Multiple Forms of Cytochrome P-450 Purified from Liver Microsomes of Phenobarbital- and 3-Methylcholanthrene-Pretreated Rabbits

II. Spectral Properties

Chikako HASHIMOTO-YUTSUDO, Yoshio IMAI, and Ryo SATO
Institute for Protein Research, Osaka University, Suita, Osaka 565
Received for publication, February 6, 1980

The spectral properties of the multiple forms of cytochrome P-450 purified or partially purified from liver microsomes of phenobarbital (PB)- and 3-methylcholanthrene (MC)-treated rabbits have been studied. Both optical absorption and EPR studies have shown that the oxidized forms of P-450₁, P-450₂ (from PB-treated animals), and P-450₃ (from MC-treated animals) are in the low spin state, having a Soret absorption peak at 417-418 nm. Oxidized P-448₁ (from both PB- and MC-treated animals), on the other hand, shows a Soret peak at 393 nm and a weak band at 646 nm. This and EPR evidence indicate that P-448₁ contains heme which is predominantly in the high spin state, though EPR studies at low temperature indicate the presence of a small amount of low spin ferric heme. The presence of tightly bound MC in P-448₁ purified from MC-treated animals is reflected by characteristic absorption peaks in the ultraviolet region, but this does not affect the absorption spectra in the Soret and visible regions. Emulgen 913, a nonionic detergent, causes the conversion of oxidized P-448₁ from the high to the low spin state, as evidenced by optical absorption and EPR results; bound MC inhibits this conversion in a noncompetitive way. Binding of ethyl isocyanide to reduced P-450₁ and P-448₁ results in the appearance of two Soret peaks in the 430 and 455 nm regions, the relative intensities of which are dependent on pH. At any pH the 455 nm peak of P-448₁ is always higher than that of P-450₁. Benzphetamine and aniline, added to oxidized P-450₁, cause Type I and Type II spectral changes, respectively, but the magnitudes of the changes are small in both cases. The Soret peak of oxidized P-448₁ at 393 nm is completely shifted to 420 nm on addition of aniline, resulting in a reverse Type I spectral change; acetanilide causes the conversion of the Soret peak to the low spin state to only a slight extent. The conversions caused by aniline and acetanilide are both inhibited by the presence of tightly bound MC. On the basis of these and other observations, the spin states of these P-450’s are discussed.

In the preceding paper (1), we described a method for the purification of multiple forms of cytochrome P-450 (P-450) from liver microsomes of phenobarbital (PB)- and 3-methylcholanthrene (MC)-treated rabbits. This method has permitted the isolation of homogeneous preparations of two

Abbreviations: P-450, cytochrome P-450; PB, phenobarbital; MC, 3-methylcholanthrene; EPR, electron paramagnetic resonance.
forms of P-450, i.e. P-450₁ and P-448₁, and a partially purified preparation of another form, termed P-450₂, from liver microsomes of PB-treated animals ("PB-microsomes"), whereas two forms of P-450, i.e. P-448₁ and P-450₀, have been obtained in homogeneous and partially purified states, respectively, from liver microsomes of MC-treated rabbits ("MC-microsomes"). Among these forms, P-450₁ and P-448₁ are most abundantly present in PB-and MC-microsomes, respectively. From studies on their molecular properties, including monomeric molecular weight, amino acid composition, and chromatographic behavior, it is clear that P-450₁ is an entity which is different from P-448₁. Although P-450₀ is similar to P-450, in molecular weight, they differ from each other in chromatographic behavior. P-450₀ resembles P-450₁ in chromatographic behavior, but they differ in molecular weight. It has also been shown that P-448₁ from MC-microsomes is different from that from PB-microsomes in that the former contains tightly bound MC (0.18-0.88 mol/mol), though they are identical with each other in other respects.

The purpose of this communication is to report the spectral properties of the P-450 preparations isolated in the preceding paper (1) and to compare them with one another as well as with those reported for P-450's purified from rabbit liver microsomes by previous workers (2). The results of EPR measurements on P-450₀ and P-448₁ are also described, and a hypothesis is presented to explain the spin states of hepatic microsomal P-450's.

MATERIALS AND METHODS

The P-450 preparations purified or partially purified in the preceding paper (1) were used. Ethyl isocyanide was synthesized as described by Jackson and McKusick (3). Phenyl isocyanide and benzphetamine were generous gifts from Dr. Y. Miyake and Dr. T. Kamataki, respectively. Emulgen 913 was kindly supplied by Kao-Atlas Co., Tokyo. The other chemicals used were of reagent grade.

Absolute and difference absorption spectra were measured at room temperature using a Cary 14 or 219 spectrophotometer. Electron paramagnetic resonance (EPR) spectra of P-448₁ in the low field region were determined at liquid helium temperature in a JES-ME spectrometer at a microwave frequency of 9.19 GHz and a modulation amplitude of 20 gauss. EPR spectra of P-450₀ and P-448₁ in the high field region were measured at liquid nitrogen temperature in the same spectrometer at a microwave frequency of 9.30 GHz and a modulation amplitude of 10 gauss. Protomer in P-450₀ and P-450₂ was determined by the reduced pyridine hemochrome method of Paul et al. (4). For determination of protomerin P-448₁, however, the method was modified in that the concentrations of NaOH and pyridine were changed to 0.5 M and 25% (v/v), respectively.

RESULTS

Absorption Spectra (Visible and Soret Regions)

—Figure 1 shows the absolute absorption spectra of three homogeneous preparations of P-450 isolated from rabbit liver microsomes, i.e. P-450₀, P-448₁ from PB-microsomes, and P-448₁ from MC-microsomes, in the oxidized, dithionite-reduced and reduced CO complex forms. Prior to spectrophotometer determinations, all the preparations were freed from Emulgen 913 as described in the preceding paper (1). Oxidized P-450₀ shows a Soret absorption peak at 418 nm together with α- and β-bands at 570 and 534 nm, respectively, and the general shape of the spectrum is characteristic of low spin hemoproteins. In the oxidized form of P-448₁ (from both PB- and MC-microsomes), on the other hand, the Soret band is broad, less intense than that of P-450₀, and blue-shifted to 393 nm. Furthermore, in the visible region the α- and β-bands are not well resolved and a weak band attributable to a charge transfer in the high spin state is seen at 646 nm. These spectral features suggest that the oxidized form of P-448₁ is in the high spin state. In the reduced form both P-450₀ and P-448₁ exhibit a Soret peak, which is less intense than that of the oxidized form, in the 412 nm region and only one band is seen in the visible region. The reduced CO complexes of both preparations show an intense Soret peak at 418 nm together with distinct α- and β-bands at 570 and 534 nm, respectively, and the general shape of the spectrum is characteristic of low spin hemoproteins. In the spectrum of the oxidized form of P-448₁ (from both PB- and MC-microsomes), on the other hand, the Soret band is broad, less intense than that of P-450₀, and blue-shifted to 393 nm. Furthermore, in the visible region the α- and β-bands are not well resolved and a weak band attributable to a charge transfer in the high spin state is seen at 646 nm. These spectral features suggest that the oxidized form of P-448₁ is in the high spin state. In the reduced form both P-450₀ and P-448₁ exhibit a Soret peak, which is less intense than that of the oxidized form, in the 412 nm region and only one band is seen in the visible region. The reduced CO complex of both preparations show an intense Soret peak in the 450 nm region and only one band in the visible region. However, the Soret peak of the reduced CO complex of P-450₀ is located at 451 nm, whereas that of P-448₁ is at 448 nm. Similarly, the peaks of the reduced form are also blue-shifted by 2-3 nm in P-448₁. Thus, the spectra of
Fig. 1. Absorption spectra of purified P-450₁ (A) and three preparations of purified P-448₁ (B). All the preparations were freed from Emulgen 913 as described in the preceding paper (1) and dissolved in 150 mm (for P-450₁) or 300 mm (for P-448₁) potassium phosphate buffer (pH 7.25) containing 20% glycerol. Two preparations of P-448₁ were purified from MC-microsomes (MC contents, 0.88 and 0.18 mol/mol heme; curves a and b, respectively) and one preparation was purified from PB-microsomes (containing no MC; curve c). The three preparations of purified P-448₁ show the same absorption spectra in the visible and Soret regions.

---, oxidized form; ----, reduced form; -.-, reduced CO complex.

P-450₁ in the Soret and visible regions (Fig. 1A) are essentially identical with those reported for P-450LM2 purified from the same source (2), supporting the conclusion that the two preparations represent the same protein (1). However, the shoulder in the 420 nm region in the reduced CO complex is significantly higher in P-450LM2 than in P-450₁, suggesting that the former preparation contained a small amount of cytochrome P-420, the denatured form of the hemoprotein (5). The spectra of the three P-448₁ preparations in the Soret and visible regions (Fig. 1B) are practically the same and are very similar to those reported for P-450LM4 purified from liver microsomes of β-naphthoflavone-treated rabbits (2), a finding which is consistent with the view presented in the preceding paper (1) that P-448₁ and P-450LM4 are identical with one another.

Absorption maxima and their molar extinction coefficients in the spectra of P-450₁ and P-448₁ are summarized in Table I, which also includes the data for P-450₂. The extinction coefficients given are based on the protoheme content determined by the reduced pyridine hemochrome method. The molar extinction increment between 451 (or 448) nm and 490 nm in the CO difference spectra of both P-450₁ and P-448₁ was estimated to be 91 mm⁻¹cm⁻¹, subject to experimental error, as originally determined for liver microsomes by an indirect method (6). In the cases of P-450₁ and P-448₁ from PB-microsomes, the equilibrium dissociation constants for the CO complexes were determined to be 0.4 μM and 4 μM, respectively, by

<table>
<thead>
<tr>
<th>P-450</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Reduced CO complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
<td>ε (mm⁻¹cm⁻¹)</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</td>
</tr>
<tr>
<td>P-450₁</td>
<td>418</td>
<td>107</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>534</td>
<td>11.3</td>
<td>545</td>
</tr>
<tr>
<td></td>
<td>570</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>P-448₁</td>
<td>393</td>
<td>95.9</td>
<td>411</td>
</tr>
<tr>
<td></td>
<td>512</td>
<td>13.9</td>
<td>545</td>
</tr>
<tr>
<td></td>
<td>646</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>P-450₂</td>
<td>417</td>
<td>109</td>
<td>412</td>
</tr>
<tr>
<td></td>
<td>535</td>
<td>12.2</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>570</td>
<td>12.5</td>
<td></td>
</tr>
</tbody>
</table>
a titration experiment in which aliquots of CO-saturated buffer were added to a known amount of P-450₁ or P-448₁ and the spectral changes occurring on addition of dithionite were measured. The estimated dissociation constant for P-450₁ is in good agreement with that reported for microsomal bound P-450 (7).

Haugen and Coon (2) have reported that the spectrum of oxidized P-450₁₄, but not that of P-450₁₅₂, was affected by the protein concentration and that the ratio of absorbance at 418 nm to that of 394 nm \( \frac{A_{418}}{A_{394}} \) increased as the cytochrome concentration was increased above 1.5 \( \mu \text{M} \). In our hands, however, no such effect was observed. Even at a concentration as high as 30 \( \mu \text{M} \), the \( \frac{A_{418}}{A_{394}} \) ratio of the oxidized form of our P-448₁ preparations remained at about 0.65, a value which was even lower than that determined for P-450₁₄ at 1.5 \( \mu \text{M} \). As had been reported by Hashimoto and Imai (8) and Haugen and Coon (2) and will be described below, nonionic detergents such as Renox 690 and Emulgen 913 cause a spectral shift of the Soret peak of oxidized P-448₁ and P-450₁₄₄ from 393 to 418 nm. The effect of protein concentration observed for P-450₁₄, therefore, suggests that this preparation was not free from the detergent. Our P-448₁ preparations, on the other hand, seemed to be practically free from the detergent.

Although the data are not shown, the absolute absorption spectra of partially purified P-450₁ (from PB-microsomes) and P-450₁ (from MC-microsomes) in the Soret and visible regions are very similar to those of P-450₁. A noticeable difference is that in both P-450₁ and P-450₁, the Soret peak of the reduced CO complex is located at 450 nm, instead of at 451 nm for P-450₁. Similarly, the Soret peak of the oxidized form is also blue-shifted by 1–1.5 nm (to 417–416.5 nm) in P-450₁ and P-450₁. These differences, though small in magnitude, were reproducible.

Absorption Spectra (Ultraviolet Region)—In the ultraviolet region detergent-free P-450₁ shows an absorption peak at 276 nm, together with a fine structure extending from 270 to 255 nm (Fig. 1A). Such a fine structure has been observed with proteins having high phenylalanine contents (11) and thus is consistent with high phenylalanine (36 out of total 430 residues) and low tryptophan contents in P-450₁ (1).

Although P-448₁ from PB-microsomes and that from MC-microsomes possess essentially the same absorption spectra in the Soret and visible regions as mentioned above, the spectra in the ultraviolet region are clearly different from each other (Fig. 1B). Thus, the spectrum of P-448₁ from MC-microsomes, but not that from PB-microsomes, exhibits several sharp peaks superimposed on the absorption due to aromatic amino acid residues. As reported in the preceding paper (1), this anomaly was found to be a reflection of the presence of tightly and specifically bound MC in the preparation purified from MC-microsomes. The MC content in the preparations so far purified varied from 0.18 to 0.88 mol per mol of the heme, and the sharp absorption peaks due to bound MC were more intense in preparations with higher MC contents (Fig. 1B). It should be noted that the MC content has no influence on the spectra of the preparation in the Soret and visible regions. Figure 1A shows that the height of the absorption peak of P-450₁ in the ultraviolet region is about half that of the oxidized Soret peak. In contrast, the ultraviolet absorption band of P-448₁ purified from PB-microsomes is much higher even though no MC is bound to this preparation (Fig. 1B). This is probably due to the much higher tryptophan content in P-448₁ (9 mol/mol) than in P-450₁ (2 mol/mol) (1), but the possibility cannot be excluded that an endogenous substrate absorbing in this wavelength region is bound to the P-448₁ preparation.

Effect of Nonionic Detergent—As mentioned above, the absorption spectra of oxidized P-448₁ and P-450₁₄₄ have been reported to be affected by nonionic detergents of a polyoxyethylene nonylphenyl ether type (2, 8). Hashimoto and Imai (8) have observed a tendency for spectral conversion of P-448₁ to a low spin form by Emulgen 913. Haugen and Coon (2) have reported further that the addition of Renox 690 to P-450₁₄₄ causes a shift of the Soret peak from 394 to 418 nm. Therefore, the effect of Emulgen 913 on the spectrum of P-448₁ was examined in detail. As shown in Fig. 2A-1, addition of increasing concentrations of Emulgen 913 to oxidized P-448₁, purified from PB-microsomes resulted in a gradual shift of the Soret peak from 393 to 417 nm, with an isosbestic point at about 407 nm, and the conversion was almost complete at a detergent concentration of 2.27% (w/v). The spectrum finally reached showed practically no...
shoulder in the 393 nm region and was characteristic of low spin hemoproteins. The spectral conversion could be readily reversed by decreasing the detergent concentration by dilution or other means. The detergent-induced spectral change (Fig. 2B-1) has a maximum at 420 nm and a minimum at 386 nm and is typical of a reverse Type I difference spectrum caused by the binding of certain substrates to oxidized P-450 (9). Addition of Emulgen 913 to a P-448, preparation containing about 0.2 mol of bound MC per mol of the heme also caused a similar spectral shift (Fig. 2A-2 and B-2) but in this case the conversion was incomplete even at the detergent concentration causing an almost complete shift in the MC-free P-448, preparation, suggesting that the bound MC was inhibitory to the detergent-induced spectral conversion. When a P-448, preparation containing about 0.9 mol of bound MC per mol of heme was used, the detergent-induced spectral conversion was only slight (data not shown). As shown in Fig. 3, double-reciprocal plots of the intensity of spectral change induced against the detergent concentration indicated that the spectral dissociation constants of the P-448, preparations for Emulgen 913 were the same (1.26 mm) and suggested that the bound MC acted as a noncompetitive inhibitor toward the spectral change induced by the binding of the Emulgen to oxidized P-448, In contrast to P-448, the spectra of P-450, P-450a, and P-450b were not significantly affected by the detergent.
Fig. 4. Effects of pH on the absorption spectra of ethyl isocyanide complexes of reduced P-450, (A) and reduced P-448, from PB-microsomes (B). P-450, and P-448, (final concentrations, 3.17 and 3.15 μM, respectively) were dissolved in 200 mM potassium phosphate buffers of the indicated pH containing 20% glycerol and 0.8 mM ethyl isocyanide. ——, pH 8.0; ----, pH 7.0; ——, pH 6.0.

Alkyl Isocyanide Binding—Previous studies with intact microsomes have shown that alkyl isocyanide binding to reduced P-450 leads to the appearance of two Soret absorption bands at about 430 and 455 nm (5, 10-13) and that the relative intensities of these two Soret peaks are dependent on pH (11, 12). Similar observations have been reported for a P-450 preparation partially purified from liver microsomes of PB-treated rabbits (14). Based on these findings, it has been suggested that reduced P-450, when in combination with the isocyanide, exists in two interconvertible states which are in a pH-dependent equilibrium (11, 12). Figure 4 shows the absorption spectra of the ethyl isocyanide complexes of reduced P-450, and P-448, (containing no bound MC) at various pH values. As can be seen, the isocyanide complexes of both preparations exhibited two Soret peaks; however, the peaks of P-450, are located at 430 and 455 nm, whereas those of the P-448, complex are at 430 and 453 nm. In both cases, the 455 (or 453) nm peak was intensified at higher pH values, whereas the height of the 430 nm peak decreased as the pH was increased. A concomitant pH-dependent alteration could also be observed in the visible region, as reported for the partially purified preparation (14). From the pH-dependent behavior of the four bands in the visible region, those at 580 and 555 nm could be concluded to be α- and β-bands associated with the 455 (or 453) nm state, whereas the 430 nm state seemed to have α- and β-bands at 560 and 533 nm, respectively.

The ethyl isocyanide complexes of the two preparations were different from each other in that at any given pH value the intensity of the 453 nm peak of the P-448, complex was considerably higher than that of the 455 nm peak of the P-450, complex. This can be more clearly visualized in Fig. 5, in which the ethyl isocyanide difference spectra of reduced P-450, and P-448, at pH 7.0 are compared. This observation is consistent with the finding reported for liver microsomes of PB- and MC-
pretreated rats (13). It was also noticed that the intensity ratio of the 455 nm to the 430 nm peak of the isocyanide complex of reduced P-450$_1$ was somewhat increased by addition of Emulgen 913 at a given pH. Phenyl isocyanide complexes of reduced P-450 and P-448$_1$ exhibited spectral features similar to those of the ethyl isocyanide complexes, but the equilibrium between the two states appeared to be shifted toward the 455 (or 453) nm state by about one pH unit (data not shown), in coincidence with the observation reported for intact microsomes (15).

Ethyl isocyanide could also bind to the oxidized forms of both P-450$_1$ and P-448$_1$, as reported for microsomal bound P-450 (10); the isocyanide complex of oxidized P-450, exhibited absorption maxima at 360, 431 (Soret), and 552 nm.

Effects of Substrates—Addition of various substrates of the P-450 monooxygenase system to liver microsomes causes characteristic spectral changes due to substrate binding to oxidized P-450 (16, 17). The spectral changes can be classified into 3 categories, Type I, Type ‡U, and reverse Type I (modified Type II), depending on the shape of the induced difference spectra (9, 17). It has also been reported that the substrate (camphor) complex of the oxidized form of bacterial P-450$_{cam}$ exists in the high spin state having a Soret peak at 391 nm, whereas the substrate-free form is in the low spin state, exhibiting a peak at 417 nm (18, 19). A similar conversion of the spin state upon binding of the substrate has been shown to occur with two species of P-450 purified from bovine adrenocortical mitochondria (20, 21). Binding of the substrates to these P-450's, therefore, results in a spectral change which is similar to the Type I spectral change in hepatic microsomal P-450.

As shown in Fig. 6, benzphetamine and aniline, both of which are metabolizable by P-450$_1$ (unpublished results), induced Type I and Type II difference spectra, respectively, when added to the oxidized form of purified P-450$_1$. In contrast to the cases of the bacterial and adrenocortical P-450's, however, the benzphetamine-induced spectral change was very slight. Even after addition of a saturating concentration of the substrate, the Soret peak at 418 nm was still clearly seen in the absolute spectrum, though its intensity was decreased to a small extent and a slight increase in absorbance was noticed in the 393 nm region. From the double-reciprocal plot of the benzphetamine concentration against the absorbance increment between 385 nm (peak) and 420 nm (trough) in the difference spectrum, the spectral dissociation constant of benzphetamine was estimated to be 0.15 mM. The type II spectral change caused by aniline was also small in magnitude; in the absolute spectrum aniline caused a slight decrease in the intensity of the Soret peak and its shift to 420 nm.

![Fig. 6. Effects of benzphetamine and aniline on the absorption spectrum of oxidized P-450](image-url)

Vol. 88, No. 2, 1980
Figure 7 shows the effects of aniline and acetanilide, both of which can be metabolized by P-448₁ (unpublished results), on the spectrum of oxidized P-448₁ purified from PB-microsomes. Addition of 40 mM aniline to this preparation resulted in a complete shift of the Soret peak to 420 nm; the resultant difference spectrum was a reverse Type I. On the other hand, the spectral change induced by 4.0 mM acetanilide was not as profound as that caused by aniline; the peak at 393 nm was decreased to a certain extent and at the same time the absorbance in the 418 nm region was increased, suggesting that a partial conversion to the low spin state had taken place. The effect of higher concentrations of acetanilide could not be examined because of its low solubility. Benzphetamine, which is only poorly metabolizable by P-448₁, caused practically no spectral change (data not shown). When a P-448₁ preparation containing about 0.9 mol of bound MC per mol of the heme (purified from MC-microsomes) was used, the aniline-induced shift of the Soret peak to 420 nm was incomplete, even at an aniline concentration as high as 40 mM (Fig. 8), suggesting that the bound MC was inhibitory to aniline binding to P-448₁. Similarly, the spectral change caused by acetanilide was also less marked in this P-448₁ preparation than in the absence of bound MC (data not shown).

EPR Spectra—Figure 9 shows the EPR spectra of oxidized P-450₁ and P-450₀ at liquid nitrogen temperature. The spectrum of P-450₁, showing signals at \( g = 2.42, 2.23, \) and 1.91, can be ascribed...
Fig. 8. Effect of bound MC on the aniline-induced spectral change of P-4481. P-4481 from MC-microsomes (MC content, 0.88 mol/mol heme) was dissolved in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol to a concentration of 2.3 µM. The concentration of aniline added was 40 mM. A, absolute spectra (—, before addition; ----, after addition of aniline); B, aniline-induced difference spectrum.

Fig. 9. EPR spectra of P-4501, P-4502, and P-4481 at liquid nitrogen temperature. The spectra were taken at a sweep rate of 1,000 Gauss in 20 min and at a time constant of 1 s. P-4501 and P-4502 were diluted to 65 and 38 µM, respectively, in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol. P-4481 from MC-microsomes was diluted to 66 µM in 300 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol. Amplitude: P-4501 (—), 2.5 × 10^3; P-4502 (----), 4 × 10^3; P-4481 (—.—), 1.6 × 10^3.

to low spin ferric heme and is essentially identical with that reported for liver microsomes from untreated animals (22, 23). Addition of benzphetamine caused only a slight decrease in the signal height; this appeared to correspond to a slight change in the absorption spectrum. On the other hand, in the presence of aniline (10 mM) a new signal at \( g = 2.37 \) appeared. The EPR spectrum of oxidized P-4502 is also typical of low spin ferric heme and resembles that of P-4501, except that the former has a signal at \( g = 2.37 \) in addition to those at \( g = 2.41, 2.23, \) and about 1.9. In contrast to the case of P-4501, addition of aniline (10 mM) to P-4502 caused the disappearance (rather than appearance) of the signal at \( g = 2.37 \).

The EPR spectrum of oxidized P-4481 (containing 0.2 mol of MC/mol) measured at liquid helium temperature is shown in Fig. 10. The
spectrum is characterized by prominent signals at \( g = 7.88 \) and 3.85, both of which are attributable to high spin ferric heme, a finding which is consistent with the high spin type absorption spectrum of the same preparation. The \( g \) values obtained agree with those reported for liver microsomes from MC-induced animals (24, 25) and a homogenous preparation of P-450LM4 purified from liver microsomes of \( \beta \)-naphthoflavone-pretreated rabbits (2). The P-448\(_1\) preparation also showed EPR signals at \( g = 2.42, 2.23, \) and 1.91, indicating the presence of low spin ferric heme (Fig. 9). Assuming that these signals have the same intensities as those observable in the spectrum of oxidized P-450\(_2\), it was estimated from the signal heights that about 10\% of the total heme in the P-448\(_1\) preparation was in the low spin state at the temperature where EPR measurements were performed. When Emul- 

gen 913 was added to oxidized P-448\(_1\), the intensities of the low spin signals were increased (data not shown), in agreement with the observation that the detergent converted the absorption spectra of P-448\(_1\) preparations to the low spin type.

**DISCUSSION**

In the preceding paper (1), it was concluded that P-450\(_1\), P-450\(_2\), and P-448\(_1\) are different molecular species based on studies of their molecular properties, including molecular weight, amino acid composition, and chromatographic behavior. This conclusion is further supported by their spectral properties described in this paper. Thus, the oxidized forms of P-450\(_1\) and P-450\(_2\) exhibit optical absorption and EPR spectra that are characteristic of low spin hemes, whereas the spectral properties of oxidized P-448\(_1\) are indicative of its high spin nature. Although the spectra of P-450\(_1\) and P-450\(_2\) are similar to each other, there are small, but clearly detectable, differences between the two. P-450\(_1\), on the other hand, is indistinguishable from P-450\(_2\) in spectral properties, but their chromatographic behavior and molecular weights are clearly different, as reported in the preceding paper (1). P-448\(_1\) purified by Haugen and Coon (2) from liver microsomes of PB-treated rabbits, and P-448\(_1\) is essentially the same in terms of spectral properties as P-450LM4 purified from liver microsomes of \( \beta \)-naphthoflavone-pretreated rabbits (2). These observations provide further support to the view presented in the preceding paper (1) that P-450\(_1\) and P-448\(_1\) are identical with P-450LM2 and P-450LM4, respectively.

Optical absorption and EPR spectra of hemoproteins reflect the electronic structure of the heme and its vicinity. The absorption spectrum of oxidized P-450\(_1\), measured at room temperature, suggests that the heme in this preparation is essentially in the ferric low spin state. EPR studies also indicate the almost completely low spin nature of the heme of oxidized P-450, at liquid nitrogen temperature. However, evidence reported in recent years has shown that the heme iron of microsomal P-450's is an equilibrium mixture of the low and high spin states and that low temperature favors the low spin state (26, 27). In view of this, it seems reasonable to assume that, at least at room temperature, oxidized P-450\(_1\) contains a small amount of high spin heme iron which is in equilibrium with a large amount of low spin iron. Recent studies have provided further evidence that in low spin P-450's an oxygen atom from an adjacent hydroxyamino acid residue or a water molecule is coordinated to the heme iron as the sixth ligand (28-31) rather than a nitrogen atom from histidine or some other residue (32). Type II compounds which have ligand field strengths much higher than a hydroxyl group or water are expected to readily replace the sixth ligand, leading to another type of low spin state (29, 30). The small Type II spectral change induced on addition of aniline, a Type II substrate, to oxidized P-450\(_1\) is, therefore, explainable as due to the conversion of all the heme iron (both low spin and high spin) into this low spin state in which the nitrogen atom of aniline is strongly coordinated to the heme. This new state shows a Soret absorption peak at 420 nm rather than at 418 nm for the substrate-free, low spin state. Like many Type I substrates, benzphetamine, which is a good substrate of P-450\(_1\) (33), causes a Type I difference spectrum when added to oxidized P-450\(_1\), suggesting an increase in the content of high spin iron. However, this conversion proceeds to only a slight extent, in contrast.
to the almost complete conversion observable on substrate additions to Pseudomonas putida P450\textsubscript{cam} (18, 19) and to adrenal cortex mitochondrial P-450\textsubscript{acc} and P-450\textsubscript{11}\textbeta (20, 21), all of which are also essentially in the low spin state in the substrate-free form. It can be suggested that binding of benzphetamine to P-450 results in only partial removal of the sixth ligand (an oxygen atom) which is responsible for the low spin nature of the hemoprotein. It should be noted that the benzphetamine-induced spectral change in purified P-450\textsubscript{1} is considerably smaller in magnitude than that observable in liver microsomes of PB-treated rabbits (unpublished results). That is indicative of significant effects of membrane structure on the spin state of P-450, as has been reported previously (27). Another less likely possibility is that in the purified preparation the substrate is not accessible to all the P-450 molecules because of their dispersion state.

Oxidized P-448\textsubscript{1}, on the other hand, appears to be essentially in the high spin state at room temperature, judging from its absorption spectrum. Its EPR spectrum, however, indicates that about 12\% of the heme in P-448\textsubscript{1} is in the low spin state at liquid nitrogen temperature. Therefore, in this case too, it seems that a temperature-dependent equilibrium exists between the high and low spin states, and that most of the heme is in the high spin state at room temperature. An interesting finding is that P-448\textsubscript{1} purified from PB-microsomes shows the same absorption spectra in the Soret and visible regions as the cytochrome purified from MCMicrosomes, even though the former contains no bound MC. It is thus clear that MC binding has no influence on the spin state of P-448\textsubscript{1}. The predominantly high spin nature of P-448\textsubscript{1}, therefore, seems to be an intrinsic property of the protein rather than a result of substrate binding, although the possibility cannot be entirely excluded that an endogenous substrate is bound to P-448\textsubscript{1} from PB-microsomes.

The spin state of P-448\textsubscript{1} can, however, be shifted towards low spin by several means even at room temperature. For instance, aniline causes a conversion of the heme to a low spin state which is apparently similar to that assumed by the aniline adduct of P-450\textsubscript{1}; this state also absorbs at 420 nm. A reversible conversion of P-448\textsubscript{1} to the low spin state is also inducible by Emulgen 913, though the final state reached shows a Soret peak at 417 nm rather than at 420 nm. It is, however, unclear at this time whether Emulgen 913 provides an oxygen atom as the sixth ligand or whether the detergent causes a conformational change of the protein leading to the coordination of a hydroxyl group from an adjacent amino acid residue or a water molecule. A conversion to the low spin state with a Soret peak at 417 nm can also be observed on addition of acetanilide, which is moderately metabolizable by P-448\textsubscript{1} (unpublished results). However, this conversion is quite incomplete even at a saturating concentration of the substrate. No satisfactory explanation is available for this partial conversion, but it is certain that acetanilide can shift the spin equilibrium of P-448\textsubscript{1} towards low spin to a certain extent.

As mentioned above, the tight and specific binding of MC to P-448\textsubscript{1} seems to exert no effect on the spin state of the oxidized hemoprotein. Nevertheless, the bound MC inhibits the Emulgen 913-induced spin conversion from the high to the low spin state in a noncompetitive fashion. Furthermore, the spectral shifts caused by aniline and acetanilide are partially prevented by the presence of bound MC. These observations suggest that MC is bound to P-448\textsubscript{1} at a site near the heme. It seems that this binding does not cause any alteration in the spin state, but interferes sterically with the spin conversion induced by Emulgen 913 and the substrates.

In confirmation of previous results obtained with liver microsomes (5, 10–12) and a partially purified P-450 preparation from PB-pretreated rabbits (14), highly purified P-450\textsubscript{1} and P-448\textsubscript{1}, in the reduced state, exhibit two Soret absorption peaks at 430 and 455 (453 for P-448\textsubscript{1}) nm upon combining with ethyl isocyanide. The relative intensities of these two Soret peaks are dependent on pH, again in agreement with earlier reports on microsomes (11, 12) and a partially purified P-450 preparation (14). As suggested previously (11, 12), it is highly likely that the ethyl isocyanide complexes of reduced P-450\textsubscript{1} and P-448\textsubscript{1} (probably also the other forms of P-450 isolated in this series of studies) exist in two interconvertible states which are in a pH-dependent equilibrium. However, elucidation of the precise electronic structures of these two states must await further investigations. The reasons for the different responses to pH of
the isocyanide complexes of P-450, and P-448, are
not known either, but this provides further support
to the view that there is a molecular difference
between the two hemoproteins.

We are grateful to Drs. M. Tamura and H. Hori for
conducting EPR measurements.

REFERENCES

1. Imai, Y., Hashimoto-Yutsudo, C., Satake, H.,
489–503

251, 7929–7939

Syntheses (Cairns, T.L., ed.) Vol. 35, pp. 62–64,
John Wiley & Sons, Inc., New York

Acta Chem. Scand. 7, 1284–1287

5. Omura, T. & Sato, R. (1964) J. Biol. Chem. 239,
2370–2378

6. Omura, T. & Sato, R. (1964) J. Biol. Chem. 239,
2379–2385

7. Omura, T., Sato, R., Cooper, Y., Rosenthal, O., &
1189

Res. Commun. 68, 821–827

9. Schenkman, J.B., Cinti, D.L., Orrenius, S., Moldeus,
4250

Biochim. Biophys. Acta 118, 651–654

Commun. 23, 5–11


Biophys. Res. Commun. 24, 668–674


Acta 153, 753–765


17. Schenkman, J.B., Remmer, H., & Estabrook, R.W.

18. Tyson, C.A., Lipscomb, J.D., & Gunsalus, I.C.
(1972) J. Biol. Chem. 247, 5777–5784

678–693

20. Takemori, S., Sato, H., Hashimoto, S., Hashi-
moto, M., Satoko, S., Gomi, T., & Katagiri, M. (1995)

21. Takemori, S., Sato, H., Gomi, T., Suhara, K., &
mun. 67, 1151–1157

J. Biol. Chem. 237, PC3843–3844

Federation Proc. 24, 1172–1180


J. Biol. Chem. 248, 7637–7647

26. Rein, H., Ristan, O., Friedrich, J., Jänig, G.R., &

27. Cinti, D.L., Sligar, S.G., Gibson, G.G., & Schen-
man, J.B. (1979) Biochemistry 18, 36–42

250, 6445–6451

455–468


31. Ristan, O., Rein, H., Jänig, G.R., & Ruckpaull, K.

J. Biol. Chem. 252, 3637–3645

82, 1237–1246

J. Biochem.