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ASSOCIATION OF HYDROPHOBIC SUBSTANCES WITH HEMIN

CHARACTERIZATION OF THE REVERSE TYPE I BINDING SPECTRUM AND ITS RELATIONSHIP TO CYTOCHROME P-450

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Abstract—When hydrophobic compounds were added to a solution of protoferriheme, a reverse type I spectral change was produced when observed by difference spectroscopy. The spectrum had a peak at 422 nm and a trough at 387 nm, and the characteristics were dependent on the pH of the sample. An association constant for the complex could be determined and was also found to be pH sensitive, with the association constant dropping to zero at values below pH 7.0 and above pH 8.5. The determination of the ΔAbs_{max} for the ethylbenzene–hemin complex at various hemin concentrations indicates monomeric heme to be the species responsible for binding the hydrocarbon with the concomitant generation of the reverse type I spectral change.

The addition of substrates to cytochrome P-450 produces perturbations of the spectrum of the hemoprotein. These spectral changes have been studied using difference spectroscopy and were categorized into three basic groups: type I, type II and modified type II spectral changes. Most substrates of cytochrome P-450 (such as benzphetamine and ethylmorphine) produce the type I spectral change. When examined by difference spectroscopy, type I compounds produce a peak at 385 nm and a trough at 420 nm [1–4]. Substrates containing an amino group (e.g. aniline) produce what has been called a type II spectrum with a peak between 425 and 435 nm and a broad trough between 390 and 410 nm [2, 4]. Substrates containing a hydroxyl function (e.g. methanol and ethanol) produce a modified type II spectral change with a peak at 420 nm and a trough at 385 nm [5, 6]. These spectral changes have subsequently been correlated with changes in the spin equilibrium of the heme, with a type I change corresponding to a low to high spin shift in the spin state of the heme [7–11].

Since the spectral interactions observed with cytochrome P-450 are the result of either direct or indirect interactions with the heme, protoferriheme has been used as a model system in an attempt to explain some of the characteristics unique to cytochrome P-450. Spectral perturbations with cytochrome P-450 have been mimicked using hemin solutions by differentially varying the pH of the reference and sample cuvettes [12] and by adding hydrophobic substances to the protoferriheme solution [13]. Substrate-induced spectral changes similar to those produced by cytochrome P-450 have been obtained more recently by adding substrates to glutathione–hemin complexes. Hemin complexes have also been used as model systems for certain cytochrome P-450-dependent metabolic reactions, such as the aromatic hydroxylation of *p*-toluidine and aniline [14, 15], the hydroxylation of acetanilide [16], olefin hydroxylation [17], and reduction of halogenated compounds, arene-oxides, and nitro- and nitrosoaromatics [18].

In a previous communication, this laboratory reported the production of a reverse type I spectral change (422 nm peak; 387 nm trough) as a result of addition of aromatic hydrocarbons to a hemin solution [13]. Brown and coworkers [19] studied the aggregation characteristics of protoferriheme and determined that it existed as an equilibrium mixture of monomer and dimer in solution with the equilibrium constant in favor of dimer formation. With these results in mind, the hydrocarbon-induced reverse type I spectral change has been further characterized with regard to substrate specificity, pH effects, hydrophobic influences on binding, and whether the monomer or the dimer is the species responsible for the spectral change.

METHODS

Protoferriheme (Hemin, Eastman) was dissolved in 0.1 N sodium hydroxide to a concentration of 2 mM. The sample was then diluted further to a concentration of 40 μM with 100 mM sodium phosphate buffer (pH 7.5) or 50 mM Tris–HCl, 0.15 M KCl (pH 7.5). Difference spectra were obtained using either a Cary 17 or an Aminco DW-2c spectrophotometer. With the protoferriheme solution in each cuvette, multiple additions of hydrocarbons

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(diluted in ethanol) were added to the sample cuvette, while equivalent volumes of ethanol were added to the reference cuvette [13]. After each addition the spectrum was recorded.

In the experiments to determine whether the monomer or dimer of protoferriheme was responsible for the spectral change, the 2 mM stock solution was diluted to different final concentrations of hemin, the pH was adjusted to 7.7, and a spectral titration was performed as described above. The concentrations of monomer and dimer present at given ferriheme concentrations were calculated from an equilibrium constant of $2 \times 10^8\ M^{-1}$ for the dimerization of heme at pH 7.7 as determined by Brown and coworkers [19]. Using the following equation

$$K_{obs} = \frac{[dimer]}{[monomer]^2} \quad (1)$$

the concentration of dimer and monomer can be readily obtained for each concentration of hemin used.

RESULTS

As shown previously, the addition of hydrophobic substrates to protoferriheme solutions produced a spectrum with an absorption maximum at 422 nm and a trough at 387 nm when observed by difference spectroscopy (Fig. 1). The response to the addition of these substances is a binding isotherm analogous in some ways to enzyme–substrate complex formation from free enzyme and substrate. Spectral association constants (K_s) were determined by analogy with the Lineweaver–Burk plot (Fig. 1, inset). Such results were obtained either with 0.1 M phosphate buffer (pH 7.7) (Fig. 1) or with 50 mM Tris–HCl, 0.15 M KCl (pH 7.7) [13]; however, the K_s for ethylbenzene was found to be more than 7-fold larger in phosphate buffer ($300\ M^{-1}$) than in Tris–KCl ($40\ M^{-1}$). When the oxygen content of the buffer was too high, the 422 nm peak in the difference spectrum disappeared. Bubbling the buffers with nitrogen before use ensures the difference spectrum shown in Fig. 1.

Table 1. Association of substances with protoferriheme*

Substance	K_s (M^{-1})	ΔAbs_{max}
Benzene	10	0.48
Toluene	18	0.77
p-Xylene	32	0.77
Ethylbenzene	39	1.30
Naphthalene	109	2.21
Hexane	49	0.28
Decane	155	0.17
Dodecane	297	0.12
Benzyl alcohol	35	0.91
Cyclohexane	21	0.12
Cyclohexanol	55	0.39
Cyclohexanene	24	1.33
2-Propanol	2.1	0.08
2-Propanone	2.6	4.00

* Hemin was diluted to a final concentration of 40 μM in 0.15 M KCl, 50 mM Tris (pH 7.7) as described in Methods.

A number of compounds were tested which produced this "characteristic" spectral change and they are shown in Table 1. Both aliphatic and aromatic hydrocarbons produced this effect. Alcohols such as 2-propanol, cyclohexanol and benzyl alcohol also elicited the response, as did some ketones. Common drug substrates for cytochrome P-450 (e.g. ethylmorphine and hexobarbital) produced small distorted spectral changes when added to hemin, indicating that the characteristic reverse type I spectral change was found with hydrophobic substances which contained no charged functional groups. Unusual spectra were also found with progesterone, chloramphenicol and glycerol. In these experiments, the hydrocarbons were diluted in ethanol and small amounts of these stock solutions were added to the hemin. Ethanol was not required for production of this spectral change; ethylbenzene produced a reverse type I spectral change when added without prior dilution. Also, when dilution was required, the hydrocarbons were diluted in ethanol only to the extent necessary for accurate addition of the small

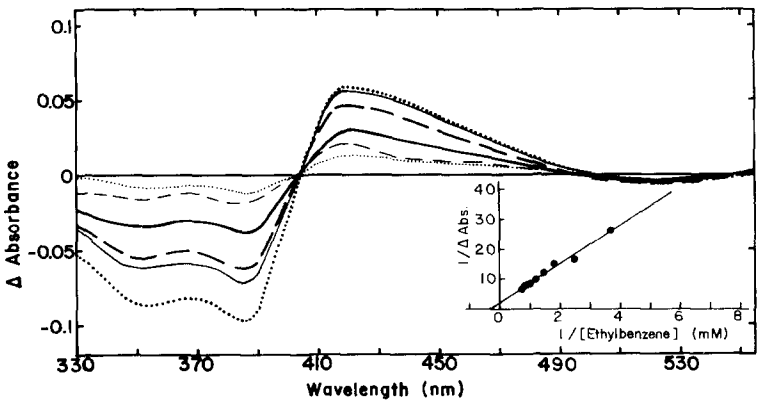

Fig. 1. Difference spectrum resulting from ethylbenzene addition to protoferriheme. Ethylbenzene was diluted to a stock concentration of 81.7 mM in ethanol. Microliter increments were added to the sample cuvette with an equivalent volume of ethanol added to the reference. The final ethylbenzene concentration ranged from 0.136 to 1.37 mM. Inset: Double-reciprocal plot of data. The apparent K_s was $300\ M^{-1}$. The hemin was diluted in 100 mM potassium phosphate, pH 7.7.

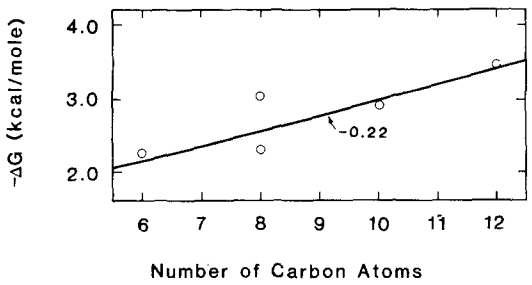

Fig. 2. Size dependence for a series of straight chain aliphatic hydrocarbons. The hydrocarbons were (6) hexane, (8) octane, (8) isooctane, (10) decane, and (12) dodecane. Experiments were done in 50 mM Tris, 0.15 M KCl (pH 7.7).

amounts of substrate, and the same hydrocarbon to ethanol ratios were used for the stock solutions used to minimize any influence ethanol might have on the binding constants. Finally, ethanol has been shown to produce a reverse type I spectral change with an association constant so small that it could not be measured accurately ($\bar{K}_s < 1\,M^{-1}$). Due to the low affinity of the solvent for hemin, the effects on the binding constants for the hydrocarbons were expected to be minimal.

As reported previously with a series limited to aromatic hydrocarbons [13], the free energy of binding to protoferriheme (calculated from $\bar{K}_s$) increased linearly with hydrocarbon size. A slope of -0.36 kcal (mole)$^{-1}$ (methylene group)$^{-1}$ was obtained. The size dependence for a series of straight chain aliphatic hydrocarbons is shown in Fig. 2, where a linear increase in the free energy of binding with hydrocarbon size was also observed. A slope of -0.22 kcal (mole)$^{-1}$ (methylene group)$^{-1}$ was obtained with this

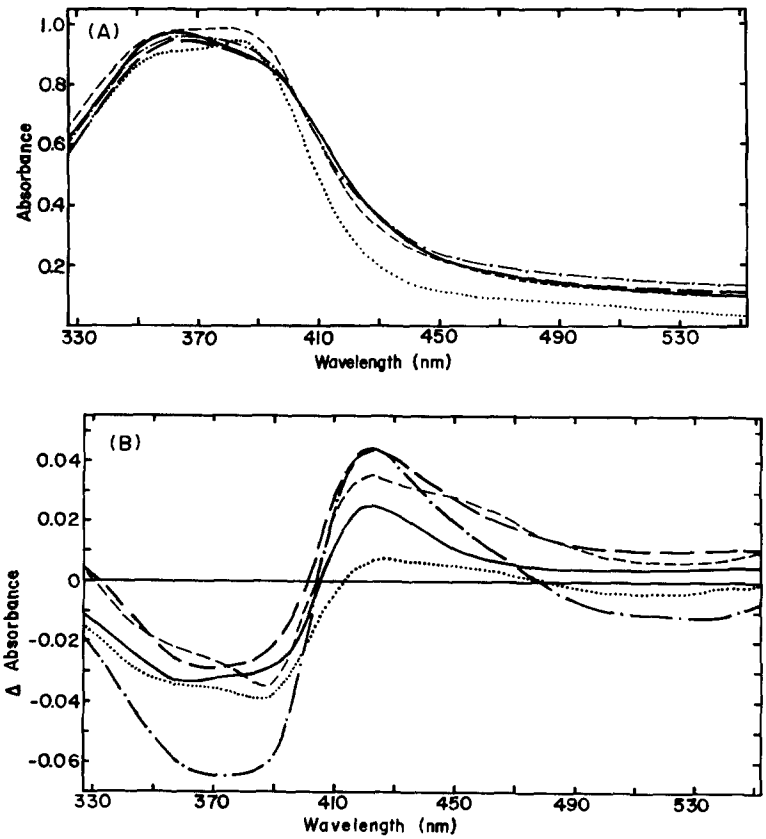

Fig. 3. The pH dependence for protoferriheme. (A) Absolute spectra for protoferriheme at various pH values ranging from 6.5 to 8.1. Identification is as follows: pH 6.5 (——), pH 6.9 (— —), pH 7.2 (—·—), pH 7.7 (----) and pH 8.1 (. . . . .). The hemin concentration was 0.2 mM in 0.1 M potassium phosphate buffer at the indicated pH values. (B) Ethylbenzene-induced difference spectrum at various pH values: pH 6.5 (——), pH 6.9 (— —), pH 7.2 (—·—), pH 7.7 (----) and pH 8.1 (. . . .). The ethylbenzene concentration was 1.36 mM.

series, a value somewhat smaller than that obtained with the aromatic hydrocarbons.

Variation of pH caused a change in both the absolute spectrum as well as the substrate-induced difference spectrum. The absolute spectra of hemin solutions at a series of pH values ranging from 6.5 to 8.1 are shown in Fig. 3A. At low pH values (pH 6.5), the absolute spectrum exhibited a peak at 365 nm with a shoulder at 385 nm. As the pH was increased, the absorbance at 385 nm increased and the 365 nm absorbance declined. When examined by difference spectroscopy, a complex pattern of absorbance changes was also observed. At each pH examined, an absorption maximum was observed at about 422 nm. At pH 6.5 a broad trough was observed at 365 nm with a shoulder at about 385 nm which became larger while the trough at 365 nm became less pronounced (Fig. 3B). The magnitude of the spectral change (422 nm trough) increased as the pH increased, reaching a maximum at pH 7.2. As the pH was increased further, the magnitude of the spectrum was diminished. Spectral association constants for the ethylbenzene–protoferriheme complex were determined for a range of pH values from 6.3 to 10.5 (Fig. 4). As shown in this figure, the $\bar{K}_s$ reached a maximum at pH 7.7, with the K_s value rapidly dropping below pH 7.4 and above pH 7.9. Outside this relatively narrow range (above pH 8.5 or below pH 7), non-linear double-reciprocal plots and/or apparent negative $\bar{K}_s$ values were obtained.

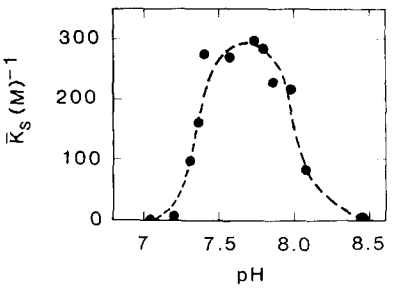

Fig. 4. Dependence of the $\bar{K}_s$ for ethylbenzene on pH. Conditions are described in Fig. 3 and in Methods.

Protoferriheme in solution exists as a monomer–dimer equilibrium with the equilibrium constant largely in favor of the dimer ($K_{eq} = 1.97 \times 10^8$ at pH 7.7, see Ref. 19). Since cytochrome P-450 contains a single heme, it was of interest to determine whether the monomeric or dimeric species of protoferriheme was responsible for the reverse type I spectral change. By using the equilibrium constant determined by Brown *et al.* [19] for protoferriheme, both the monomer and dimer concentrations can be calculated at any given hemin concentration. Pro-

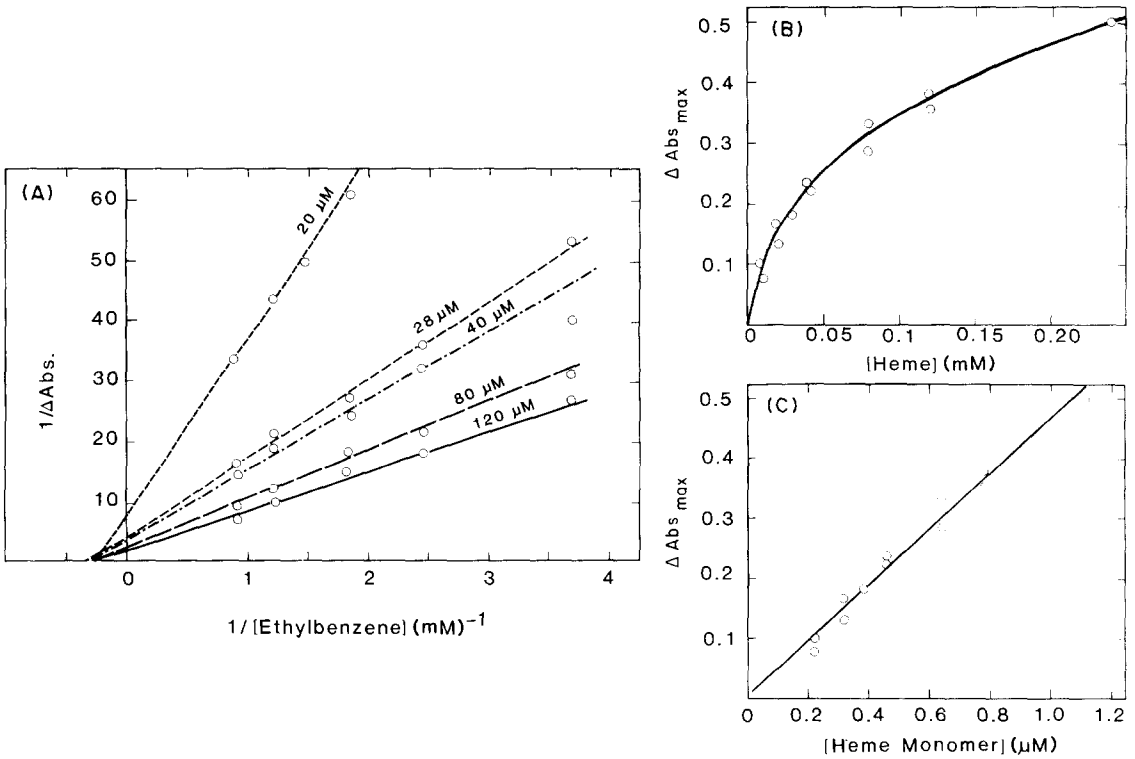

Fig. 5. Determination of hemin species responsible for the reverse type I spectral change. Spectral titrations were performed at various hemin concentrations in order to differentially vary monomer and dimer concentrations. Hemin was diluted in 0.1 M potassium phosphate (pH 7.7). (A) Double-reciprocal plots for ethylbenzene binding at a series of hemin concentrations. (B) Dependence of ΔAbs_{max} from panel A on dimer concentration and on total hemin concentration. The dependence of ΔAbs_{max} on the dimer concentration is shown. Virtually the same curve was obtained when the total heme concentration was used due to the extremely large K_{eq} for the $2M \rightleftharpoons D$ equilibrium. (C) Dependence of ΔAbs_{max} from panel A on the monomer concentration.

toferriheme solutions at various concentrations were titrated with ethylbenzene, the results of which are shown in Fig. 5A. As the concentration of protoferriheme was diminished, the maximal absorbance (ΔAbs_{max}) was also decreased. The association constant for the hemin–ethylbenzene complex was unaffected. The value for ΔAbs_{max} was then plotted against total protoferriheme concentration, monomer concentration, and dimer concentration to determine if a direct relationship exists. The dependence of ΔAbs_{max} on both the total protoferriheme concentration and dimer concentration is shown in Fig. 5B. A non-linear relationship was found in each case. However, when ΔAbs_{max} was plotted against monomer concentration, a linear relationship was obtained (Fig. 5C). The direct relationship between monomer concentration and ΔAbs_{max} indicates that the monomeric species was responsible for the spectral change. The difference extinction coefficient for the ethylbenzene–hemin complex was obtained from the slope of the line in Fig. 5C (470 $mM^{-1}\,cm^{-1}$).

DISCUSSION

The results presented in this paper demonstrate the production of a reverse type I spectral change by adding various substances to a protoferriheme solution. The spectrum was produced by a number of different compounds with hydrophobicity being a common and necessary requirement for its production. When typical cytochrome P-450 drug substrates (e.g. hexobarbital and ethylmorphine) were examined, distorted spectra were produced.

The shape of the substrate-induced difference spectrum was influenced by the pH of the solution. Each of the difference spectra exhibited a peak at 422 nm and a broad trough ranging from 365 to 385 nm which appeared to be made up of two components. At pH values lower than 7.2, the 365 nm compound was predominant, with the 385 nm component predominating at pH values above 7.2. Also, the magnitude of the absorbance change produced was dependent on the pH of the solution with the largest change being produced when the hemin was at pH 7.2 (Fig. 3B).

The size dependence studies indicate that hydrophobic interactions are involved with association of hydrocarbons with protoferriheme. For partitioning of a series of hydrocarbons from an aqueous environment into an organic solvent such as octanol, the slope of the change in free energy for each methylene group would be approximately −0.7 kcal/mole [20, 21]. The size dependence for aromatic hydrocarbon binding to the spectral site of cytochrome P-450 both from untreated and phenobarbital-pretreated rats was about −0.7 kcal/mole [21], strongly suggesting complete transfer of the hydrocarbon from the aqueous to the organic (enzyme) phase. By contrast, the size dependence of association of aromatic hydrocarbons with protoferriheme [13], as well as that for the straight chain aliphatic hydrocarbons as shown in Fig. 2 (−0.36 kcal $(mole)^{-1}$ $(methylene\ group)^{-1}$ and −0.22 kcal $(mole)^{-1}$ $(methylene\ group)^{-1}$ respectively), indicate that these substrates are not completely extracted from the aqueous phase, and therefore remain partially exposed to water.

The direct relationship between the ΔAbs_{max} for the ethylbenzene–protoheme complex and the concentration of monomeric heme strongly indicates that the monomeric species is indeed responsible for the spectral change (Fig. 5C). The difference extinction coefficient for the hydrocarbon–protoferriheme complex (422 − 387 nm), as calculated from the slope in Fig. 5C, is 470 $mM^{-1}\,cm^{-1}$ within the same order of magnitude but about 4-fold larger than the difference extinction coefficient for the type I binding to cytochrome P-450 of 126 $mM^{-1}\,cm^{-1}$ [11, 21, 22].

The results presented here illustrate a number of similarities between protoferriheme and cytochrome P-450 concerning the spectral binding of substrates. The spectrum produced with protoferriheme is approximately the reverse of the substrate-induced type I binding spectrum observed with cytochrome P-450. The wavelengths of the peaks and troughs correspond in each system. Additionally, monomeric heme is involved in the production of the spectrum, with the difference extinction coefficient for the heme–substrate complex being about four times larger than that of the cytochrome P-450–substrate complex. This difference may be due in part to the difficulty in experimentally determining the equilibrium constant for the monomer–dimer equilibrium because of its extremely large value [19]. However, unless there are gross differences in the monomer–dimer equilibrium caused by slightly different experimental conditions than reported by Brown *et al.* (e.g. a different source of hemin), this value is indeed larger than that of the difference extinction coefficient for the spin transition with cytochrome P-450. For example, a 4-fold error in the extinction coefficient would require at least a 10-fold error in the calculated value for K_{obs} as calculated from equation 1. Further, other compounds (e.g. naphthalene) produced an even larger spectral change than ethylbenzene (Table 1), indicating that the extinction coefficient for a high to low spin transition with hemin is significantly larger than that found for cytochrome P-450 spin transitions.

The spectral changes observed from hydrocarbon association with hemin were opposite from those obtained from substrate association with cytochrome P-450. When substrates bind to cytochrome P-450, a shift in the spin equilibrium from the low spin to the high spin form occurs. This change in configuration of the heme has been correlated with the type I spectral change [7–11]. When examining hemin solutions, the observed reverse type I spectral change likely represents a shift in the spin equilibrium of the heme monomer from the high spin to low spin state, suggesting that the fraction of heme which exists in the monomer probably exists predominantly in the high spin state. This high to low spin transition is brought about by its interaction with a hydrophobic substance. This association is not thought to be a direct interaction with the iron since simple hydrocarbons can produce such a change, but might be an association with the plane of the porphyrin ring and displacement of water.

With cytochrome P-450 the spectral change might

be brought about in a similar manner. In this case cytochrome P-450 might have a hydrophobic substance associated with the heme in the substrate-free state. This hydrophobic substance would not be a substrate, but would likely be part of the enzyme such as a string of hydrophobic amino acids or perhaps even a trapped phospholipid moiety. This hydrophobic region could interact with part of the porphyrin ring in the absence of substrate (free enzyme) in a manner similar to the interaction between hemin and hydrocarbon as described above. Addition of substrate to cytochrome P-450 may then produce a conformational change in the protein that could cause this hydrophobic region to move away from the heme and a type I spectral change would result. In a recent report by Poulos and coworkers [23], the crystal structure of substrate bound cytochrome P-450_{cam} was determined. A hydrophobic stretch of amino acids (residues 244–249) was indeed shown to run close to the distal heme-surface. Movement of these amino acids closer to the heme might occur as a result of dissociation of substrate from the active site. Substrate binding might bring about the movement of this hydrophobic region away from the heme and thus indirectly cause the typical type I change. This is probably the most simplistic suggestion that we might make; the eventual explanation might be more complex. Further information on the nature of the substrate-free enzyme might be illuminating in this regard. A point which we wish to emphasize is that the spectral change observed with cytochrome P-450 can be accounted for in terms of medium effects in the vicinity of the heme; no covalent bonds are necessarily involved.

The interaction of alcohols with hemin might be more complex than that of the simple hydrocarbons. These compounds by virtue of the hydroxyl function might be thought to associate directly with the heme iron, producing the modified type II spectral change which has been shown previously for the binding of alcohols to cytochrome P-450 [5, 6]. In this study, ethanol was shown to associate with hemin in solution; however, the association constant was extremely small ($K_s < 1.0\,M^{-1}$). Dual association of ethanol with both the porphyrin ring and the heme iron might indeed occur, but its small association constant for hemin might make differentiation of these effects difficult to analyze clearly. It is important to note that, without invoking complex mechanisms, hydrophobicity appears to be the predominant factor involved in the production of this spectral change with hemin.

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