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A REVERSE TYPE I SPECTRAL CHANGE OBSERVED WITH HEMIN AND ITS POSSIBLE SIGNIFICANCE WITH RESPECT TO CYTOCHROME P-450

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Many substances combine with oxidized cytochrome P-450, changing its absorbance. It is possible to estimate an apparent binding or affinity constant from this type of data [1,2]. As a rule, two kinds of changes may be observed. One class of substance such as ethylmorphine produces a minimum in the difference spectrum at 420 nm and a maximum at 385-390 nm. These are type I compounds. Other compounds exemplified by aniline or nicotinamide bring about a maximum at approximately 430 nm and a minimum at 390-410 nm. These are type II compounds. The mechanism by which such spectral changes are brought about is not entirely clear.

Imai and Sato [3] noted similarities between protoferriheme and cytochrome P-450 with ethylisocyanide and pyridine induced spectral interactions. Schenkman and Sato [4] also used protoferriheme as a model for cytochrome P-450, inducing a type I change by increasing its pH. In this communication a reverse type I spectral change is induced by the addition of hydrophobic substances to protoferriheme. Its significance with respect to the spectral changes observed with cytochrome P-450 is discussed.

MATERIALS AND METHODS

All hydrocarbons were purchased from Eastman. A stock solution of 2 mM protoferriheme (Hemin, Eastman, 97 + % by spectral analysis) was made up in 0.1 N sodium hydroxide. The working solution was prepared by diluting 1 ml of stock solution to 25 ml with Tris-KCl(50 mM) buffer, giving a final concentration of 8×10^{-5} M. The hydrocarbon solutions were made up in alcohol; it was ascertained that alcohol itself produced no appreciable spectral change with protoferriheme. The pH was adjusted to 7.68 with a few µl of 1 N NaOH.

The spectral changes were measured by means of a Cary 17 spectrophotometer. The volume of the protoferriheme solution in the cuvette was 3 ml. The hydrocarbon solution was added in alcohol to the cuvette in the sample compartment by means of a micropipette. A like amount of alcohol was added to the reference compartment and the spectral change determined. The process was repeated until 80 µl alcohol or alcoholic solution of hydrocarbon had been added to each of the corresponding cuvettes. The data were plotted as suggested by Schenkman et al. [1,2], that is to say the reciprocal of Δ Absorbance, the difference in optical density at 420 and 385 nm, was plotted against the reciprocal of the hydrocarbon concentration. The apparent spectral constants were calculated by the method of least squares. They were computed as apparent association constants for the reaction

$$E + S \underset{\leftarrow}{\overset{\rightarrow}{}} ES$$

where $K = \frac{[ES]}{[E][S]}$ at equilibrium. The apparent change in free energy, ΔG^0 was then written as $-RT \ln K$.

RESULTS

If protoferriheme is allowed to interact with hydrophobic substances such as hydrocarbons, a large spectral change takes place as is seen in Fig. 1 which illustrates the difference spectrum obtained with ethylbenzene at pH 7.68. Similar spectral changes were observed with aliphatic hydrocarbons; aromaticity is not required.

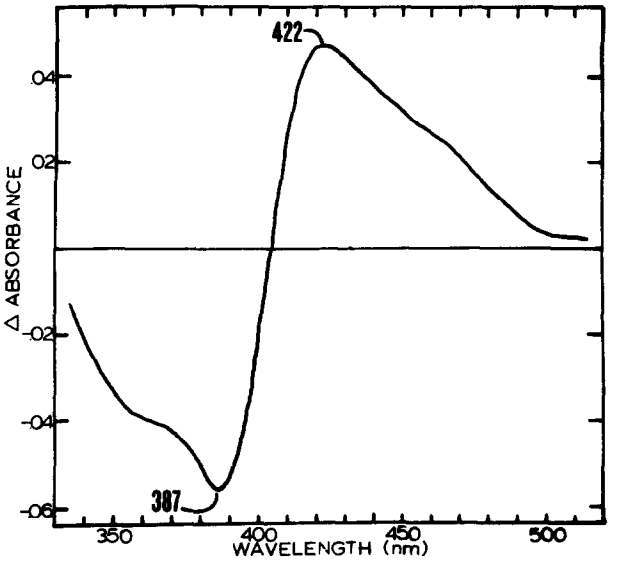

Fig. 1. Difference spectrum of ethylbenzene with protoferriheme. Ethylbenzene was added to a final concentration of 9.9×10^{-4} M, as described in Materials and Methods.

It may be observed that this spectral change is a mirror image of that obtained when cytochrome P-450 interacts with a type I substrate. In the case of protoferriheme, the peak and trough occur at 422 and 387 nm respectively, rather than 420 and 385 nm as is usually the case with the enzyme. The Δ Absorbance (difference in absorbance at 422 and 387 nm) is

proportional to the concentration of added hydrocarbon as is the case with the addition of substrate to cytochrome P-450. A plot of the reciprocal of Δ Absorbance vs the reciprocal of the hydrocarbon concentration, as applied by Schenkman et al. [1,2] to cytochrome P-450, also gives a straight line with protoferriheme, allowing the calculation of an apparent association constant for the binding of the hydrocarbon to protoferriheme. A typical example of this double reciprocal plot may be seen in Fig. 2. Typical substances which give a type I response with the enzyme such as hexobarbital or ethylmorphine give a distorted noncharacteristic spectral change with protoferriheme. Aniline causes changes similar to those produced by the hydrocarbons. The substance used must have predominantly hydrophobic character to give a good response and to bind well.

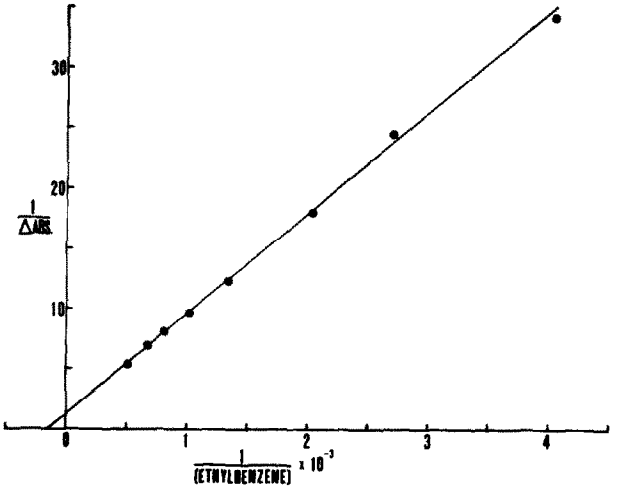

Fig. 2. Double reciprocal plot of ethylbenzene with protoferriheme. Ethylbenzene concentrations ranged from 2.48 x 10^{-4} M to 1.98 x 10^{-3} by adding 5 μl increments of a 0.0743 M solution of ethylbenzene dissolved in ethanol.

A previous communication from this laboratory [5] pointed out that the free energy of binding of a homologous series of aromatic hydrocarbons to hepatic microsomal cytochrome P-450 was linear with hydrocarbon size. Figure 3 shows that a similar result is obtained for the apparent binding of the same series of hydrocarbons to protoferriheme. The slope is 0.36, by the method of least squares, as compared to a slope of 0.39 for the hepatic microsomal cytochrome P-450. This agreement might be fortuitous; it is of interest that there is an increased negative free energy change as molecular size is increased which amounts to 0.36 kcal per mole per added carbon atom. This is a measure of the effect of added carbon atoms on the work involved in complex formation.

The results described are in agreement with the concept that in the resting state of the enzyme a hydrophobic moiety (associated with the enzyme itself) is in close proximity to the heme(s). When a type I substrate such as hexobarbital (which does not itself produce a characteristic change with heme) combines with cytochrome P-450 at some site other than in the vicinity of the heme, a conformational change or some other process occurs which

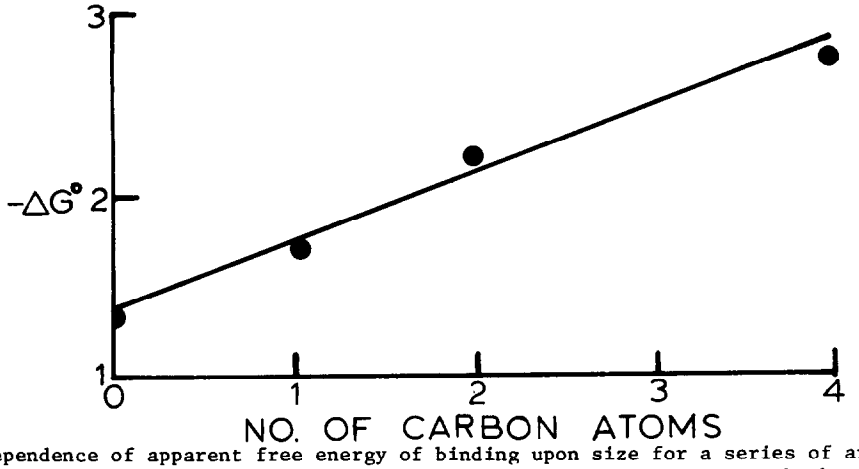

Fig. 3. Dependence of apparent free energy of binding upon size for a series of aromatic hydrocarbons. The abscissa represents the number of carbon atoms added to the benzene ring. Therefore, the points represent benzene (zero carbon atoms), toluene, ethylbenzene and naphthalene in ascending order. While naphthalene is not strictly speaking an analog, it has been shown previously [5,6] to behave as though it were.

results in the withdrawal of this hydrophobic moiety from the vicinity of the heme(s). Since introduction of hydrophobic (hydrocarbon-like) substances results in the above described inverse type I response, moving that moiety away from the heme(s) would result in a typical type I difference spectrum. Conversely, the removal of the substrate from the oxidized enzyme could cause this hydrophobic moiety to return to the original position relative to the heme, thus giving a reverse type I change. It is not possible to choose as to whether the hydrophobic moiety moves away from the heme or vice versa; a combination of both is possible.

The interaction of hydrocarbon with protoferriheme causes a reproducible increase in the pH of .01 unit after the addition of 9.9 mM ethylbenzene. These results are consistent with the work of Schenkman and Sato [4] where an increase in hydroxyl ion caused a type I change. These present results show a reverse type I change with the uptake of a proton from the medium, mediated by the interaction of ethylbenzene with the protoferriheme moiety. In other words, the reverse type I spectral change is associated with the hydrocarbon-protoferriheme complex being a stronger base than the free protoferriheme.

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