunnenberg, D. M. Dooley, H. B. Gray, I. C. Gunsalus and J. A. Peterson for helpful discussions. This is Contribution No. 5565 from the Arthur Amos Noyes Laboratory.

References

[1] Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P. and Münck, E. (1974) in: Molecular Mechanisms of Oxygen Activation (Hayaishi, O. ed) Ch. 14, Academic Press, New York.

[2] Tang, S. C., Koch, S., Papaefthymiou, G. C., Foner, S., Frankel, R. B., Ibers, J. A. and Holm, R. H. (1976) J. Am. Chem. Soc. 98, 2414–2434.

[3] Collman, J. P., Sorrell, T. N. and Hoffman, B. M. (1975) J. Am. Chem. Soc. 97, 913–914.

[4] Ogoshi, H., Sugimoto, H. and Yoshida, Z. (1975) Tetrahedron Lett. 2289–2292.

[5] Dawson, J. H., Holm, R. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C. and Tang, S. C. (1976) J. Am. Chem. Soc. 98, 3707–3709.

[6] (a) Stern, J. O. and Peisach, J. (1974) J. Biol. Chem. 249, 7495–7498;
(b) Collman, J. P. and Sorrell, T. N. (1975) J. Am. Chem. Soc. 97, 4133–4134.

[7] Chang, C. K. and Dolphin, D. (1975) J. Am. Chem. Soc. 97, 5948–5950.

[8] Collman, J. P., Sorrell, T. N., Dawson, J. H., Trudell, J. R., Bunnenberg, E. and Djerassi, C. (1975) Proc. Natl. Acad. Sci. USA 73, 6–10.

[9] Chang, C. K. and Dolphin, D. (1976) Proc. Natl. Acad. Sci. USA 73, 3338–3342.

[10] Dolinger, P. M. Kielczewski, M., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E. and Djerassi, C. (1974) Proc. Natl. Acad. Sci. USA 71, 399–403.

[11] Dawson, J. H., Dolinger, P. M., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E. and Djerassi, C. (1974) Proc. Natl. Acad. Sci. USA 71, 4594–4597.

[12] Dawson, J. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., Chiang, R. and Hager, L. P. (1976) J. Am. Chem. Soc. 98, 3709–3710.

[13] Dawson, J. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., Gouterman, M., Connell, C. R. and Sayer, P. (1977) J. Am. Chem. Soc. 99, 641–642.

[14] Dawson, J. H., Trudell, J. R., Linder, R. E., Barth, G., Bunnenberg, E. and Djerassi, C. (1978) Biochemistry 17, in press.

[15] Vickery, L., Salmon, A. and Sauer, K. (1975) Biochim. Biophys. Acta 386, 87–98.

[16] Shimizu, T., Nozawa, T., Hatano, M., Imai, Y. and Sato, R. (1975) Biochemistry 14, 4172–4178.

[17] Hashimoto, C. and Imai, Y. (1976) Biochem. Biophys. Res. Commun. 68, 821–827.

[18] A culture of *Pseudomonas putida* (strain PpG1) was kindly provided by Professor I. C. Gunsalus (University of Illinois, Urbana, IL 61801, USA).

[19] Yu, C.-A., Gunsalus, I. C., Katagiri, M., Suhara, K. and Takemori, S. (1974) J. Biol. Chem. 248, 94–101.

[20] Peterson, J. A. (1971) Arch. Biochem. Biophys. 144, 678–693.

[21] Peterson, J. A., Ishimura, Y. and Griffin, B. W. (1972) Arch. Biochem. Biophys. 149, 197–208.

[22] Ishimura, Y., Ullrich, V. and Peterson, J. A. (1971) Biochem. Biophys. Res. Commun. 42, 140–146.

[23] Vickery, L., Nozawa, T. and Sauer, K. (1976) J. Am. Chem. Soc. 98, 343–350.

[24] Cramer, S. P. (1977) Doctoral Dissertation, Stanford University, Stanford CA 94305, USA.

[25] Sharrock, M., Münck, E., Debrunner, P., Marshall, V., Lipscomb, J. D. and Gunsalus, I. C. (1973) Biochemistry 12, 258–265.

[26] Sharrock, M., Debrunner, P., Schulz, C., Lipscomb, J. D., Marshall, V. and Gunsalus, I. C. (1976) Biochim. Biophys. Acta 420, 8–26.

[27] Chevion, M., Peisach, J. and Blumberg, W. E. (1977) J. Biol. Chem. 252, 3637–3645.

[28] Marchant, L., Sharrock, M., Hoffman, B. M. and Münck, E. (1972) Proc. Natl. Acad. Sci. USA 69, 2396–2399.

[29] Antonini, E., and Brunori, M. (1971) in: Hemoglobin and Myoglobin in Their Reactions with Ligands, pp. 17–19, North-Holland, Amsterdam.

[30] Sligar, S. G., Lipscomb, J. D., Debrunner, P. G. and Gunsalus, I. C. (1974) Biochem. Biophys. Res. Commun. 61, 290–296.

[31] Ballou, D. P., Veeger, C., Van der Hoeven, T. A. and Coon, M. J. (1974) FEBS Lett. 38, 337–340.

[32] Hanson, L. K., Eaton, W. A. Sligar, S. G., Gunsalus, I. C., Gouterman, M. and Connell, C. R. (1976) J. Am. Chem. Soc., 98, 2672–2674.

[33] Chang, C. K. and Dolphin, D. (1976) J. Am. Chem. Soc. 98, 1607–1609.

[34] Nastainczyk, W., Ruf, H.-H. and Ullrich, V. (1976) Chem.-Biol. Interact. 14, 251–263.

[35] Ruf, H. H. and Wende, P. (1977) J. Am. Chem. Soc. 99, 5499–5500.

[36] Nappa, M., Valentine, J. S. and Snyder, P. A. (1977) J. Am. Chem. Soc. 99, 5799–5800.

[37] Walba, H. and Isensee, R. W. (1961) J. Org. Chem. 26, 2789–2791.

[38] Mohr, P., Scheler, W., Schumann, H. and Miller, K. (1967) Eur. J. Biochem. 3, 158.