Advances in Inorganic and Bioinorganic Mechanisms

Volume 4

edited by
A. G. Sykes

Advances in Inorganic and Bioinorganic Mechanisms

Volume 4

edited by
A. G. Sykes

Department of Inorganic Chemistry
The University
Newcastle upon Tyne, England

1986

ACADEMIC PRESS

Harcourt Brace Jovanovich, Publishers

London Orlando San Diego New York
Austin Montreal Sydney Tokyo Toronto

ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road
LONDON NW1 7DX

United States Edition published by
ACADEMIC PRESS, INC.
Orlando, Florida 32887

ISBN 0–12–023804–7

PRINTED IN THE UNITED STATES OF AMERICA

86 87 88 89 9 8 7 6 5 4 3 2 1

Contributors

JOHN H. DAWSON
Department of Chemistry
University of South Carolina
Columbia
South Carolina 29208
USA

KIM SMITH EBLE[1]
Department of Chemistry
University of South Carolina
Columbia
South Carolina 29208
USA

STEPHEN F. LINCOLN
Department of Physical and Inorganic Chemistry
The University of Adelaide
Adelaide
South Australia 5001
Australia

K. L. NASH
U.S. Geological Survey
Denver Federal Center
Denver
Colorado 80225
USA

HIROSHI OGINO
Department of Chemistry
Faculty of Science
Tohoku University
Sendai 980
Japan

[1] Present address: Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103, USA.

MAKOTO SHIMURA
Department of Chemistry
Faculty of Science
Tohoku University
Sendai 980
Japan

L. H. SKIBSTED
Chemistry Department
Royal Veterinary and Agricultural University
DK-1871 Copenhagen V
Denmark

J. C. SULLIVAN
Chemistry Division
Argonne National Laboratory
Argonne
Illinois 60439
USA

RICHARD C. THOMPSON
Department of Chemistry
University of Missouri
Columbia
Missouri 65211
USA

Contents

ADVANCES IN INORGANIC AND BIOINORGANIC MECHANISMS, VOL. 4

Cytochrome P-450: Heme Iron Coordination Structure and Mechanisms of Action

John H. Dawson and Kim Smith Eble*

Department of Chemistry
University of South Carolina
Columbia, South Carolina, USA

* Present address: Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103, USA.

I. INTRODUCTION

Oxidation–reduction reactions are of vital importance to all biological systems. Redox reactions are involved not only in the essential processes of energy storage and interconversion, but also in the biosynthesis of organic compounds that are necessary for the complete functioning of the cell. Perhaps the most intriguing of the enzymes which catalyze redox reactions are those that directly interact with molecular oxygen (dioxygen).[1] Well over 200 enzymes are known to utilize dioxygen as a substrate.[2] These enzymes have been broken down into two categories based on whether oxygen atoms from dioxygen are directly incorporated into the substrate (the oxygenases) or whether the substrate is oxidized without oxygen incorporation (the oxidases).[3] The oxygenases have been further subdivided into the dioxygenases, which incorporate both oxygen atoms of dioxygen into the organic substrate, and the monooxygenases, which only incorporate one oxygen atom (the other oxygen atom of dioxygen is reduced to water).[3]

This article will examine the most well understood of the monooxygenase enzymes: cytochrome P-450. Cytochrome P-450 is an enzyme that has something for everyone: organic chemists are fascinated by its ability to activate dioxygen, inorganic chemists by its unusual heme iron coordination structure, physical chemists by its unique spectroscopic properties, pharmacologists and medicinal chemists by its important involvement in drug metabolism, environmental chemists by its function in the breakdown of xenobiotics, molecular biologists by its inducibility and existence in multiple forms, and, of course, biochemists by all of the above as well as its role in chemical carcinogenesis, membrane detoxification, and the biosynthesis of steroid hormones, bile acids and prostaglandins. Consequently, cytochrome P-450 has been the subject of one book,[4] numerous conference proceedings,[5–9] as well as an extensive collection of review articles.[10–23] In this review, following a brief general introduction, we will focus on two topics: the coordination structure of the active site heme iron and selected aspects of the enzymatic mechanism of action. For the latter topic, particular emphasis will be placed on the transfer of electrons to the heme iron, the breakdown of the oxygenated intermediate, and the use of alternative substrates.

Cytochrome P-450 is a collective name given to a group of heme-containing enzymes with similar spectral properties, first observed in 1958 in rat liver microsomes.[24,25] The historical development of the field has been nicely reviewed by Mannering.[17] The name P-450 arises from the major absorption band of the ferrous heme–carbon monoxide enzyme complex, which, relative to other heme proteins, occurs at the unusually long wavelength of approximately 450 nm. Unlike most other cytochromes, P-450 does not function merely as an electron carrier, but is also an enzyme capable of catalyzing

oxygenation reactions. A general representation of the reaction catalyzed by cytochrome P-450 is shown in Eq. (1).

$$RH + NAD(P)H + H^+ + O_2 \rightarrow ROH + NAD(P)^+ + H_2O \quad (1)$$

The hydroxylation reaction shown here is actually an oversimplification since P-450 is also known to catalyze epoxidation reactions, N-, S-, and O-dealkylations, N-oxidations, sulfoxidations, dehalogenations, and more (Fig. 1).[23]

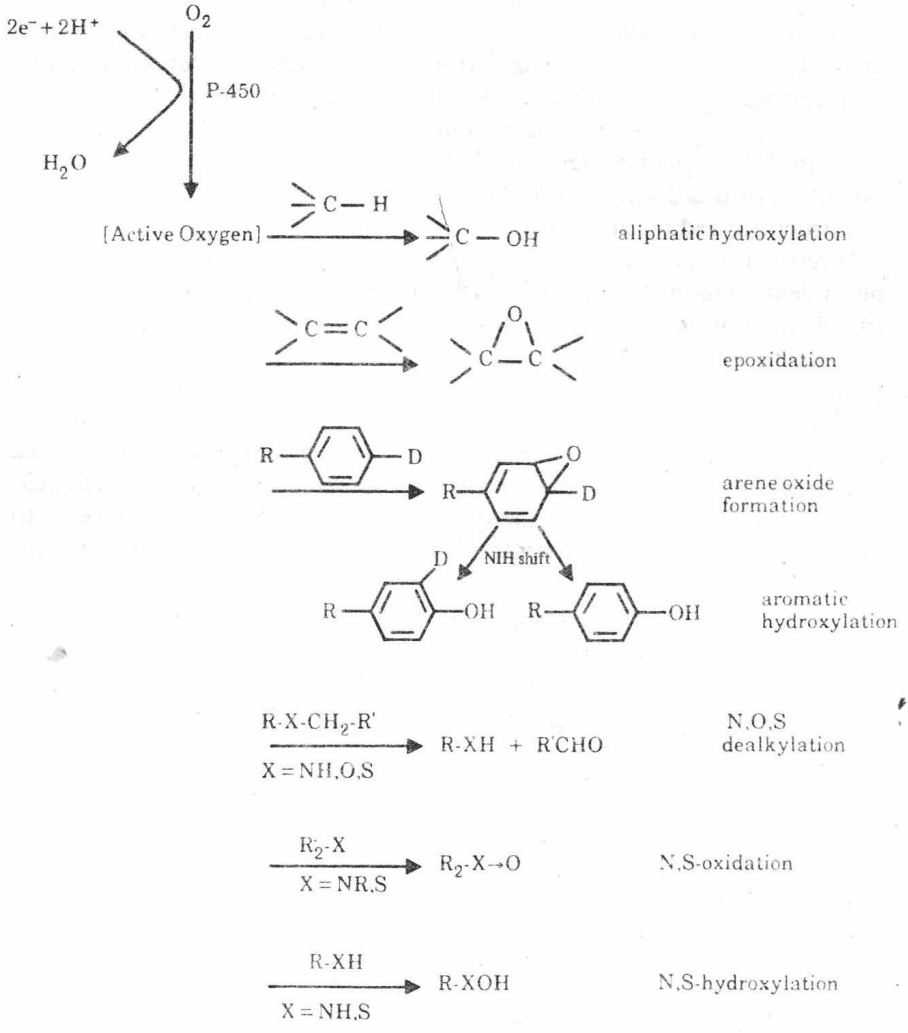


Fig. 1. Major types of oxygen incorporation reactions catalyzed by cytochrome P-450.

Cytochrome P-450 enzymes having widely varied substrate specificities have been isolated from many sources including mammalian tissues (liver, adrenal cortex, kidney, lung, skin, etc.), plants, insects, yeast, and bacteria. Multiple forms (isozymes) of P-450 have been shown to exist in several of the sources just listed; these isozymes have different, although usually overlapping, substrate specificities.[23] Studies involving the membrane-bound, insoluble mammalian P-450 enzymes have demonstrated their involvement in membrane detoxification and the solubilization of membrane-trapped nonpolar molecules, conversion of cholesterol to steroidal hormones, and drug hydroxylations. In addition to these beneficial roles, perhaps the best known group of reactions catalyzed by P-450 is the activation of chemical carcinogens. As shown in Fig. 2, benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons are converted in a series of reactions catalyzed by liver microsomal P-450 into carcinogenic dihydrodiol epoxide metabolites that can bind to DNA guanine residues.[26,27] The long-range goal of selective inhibition of this detrimental P-450 activity is the rationale behind many of the structural and mechanistic studies of cytochrome P-450.

Investigation of the physical and chemical nature of enzymes is largely dependent on the availability of purified, homogeneous protein preparations. Initial attempts to purify cytochrome P-450 from liver microsomes were unsuccessful because this membrane-bound P-450 was denatured by common purification techniques such as detergent solubilization or the use of proteases or high salt concentrations.[24,25,28] Discovery of a soluble P-450 involved in camphor degradation in the bacterium *Pseudomonas putida*[29] and the subsequent purification of this enzyme to homogeneity[30] greatly facilitated examination of the P-450 enzyme system. Since the catalytic cycle appears to be independent of the P-450 source,[14] many investigators have utilized this

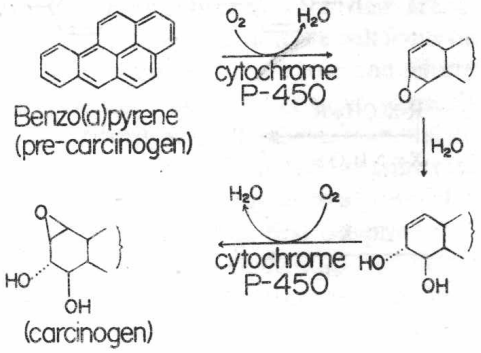
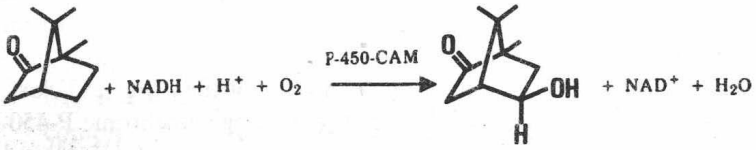


Fig. 2. P-450-catalyzed activation of chemical carcinogens. The conversion of benzo[*a*]pyrene to the dihydrodiol epoxide is a three-step reaction involving liver microsomal P-450 and epoxide hydrase. The reactive intermediate thus formed can bind to DNA guanine residues.

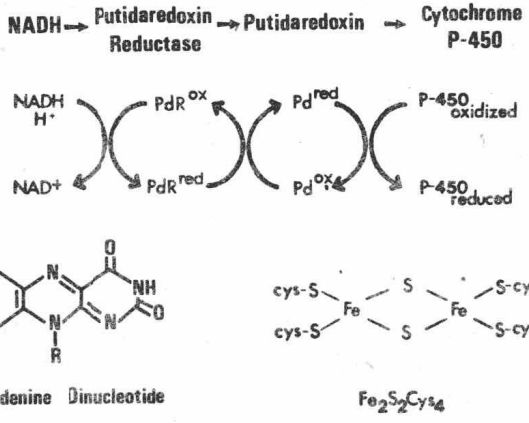
more stable and more easily purified bacterial P-450, which has come to be known as P-450-CAM.

Cytochrome P-450 belongs to a subset of the monooxygenases known as hydroxylases, since one oxygen atom from molecular oxygen is incorporated into the substrate in the form of a hydroxyl group. The P-450-catalyzed hydroxylation of camphor in *P. putida* is the first step in camphor deg-



(2)

radation as a means for providing energy to the cell. The specific reaction catalyzed by P-450-CAM is shown in Eq. (2).



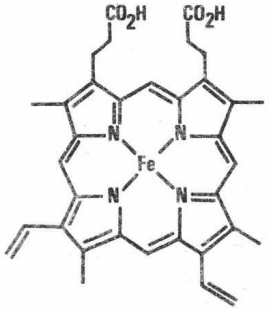


Fig. 3. The electron transport pathway from NADH to cytochrome P-450 and the structures of the prosthetic groups. Putidaredoxin reductase (PdR) contains flavin adenine dinucleotide (FAD), putidaredoxin (Pd) contains a [2Fe–2S] iron–sulfur center, and P-450 contains an iron protoporphyrin IX unit.

The substrate camphor molecule is stereospecifically and regiospecifically hydroxylated at the exo-5 position, with reduced nicotinamide adenine dinucleotide (NADH) serving as the source of reducing equivalents. As with other P-450 enzymes, P-450-CAM cannot accept electrons directly from NADH. Two other proteins are involved in the transfer of reducing equivalents from NADH to P-450-CAM. As shown in Fig. 3, two electrons are initially transferred from NADH to a flavoprotein, putidaredoxin reductase, which contains a flavin adenine dinucleotide (FAD) prosthetic group. From putidaredoxin reductase, the reducing equivalents pass in sequential one-electron steps to putidaredoxin, an iron–sulfur protein of the [2Fe–2S] class, and then to the iron protoporphyrin IX (heme)-containing cytochrome P-450-CAM. All three of these proteins have been purified to homogeneity[31–33] and reconstitution of the purified components results in a functional hydroxylase system. Analogous, short electron transport systems exist to deliver electrons from NAD(P)H to mammalian P-450 except that in some cases, such as the liver microsomal P-450, no iron–sulfur protein is required.

II. THE HEME IRON COORDINATION STRUCTURE OF CYTOCHROME P-450

A. Overview

Since a thorough understanding of the basic structural features of an enzyme active site is a prerequisite to any attempts to accurately predict the mechanism of action of the enzyme, there has been an extensive effort made to determine the heme iron coordination structure of cytochrome P-450. In fact, it is fair to say that more is known about the active site structure of this enzyme than any other enzyme for which there has not been a crystal structure reported. The reasons for this are twofold. (1) The spectroscopic properties of cytochrome P-450 are quite unusual among heme proteins. Because of this, (2) inorganic chemists interested in building structural models for the active site of heme proteins have focused considerable attention on P-450 and have succeeded in constructing models whose spectroscopic properties closely match those of the native enzyme. In this two-prong protein/model system approach, no single spectroscopic method has provided sufficient information to delineate the active site structure of P-450. Instead, the combined results from investigations with a large number of spectroscopic techniques have led to the picture of the active site that will be presented here. Although there are still some points of uncertainty to be worked out, as far as the basic metal coordination unit of cytochrome P-450 is concerned, the crystal structure of P-450-CAM should not contain any surprises.[34]

It is generally agreed that Mason was the first to suggest that some of the unusual properties of P-450 were indicative of a sulfur-containing amino acid axial ligand.[35] As shown in Fig. 4, the active site structure of P-450 has been established to consist of a cysteinate ligated heme iron which goes through a reaction cycle consisting of four states. The evidence for the sequence of intermediates in this cycle has been amply reviewed elsewhere.[4–23] Here, we will focus on the evidence for the structures of these intermediates. The cycle begins with the native substrate-free, low-spin ferric enzyme (state **1** in Fig. 4) which is converted to a high-spin form (**2**) upon substrate binding. Electron transfer leads to the high-spin ferrous state (**3**) which can bind oxygen to yield oxy-P-450 (**4**). Although it is not part of the reaction cycle, the ferrous-CO complex (**5**) is also shown because it is this form of the enzyme that has its absorption maximum near 450 nm from which the name P-450 was derived.[28]

Additional support for the suggestion of Mason that a sulfhydryl group is coordinated to the heme iron of P-450[35,36] was provided by the similarity between the EPR properties of thiolate-bound myoglobin [(His)imidazole–ferric heme–SR] and low-spin P-450 [(Cys)RS–ferric heme–L].[37–39] Despite these indirect clues as to the identity of the key axial ligand to the heme iron of P-450, in the opening paragraph of the 1975 publication of the

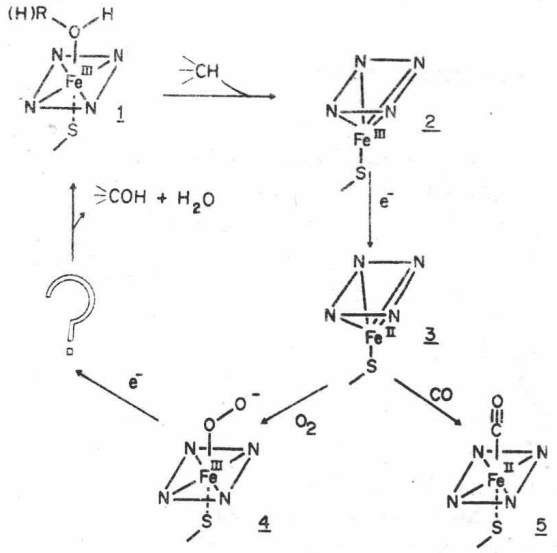


Fig. 4. Catalytic cycle of P-450, and the postulated structures of the iron site for the isolable intermediates in the P-450 catalytic cycle. Oxy-P-450 (state **4**) is shown as a complex of ferric iron and superoxide anion but could also be described as an adduct of neutral dioxygen and ferrous iron. The porphyrin ring is abbreviated as a parallelogram with nitrogens at the corners.

first crystal structure of a heme iron–thiolate complex, Holm stated, "The possibility... of axial sulfur ligation... has proved difficult to assess in the absence of fully characterized sulfur-bound porphyrins."[40] That work by Holm and co-workers,[40–42] similar investigations by Collman, Dolphin, and their co-workers[43–48] and by others,[49–53] as well as subsequent model studies most importantly by Weiss and co-workers[54–56] have provided homogeneous, often crystalline, active site analogs for each of the reaction states of P-450. Together with important parallel studies of the spectroscopic properties of the protein, their investigations have led to our current picture of the active of P-450 that is to be reviewed in this section.

Rather than discuss the evidence for each structure in Fig. 4 independently, this section has been organized by spectroscopic method with all the key results published with each particular technique grouped together. Following a discussion of the evidence for the active site structure of P-450 derived from the most commonly employed form of spectroscopy, namely electronic absorption, the remaining material will be presented in order of decreasing energy of the form of electromagnetic radiation utilized, from Mössbauer (γ rays) to NMR (radio waves) spectroscopy. Finally, evidence derived from magnetic susceptibility, from chemical modification studies, from examination of cobalt-substituted P-450, from theoretical calculations, and from proton balance ligand and redox titration experiments will be discussed. A thorough, but not exhaustive, coverage of the literature has been attempted.

B. Electronic absorption spectroscopy

The electronic absorption spectral properties of states 1–5 of cytochrome P-450 (Table 1) were what first attracted interest to this protein.[24,25] A number of these properties are unusual relative to hemoglobin/myoglobin[63] and to other heme proteins such as cytochrome c[64] and b_5.[65] Most striking, of course, is the red-shifted Soret peak for the ferrous-CO state of P-450, which is at 450 nm rather than near 420 nm. The spectrum of the ferrous-CO protein is also atypical in the presence of an intense near-UV (δ) band at 366 nm[44,66,67] and in the coalescence of the normally distinct α and β bands into a single peak. For the ferric low-spin enzyme, the slightly red-shifted Soret peak and the relative intensity of the α and β bands ($\alpha/\beta > 1$) represent spectral differences that distinguish this P-450 state from other low-spin ferric heme proteins. For high-spin ferric and ferrous P-450, the Soret peaks are substantially blue-shifted in comparison to other high-spin heme proteins.[63] Finally, the spectrum of oxy-P-450 is puzzling because its Soret peak[58,61,62] is at the same wavelength as oxymyoglobin and yet, in contrast to oxymyoglobin, it has a prominent δ band and coalesced α and β transitions.

TABLE 1

Electronic absorption spectral properties of cytochrome P-450-CAM

Structure[a]	Oxidation state	Spin state	Peak positions[b] [nm (ε_{mM}, mM^{-1}, cm^{-1})]					Ref.[c]
			δ	Soret	β	α		
1	Ferric	Low	356 (32)	417 (115)	536 (10.6)	569 (11.1)	—	[57–59]
2	Ferric	High	—	391 (102)	510 (13.0)	540 (11.2)	646 (5.4)	[31, 59, 68]
3	Ferrous	High	—	408 (87)	542 (16)		—	[31, 59, 68]
5	Ferrous-CO	Low	366 (52)	446 (120)	551 (14)		—	[60, 58, 59]
4	Ferrous-O_2	Low	353 (46)	419 (82)	554 (16)		—	[61, 58, 62]

[a] See Fig. 4.

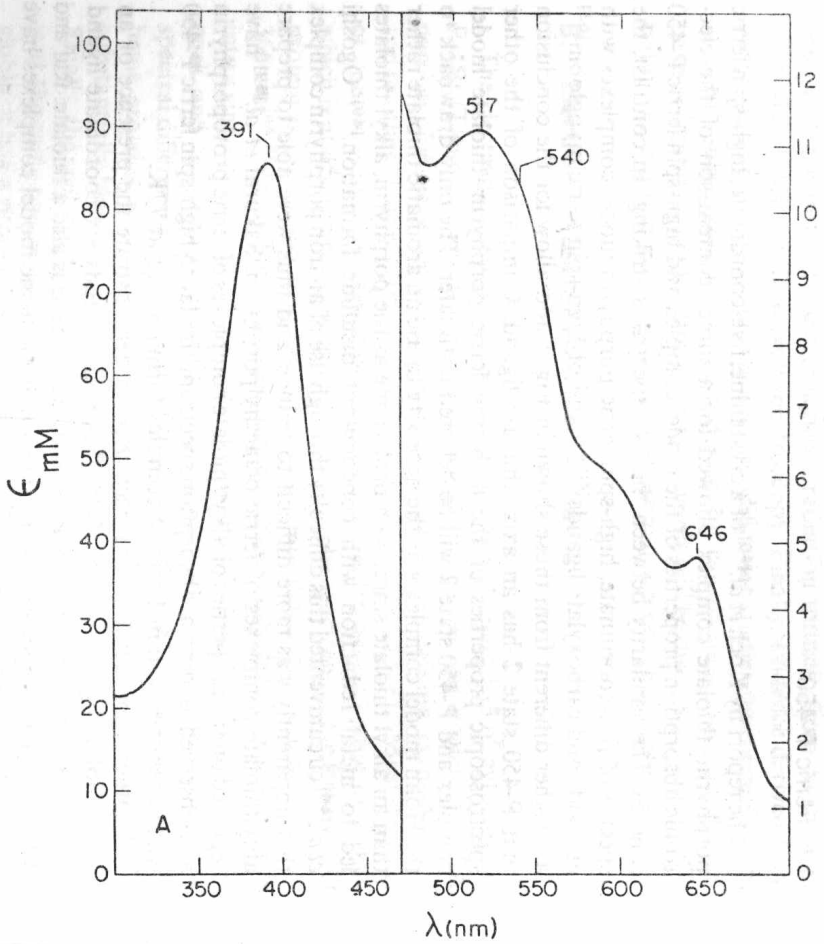
[b] Dash indicates the absence of a band in this region.

[c] The first reference listed is the one from which the data in the table are taken. The other references refer to other studies on that particular P-450 state.

1. Ferric P-450

The report by Koch *et al.*[40] of a crystalline, five-coordinate, high-spin ferric porphyrin–thiolate complex allowed for a direct comparison of the electronic absorption properties of the model complex and high-spin ferric P-450 (Fig. 5). The similarity between the two spectra is striking. In contrast, the spectra of five-coordinate, high-spin ferric porphyrin model complexes with alkoxide and carboxylate ligands[40,41] and of high-spin ferric myoglobin[63] are rather different from those shown in Fig. 5 and allow for the conclusion that P-450 state **2** has an axial thiolate ligand. Comparison of the other spectroscopic properties of the high-spin ferric porphyrin–thiolate model complex and P-450 state **2** will be deferred until later. The only drawback to the Holm model complex was the necessity to use an aromatic thiolate rather than an alkyl thiolate since, with protoheme as the porphyrin, alkyl thiolates led to metal reduction with concomitant disulfide formation.[40] Ogoshi *et al.*[49] circumvented this difficulty through use of an iron porphyrin complex that apparently was more difficult to reduce, and thus were able to prepare alkylthiolate complexes of ferric octaethylporphyrin. Sakurai *et al.*[69] have reported on the properties of alkylthiolate complexes of ferric protoporphyrin and observed an optical absorption spectrum similar to high-spin ferric P-450 when examined within 1 min of complex formation or at 77K.

Another approach that has been used to demonstrate the presence of an axial thiolate ligand to ferric P-450 has been to study six-coordinate ligand complexes of the protein itself where the added ligand is also a thiolate. Ruf and Wende[51] have reported that bisthiolate–ferric heme model complexes have



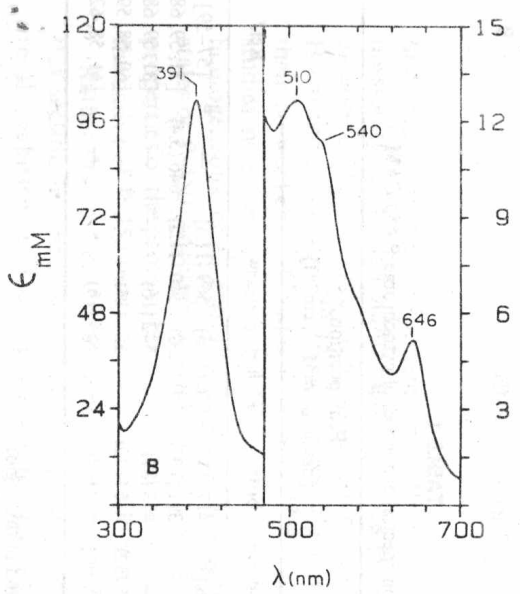


Fig. 5. (A) Electronic absorption spectrum of ferric protoporphyrin IX dimethyl ester (*para*-nitrobenzene thiolate) in toluene. (R. H. Holm, personal communication; data reported in tabular form in Ref. [41].) (B) Electronic absorption spectrum of high-spin (camphor-bound) ferric cytochrome P-450-CAM at pH 7.0. (From Ref. [68].)

very unusual absorption spectra in which there are two Soret bands (~380, ~470 nm) with equal integrated intensity. The crystal structure of such a complex has recently been published.[70] With biomimetic nitrogen, oxygen, and sulfur donor sixth ligands and a thiolate fifth ligand, the bisthiolate case is the only one giving rise to this unique split Soret (hyperporphyrin) absorption spectrum. Over the last 10 years extensive P-450 ligand binding studies have been reported by Ullrich,[52,71–74] Dawson,[57,75,76] and others.[77,78] In these investigations, only thiolate adducts of ferric P-450 have been found to reproduce the hyperporphyrin spectrum observed in bisthiolate model complexes by Ruf and Wende[51] and more recently by others.[79,80] The dramatic spectral change observed upon thiol addition to ferric P-450 is displayed in Fig. 6 for the addition of an acidic thiol, *para*-chlorobenzenethiol, to either P-450 state 1 or 2. The generation of such a hyper spectrum provides strong support for the presence of an endogeneous thiolate ligand trans to the added thiolate.

A similar approach has been used to identify the sixth ligand to P-450 state 1. The UV-visible absorption spectrum of low-spin ferric P-450 is shown in Fig. 7A. In the model system approach, Holm,[41] Ullrich,[52,73,74] and, more recently, Sakurai[81,82] and their co-workers have studied numerous low-spin six-coordinate complexes of the composition [RS–ferric heme–X] where X is nearly every conceivable biomimetic ligand. First of all, it was found that the thiolate ligand was necessary to obtain absorption spectra that at all resembled that of low-spin ferric P-450, further substantiating the presence of the endogeneous thiolate ligand. Beyond this, it was found, most convincingly by Ruf *et al.*,[52] that the best fit of the absorption spectrum of P-450 state 1 was obtained was X equal to an oxygen donor ligand.[52,73,74,81,82] Similar, equally extensive studies of the absorption spectra of ligand complexes of ferric P-450 having the composition [(Cys)RS–ferric heme–X] by Dawson and co-workers,[57,76,83] White and Coon,[78] and Yoshida *et al.*[84] have led to a similar conclusion; i.e., the sixth ligand to ferric P-450 is an oxygen donor, most likely from an alcohol containing amino acid or possibly from water. The UV-visible absorption spectrum of the 1-pentanol complex of ferric P-450 is displayed in Fig. 7B and is easily seen to be quite similar to the spectrum of P-450 state 1 shown in Fig. 7A. Competition experiments were done in the Dawson laboratory to be certain that the oxygen donor ligands were indeed coordinating to the P-450 heme iron.[57,83] Alternatively, White and Coon[78] as well as Yoshida *et al.*[84] used P-450 isozymes that are five-coordinate, high-spin as isolated; in such cases ligand binding and concomitant conversion of the heme iron to the low-spin state were easily verified by the large spectral changes that were observed. As will be discussed later, in some cases results from other laboratories using different techniques have led to further support for an oxygen donor as the sixth ligand to ferric P-450, while in other cases contradictory conclusions have been reached.