Ligand Interactions with Hemoprotein P-450. Equilibria between High- and Low-Spin Forms of P-450 in Bovine Adrenal Mitochondria*

C. R. E. Jefcoate† and J. L. Gaylor‡

ABSTRACT: The spin states of adrenal mitochondrial cytochrome P-450 were investigated using difference spectroscopy and octylamine as a ligand for the hemoprotein. Spectral evidence for two forms of cytochrome P-450 is presented, which confirms a previous report of adrenal mitochondrial P-450.

The spectra are correlated closely with earlier investigations of P-450 in liver microsomes. The binding of octylamine to adrenal mitochondrial P-450 was weaker than the similar binding to liver microsomal P-450, although with P-450 from both sources, the binding to high-spin P-450 was weaker than the binding to the low-spin form. Evidence is presented that 11-deoxycorticosterone and 11-dehydrocorticosterone occupy distinct binding sites. Spectral data suggest that these steroids may alter the spin state of the respective forms of P-450 to which they are bound. The extent of spectral changes upon steroid binding was compared with spectral changes resulting from addition of octylamine in each case; quantitative relationships between amounts of high- and low-spin forms were established which provided consistent results. Ethyl isocyanide was found to bind only to low-spin oxidized P-450 while, contrary to previous observations on P-450 of liver microsomes, the ethyl isocyanide complexes of reduced P-450 yielded an absorbance ratio (455:428 mμ) which gave no indication of the high-spin character of the oxidized state. The quantitation of the two forms of P-450 along lines described in this paper provides a basis for evaluating the relative contributions of high- and low-spin forms to P-450-dependent hydroxylation reactions in adrenal mitochondria.

Cytochrome P-450 has been implicated in a variety of mixed-function oxidations associated with the mitochondria of the adrenal cortex. In particular, both the side-chain cleavage of cholesterol (Boyd and Simpson, 1968) and 11β-hydroxylation of 11-deoxycorticosterone (Wilson *et al.*, 1965) have been shown to involve P-450 as terminal oxidase.

A steroid substrate-induced difference spectrum (λ_{max} 385 mμ, λ_{min} 420 mμ) has been observed for P-450[1] of adrenal mitochondria upon addition of either 11-deoxycorticosterone (11-DOC) (Cooper *et al.*, 1965) or 11-deoxycortisol (Oldham *et al.*, 1968). Whysner and Harding (1968) have shown that these spectral characteristics are retained by mitochondrial subparticles prepared by sonication, and these workers have also noted two additional spectral effects; 20α-hydroxycholesterol produced a difference spectrum (λ_{max} 420, 535, 570; λ_{min} 385 mμ) that was the reverse of that produced by 11-DOC. Furthermore, the addition of high concentrations of 11-DOC produced a progressive diminution of the difference spectrum exhibiting a *minimum* at 420 mμ. Both of these effects were attributed to the existence of two forms of P-450 in the adrenal mitochondria, which showed opposing spectral changes upon addition of the steroid substrate.

Williams (1968) was the first to suggest that two distinct spin states of P-450 might exist. Subsequently, a new Soret peak at 395 mμ has been assigned to a second type of P-450 (Hildebrandt *et al.*, 1968), which has been correlated with an electron paramagnetic resonance spectrum of high-spin iron (Jefcoate *et al.*, 1969). A direct relationship has been derived between octylamine difference spectra of oxidized P-450 and the relative amounts of the two forms of P-450, both of which were generally present in liver microsomes (Jefcoate *et al.*, 1969).

In support of earlier observations (Mitani and Horie, 1969a,b; Whysner *et al.*, 1969), octylamine binding studies further indicate that the two forms of adrenal mitochondrial P-450 are high- and low-spin forms of P-450 (Jefcoate *et al.*, 1970), which may change their spin state upon binding specific steroids. This manuscript describes further spectral evidence for high- and low-spin states of P-450 in adrenal mitochondria and detailed experimental evidence on the interconversion of these spin states by the use of both heme-binding ligands (octylamine and cyanide) and steroids (protein- or lipid-binding ligands). Since different binding sites are likely for these different classes of ligands, we have investigated their simultaneous action upon P-450. We describe the use of both the difference spectra from steroid binding and from octylamine binding for the quantitation of the two forms of P-450 in adrenal mitochondria.

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[1] Abbreviations used are as follows: P-450, cytochrome P-450; 11-DOC, 11-deoxycorticosterone; 11-DHC, 11-dehydrocorticosterone.

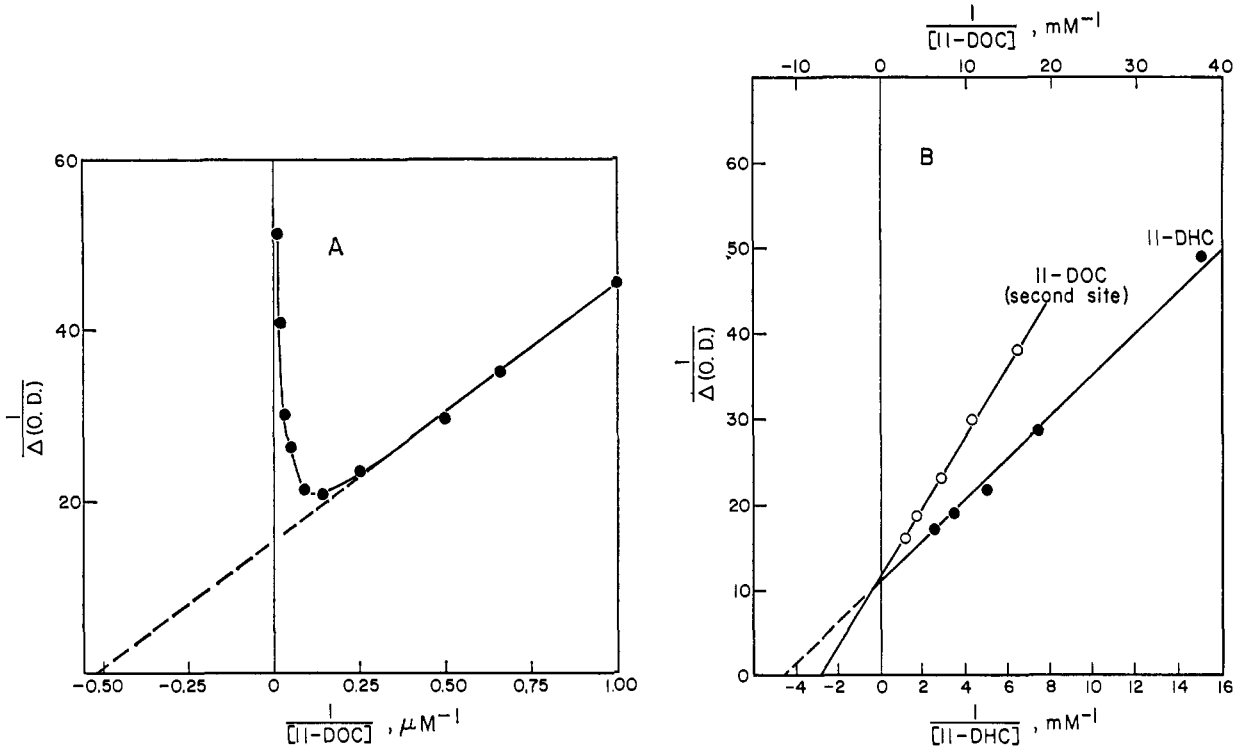

FIGURE 1: (A) Reciprocal plot of binding of 11-DOC. Subparticles of adrenal mitochondria (3 mg/ml) were suspended in 0.1 M sodium phosphate buffer. The steroid, in ethanol, was added to the sample cuvet and an equal quantity of ethanol was added to the reference cuvet. The difference in optical density at 420 and 385 mμ was measured. The extrapolated line provides the binding constant for the strong binding site. (B) Reciprocal plot of binding of 11-DHC and the secondary binding of 11-DOC. Subparticles were suspended as described in A. The Δ(OD) for secondary binding of 11-DOC was calculated from the decrease in Δ(OD) below the value obtained from the extrapolated reciprocal plot in A.

Experimental Procedures

Bovine adrenal glands were obtained from a slaughterhouse. The cortex was obtained by dissection. The homogenization and collection of mitochondria by differential centrifugation was carried out as described by Harding *et al.* (1968). Submitochondrial particles were prepared by sonic treatment of the mitochondrial suspension; each suspension, in 0.2 M phosphate buffer, was treated with a Branson Sonifier, Model 75, four times for 20 sec each time (Harding *et al.*, 1968). The submitochondrial preparations were collected by high-speed centrifugation (105,000*g* for 1 hr). The pellets were suspended in 0.1 M potassium phosphate buffer (pH 7.4). Spectral measurements were made immediately.

P-450 and P-420 were determined spectrally using millimolar extinction coefficients reported by Omura and Sato (1964). Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

For difference spectroscopy, tissue preparations were suspended in phosphate buffer. Concentrated solutions of ligands were prepared with the same buffer. Solutions were adjusted to pH 7.4 before use. Equal volumes of buffer were added to the reference cuvets to compensate for small changes in volume.

All spectra were recorded in a Cary spectrophotometer equipped with a high-intensity light source and a high-sensitivity slidewire (Jefcoate and Gaylor, 1969). Concentrations of ligands used are indicated in the appropriate tables and figures.

Ethyl isocyanide was the generous gift of Dr. M. W. Anders of Cornell University. Octylamine was obtained from Aldrich Chemical Company; it was used without further purification.

Results

Steroid Binding. 11-DOC produced a difference minimum at 420 mμ and a maximum at 385 mμ (K = 1–2 μM as shown in Figure 1A); at much higher concentrations, 11-dehydrocorticosterone (11-DHC, K = 0.22 mM) produced an exactly inverted difference spectrum of the type reported by Whysner and Harding (1968) for 20α-hydroxycholesterol from which a binding constant (K = 0.22 mM) was calculated (Figure IB). We also confirmed their observation that addition of very high concentrations of 11-DOC leads to secondary binding and diminution of the difference spectrum produced by low concentrations of 11-DOC. Significantly, the binding constant for the weaker combination of 11-DOC (0.14 mM) is close to that of 11-DHC, and the spectral change produced at saturation was the same for both steroids (Figure 1B).

The two distinct forms of P-450, which have been found in liver microsomes, have Soret peaks in the oxidized state at 415 (low spin) and 395 mμ (high spin). Thus, the difference spectrum produced by low concentrations of 11-DOC corresponds to loss of a Soret peak at 415 mμ and gain of one at 395 mμ, which is consistent with a change in the spin state of a low-spin P-450 (Williams, 1968). Similarly, high concentrations of 11-DOC or 11-DHC appear to change the spin state of a high-spin P-450.

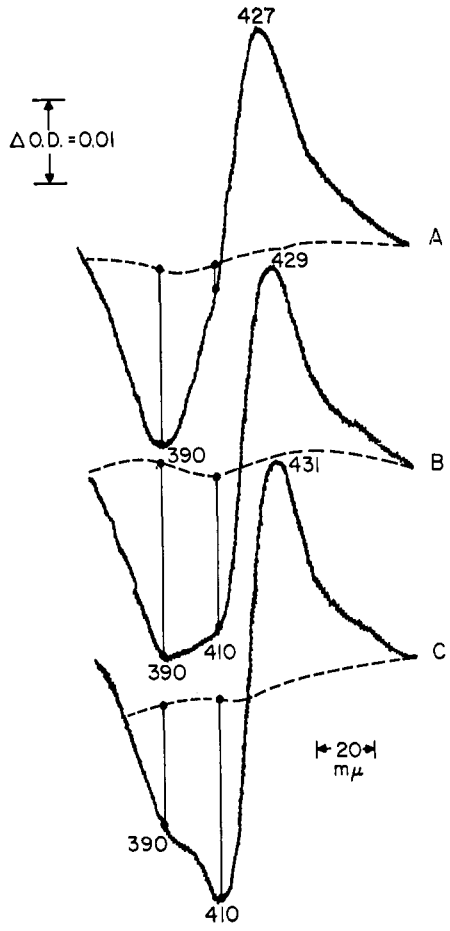

FIGURE 2: Octylamine difference spectra; effect of steroids. Subparticles of adrenal mitochondria (3 mg/ml) were suspended as for Figure 1A. Octylamine (1.95 mM) was added to the sample cuvet under the following conditions: (A) with 5 μM 11-DOC in both cuvets; (B) with no steroid added to subparticles; and (C) with 0.6 mM 11-DHC in both cuvets.

Binding of Octylamine. The binding of amines to P-450 in liver microsomes has been investigated extensively (Jefcoate *et al.*, 1969). Octylamine was bound selectively to two forms of liver microsomal P-450; type *a* or high-spin (λ_{max} 427, λ_{min} 392, K = 0.3 mM) and type *b* or low-spin (λ_{max} 432, λ_{min} 410, K = 0.007 and 0.05 mM).

When octylamine was added to either whole adrenal mitochondria or subparticles, a mixed difference spectrum of type *a* and type *b* components was observed (Figure 2B). As the concentration of octylamine was increased, changes in the difference spectra indicated that type *b* P-450 was bound first, following by binding type *a* P-450 (Figure 3). The contributions to type *a* and type *b* spectra were separated according to the method described for liver microsomes (Jefcoate *et al.*, 1969), *i.e.*, type *a* [$\Delta(OD)_{390} - 0.5\,\Delta(OD)_{410}$]; type *b* [$\Delta(OD)_{410}$], and the calculated data are represented in Figure 3. Both binding constants (type *a* = 2.0 to 2.5 mM; type *b* = 0.5 to 0.6 mM) were considerably weaker than those found for P-450 of liver microsomes, although for adrenal as well as liver, type *a* P-450 bound octylamine more weakly than did type *b* P-450.

Quantitation of Octylamine Spectra. There is an exact relationship between the relative Δ(OD) values at 390 and 410 mμ in an octylamine difference spectrum and the absolute amounts of type *a* and type *b* P-450 (Jefcoate *et al.*, 1970, and formula reproduced in the legend of Figure 5). This relationship requires determination of the three parameters, x, y, and z [$x = \Delta(OD)_{390}/\Delta(OD)_{410}$ (for a pure *b* spectrum); $y = \Delta(OD)_{410}/\Delta(OD)_{390}$ (for a pure type *a* spectrum); $z = \Delta\epsilon_{390}$(type *a*)$/\Delta\epsilon_{410}$(type *b*)].

We assume from the steroid-induced difference spectrum that saturation with 11-DHC resulted in a pure type *b*, oxidized P-450 spectrum, while saturation of 11-DOC in the strong binding site (primary) resulted in a type *a* spectrum of oxidized P-450. The effect of 11-DOC and 11-DHC upon the octylamine difference spectra is shown in Figure 2A and C, respectively. Clearly, 11-DOC has increased the proportion of the type *a* spectrum, while 11-DHC has increased the type *b* spectrum. The limit of solubility of 11-DHC and the opposing effect of secondary binding by 11-DOC, however, mean that about 90% of saturation is the limit to which either of these steroids can be added for this experiment; therefore, extrapolation to the saturated values was necessary. Thus, octylamine difference spectra were measured for suspensions in which the proportions of type *a* and type *b* P-450 were varied by increasing concentrations of either 11-DHC or 11-DOC.

In Figure 4A, the spectral ratio $\Delta(OD)_{410}/\Delta(OD)_{390}$ is plotted against the percentage of P-450 bound by the steroid relative to saturation (obtained by extrapolation). Since saturation of 11-DHC binding and 11-DOC strong binding would result in complete conversion into, respectively, type *b* and type *a* P-450, extrapolation gives values for the parameters x and y, respectively (x = 1/intercept = 0.5; $y = -0.05$; see definitions of x, y, and z above). The value for z (1.9) was obtained by a similar extrapolation procedure (Figure 4B) which provided $\Delta(OD)_{410}$ at saturation of 11-DHC (pure type *b*) and $\Delta(OD)_{390}$ at saturation of the strong site of 11-DOC (pure type *a*). The ratio of these spectral changes is identical with the ratio of the corresponding extinction coefficients, since the concentration of P-450 has remained constant; *i.e.*, the P-450 has been converted completely into either type *a* or type *b* P-450.

To check the accuracy of this calculation, the proportion of type *a* P-450 was related to the octylamine spectral ratio (Figure 5). Points represent experimental spectral ratios obtained on solutions containing various amounts of either 11-DHC or 11-DOC, while the line was calculated from the formula given in the legend of Figure 5. The proportions of type *a* and type *b* P-450 were calculated from the respective steroid equilibrium constants that were determined for that particular preparation of adrenal mitochondria subparticles. The fit of experimental points to the theoretical line confirms the suitability of the extrapolation procedure.

Since the two types of steroid difference spectra are exact inverses, the Δε (420–385 mμ) is probably the same in each case. Thus, we have assumed that type *a* P-450 is measured by the saturated difference spectrum provided by 11-DHC and type *b* P-450 is measured by the difference spectrum obtained by saturation of the primary binding site for 11-DOC.

In Table I, ratios of type *a* to type *b* P-450 in three different preparations (five adrenal glands each) were obtained by both steroid and octylamine titration methods. There was

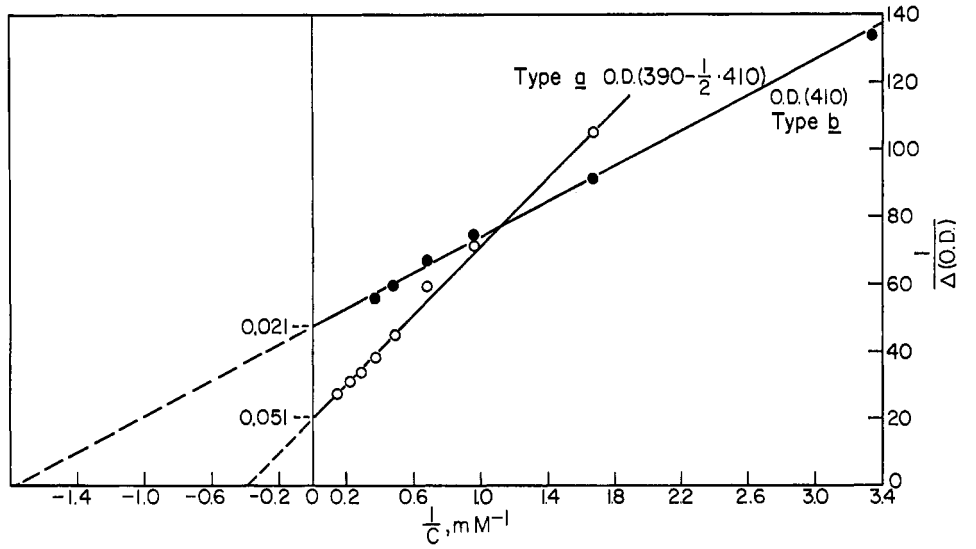

FIGURE 3: Reciprocal plot of binding of octylamine to P-450 in subparticles from adrenal mitochondria. Conditions were as described in Figure 1A. Δ(OD) at 390 and 410 mμ were measured relative to an isosbestic point at 500 mμ. P-450 content = 1.2 mμmoles/ml.

close agreement between the two methods of estimation over a range of about 0.6–1.4 (*a*/*b*). When mitochondria from four glands were prepared and examined individually, the octylamine ratio varied to a much smaller extent (0.75, 0.83, 0.86, 0.94). These glands had been stored at 4° for several days, and it has been our experience that the proportion of type *a* P-450 decreases with aging.

Cyanide Binding. Cyanide ions bind weakly to both forms of P-450 in liver microsomes, although the affinity for type *b* P-450 (K = 5 mM) is somewhat stronger than for type *a* P-450 (K >20 mM). The difference spectrum observed for type *b* binding (λ_{max} 446, λ_{min} 410) showed a shift of both maximum and minimum to shorter wavelength as the cyanide concentrations became sufficient to produce significant type *a* binding. Thus, in Figure 6, three different stages of cyanide binding to adrenal mitochondrial subparticles have been represented. At low concentrations (0.2 mM) a small difference spectrum was obtained (λ_{max} 445, 425; λ_{min} 406) (Figure 6A), which was saturated at this concentration. Hemoglobin or cytochrome oxidase are possible sources for this spectrum. At 4 mM cyanide, a sharp minimum at 410 mμ is obtained with a broad maximum at 442 mμ (Figure 6B), which is similar to the above values for type *b* of P-450 of liver microsomes. At 20 mM, the spectrum shows an appreciable shift of the spectrum to shorter wavelength (Figure 6C). Thus, for the cyanide difference spectrum, the ratio $\Delta(OD)_{390}/\Delta(OD)_{410}$ changed from 0.72 to 1.05 as the concentration of cyanide increased from 4 to 20 mM. This change in ratio is attributed to binding of cyanide to type *a* P-450 at higher concentrations of cyanide.

Ethyl Isocyanide Binding. When ethyl isocyanide was added to oxidized P-450 in adrenal mitochondria, a difference spectrum (λ_{max} 432, λ_{min} 412) was observed that was similar to that reported by Imai and Sato (1967a) for binding to P-450 in liver microsomes (Figure 7), although the spectrum was somewhat distorted by a yellow contaminant in the ethyl isocyanide solution. The binding constant could not be determined accurately due to the impure quality of the ethyl isocyanide; however, an approximate value of 0.4 mM was determined. Significantly, the difference minimum at saturation showed almost the same Δ(OD) relative to 500 mμ at saturation of type *b* P-450 with octylamine. The sharpness of the ethyl isocyanide difference spectrum, together with the similarity of difference maxima and minima with the type *b* octylamine spectrum strongly suggest that ethyl isocyanide binds only to type *b* of oxidized P-450.

The ethyl isocyanide difference spectra of reduced P-450 in liver microsomes have also provided evidence for two forms of P-450 (Imai and Sato, 1967b; Sladek and Mannering, 1966).

When the oxidized P-450 content of adrenal mitochondria was apparently 40% of type *a* (as indicated by the octylamine difference spectrum in Figure 7), the reduced P-450–ethyl isocyanide complex showed a ratio $\Delta(OD)_{428}/\Delta(OD)_{455}$ of approximately 4.7 which, for liver microsomal P-450, corresponds to pure type *b* (Jefcoate *et al.*, 1970). Furthermore, the reduced P-450–CO spectrum showed no significant quantity of P-420 or hemoglobin, and the Soret difference peak was exactly at 450 mμ. Similar spectra were obtained both before and after sonication of the mitochondria. Thus, the reduced state of P-450 from adrenal mitochondria shows

TABLE I: Estimation of Relative Amounts of Adrenal Mitochondrial Type *a* and Type *b* of P-450 by Two Different Methods.

Preparation	[Type *a*]/[Type *b*]	
	From Octylamine Binding	From Steroid Binding
I	0.6	0.6
II	1.7	1.4
III	1.1	1.0

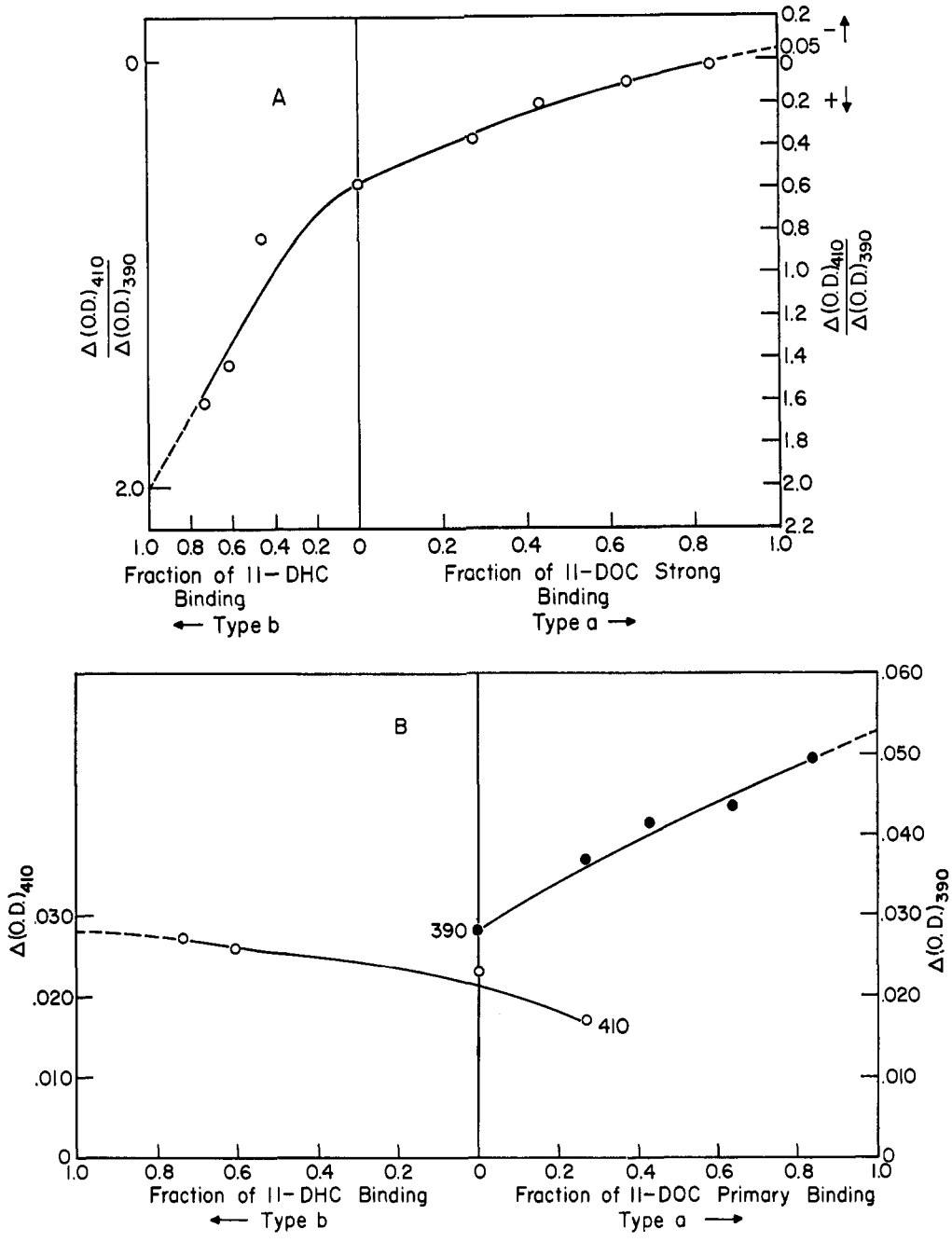

FIGURE 4: Determination of the characteristics of the octylamine difference spectra for separate high- (type *a*) and low- (type *b*) spin forms of P-450. (A) Spectral ratio $\Delta(OD)_{410}/\Delta(OD)_{390}$ (x, y); (B) $\Delta(OD)_{410}$ of pure low-spin P-450 and $\Delta(OD)_{390}$ of pure high-spin P-450. Pure high spin P-450 corresponds to complete 11-DOC binding at the primary site; pure low-spin P-450 corresponds to complete 11-DHC binding. Difference spectra were determined for subparticles of adrenal mitochondria as described in Figure 1.

no evidence of the spectral characteristics corresponding to the type *a* form of oxidized P-450 of liver microsomes.

Effect of Heme-Coordinating Ligands on Steroid Binding. The binding of 11-DOC was examined in the presence of varying amounts of *n*-octylamine. The difference minimum was progressively shifted from 418 to 422 mμ while the steroid binding constant was increased approximately according to competitive inhibition (Table II). Conversely, 10 μM 11-DOC inhibited the binding of *n*-octylamine to type *b* P-450 by a factor of five. The effect of octylamine addition on the binding of 11-DHC was minimal; the small effect was not observed when pregnenolone, a stronger-binding steroid, was substituted for 11-DHC.

Discussion

The observation of two opposite types of difference spectra from the interaction of steroids with P-450 in the mitochondria of the adrenal cortex has been interpreted as due to two distinct forms of P-450 (Whysner and Harding, 1968).

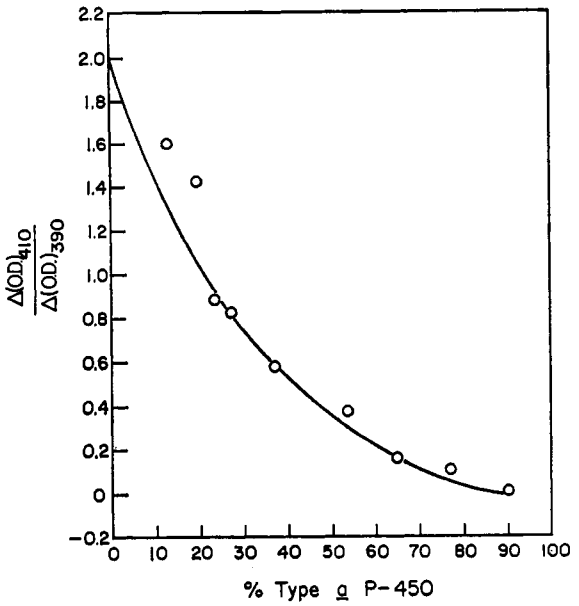

FIGURE 5: Fit of experimental estimation of the percentage high-spin P-450 (type *a*) from octylamine difference spectra. The line was calculated from the formula

$$\frac{[\text{type } a \text{ P-450}]}{[\text{type } b \text{ P-450}]} = \frac{\Delta(\text{OD})_{390} - x\Delta(\text{OD})_{410}}{z[\Delta(\text{OD})_{410} - y\Delta(\text{OD})_{390}]}$$

with the experimental values for x (0.5), y (−0.05), and z (2.0) (see Figure 4). Points represent experimental spectra for submitochondrial particles in which the proportions of high- and low-spin P-450 were controlled by the addition of specific concentrations of either 11-DHC or 11-DOC. Conditions are as described in Figure 1.

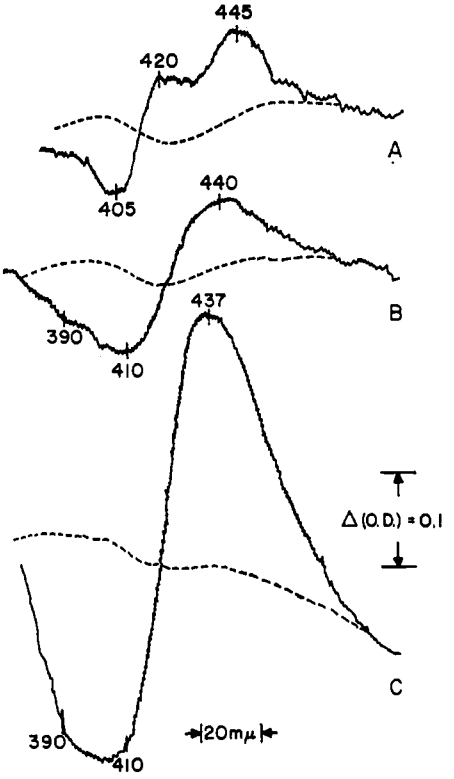

FIGURE 6: Binding of cyanide to P-450 in submitochondrial particles. (A) [CN^-] = 0.2 mM; (B) [CN^-] = 4 mM minus reference [CN^-] = 0.2 mM; (C) [CN^-] = 20 mM minus reference [CN^-] = 0.2 mM and (----) = base line. Submitochondrial particles from bovine adrenal cortex (3 mg/ml) were suspended as described in Figure 1.

Recently, Whysner *et al.* (1969) have shown that 11-deoxycorticosterone and 20α-hydroxycholesterol, respectively, diminish or increase the low-spin electron paramagnetic resonance signal of P-450 from adrenal mitochondria.

Two forms of P-450 in adrenal mitochondria have been indicated in this study by the binding properties of octylamine, which closely parallel the binding of octylamine to P-450 of liver microsomes. Since the octylamine difference spectra of liver microsomes have been correlated with the electron paramagnetic resonance spectra of high- and low-spin hemes (Jefcoate *et al.*, 1969), octylamine binding to adrenal P-450 agrees with the conclusion that P-450 of adrenal mitochondria exists in two spin states. In addition, the sequential binding of the two forms by octylamine suggests that these two forms of P-450 are not in equilibrium but correspond to distinct proteins.

Furthermore, the secondary binding site of 11-DOC and the site for 11-DHC are probably identical. Whysner and Harding (1968) showed similarly that 20α-hydroxycholesterol, which produces a difference spectrum identical with 11-DHC, prevents the secondary effect of 11-DOC.

The pair of inversely related difference spectra that were discussed above suggests the presence of two different forms of P-450 characterized by absorption spectra which can interchange in the presence of steroids

$$\text{P-450(B)} + \text{11-DOC} \rightleftharpoons \text{P-450(A)}\cdot[\text{11-DOC}] \quad (1)$$

$$\text{P-450(A)} + \text{11-DHC} \rightleftharpoons \text{P-450(B)}\cdot[\text{11-DHC}] \quad (2)$$

The suffixes A and B refer only to the spectral types which are governed by the heme region; no evidence has been supplied for more extensive equilibration of P-450 proteins.

The weaker binding of octylamine by both forms of P-450 in adrenal mitochondria compared to the corresponding forms of P-450 in liver microsomes contrasts

TABLE II: Influence of Heme Ligands on Steroid Binding.

Ligand (mM)	[Ligand] (mM)	Steroid (mM)	*K* (Steroid) (μM)
		11-DOC	1.2
n-Octylamine	0.3	11-DOC	1.8
n-Octylamine	0.6	11-DOC	3.8
n-Octylamine	1.2	11-DOC	4.8
		11-DHC	290[a]
n-Octylamine	1.5	11-DHC	160[a]
CN^-	20	11-DOC	2.0

[a] With repetition of these experiments in which a stronger binding steroid, prenenolone ($K \sim 1.4 \times 10^{-5}$ M), was substituted for 11-DHC, no effect of *n*-octylamine on the binding constant was observed. The binding constant for pregnenolone is lower and may be determined much more accurately.

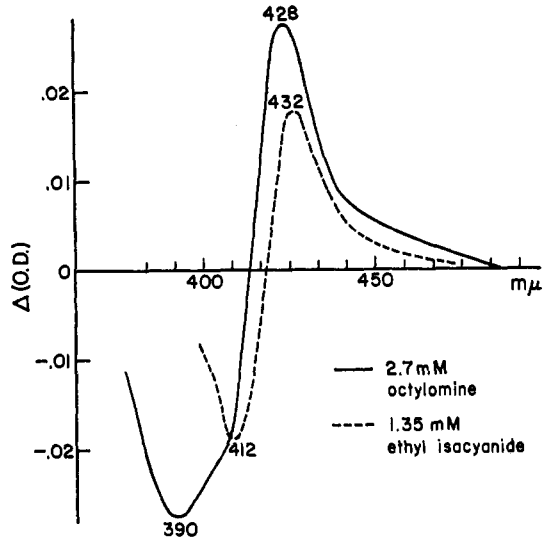

FIGURE 7: Binding of ethyl isocyanide to oxidized P-450 from subparticles of adrenal mitochondria. Submitochondrial particles were suspended (3 mg/ml) as described in Figure 1: (——) [octylamine] = 2.7 mM; (----) [ethyl isocyanide] = 1.35 mM.

with the close similarity of cyanide binding constants. However, in each case, the heme binding ligand shows a preference for low-spin P-450. The relatively weak affinity of the P-450 species in adrenal mitochondria for octylamine may be a reflection of the higher specificity of the heme region for certain steroids compared to P-450 in liver microsomes.

In all probablilty, octylamine, cyanide ions, and ethyl isocyanide change the spectrum of oxidized P-450 by direct coordination to the heme iron. Steroids, however, have no coordinating ligand, and, therefore, the large spectral changes that they produce can only arise by a modification of protein structure which affects the heme-coordinating ligands of the protein.

Studies of steroid binding confirmed the observations of Whysner *et al.* (1969) that two steroid binding sites exist in adrenal mitochondria: one appears specific to steroid substrates, *e.g.*, 11-deoxycorticosterone; and a second site, which in most cases exhibits weak binding, is much less specific. The binding at the second site is much weaker than that expected for simple hydrophobic binding, thus indicating adverse steric or polar effects from the structure of this form of P-450. These effects appear to be partially relieved upon the additional binding of octylamine. By contrast, the binding of octylamine to the heme competitively inhibits the binding steroid substrates at the higher specificity site, suggesting that the latter is also located near to the heme. The effect of octylamine on the two steroid binding sites can be summarized in eq 3 and 4

$$\text{P-450(B)} + \text{11-DOC} \rightleftharpoons [\text{P-450(A)}\cdot\text{11-DOC}] \quad (3)$$

$$[\text{P-450(A)}\cdot\text{11-DOC}] + \text{RNH}_2 \rightleftharpoons [\text{P-450(A)}\cdot\text{RNH}_2] + \text{11-DOC}$$

$$\text{P-450(A)} + \text{11-DHC} \rightleftharpoons \text{P-450(B)}\cdot\text{11-DHC} \quad (4)$$

$$[\text{P-450(B)}\cdot\text{11-DHC}] + \text{RNH}_2 \rightleftharpoons [\text{P-450(B)}\cdot\text{11-DHC}\cdot\text{RNH}_2]$$

Quantitation of the difference spectra produced by adding octylamine to mitochondrial subparticles containing either 11-DOC or 11-DHC confirmed that these steroids effect changes in the spin state of P-450. A close correlation appears between the ratios of high- and low-spin P-450, as calculated from the two types of steroid binding or octylamine difference spectra. Parameters for the equation relating the octylamine difference spectral changes to the ratio of spin types of P-450 (see legend of Figure 5) were estimated by varying the latter ratio by means of steroid binding and these parameters closely matched those obtained from the electron paramagnetic resonance spectra of liver microsomes. However, these parameters were determined approximately for liver microsomes, since the proportions of the two forms for one suspension was established by an electron paramagnetic resonance investigation of their respective electron spin resonance spectra. An independent, confirmatory, and more accurate method has been used here.

Difference spectra indicated that ethyl isocyanide was bound exclusively to low-spin form of adrenal P-450. Since π-acceptor ligands of this type generally produce a low-spin state upon binding iron, an initial preference for this spin state was not unexpected. The difference spectrum of reduced P450 with ethyl isocyanide was, by analogy with liver microsomal P-450, that of pure type *b* P-450 (Imai and Sato, 1967b; Jefcoate *et al.*, 1969). The total absence of any extension of the properties of type *a* oxidized P-450 to the reduced state of P-450 in adrenal mitochondria suggests that perhaps the distinctions between the different forms of P-450 in the oxidized state may be controlled by additional components in the mitochondria which no longer exert an influence in the reduced state. Apparently, liver microsomes do not contain these factors, because isocyanide binding to reduced liver microsomal P-450 can be described by a representation of two forms of P-450 (Miyake *et al.*, 1969) whose proportion directly relates to the proportion of high- and low-spin P-450 in the oxidized state (Jefcoate *et al.*, 1970). However, there is no adequate theoretical explanation for this correlation.

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Isolation and Properties of a Chloroplast Coupling Factor and Heat-Activated Adenosine Triphosphatase*

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ABSTRACT: The purification to homogeneity of a coupling factor (CF_1) from spinach chloroplasts is described. Conversion of the coupling factor, a latent ATPase, to a manifest ATPase by mild heat treatment in the presence of ATP yields a protein which is identical with CF_1 with regard to (a) electrophoretic mobility on polyacrylamide gel, (b) sedimentation patterns in the analytical ultracentrifuge, and (c) amino acid composition. The molecular weight of the native coupling factor and the heat-activated ATPase determined by high-speed sedimentation to equilibrium was found to be 325,000. In the presence of 5 M guanidine-HCl the apparent molecular weight was found to be 62,000 by high-speed sedimentation to equilibrium. From amino acid analysis a minimal molecular weight of 28,000 was calculated.

Studies on a coupling factor from chloroplasts (Avron, 1963; Vambutas and Racker, 1965) have revealed considerable similarities to a coupling factor isolated from beef heart mitochondria (Pullman *et al.*, 1960). Both proteins are cold-labile in solution (Penefsky *et al.*, 1960; McCarty and Racker, 1966) and show similar structures in the electron microscope (Vambutas and Racker, 1965). The soluble mitochondrial coupling factor has ATPase activity (Penefsky *et al.*, 1960), whereas, the chloroplast factor is a latent ATPase and exhibits hydrolytic activity only after treatment with trypsin or heat (Vambutas and Racker, 1965). Pullman and Monroy (1963) have isolated a trypsin-sensitive protein from mitochondria which is a specific inhibitor of mitochondrial ATPase. This inhibitor forms a soluble complex with mitochondrial ATPase which has no hydrolytic activity, but serves as a coupling factor. The masked ATPase activity of this complex can be reactivated by heat treatment.

These findings raised the possibility that CF_1[1] may represent a firmly associated ATPase–inhibitor complex. However, efforts to isolate an inhibitor from CF_1 or from chloroplasts by a procedure similar to that of Pullman and Monroy were unsuccessful (Farron, 1969). Efforts were therefore made to establish whether any part of the coupling factor dissociates irreversibly during heating. In order to do this without ambiguity it was imperative to work with a homogeneous preparation. In the present paper the purification of the coupling factor to homogeneity is described, together with studies of some physicochemical and chemical properties of the protein before and after heat activation. The data indicate that the conversion of latent into manifest ATPase by heat treatment does not entail gross changes in conformation, in the state of aggregation, in electrophoretic mobility, or in amino acid composition.

Experimental Procedure

Materials. Gd·HCl (Ultra Pure) was purchased from Mann Research Laboratories and was used without further purification.

Methods. Amino acid analyses were carried out on a Beckman Spinco Model 120C amino acid analyzer by the method of Spackman *et al.* (1958). Disc gel electrophoresis was performed according to Ornstein and Davis (1964); instead of mixing the sample with spacer gel the sample was

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: chloroplast coupling factor (CF_1) and guanidine hydrochloride (Gd·HCl).