MOLECULAR PHARMACOLOGY, 11, 818-823

A Re-evaluation of the Optical Titrations of the 430 and 455 nm Chromophores of Ethyl Isocyanide Complexes of Mammalian Hepatic Cytochrome P-450

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(Received February 11, 1975)

SUMMARY

PEISACH, J. & MANNERING, G. J. (1975) A re-evaluation of the optical titrations of the 430 and 455 nm chromophores of ethyl isocyanide complexes of mammalian hepatic cytochrome P-450. *Mol. Pharmacol.*, 11, 818–823.

A computational method is presented from which one may calculate the pK for the pH-induced spectral changes of the 430 and 455 nm chromophores of ethyl isocyanide complexes of rat liver microsomal cytochrome P-450. For the rat liver heme protein from control and phenobarbital- or 3-methylcholanthrene-treated animals, the pK for the loss of the 430 nm absorption is equal to the pK for the gain of the 455 nm absorption, establishing that the two chromophores are in pH equilibrium with one another in all three preparations. This equilibrium is maintained even when the change in pH occurs after the addition of ethyl isocyanide. It is concluded that the appearance of 430 nm absorption after the addition of ethyl isocyanide to reduced cytochrome P-450 does not necessarily represent conversion of this cytochrome to cytochrome P-420.

INTRODUCTION

Ever since the initial observations of Imai and Sato on reduced liver microsomal cytochrome P-450 (1), the unusual optical difference spectrum in the Soret region formed after the addition of ethyl isocyanide has been used to differentiate this type of heme protein from others. A typical Soret maximum is observed at 430 nm, and a second peak, extraordinary for EtNC[2] complexes of hemoproteins, is seen at 455 nm. When the pH of the reduced microsomal suspension is raised, EtNC addition leads to a smaller 430 nm absorption while the 455 nm peak is increased in intensity. Imai and Sato suggested that the interconversion of the 430 and 455 nm chromophores is pH-dependent and were able to demonstrate this phenomenon with microsomes from PB-induced rabbits. If the cytochrome P-450 is denatured to form cytochrome P-420, only the 430 nm absorption difference is seen after the addition of EtNC, regardless of the pH of the preparation (2).

This investigation was supported in part by United States Public Health Service Grants HL-13399 (J. P.), National Cancer Institute Contract NO1-CP-55606 (J. P.) and GM-15477 (G. J. M.) and is Communication 323 from the Joan and Lester Avnet Institute of Molecular Biology.

[1] Recipient of Public Health Service Research Career Development Award 2-K3-GM-31,156.

[2] The abbreviations used are: EtNC, ethyl isocyanide; PB, phenobarbital; 3-MC, 3-methylcholanthrene.

In subsequent studies, using rat liver microsomal cytochrome P-450, Sladek and Mannering (3) observed that the crossover of a plot of optical density vs. pH for both the 430 and 455 nm absorptions obtained with microsomes from 3-MC-treated animals was not the same as that observed with preparations from either control or PB-treated animals. These authors, however, suggested similar pH-dependent interconversions of the 430 and 455 nm chromophores in all preparations, even though the crossovers of the respective titration curves were different.

In a recent study (4) a new analysis of published optical data for the effect of pH on the EtNC complexes of cytochrome P-450 in liver microsomes (1, 3) and in partially purified preparations (5, 6) was presented. Computer-aided fitting of these data to a titration equation used to relate optical density, pH, and pK demonstrated that the 430 and 455 nm absorptions of EtNC-treated cytochrome P-450 were indeed in pH equilibrium in the case of the microsomal cytochrome from control or PB-treated animals and in some of the partially purified preparations. Here the pK for the loss of the 430 nm absorption with increasing pH was equal to the pK of the gain of the 455 nm absorption. On the other hand, in preparations from 3-MC-treated animals the respective pK values for the 430 and 455 nm absorption changes as a function of pH were so different from each other as to suggest that the pH-dependent equilibrium did not exist. In that study the lack of pH dependence was attributed either to the formation of an intermediate state or to the paucity and possible inaccuracy of the data.

In our present study we have carefully re-examined the effect of pH on the Soret maxima of the EtNC complexes of cytochrome P-450 from control and PB- and 3-MC-treated rats. In all instances the 430 and 455 nm chromophores were demonstrated to be in pH equilibrium. A careful study was also made of the crossover points for the titration curves, and these differ significantly from those previously published (3). Finally, we show that the pH-dependent equilibrium of the 430 and 455 nm chromophores is maintained even when the pH is altered after the addition of EtNC to reduced microsomes.

MATERIALS AND METHODS

Cytochrome P-450 concentrations were determined by the method of Omura and Sato (2).

Male Holtzman rats weighing 90–110 g were divided into three groups. For a 4-day period members of the first group were injected daily with a 1.6% solution of sodium PB (40 mg/kg) in NaCl; members of the second group, with a 0.8% solution of 3-MC (20 mg/kg) in corn oil; and the third, control group, with corn oil. About 20 hr after the last injection, three to four animals from a single group were killed by decapitation, and washed liver microsomes were prepared according to the method of Sladek and Mannering (7). Microsomes were diluted to a final concentration of 1 mg of protein per milliliter with 0.2 M phosphate buffer. $Na_2S_2O_4$ in excess of that needed to reduce the cytochromes completely was added, and the sample was divided into two cuvettes. EtNC (4.24 nM) was added to one cuvette, and the optical spectral difference in the Soret region was recorded on an Aminco-Chance DW-2 spectrophotometer. Individual aliquots of the same microsomal suspension were diluted in phosphate buffer in order to study the effect of pH in the range of 6.0–8.0 so that nine equally spaced values of pH were used for the titration.[3] Each titration, using microsomes from a particularly treated animal, was repeated three times, each time using a new preparation of microsomes pooled from three to four livers. Optical densities of the difference absorption maxima at both 430 and 455 nm for each titration were iterated and fitted by least-squares analysis to the algorithm

$$Y = A + \frac{B}{1 + 10^{(X-C)}}$$

where Y is the optical density at any pH, A is the optical density at the end of the titration, B is the computed optical density difference at the pH extrema of the titra-

[3] In the amounts used in these experiments, EtNC and $Na_2S_2O_4$ did not alter the pH of any of the strongly buffered microsomal suspensions.

tion (low pH value minus high pH value), X is the pH at which the spectrum is observed, and C is the computed pK. Data were plotted on a Hewlett-Packard model 7004B x-y recorder interfaced to a Honeywell model 6000 computer with a Time Share Peripheral plotter controller. The crossovers of the titration curves were read directly from the computer-generated plots.

In another type of study, the spectral difference between reduced liver microsomes (1 mg of protein per milliliter) treated with EtNC and untreated reduced liver microsomes was determined in 1 ml of 25 mM phosphate buffer, pH 6.5. Concentrated phosphate buffer was added to each cuvette in order to raise the pH to about 7.5, and the spectral difference was once again determined. In both cases the spectral ratios of the 430 and 455 nm absorptions were calculated. In a converse experiment the difference spectrum of the EtNC complex was determined in 25 mM phosphate buffer, pH 7.5.[4] Concentrated buffer was added in order to lower the pH to about 6.5, and the spectrum was once again recorded. Each experiment was performed in triplicate, using hepatic microsomal preparations from control and PB- and 3-MC-treated animals.

RESULTS

Least-squares fits of titration data to the algorithm for control and PB- and 3-MC-induced rat liver microsomal cytochrome P-450 are shown in Fig. 1. As can be seen from the analysis presented in Table 1, the pK for the loss of the 430 nm absorption is equal to the pK for the increase of the 455 nm absorption in each of the three preparations, thus confirming the original conclusions of Imai and Sato (1) and Sladek and Mannering (3). The computed ratios of extinctions for both high- and low-pH forms of each chromophore, ΔA_{430} and ΔA_{455}, as well as the pH crossing, are also given in Table 1. It should be noted that the pH crossover as well as the pK values for the preparations from control and PB-treated animals are the same but differ significantly from those for the preparation from 3-MC-treated animals.

In the pH jump experiments, the spectral ratio of the 430 to 455 nm absorptions is constant for a particular preparation at any pH; differences in the ratio depend only upon the treatment of animals (Table 2). Thus the pH-dependent equilibrium demonstrated for the 430 and 455 nm chromophores is maintained even after the cytochrome P-450 is ligated to EtNC.

DISCUSSION

The presence of both 430 and 455 nm chromophores is ubiquitous to EtNC complexes of all preparations of cytochrome P-450, whether bacterial (8, 9) or mammalian (1, 2) in origin. The 430 nm chromophore cannot necessarily be ascribed to the presence of cytochrome P-420 since it is observed even when cytochrome P-420 is shown to be absent.

From stopped-flow experiments, Imai and Mason (10) demonstrated that the addition of EtNC to reduced cytochrome P-450 from a mammalian source leads first to the appearance of the 455 nm absorption, followed by the one at 430 nm. It can be argued that EtNC is a chaotropic reagent which is sufficiently disruptive to protein structure, much like salts (11), urea,[5] and other organic reagents (11, 12), as to convert it to cytochrome P-420 which exhibits the characteristic 430 nm absorption (2). An argument against this hypothesis is that for all microsomal preparations, whether from control, PB-treated, or 3-MC-treated rats, the pH dependence of the relative intensities of the 430 nm and 455 nm chromophores was the same whether changes in pH were made before or after the addition of EtNC (Fig. 1 and Table 1). Thus the pH-dependent equilibrium responsible for generating the two chromophores is not perturbed by the presence of EtNC, and the appearance of the 430 nm absorption after the addition of EtNC is not necessarily indicative of protein denaturation.

[4] The addition of microsomes altered the pH, but no further change was observed after the addition of $Na_2S_2O_4$ or EtNC. Recorded pH values were those observed after the addition of reagents.

[5] J. O. Stern and J. Peisach, unpublished observations.

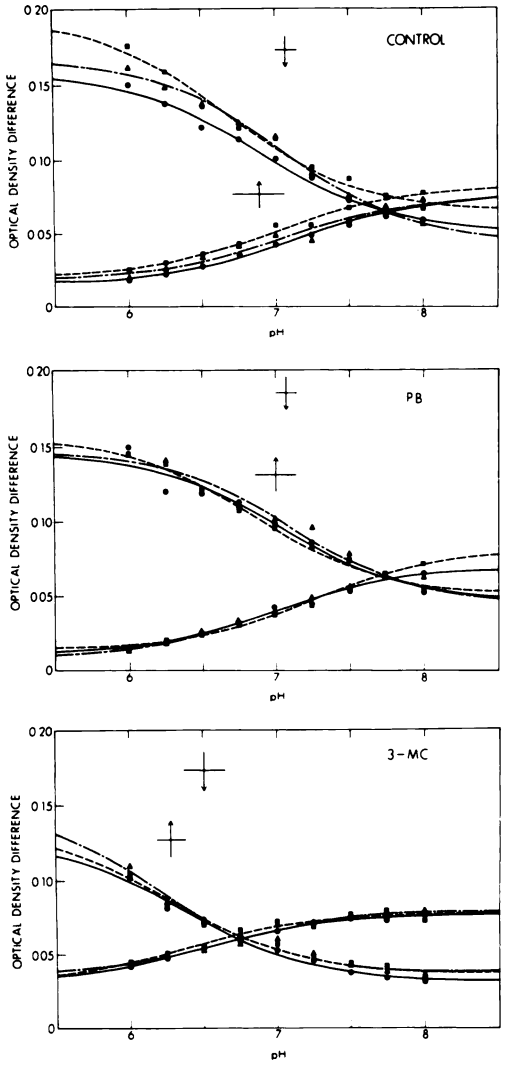

FIG. 1. *Computer-generated titration curves for effect of pH on 430 and 455 nm chromophores of ethyl isocyanide complexes of cytochrome P-450 in microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats.*

The optical data, expressed as optical density difference (compared to reduced cytochrome P-450) per nanomole of cytochrome per centimeter of light path, were fitted to the algorithm as described in the text. Individual symbols (●, ▲, ■) represent the data obtained for a titration using a single preparation of microsomes pooled from three or four animals in a single treatment group. Thus, for each type of preparation, three titration curves are shown. The upward arrow is at the pK for the decrease of the 430 nm absorption, whereas the downward arrow is at the pK for the increase of the 455 nm absorption, both as a function of pH. The horizontal lines crossing the arrows represent the standard deviation of each computed pK value. The computed difference in optical density of a particular chromophore at high and low pH, ΔA, as well as the pK value and pH titration curve crossover, is given in Table 1. The cytochrome P-450 concentrations in microsomes from control and phenobarbital- and 3-methylcholanthrene-treated rats were 0.99 ± 0.11, 2.90 ± 0.19, and 2.31 ± 0.05 nmoles/mg of protein, respectively.

To what structures, then, do we ascribe both the 430 and 455 nm chromophores? Much evidence has been presented to indicate that cytochrome P-450 preparations are heterogeneous (3, 13–16). The heterogeneity may extend down to the local environment of the heme (17). For the ferric protein, the presence of an alkyl thiol, such as from cysteine, has been implicated from EPR studies (18, 19). Peisach *et al.* (20) have suggested that the formation of the 430 or 455 nm chromophores after the addition of EtNC to the ferrous protein is dependent upon the relative bond strengths of the two axial ligands of the heme. That is, displacement of the sulfur-containing ligand would lead to the formation of a 430 nm chromophore whereas displacement of the *trans* axial ligand would lead to the formation of the 455 nm chromophore. The differences in reactivity elicited by EtNC which are dependent upon the type of animal treatment may reflect differences in the chemical structure of the heme ligand *trans* to the thiol (20). On the other hand, if the chemical structure of this as yet unknown ligand is the same in all preparations of hepatic cytochrome P-450, then the pH-dependent optical phenomena discussed here for EtNC ligation are either governed by differences in localized accessibility of the heme site to EtNC or more likely by internal ligand exchange reactions which themselves are pH-dependent as are found in cytochrome *c* (21).

What, then, of previous studies of liver microsomal cytochrome P-450 from 3-MC-treated rats, in which the pK values for the disappearance of the 430 nm chromophore and the appearance of the 455 nm chromophore are different from one another (4)? The most likely explanation is that the calculated difference is within the

TABLE 1

Analysis of computed titration curves for ethyl isocyanide complexes of preparations of microsomal cytochrome P-450 from control and phenobarbital- and 3-methylcholanthrene-treated rats

The average values for the three titrations of any particular group and their respective standard deviations of computer-determined pK values are given for the 430 nm (pK_{430}) and 455 nm (pK_{455}) chromophores. σ denotes the standard deviation of mean differences between any pair of pK values determined at 430 and 455 nm and is an indication of the reliability of the congruence of the pK values. The pH crossover determined visually from the titration curves given in Fig. 1 refers to the pH, and its standard deviation, at which the computer-generated curves for a single preparation expressing the loss of 430 nm absorption and the gain of 455 nm absorption, as a function of increasing pH, cross. The terms ΔA_{430} and ΔA_{455} are the average computed differences and their standard deviations, expressed in units of optical density per nanomole of cytochrome P-450 per centimeter of light path, of the individual chromophores at pH extrema in the titration curves, while the term ΔA_{430}:ΔA_{455} is the average ratio and standard deviation of these optical density differences.

Preparation	pK_{430}	pK_{455}	σ	pH crossover	ΔA_{430}	ΔA_{455}	ΔA_{430}:ΔA_{455}
Control	6.89 ± 0.18	7.07 ± 0.07	0.15	7.75 ± 0.05	0.120 ± 0.011	0.061 ± 0.002	1.98 ± 0.20
PB	7.00 ± 0.14	7.07 ± 0.17	0.20	7.76 ± 0.05	0.104 ± 0.003	0.063 ± 0.005	1.69 ± 0
3-MC	6.28 ± 0.10	6.51 ± 0.14	0.19	6.74 ± 0.02	0.103 ± 0.009	0.046 ± 0.002	2.24 ± 0.27

TABLE 2

Effect of pH on spectral ratio of 430 and 455 nm absorptions of ethyl isocyanide complexes of cytochrome P-450 from control and phenobarbital- and 3-methylcholanthrene-treated rats

For any given microsomal preparation, the optical difference spectrum of the ethyl isocyanide complex of reduced cytochrome at pH 7.5 was determined and the spectral ratio A_{430}:A_{435} was calculated. Concentrated buffer (1 M) was added to lower the pH to 6.5, and the difference spectrum and spectral ratio were again determined (Decreasing pH). In another set of experiments the initial pH of the preparations of 6.5 was raised to 7.5 (Increasing pH). For each initial pH and for every microsomal preparation, experiments were performed in triplicate. Within the experimental error, the spectral ratio determined at any pH for a particular preparation remains constant.

Preparation	Decreasing pH		Increasing pH	
	pH	A_{430}:A_{455}	pH	A_{430}:A_{455}
Control	7.42	1.05 ± 0.05	7.58	0.98 ± 0.15
	6.49	5.51 ± 0.16	6.44	5.54 ± 0.58
PB	7.47	1.06 ± 0.08	7.51	1.12 ± 0.10
	6.51	4.87 ± 0.26	6.52	5.44 ± 0.56
3-MC	7.46	0.56 ± 0.10	7.54	0.49 ± 0.05
	6.52	1.31 ± 0.22	6.53	1.46 ± 0.16

experimental error of the study. For the analysis based on the work of Sladek and Mannering (3), only five data points were published for each preparation. Our present analysis, based on many more data points and on experiments performed in triplicate, would suggest that even with cytochrome P-450 from 3-MC-treated rats (Table 1) the 430 and 455 nm chromophores are in pH-dependent equilibrium with one another. A similar treatment of published data of Imai and Siekevitz (12) on EtNC complexes of rat liver microsomal cytochrome P-450 from control and 3-MC-treated animals leads to the same conclusion (Table 3), even though only seven pH values were used in that study. It is interesting, however, that the relative extinction coefficients (expressed as a function of change in optical density in Table 1) are

TABLE 3

Analysis of computed titration curves for ethyl isocyanide complexes of rat liver microsomal cytochrome P-450 from control and 3-methylcholanthrene-treated rats, based on published data of Imai and Siekevitz (12)

The published titration data were photographically enlarged and printed on grid paper. The coordinates for seven values of pH, determined visually, were then fitted to the algorithm as described in the text. The pK for the loss of the 430 nm absorption (pK_{430}) and that for the increase of the 455 nm absorption (pK_{455}) as a function of increasing pH, as well as the pH at which the generated titration curves cross, are given below.

Preparation	pK_{430}	pK_{455}	pH crossover
Control	6.94	6.97	7.89
3-MC	6.42	6.32	6.82

quite different for the various preparations. Furthermore, the titration curve crossovers determined in this study are significantly different from those determined previously (3). These new values are also close to those determined recently (6) for partially purified soluble preparations of cytochromes P-450 obtained from PB-treated (PB III) or 3-MC-treated (3-MC III) rats. It is our contention that these differences in the physical properties of the 430 and 455 nm chromophores are based on structural differences in the vicinity of the prosthetic group of the cytochromes P-450 in the various preparations, and will ultimately be related to enzymatic properties.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Miss Viola Abbott.

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