# EVIDENCE FOR TWO FORMS OF P-450 HEMOPROTEIN IN MICROSOMAL MEMBRANES

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The addition of ethyl isocyanide to dithionite-treated liver microsomes causes an unusual spectral change possessing two bands in the Soret region (Omura and Sato, 1962; 1964a). Several lines of evidence have been presented that a single hemoprotein called P-450 is responsible for this difference spectrum (Omura and Sato, 1964a; Nishibayashi et al., 1966). Such an anomalous spectrum has, however, not yet been reported for any hemoproteins and the reason for this anomaly has remained unelucidated. This paper describes evidence that the two-banded Soret spectrum arises from the possible presence in microsomes of two forms of P-450, which appear to be in a pH-dependent equilibrium and differ from each other not only spectrally but also functionally.

Rabbit liver microsomes were prepared free from adsorbed hemoglobin as described by Omura and Sato (1964a). Partially purified P-420 described in a previous paper (Imai and Sato, 1966) was used in the present study. Difference spectra were recorded at room temperature with a Cary 14 spectrophotometer, using cuvettes of 1 cm light path. Ethyl isocyanide was synthesized according to Jackson and McKusick (1955).

As reported previously (Omura and Sato, 1962; 1964a), the ethyl isocyanide difference spectrum of dithionite-treated microsomes, when measured at pH 7, showed two Soret peaks, i.e. the main peak at 430 mμ ("430 peak") and the anomalous small peak at 455 mμ ("455 peak"). While studying the nature of these peaks, it was found that pH exerts a profound influence on their relative heights.

As shown in Fig. 1 and Fig. 2 (Curves A and B), only the 430 peak was seen in the spectrum obtained at pH 6. The 455 peak became observable at more alkaline reactions; it was intensified progressively as pH was increased. This intensification of 455 peak was accompanied by a corresponding decrease in the height of 430 peak, suggesting an interrelationship between the two peaks. As a result of such an inverse pH effect, the 455 peak became even higher than the 430 peak at pH 8. Although relative peak intensities were affected by pH, the heights of both peaks were similarly dependent on the ethyl isocyanide concentration regardless of the pH employed (pH 6 to 8). An apparent dissociation constant of about 6 μM was obtained from the effects of ethyl isocyanide concentration on the intensities of both peaks in the entire range of pH examined. This value is somewhat different from that (1 μM) previously determined at pH 7 (Omura and Sato, 1964a), but falls in the same order of magnitude.

Reduced P-450 in dithionite-treated microsomes, when bound to CO, produces a difference spectrum having only one peak at 450 mμ (Omura and Sato, 1962; 1964a). In contrast to the peaks induced by ethyl isocyanide, this CO peak was not appreciably affected in shape and intensity by the change in pH from 6 to 8 (Fig. 2, Curve C). However, clear spectral competition could be demonstrated between these two ligands, and the two peaks in the ethyl isocyanide spectrum were affected to the same extent by CO at any pH and ethyl isocyanide concentration. This provided further evidence that the same CO-binding hemoprotein, P-450, is involved in the appearance of the two Soret peaks in the ethyl isocyanide spectrum.

Although the observations described above may seem rather complicated, they could be explained satisfactorily by the following assumptions. (a) Microsomal P-450, at least in the reduced state, exists in two interconvertible forms which are in a pH-dependent equilibrium. (b) These two forms of the reduced pigment, having the same affinity for ethyl isocyanide, give rise to two Soret

peaks at 430 and 455 mμ. They will be called "430 form" and "455 form", respectively. (c) Either the same peak at 450 mμ is produced on combination of CO with both forms or CO combines only with one form possessing a far greater affinity for CO than the other. In the latter case, a shift of equilibrium should occur in favor of the formation of CO compound. These assumptions seem to fit very well the observed phenomena, but it is not yet possible to exclude the possibility that the postulated two forms are an artifact caused by the combination of ethyl isocyanide with reduced P-450.

In a previous paper it was demonstrated that oxidized P-450 in aerobic microsomes can also interact with ethyl isocyanide to give a difference spectrum having a Soret peak at 434 mμ (Nishibayashi et al., 1966). As shown in Fig. 2 (Curve D), pH showed no effect on this peak. Two alternative explanations may be offered for this observation. (a) Oxidized P-450, in contrast to the reduced pigment, occurs in microsomes in a single form. (b) As in the case of reduced P-450, the oxidized hemoprotein do exist as an equilibrium mixture of two forms. In the latter case the pH-independent nature of spectrum may be interpreted in the same way as mentioned above for the CO binding by the two forms of reduced pigment.

It has been established that P-450 is involved as the oxygen activating enzyme in NADPH-dependent hydroxylations of aniline and other drugs by liver microsomes (Sato et al., 1965; Cooper et al., 1965; Omura et al., 1965). Recent studies have further demonstrated that these drugs interact with oxidized P-450 producing characteristic spectral changes (Imai and Sato, 1966; Remmer et al., 1966). In view of these facts it is clear that the observations described above have important bearings on the hydroxylation mechanisms. This is especially so because the 455 form of P-450 is present in a quantity comparable to the 430 form in microsomes placed at pH 8, where aniline hydroxylation proceeds maximally. Furthermore, the pH-activity curve for aniline hydroxylation was found to resemble

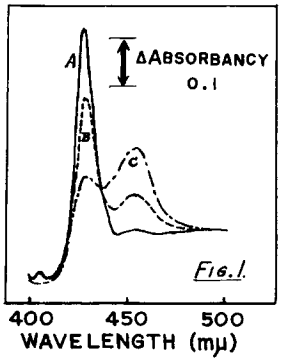

Fig. 1.

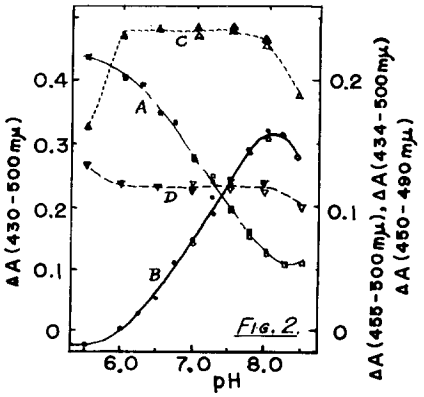

Fig. 2.

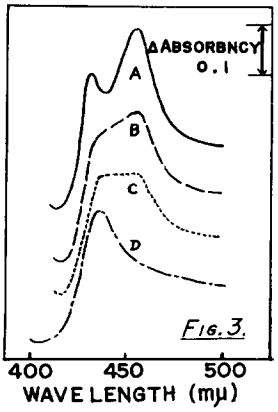

Fig. 3.

Fig. 1. Ethyl isocyanide difference spectra of dithionite-treated liver microsomes measured at different pH's. Microsomes (1.8 mg of protein or 2.7 mμmoles of P-450 per ml) suspended in 0.25 M K-phosphate buffer were used. The final concentration of ethyl isocyanide was 3.45 mM. Curve A, pH 6. Curve B, pH 7. Curve C, pH 8.

Fig. 2. Dependence on pH of peaks in microsomal difference spectra. Liver microsomes (1.8 mg of protein or 2.7 mμmoles of P-450 per ml) suspended in either 0.25 M K-phosphate buffer (■,●,▲,▼) or 0.25 M Tris acetate buffer (□,○,△,▽) were used. Ethyl isocyanide difference spectra of both dithionite-treated and aerobic microsomes were measured with 3.45 mM (final concentration) of ethyl isocyanide. CO was saturated to the sample to obtained the CO difference spectrum. Peak intensity was expressed by the absorbancy increment between the peak position and an appropreate reference wavelength, e.g. Δ A(450 - 490 mμ) for the 450 mμ peak in the CO spectrum. Curve A, 430 mμ peak in ethyl isocyanide spectrum of dithionite-reduced microsomes. Curve B, 455 mμ peak in the same spectrum as in A. Curve C, 450 mμ peak in CO spectrum of reduced microsomes. Curve D, 434 mμ peak in ethyl isocyanide spectrum of aerobic microsomes.

Fig. 3. Ethyl isocyanide difference spectra of liver microsomes in presence of NADPH. Microsomes (2.1 mg of protein or 3.8 mμmoles of P-450 per ml) were suspended in 0.2 M Tris acetate buffer (pH 8.0) containing 2.5 mM $MgCl_2$, 10 mM nicotinamide, 5 mM glucose-6-phosphate, an excess of glucose-6-phosphate dehydrogenase, and various amounts of NADP. The difference spectrum induced in this suspension by 3.45 mM of ethyl isocyanide was recorded either in the presence or in the absence of oxygen. Curve A, anaerobic spectrum, NADP 0.5 mM. Curve B, aerobic spectrum, NADP 0.5 mM. Curve C, aerobic spectrum, NADP 0.05 mM. The ethyl isocyanide(3.45 mM) difference spectrum of aerobic microsomes is shown in Curve D for reference.

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the curve showing the dependence on pH of the concentration of 455 form (Fig. 2, Curve B) in the pH range from 6 to 8.

To elucidate the relations between hydroxylation reactions and the two forms of P-450, the effects of NADPH (added in the form of an NADPH-generating system) on the ethyl isocyanide spectrum were studied. To facilitate comparison, the experiments were performed at pH 8.0 in the same reaction mixture as used for measuring aniline hydroxylation (Imai and Sato, 1959), except that aniline was omitted and the NADP concentration was varied. Curve A of Fig. 3 shows that the addition of ethyl isocyanide to microsomes which had been treated with NADPH under anaerobic conditions produced a difference spectrum with two Soret peaks; this spectrum was identical with that obtained at the same pH in the presence of dithionite (Fig. 1, Curve C). This confirmed the earlier conclusion (Omura and Sato, 1964a) that microsomal P-450 is fully reducible by NADPH in the absence of oxyger. On the other hand, difference spectra as illustrated in Curves B and C of Fig. 3 were obtained when ethyl isocyanide was added to microsomes in the preserce of both oxygen and NADPH. As will be seen, the absorption around 455 mμ became more pronounced on increasing the NADPH concentration. An analysis of these spectra suggested strongly that they are composed of two bands of different origir, i.e. the peak at 434 mμ (Fig. 3, Curve D) arising mainly from the ethyl isocyanide interaction with oxidized P-450 (Nishibayashi et al., 1966) and the 455 peak caused by the reagent combination with the 455 form of reduced P-450. It may therefore be concluded that in the steady state established under these conditions P-450 is partially reduced; the reduced pigment exists mostly in the 455 form, though the 455 peak is not yet fully developed as compared with the case of dithionite treatment. The 430 form of reduced P-450, if produced to any extents, may be reoxidized almost instantaneously probably due to its high affinity for oxygen. It was thus suggested that the two forms of P-450 differ from each other not only in their ethyl isocyanide spectra but also in their oxidation-reduction behaviors. Although it is still premature to draw any conclusions, these findings appear to be of importance in elucidating the microsomal

hydroxylation mechanisms. The peaks observed in the above aerobic experiments also changed its intensity depending on the ethyl isocyanide concentration, and an apparent dissociation constant of about 0.3 mM was obtained for the peak at 455 mμ. This value is much higher than that (about 6 μM) determined for fully reduced P-450 in dithionite-treated microsomes. Such a low affinity under aerobic conditions may be due to the oxygen competition with ethyl isocyanide and autoxidation of reduced pigment.

Omura and Sato (1964a,b) have reported that solubilization and other treatments of microsomal bound P-450 result in its conversion to another form called P-420 which behaves spectrally as a normal hemoprotein; reduced P-420 gives only one Soret peak on combination with ethyl isocyanide. Using a partially purified preparation of P-420, it was further found that pH has no effect on this Soret peak. These and other findings apparently indicate that the spectral anomaly of P-450 disappears when it is converted to P-420, though a certain degree of anomaly was detected in the effect of ethyl isocyanide concentration on spectra of P-420. This will be dealt with in a later publication.

From the data reported in this paper it appears that P-450, at least in the reduced state, exists in microsomes in two forms. As pointed above, this phenomenon may be an artifact induced by the combination of the hemoprotein with ethyl isocyanide. However, since this anomaly is observable only when the pigment is in the P-450 state and disappears on its conversion to the P-420 state, this seems to provide an important clue to the understanding of the special state of the P-450 or metabolically reactive state of the hemoprotein. In view of the apparently selective formation of one of the two forms of reduced P-450 in the presence of oxygen and NADPH, it is very likely that the two forms play different roles in the hydroxylation mechanisms.

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