The Relationship between the pH-Induced Spectral Change in Ferriprotoheme and the Substrate-Induced Spectral Change of the Hepatic Microsomal Mixed-Function Oxidase

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SUMMARY

Studies on the spectral change (type I) accompanying the formation of the enzyme-substrate complex of the hepatic microsomal mixed-function oxidase have been made to ascertain the relationship of the heme of cytochrome P-450 to this spectral change. Previously work has suggested that the type II spectral change was due to ferrihemochrome formation. Using ferriheme solutions as a model system, we have shown that spectral changes could be induced by alteration of the electronegativity, or polarity, of one ligand of the heme by variation of pH between 5.0 and 8.0. The spectral changes observed were similar with respect to wavelengths of the peak, the trough, and the isosbestic point, as well as in magnitude in relation to the heme concentration, when compared with the type I spectral change of microsomes containing cytochrome P-450 as the only heme constituent. These findings suggest that the two phenomena result from the same process: an alteration of the electronegativity of the sixth ligand of the heme. It is suggested that in microsomes the spectral changes are due to the displacement of the sixth ligand from a hydrophobic region of the apoenzyme (possibly the active site) by the substrate. It is suggested that two forms of cytochrome P-450 exist: one form is the unreacted enzyme, and the other is the substrate-bound enzyme. In the course of the metabolism of the substrate, the first form is converted to the second form in the presence of substrate, and this transition is accompanied by the type I spectral change.

INTRODUCTION

Since about 1964, attempts have been made to understand the nature of an unusual hemoprotein, cytochrome P-450, present in microsomes: the reason why the absorption maximum of its reduced carbon monoxide complex is displaced to the blue region (450 mμ) of the spectrum (1, 2), the causes of its unusual reduced minus oxidized spectrum (3), and the manner in which cytochrome P-450 functions in the mixed-function oxidase reaction (4).

In 1964, Omura and Sato (3) showed that the hepatic microsomal pigment which binds CO contained protoheme. Evidence that this pigment was a type *b* cytochrome was obtained by conversion to a derivative hemoprotein, by pyridine-hemochrome formation, and by removal of the heme with acid-acetone.

In 1963, Narasimhulu (5) observed that addition of substrate to the adrenal cortex microsomal suspension caused an alteration

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of the difference spectrum (6). The same type of spectral change was observed by Schenkman *et al.* (7, 8) and Remmer *et al.* (9) with rat liver microsomes, and by Imai and Sato (10, 11) with rabbit liver microsomes. These spectral changes were termed type I[2] spectral changes (8, 9). Another type of spectral change, termed type II[3] (8, 9), was ascribed to ferrihemochrome formation because of its characteristics and because the chemicals causing it were all amines and caused the displacement of CO from cytochrome P-450 (8).

The type I spectral change was shown to be due to formation of an enzyme-substrate complex between the mixed-function oxidase and its substrates (8, 11), and to precede the oxidation reaction. Although the concentration of cytochrome P-450 in liver microsomes roughly parallels the content of mixed-function oxidase activity, even following treatment of animals with phenobarbital (12), the quantitative correlation between enzyme activity and the type I spectral change is more consistent (13).

In the course of studies on the nature of the substrate-induced spectral change and its relationships to the mixed-function oxidase, it was noticed that ferriheme could serve as a model for ferricytochrome P-450, and that the spectral change could be imitated by altering the pH of the ferriheme solution. On the basis of information obtained with ferriheme, and from earlier studies, the mechanism of interaction between substrates of the mixed-function oxidase and cytochrome P-450 is described, and a possible mechanism of action of P-450 in the drug-oxidase reaction is suggested.

[2] Type I spectral change is characterized by a trough in the difference spectrum at 420 mμ and a peak at about 390 mμ. Type I compounds are substrates, such as hexobarbital, aminopyrine, and chlorpromazine (8), of the mixed-function oxidase which cause the type I spectral change.

[3] Type II spectral change is characterized by a trough in the difference spectrum at about 392 mμ and a peak in the Soret region between 425 mμ and 435 mμ. Type II compounds cause this type of spectral change and, with the exception of aniline, are not substrates of the mixed-function oxidase. Other type II compounds are pyridine, nicotine, and nicotinamide, (8).

MATERIALS AND METHODS

Microsomes were prepared as previously described (8) from 0.25 M sucrose homogenates by differential centrifugation. Spectra were recorded with a Cary model 14 recording spectrophotometer. Hemin was obtained from Eastman Kodak and was purified by dissolving it in chloroform. It was washed eight times with 50% aqueous methanol containing 0.5 M HCl, as described by Fox and Thomson (14). Chloroform was removed from the hemin by drying under vacuum. Hemin was added to 0.1 M NaOH just prior to use, at a concentration of about 0.09 mM, and 0.1 ml of this solution was diluted to 40 ml in 0.01 M potassium phosphate buffer. Spectra were determined within 5 min of dilution. Cells with a 10-cm light path were used to enable detection of spectral absorption changes in dilute solutions. The effect of pH on the absorption spectrum could be observed by making microliter additions of 4 M NaOH or 4 M HCl to the medium while monitoring the pH of the solution. The extinction coefficient of cytochrome P-450 in the reduced CO difference spectrum ($A_{450} - A_{500}$) was taken as 91 mM^{-1} cm^{-1}, and the difference in extinction coefficient of the pyridine-hemochrome between the reduced and oxidized forms was assumed to be 32.4 mM^{-1} cm^{-1} between 557 mμ and 575 mμ (3).

RESULTS

Microsomal type I spectral change. It had previously not been possible to observe the effect of substrates on the absolute spectrum of cytochrome P-450 because of the presence of the large amount of heme (cytochromes b_5 plus P-450) in microsomes, and because of the relatively small change in absorption that occurs on addition of substrates to liver microsomes. This problem has been circumvented in the present work by the use of rabbit liver microsomal preparations from which the cytochrome b_5 had been removed. The resultant particles, termed "P-450 particles," contain cyto-

chrome P-450 as the only source of heme, and were kindly supplied by Miss H. Nishibayashi (15).

The absolute spectrum of cytochrome P-450 in 0.1 M Tris-HCl (pH 7.5) was determined by blanking out the contribution to absorption by the microsomal particles with a preparation of particles bleached free of heme with H_2O_2. The resultant absorption spectrum, which has a maximum at 419 mμ, is shown in Fig. 1 (solid curve). The addition of hexobarbital to the substrate saturation level causes a decrease in the magnitude of the Soret absorption, with a slight shift of the maximum to 415 mμ (dashed curve). Simultaneously, there is a slight increase in absorption below 400 mμ, with isosbestic points appearing at 407 and 365 mμ. In the visible region there are decreases in absorption at about 533 mμ and around 570 mμ, as shown previously by Schenkman *et al.* (8) in difference spectra. Both the decrease in absorption in the Soret region and the increase in absorption in the near ultraviolet regions of the spectrum are small, less than 10% of the absolute absorption, and are better seen in the difference spectrum (Fig. 2). The difference spectrum shows the typical type I spectral change, as described elsewhere (8). Although the

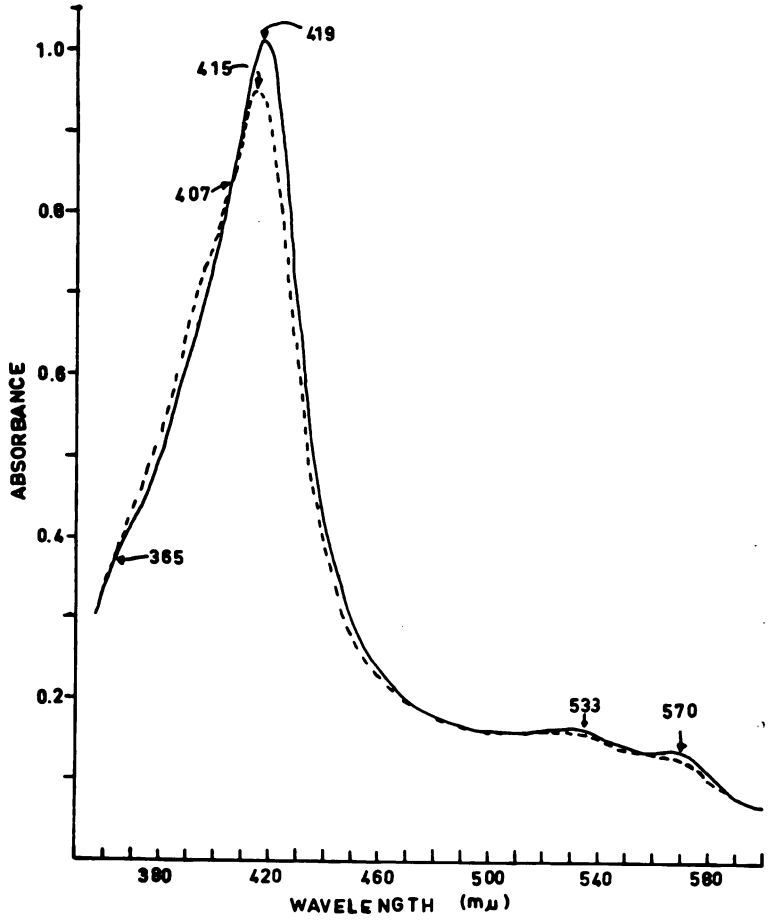

FIG. 1. *Spectral changes caused by the addition of hexobarbital to rabbit liver microsomal P-450 particles*

Particles, containing P-450 in the ferric form, were suspended in 0.1 M Tris-HCl buffer (pH 7.5) to a concentration of 1.4 mg of protein per milliliter (6.9 μM P-450), and spectra were recorded at room temperature against bleached particles in the spectrophotometer, in the presence (– – –) and absence (——) of 1.6 mM hexobarbital.

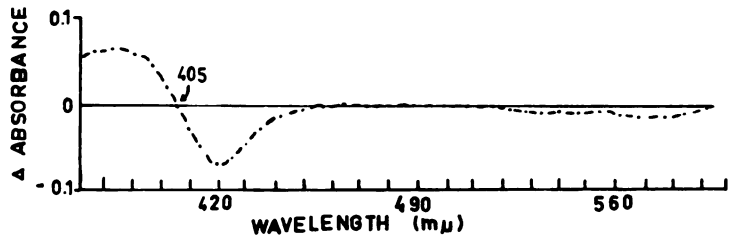

FIG. 2. *Difference spectrum between samples of P-450 particles in the presence and absence of 1.6 mM hexobarbital*
The two preparations shown in Fig. 1 were used.

P-450 particles show the type I spectral change, no drug-oxidase activity could be demonstrated, owing to destruction of the capacity to reduce cytochrome P-450 by NADPH during the removal of cytochrome b_5.

Model for type I spectral change. The type I spectral change could be duplicated by using purified ferriheme in place of cytochrome P-450, and by raising the pH in the pH range of 5.0–8.0 instead of adding substrate. Figure 3 shows the effect of altering the electronegativity of one ligand of the heme by changing the pH of the buffer solution; since the pK'_1 of the first hydroxyl on the iron is 7.4–7.6 (16), the effect is due to the replacement of a water ligand by a OH^- ligand on the iron of the heme. The absolute spectrum of a ferriheme solution at different pH values is shown in Fig. 3. Note the appearance of an isosbestic point at 407 mμ (shown more clearly in Fig. 4), embracing curves for ferriheme absorption in the pH range from 5.0 to 7.7. Above pH 8 there is a shift of the absorption maximum to lower wavelengths (up to 10 mμ) as the pH is raised further; the resultant curves no longer intersect the isosbestic point, indicating that a new species is forming that differs slightly from the two observable at lower pH values.

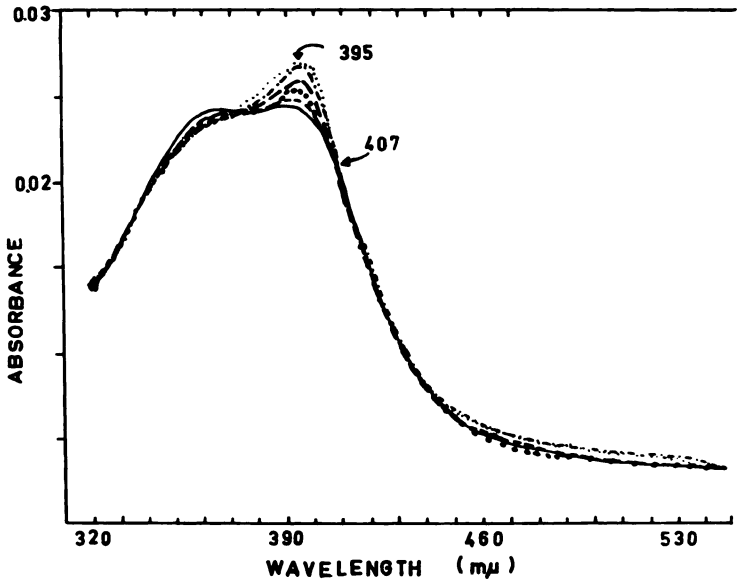

FIG. 3. *Spectral change caused by alteration in the pH of a solution of ferriheme*
Ferriheme (0.223 μM) in 0.01 M phosphate buffer was titrated with 4 M HCl or 4 M NaOH, and spectra were recorded at room temperature (18°) at pH 5.2 (——), 5.9 (- - - -), 6.3 (· · · ·), 6.6 (- - -), 6.9 (- · - ·), and 7.1 (· · · ·).

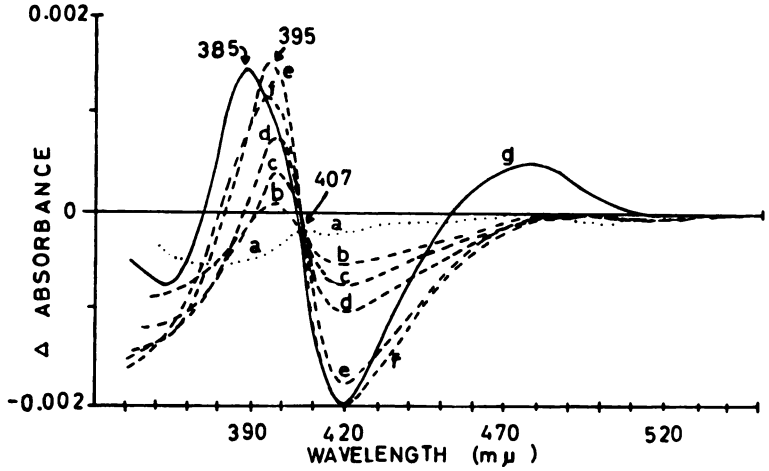

FIG. 4. *Difference spectra of the effects of pH on ferriheme in solution*

The solutions shown in Fig. 3 were used, the different solutions being compared with solutions of ferriheme at pH 5.2. One solution at pH 5.4 was compared with a portion of the same solution at pH 3.9 (*a*). The pH values for the sample and reference solutions of the different spectra were:

Curve	Sample pH	Reference pH
a	5.4	3.9
b	5.9	5.2
c	6.3	5.2
d	6.6	5.2
e	7.5	5.2
f	7.7	5.2
g	10.4	5.2

The curves were normalized to the baseline at 500 mμ, a region where they were relatively flat, because slight differences in over-all absorption (baseline shifts) occurred on the expanded scale, and turbidity changes were also magnified by the use of 10-cm cuvettes.

In the pH regions embraced by the isosbestic point there is a continuous increase in absorption at 395 mμ as the pH is raised, with a continuous decrease in absorption at 419 mμ of approximately equal magnitude. This is shown more clearly in difference spectrum (Fig. 4) relative to pH 5.2. The maximal decrease in absorption at 419 mμ occurs at pH 7.7, but at that pH a shift in the absorption peak at 395 mμ is already beginning (curve *f*). At pH 10.4 the change at 419 mμ is not further increased, but the absorption peak has been shifted to 385 mμ (solid curve), and another absorption peak appears in the blue region of the spectrum; this curve does not intersect the 407 mμ isosbestic point.

As with the spectral change on addition of substrates to liver microsomes, the magnitude of the pH-induced spectral changes in the Soret and near ultraviolet regions is low, representing less than 15% of the absorption at the maximum. In both cases the change in the Soret region is about equal in magnitude to that in the near ultraviolet region.

The decrease in absorption at 419 mμ of the ferriheme solution ($A_{419} - A_{500}$), when expressed on the basis of ferriheme concentration, ranged from 9.5 to 10.8 mM^{-1} cm^{-1} at the maximum change (pH 7.7 — pH 5.2). When expressed in a similar manner, on the basis of the cytochrome P-450 content, the magnitude of the spectral change ($A_{420} - A_{500}$) obtained on addition of hexobarbital to suspensions of P-450 particles, rabbit liver microsomes, or rat liver microsomes was also about 11.0 mM^{-1} cm^{-1}.

DISCUSSION

In this study, ferriheme solutions ranging in concentration from 0.05 to 15 μM were examined. The extinction coefficient above 10 μM concentration at pH 7.5 was about 65 mM^{-1} cm^{-1}, but, in agreement with Inada and Shibata (17), in more dilute solutions (0.05–0.95 μM) the extinction coefficient was 120 mM^{-1} cm^{-1} at pH 7.3. As shown in Figs. 3 and 4, the extinction coefficient is pH-dependent, increasing with the pH value to a maximum at about pH 7.5 and decreasing thereafter. The decrease in extinction coefficient with increasing concentration of ferriheme is accompanied by a shift in the absorption maximum to shorter wavelengths, similar to that seen on increasing the pH value above 8. Although the extinction coefficient is decreased at higher concentrations and the absorption maximum is shifted to a lower wavelength (about 390 mμ), the same spectral change with pH is observed when examined as a difference spectrum against a solution of ferriheme at pH 5.5. Most probably this hyperchromicity is the result of polymerization and is similar to the phenomenon observed with polynucleotides (18), since with more concentrated solutions the ferriheme can be removed from solutions at pH 5.0 by centrifugation at only $600 \times g$ for 15 min. In more dilute solutions, however, the pH of the reference solution could be reduced to as low as 3.9 (Fig. 4) without inducing a significant spectral change.

In partial agreement with Inada and Shibata (17) was the finding that there is a time-dependent decrease in the magnitude of ferriheme absorption in the near ultraviolet. In 55 min, at pH 7.3, there was a 3–8% decrease in the 395 mμ absorption peak, but at pH 8.6 there was essentially no decrease in absorption of ferriheme solutions at concentrations of 0.115–1.15 μM.

Earlier (8) suggestions as to the manner of substrate interaction with ferricytochrome P-450 of liver microsomes proposed that the compounds producing type II spectral changes interact with the iron at the CO-binding site of the heme, whereas the site of interaction of the type I compounds was considered to be different. The current investigation gives insight into the manner of interaction of the type I compounds, and suggests that cytochrome P-450 exists in two related forms, both present in liver microsomes from normal animals in about equal amounts.

The duplication of the type I spectral change of ferriheme solution with respect to wavelength of the absorption peak, trough, isosbestic point, and magnitude (on the basis of heme concentration) strongly suggests that the effects in ferriheme and in ferricytochrome P-450 result from the same phenomenon, an increase in the electronegativity or polarity of the sixth ligand[4] of the heme. This spectral change was accomplished with ferriheme by changing the ligand from water to OH^-, and with ferricytochrome P-450 by possibly displacing the sixth ligand from one part of the enzyme to a more polar region. The chemical nature of the sixth ligand to the heme is at present unknown. It is possible that the sixth ligand is displaced from the active site of the enzyme itself. Since the many substrates of the mixed-function oxidase of liver microsomes have in common only the property of being lipid-soluble (19), it is assumed that the active site of the enzyme is in a hydrophobic region of the apoenzyme, as suggested by Imai and Sato (11). The sixth ligand is probably not released to the medium, since alteration of the pH of the medium containing microsomes does not cause a spectral change, nor does it alter the substrate-induced spectral change[5]. In contrast, changes in pH profoundly alter the ethylisocyanide-binding spectrum of ferrocytochrome P-450 when the heme is reduced with dithionite (20).

From the similarity between the magnitude of the change in absorption at 420 mμ (based on ferriheme concentration) with pH and ferricytochrome P-450 with hexobarbital, it would appear that the fraction of heme reacting to cause the spectral

[4] The ligand of the microsomal heme which interacts with oxygen and CO has arbitrarily been designated as the sixth ligand for ease in referring to it, as has the ligand of ferriheme which is replaced by OH^-.

[5] Unpublished observations.

change in both cases is the same. Thus, if all the ferriheme is present in the alkaline form at pH 7.7, when conversion to a third species begins, the optical density change per millimolar concentration of heme per centimeter is an accurate measure of the maximal change in absorbance at 419 mμ. If appreciable amounts of the third species or acid form of ferriheme exist, however, the optical density value per millimolar concentration of heme per centimeter would actually be greater, and, by analogy, the corresponding value in liver microsomes (11 mM^{-1} cm^{-1}) would reflect an interaction between hexobarbital and a fraction of the ferricytochrome P-450 molecules present in the microsomes. Although addition of aminopyrine (3 mM) to a rat liver microsome suspension containing saturating amounts of hexobarbital (3 mM) does not increase the magnitude of the type I spectral change further, the possibility exists that only a part of the heme is interacting, since part of the hemoprotein may already have endogenous substrates bound to it.

We have thus described two forms of ferricytochrome P-450. One form, with an intact sixth ligand and relatively greater absorbance at 420 mμ, is the one that interacts with substrates of the mixed-function oxidase prior to their oxidation. After interaction with substrates, the first form is converted to the second form, which differs in that it has substrate bound to the apoenzyme, the sixth ligand of the heme is altered, possibly at a different site on the protein, and the absorption at 390 mμ is greater. These two forms are interconvertible, as shown by the variation in ferriheme absorption with changes in pH, and after removal of substrate from the microsomes (8). The relative amounts of the two forms present in isolated liver microsomes would depend on the solubility of the endogenous substrates in the homogenizing and washing media, and on how much of the substrate remained bound to the enzyme during the preparation of the microsomes.

A possibility which must be considered is that cytochrome P-450 exists as a dimer in which the hemes interact, as suggested by at least two authors (21, 22). From the similarity between the ferriheme spectral change and the ferricytochrome P-450 spectral change, it would be expected that the molar heme contents of the two would be similar. Unfortunately, there is still controversy over whether the basic unit of ferriheme in solution is a monomer (17, 23) or a dimer (16, 24).

Whether cytochrome P-450 exists as a monomer or a dimer with respect to heme, it is clear that the spectral change caused by the addition of substrate to the mixed-function oxidase depends on the presence of substrate in the molecule. Preliminary data suggest that the alteration of the sixth ligand of the heme permits a faster flow of reducing equivalents to the heme.[5] After reduction, the sixth ligand is free from the apoenzyme, as evidenced by its ability to combine with CO or O_2.

It may be suggested that the sequential steps in drug metabolism are the following. (a) The substrate interacts with the apoenzyme, altering the sixth ligand of the ferriheme, possibly shifting it to a more polar region of the apoenzyme. (b) Reducing equivalents flow from NADPH to the heme, resulting in a release of the sixth ligand from the enzyme. (c) The sixth ligand interacts with oxygen (or carbon monoxide) in the medium, with a rapid release of electrons to the oxygen. (d) The "activated oxygen" interacts with the adjacent substrate. (e) The oxidized substrate, which is now more polar, is released from the enzyme, and the hemoprotein returns to its previous state.

Steps (c) and (d) must be exceedingly rapid relative to step (b), since attempts to observe an oxygenated intermediate by spectral and electron spin resonance methods have been unsuccessful. The rate of reduction of cytochrome P-450 is rather slow, since only about 2–3 mμmoles are reduced per minute per milligram of microsomal protein in normal rats at 18°.[5]

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