help with the hydrophobic interaction chromatography.

Registry No. Calcium, 7440-70-2.

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Kinetics of Cytochrome P-450 Reduction: Studies in Bovine Adrenocortical Microsomes†

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ABSTRACT: The results of the present study indicate first that in the microsomal preparation, the components of the P-450 reduction system are heterogeneously distributed, comprising dissociable and nondissociable parts. Second, the P-450 reduction curve can be adequately described by a sum of two exponential functions, indicating two concurrent first-order reactions. Third, the two phases can be altered independently. The addition of the substrate increased the extent of the fast phase while it had little or no effect on that of the slow phase. Changes in the interaction of the dissociable and nondissociable components affected the extent of the slow phase while they were without effect on that of the fast phase. Experiments with different steroids indicated that the independence of the two phases is not due to functionally different P-450's and that the cytochrome reduced in both phases is essentially P-450$_{C\text{-}21}$. The results are interpreted as follows: Transformation of P-450 from the low- to the high-spin state controls the total P-450 reduced. The rate and the biphasicity of the reduction are functions of the interaction of P-450 and the reductase.

Previous investigations (Narasimhulu et al., 1966, 1971a,b) indicated that the substrate-produced type I spectral change in cytochrome P-450 represents transformation of the cytochrome from an "inactive" to an "active" state and that this transformation is essential for the enzymatic reduction of the cytochrome. It is now generally agreed that the spectral change is associated with the transformation of the heme from a low-spin to a high-spin state and that this is an obligatory step for the transfer of the first electron to the cytochrome (White & Coon, 1980). The process of the first electron transfer has been investigated under anaerobic conditions in various laboratories. Gigon et al. (1969) first noted that in

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hepatic microsomes the anaerobic reduction of the cytochrome P-450 follows biphasic kinetics. In addition, they noted that the type I compounds stimulated the initial rate of the reduction. Subsequently, similar biphasic reduction was also observed in bovine adrenocortical mitochondria (Harding et al., 1970; Cheng & Harding, 1973) as well as in the microsomes (Narasimhulu, 1973). In addition in the microsomes, graphic resolution of the two phases (Narasimhulu, 1973) indicated that type I steroids increased the extent of the fast phase, resulting in an apparent increase in the initial rate of reduction. Recently, Kominami & Takemori (1982) have demonstrated the biphasic reduction of $P\text{-}450_{C\text{-}21}$ by using the highly purified enzymes from bovine adrenocortical microsomes. The biphasic kinetics have also been observed in the purified and reconstituted hepatic enzyme systems (Coon et al., 1971, 1973; Vatsis & Coon, 1979; Iyanagi et al., 1978).

Different models have been proposed to explain the biphasic kinetics. On the basis of the result that type I compounds accelerated the initial rate of reduction, Gigon et al. (1969) interpreted the fast phase as being due to the reduction of the substrate-bound P-450 and the slow phase as due to the substrate-free P-450. Since no break was observed in an Arrhenius plot of the rate constant of the fast phase, Peterson et al. (1976) proposed a model in which lateral diffusion is not required for the interaction of the reductase and P-450 in the fast phase. According to this model, the fast phase is produced by a cluster of about 8–10 P-450 molecules around each reductase molecule. Cytochrome P-450 molecules that are not a part of the cluster produce the slow phase of reduction, by association with the reductase only after lateral diffusion within the membrane. In a third model proposed by Oprian et al. (1979) based on their studies with purified $P\text{-}450_{LM4}$ and the reductase, a 1:1 complex of P-450 and the reductase is supposed to undergo biphasic reduction, and the biphasic kinetics are a property of the reductase. It is thought that reduction of the uncomplexed P-450 constitutes a slow third phase. While the models proposed by Peterson et al. (1976) as well as Oprian et al. (1979) require complex formation between P-450 and the reductase, Duppel & Ulrich (1976), Yang et al. (1977), and Taniguchi et al. (1979) favor interaction of the two proteins by lateral diffusion and random collision. Recently Backes et al. (1982), based on their studies with rat liver microsomes, have proposed still another model in which the two phases result from two sequential first-order processes which result in burst kinetics. In addition, they propose that the change in the spin state of cytochrome P-450 controls the rate of reduction. In certain other instances, P-450 reduction has been viewed as simultaneous first- and second-order processes (Diehl et al., 1970) and four simultaneous first-order processes (Ruf et al., 1980).

In the present study, the P-450 reduction kinetics have been investigated in bovine adrenocortical microsomes. The results indicate, first, that in the microsomal preparation, the components of the P-450 reduction system are heterogeneously distributed, comprising nondissociable and dissociable parts. Second, the time course of P-450 reduction is adequately describable by a sum of two exponential functions indicating two concurrent first-order reactions. Third, the extents of the two phases can be altered independently. We interpret the results as follows: The transformation of P-450 from the low- to high-spin state controls the total P-450 reduced. The rate of reduction and the biphasicity of the reduction are functions of the interaction of P-450 and the reductase.

Materials and Methods

Materials

Glucose, glucose-6-phosphate dehydrogenase (type II), NADP, steroids, and crystallized bovine serum albumin were obtained from Sigma Chemical Co. Bovine adrenal glands were transported from a nearby slaughterhouse.

Methods

Isolation of the Microsomal Fraction. The adrenal glands were bisected and demedullated, and the cortex tissue was scraped off the capsule. A 20% homogenate of the scrapings in 0.25 M sucrose containing 0.05 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.2, and 1 mM ethylenediaminetetraacetic acid (EDTA) was prepared. The microsomal fraction was isolated by differential centrifugation essentially as described previously (Narasimhulu, 1971a). The final sediment was suspended in 0.25 M buffered sucrose to approximately 20 mg/mL and stored at −70 °C until use.

Procedure for Washing the Microsomes. Unless otherwise specified, the microsomes were diluted with 1.8% bovine serum albumin (BSA) containing glycylglycine buffer, pH 7.4 (Rosenthal & Narasimhulu, 1969), to a protein concentration of approximately 0.5 mg/mL. They were then lightly homogenized and centrifuged at 6700*g* for 15 min. The sediment was resuspended and the washing procedure repeated for the second time.

Procedure for Enzyme Assay. A 3.0-mL aliquot of a mixture of the adrenal microsomal preparation and 1.8% BSA-containing glycylglycine buffer, pH 7.4, was placed in an Aminco anaerobic cuvette equipped with a plunger assembly to make additions under anaerobic conditions. This mixture was bubbled for 7 min with carbon monoxide that had been passed through Fieser's deoxygenizing mixture (0.5% sodium dithionite and 0.05% sodium anthroquinone-2-sulfonate in 0.1 N NaOH) as described by Gigon et al. (1969). At the end of the gassing period, the O_2, scavenging system catalase (30 units/mL) plus glucose (5 mM) plus glucose oxidase (1.5 units/mL) was added to the assay system in this order. While the gassing continued, the plunger assembly containing 40 μL of a deoxygenated NADPH-generating system sufficient to give final concentrations of 3.3 mM glucose 6-phosphate, 0.2 unit/mL glucose-6-phosphate dehydrogenase, and 0.6 mM NADP was inserted. The air vent in the plunger assembly was closed after gassing for an additional 3 min. The cuvette was then placed in an Aminco-Chance dual-wavelength spectrophotometer. After sufficient time (predetermined) was allowed for temperature equilibration, the base line of equal output of the two photomultipliers was recorded. The reaction was then started by quickly lowering the plunger. The addition time is estimated to be 1 s. The absorbance between 450 and 490 nm was recorded as a function of time.

Data Analysis. The time course of P-450 reduction was digitized. Computer analysis of the digitized data was carried out by fitting a double exponential function of the form

$$C = A_1(1 - e^{-B_1 t}) + A_2(1 - e^{-B_2 t})$$

using a nonlinear least-squares procedure based on a search program of Hooke & Jeeves (1961). The goodness of fit of the theoretical curve to the experimental data was evaluated by determining the sum of squares of the residuals and the standard error of the estimate. A conveniently digitizable portion of the curve (usually up to 100–150 s) was used for analysis. However, in several experiments (the ones shown in Table IV and others not reported in this paper), the curves

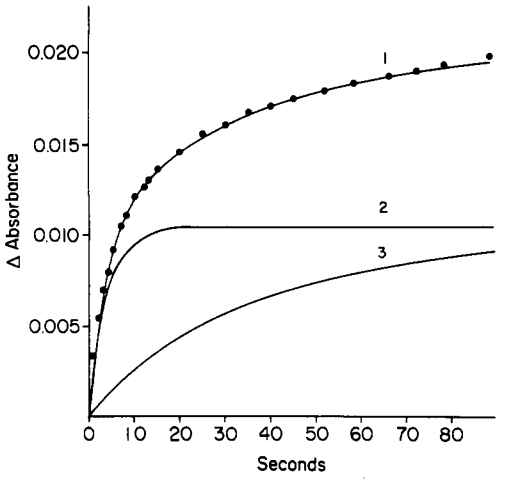

FIGURE 1: Resolution of the time course curve of P-450 reduction in washed microsomes. The details of the procedure, assay system, and data analysis are described under Methods. The assay system contained 0.56 μM dithionite-reducible P-450 and 40 μM 17α-hydroxyprogesterone. In curve 1, the closed circles are experimental points, and the solid line is the theoretical function. Curves 2 and 3 are the resolved fast and slow phases, respectively. The kinetic parameters of this curve are shown in row 2 of Table III.

up to 245 s were used for analysis. In all instances, the curves could be adequately described by a sum of two exponential functions. After these long periods of time, the increase in absorbance was so small that it became difficult to measure accuretely. It should be pointed out that the figures do not show the entire portions of the curves used for analysis. The first 4 s of the data was usually omitted from the analysis because several of these early data points deviated erratically from a smooth curve by more than twice the standard error of estimate. This type of deviation of the initial points is not unexpected, due to the time required to attain homogeneity after mixing the reactants. The analysis used made it possible to resolve the two phases and evaluate the rate constants as well as the extents of both phases without interference from the overlap of the two phases.

Results

Biphasic Reduction of Cytochrome P-450 in Adrenocortical Microsomes. The time course curves of P-450 reduction were analyzed as described under Methods. The results indicate that a sum of two exponential functions of the form

$$C = A_1(1 - e^{-B_1 t}) + A_2(1 - e^{-B_2 t})$$

can adequately describe the entire curve under the present experimental conditions. In the theoretical function, when viewed in terms of the integrated rate equation, C is the concentration of P-450 reduced expressed as ΔA(450–490 nm). A_1 and A_2 are the extents of the two phases, that is, the concentrations of P-450 reduced in the fast and the slow phases, respectively. The interpretation of B_1 and B_2 depends upon the model describing the biphasic kinetics. In the present case in which two phases are apparently independent, B_1 and B_2 represent first-order rate constants. They would be pseudo-first-order rate constants under the present experimental conditions. The results of resolution of a representative time course curve obtained in the presence of a saturating concentration (40 μM) of 17α-hydroxyprogesterone are shown in Figure 1. The figure shows the fit of the theoretical curve (curve 1, solid line) to the experimental points (curve 1, closed circles) obtained by digitizing the time course curve. Curves 2 and 3 are the resolved theoretical curves representing the fast and slow phases, respectively. There is considerable

Table I: Effect of Microsome Concentration on the Kinetic Parameter of Cytochrome P-450 Reduction[a]

prepn	concn (mg of protein/mL)	ΔA(450–490 nm) A_1[b]	A_2[b]	B_1[c]	B_2[c]	SEE[d] (ΔA)
I	1.8	0.033	0.051	0.301	0.02	0.00023
II	0.45	0.0077	0.0067	0.282	0.026	0.000092
I/II	4	4.3	7.6	1.07	0.78	

[a] The details of the procedure, assay system, and data analysis are described under Methods. The concentration of 17α-hydroxyprogesterone was 40 μM. The concentration of the dithionite-reducible P-450 was 0.57 nmol/mg of protein (E = 91 mM^{-1} cm^{-1}). The temperature was 25 °C. [b] A_1 and A_2 are the change in absorbancies. [c] B_1 and B_2 are rate constants (s^{-1}). [d] SEE = standard error of estimate for the entire curve.

overlap of the two phases. The slow phase is present from the starting point of the reaction observable by the present technique. The fast phase is 97% complete within 10 s while the slow phase extended to longer than 120 s under the present experimental conditions. The goodness of fit of the theoretical curves to the experimental data is indicated by the standard errors of estimates being 2 orders of magnitude lower than the values for absorbance (see the tables in this paper). Due to the strong overlap of the two phases, the customary graphic procedure was not used for determining the kinetic parameters.

Effect of Microsome Concentration on Biphasic Reduction. The kinetic parameters of the two phases of P-450 reduction in the presence of a saturating concentration of the substrate 17α-hydroxyprogesterone obtained at two different concentrations of the microsomes are shown in Table I. It is noted that dilution of the microsomes from 1- to 4-fold results in a corresponding decrease in the extent of the fast phase (A_1). However, the extent of the slow phase (A_2) decreased to nearly one-eighth. This dilution effect is not due to the oxygen sensitivity of the slow phase (Gigon et al., 1969) because an oxygen scavenging system was present in the reaction medium and allowed to equilibrate for long periods of time to ensure anaerobic conditions. In addition, the dilution resulted in a nearly 30% increase in the rate constant (B_2) of the slow phase and decreased the concentration reduced in this phase. If it were due to oxygen sensitivity, one would expect a higher rate constant at the higher microsome concentration due to the higher rate of consumption of the residual oxygen. This dilution effect would be consistent if the slow phase components were dissociable. Therefore, attempts were made to physically separate the slow phase components, after diluting the microsomes with the albumin-containing buffer, by the washing procedure described under Methods.

Effect of Washing the Microsomes on Biphasic Reduction. Washing the microsomes preserved the biphasicity of the reduction. However, unlike the case of the original microsomes, the slow phase did not exhibit the dilution effect. In other words, washed microsomes behaved similar to diluted microsomes. The kinetic parameters of the washed microsomes, the washings, and the reconstituted system obtained in the presence of a saturating concentration of 17α-hydroxyprogesterone are shown in Table II. The relative extents of the two phases (A_1/A_2) in the three instances are different. The extent of the slow phase (A_2) and also the rate constant (B_2) in the washed microsomes are nearly the same as those in the washings. However, the extent of the slow phase is considerably more than additive when the two fractions are combined (Table II, row 3), indicating an additional amount of P-450 is reduced by the interaction of the dissociable and nondissociable components of the slow phase. Such interactions are

Table II: Effect of Washing Microsomes on the Kinetic Parameters of Cytochrome P-450 Reduction[a]

prepn	microsomes	ΔA(450-490 nm) A_1	A_2	B_1	B_2	SEE (ΔA)
I	washed	0.010	0.010	0.164	0.018	0.00013
II	washings (supernatant)	0.0040	0.0082	0.141	0.017	0.000083
III	recombined	0.016	0.032	0.101	0.007	0.00029

[a] The procedure for washing the microsomes is described under Methods. The assay conditions are as described in the legend of Figure 1 and under Methods. Microsome concentrations derived from the starting concentration of the microsome suspension are the following: (I) ~1.0 mg of protein/mL; (II) supernatant obtained by centrifuging a suspension of 2.5 mg of the microsomal protein/mL in BSA-containing buffer; (III) ~1.0 mg of protein/mL. Total dithionite-reducible P-450 in terms of ΔA(450-490 nm) is (I) 0.048, (II) 0.012, and (III) 0.0608.

probably not involved in any significant degree in producing the fast phase (A_1) because the extent of the fast phase in the case of the combined fractions (Table II, row 3) is nearly additive. This is also supported by the lack of a dilution effect on the extent of the fast phase (Table I, row 1). In subsequent experiments, the interaction between the dissociable and nondissociable parts was minimized by washing the microsomes and keeping the microsome concentration to a minimum.

Effect of Steroids on the Biphasic Reduction of Cytochrome P-450. The effects of 17α-hydroxyprogesterone, the preferred substrate for the C-21 hydroxylase, on the kinetic parameters of P-450 reduction are shown in Table III. The results obtained with two different batches of the microsomal preparation are presented. In all instances, the presence of the substrate strikingly increased the extent of the fast phase (A_1) and correspondingly increased the total ($A_1 + A_2$) P-450 reduced at infinite time while the extent of the slow phase (A_2) remained essentially unaltered. This result indicates that in the absence of the added substrate, the concentration of the enzymatically reducible P-450 is much less. At the saturating concentration used, the substrate decreased the rate constants (B_1 and B_2). The reason for this is not known. It is interesting that in the diluted as well as the washed microsomes, in the presence of a saturating concentration of the substrate, the extent of the fast phase (A_1) is equal or not far from being equal to that of the slow phase (A_2). The near-equality of the extents of the two phases in several batches of the microsomes prepared on different days from different lots of the adrenal glands is shown in the Figure 2. It is emphasized that these experiments were performed under conditions which minimize the interaction between the dissociable and nondissociable components. For reasons not clear, the rate constants have varied from preparation to preparation. However, the extents of the two phases are nearly equal as indicated by the point of intersection on the y axis being not far from 50%. The ratio A_2/A_1 ranged between 1.0 and 1.3.

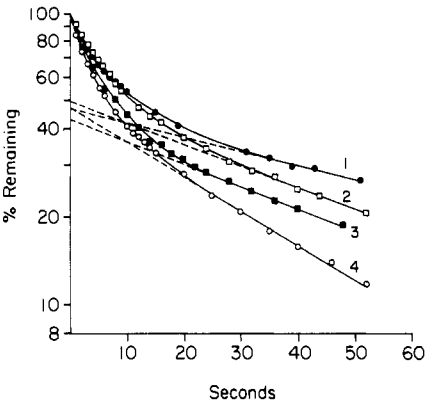

FIGURE 2: First-order plots of the P-450 reduction curve showing the near-equality of the extents of the two phases of cytochrome P-450 reduction. Curves 1–4 were obtained on different days by using the washed microsomes prepared from different lots of the adrenal glands. The P-450 concentration in the assay system ranged from 0.4 to 0.55 μM. The 17α-hydroxyprogesterone concentration was 40 μM.

Since the adrenal microsomes contain 17α-hydroxylase activity in addition to the C-21 hydroxylase, it was important to determine the extent of contribution by P-450$_{17\alpha}$ to the observed biphasic reduction. Therefore, the effects of progesterone (the substrate for 17α-hydroxylase as well as C-21 hydroxylase) and progesterone plus 17α-hydroxyprogesterone (the substrate for C-21 hydroxylase) were studied. The results are shown in Table IV. The total P-450 reduced in the presence of 17α-hydroxyprogesterone alone is about 11% lower than that in the presence of progesterone alone and 8.4% lower than that in the simultaneous presence of the two steroids. When the two phases are considered separately, in the presence of 17α-hydroxyprogesterone, A_1 is about 8% lower and A_2 is 13% lower than those in the presence of progesterone alone. They were 7% and 9% lower, respectively, than those in the simultaneous presence of the two steroids. These differences are all small. Accordingly, when compared with the control (not shown in the table), the effects of the two steroids on the two phases were very similar. They were similar in that they both produced a striking increase in the extent of the fast phase and a very small increase in that of the slow phase. However, the increases produced by progesterone were somewhat higher than those produced by 17α-hydroxyprogesterone.

Discussion

Since the sum of only two exponential functions of the form indicated was sufficient to adequately describe the P-450 reduction curve under the present experimental conditions, it is considered that the reduction in the adrenal microsomes follows pseudo-first-order biphasic kinetics. In the microsomal preparation, the fast phase components are apparently in an undissociable complex while those of the slow phase are partly dissociable. This is indicated by the result that upon dilution the extent of the fast phase decreased in proportion to the dilution while that of the slow phase decreased much more than

Table III: Effect of Substrate on the Kinetic Parameters of Cytochrome P-450 Reduction in Washed Microsomes[a]

prepn	ΔA(450-490 nm), A_1 C[b]	A_1 +S[c]	B_1 C	B_1 +S	ΔA(450-490 nm), A_2 C	A_2 +S	B_2 C	B_2 +S	$A_1 + A_2$ C	$A_1 + A_2$ +S	SEE (ΔA) C	SEE (ΔA) +S
I	0.0015	0.0113	0.252	0.199	0.0075	0.0086	0.026	0.0177	0.0095	0.0199	0.000055	0.0001
II	0.0021	0.0116	0.348	0.252	0.0090	0.0101	0.0327	0.0197	0.0101	0.0217	0.00009	0.00012

[a] The concentration of 17α-hydroxyprogesterone was 40 μM. The concentration of the dithionite-reducible P-450 in terms of ΔA(450-490 nm) was (I) 0.045 and (II) 0.051. [b] C = in the absence of the added substrate. [c] +S = in the presence of a saturating concentration of 17α-hydroxyprogesterone.

Table IV: Extent of Cytochrome P-450 Reduced in Microsomes in the Presence of Steroids[a]

steroid	ΔA(450–490 nm) A_1	A_2	A_1+A_2	SEE
progesterone	0.0138	0.0197	0.0335	0.000126
17α-hydroxyprogesterone	0.0127	0.0171	0.0298	0.000162
17α-hydroxyprogesterone + progesterone	0.0128	0.018	0.0307	0.000169

[a] The assay system contained 1 mg of microsomal protein/mL. The concentrations of the steroids were 40 μM.

accountable by the dilution. In addition, the slow phase could be physically separated from the fast phase to a considerable extent. This is indicated by the result that the relative extent of the slow phase (A_2/A_1) in the washed microsomes (0.98) is much less than that in the washings (2.1) and that in the reconstituted system (2.0). When P-450 reduction was measured after the washed microsomes and the washings were recombined, the extent of the slow phase was much more than additive. This result as well as the dilution effect on the slow phase indicates that there is interaction between the dissociable and nondissociable components. The nature of the dissociable components is not known. However, in view of the stimulation of steroid C-21 hydroxylation by certain cytosolic proteins (Ponticorvo et al., 1980), the existence of carrier proteins for lipid-soluble substrates including steroids (Scallen et al., 1974; Dempsey, 1974), and the enhancement of binding of substrates to $P\text{-}450_{scc}$ by certain cytosolic factors such as poly(phosphoinositides) (Farese & Sabir, 1979), several possibilities exist. Washing of the microsomes with buffer containing BSA and salts could remove a variety of factors including lipids and proteins which may be involved in the hydroxylation reaction. In the present study, the observed partial reduction of P-450 in the washed microsomes and the increased reduction upon readdition of the washings would be consistent if dissociable factors such as those which can enhance steroid binding are present in the microsomal preparation. Such factors can increase in total P-450 reduced by increasing the concentration of the substrate-bound P-450. As indicated earlier, the increase occurs only in the slow phase. In this connection, it is interesting that in the present experiments the rate constant for the slow phase in the washed microsomes ranged from 1.02 to 1.18 min^{-1}. These values are not too far from the range (1.2–3.3 nmol of product min^{-1}) of values obtained at 25 °C for the overall hydroxylation reaction in the washed as well as sucrose density gradient centrifuged microsomes (Narasimhulu, 1978) prepared from different lots of the adrenal glands in different years. In addition, similar to the extent of the slow phase, the rate of C-21 hydroxylation is sensitive to the dilution of the microsomal preparation; that is, the activity decreases more than is accountable for by the dilution (Chaslow & Lieberman, 1979). These observations suggest the possibility that the slow phase reflects the rate-limiting step of the overall hydroxylation reaction. Since in the washed microsomes the slow phase could not be decreased any further upon dilution, the two phases are considered as integral parts of the reduction system in microsomal membranes.

As indicated earlier, the reduction of P-450 in the two phases follows pseudo-first-order kinetics under the present experimental conditions. The nature of the rate-limiting step is not known. Substrate binding is very much faster than the rate of reduction, as indicated by the substrate-produced type I spectral change which occurs within less than a second (estimated substrate addition time). This is the lower limit of the present technique used for measuring P-450 reduction. In addition, at the saturating concentration of substrate used in the present experiments, the presence of the substrate decreases the rate constant. Therefore, in the adrenal microsomes, the substrate cannot control the rate-limiting step in the manner suggested in the case of liver microsomes (Backes et al. 1982). It controls, however, the concentration of P-450 reduced, probably by increasing the concentration of the enzymatically reducible form of P-450 (the high-spin form). This increase is observable only in the fast phase while the concentration reduced in the slow phase remains essentially unaltered. Thus, an increase in the extent of the fast phase occurs without a corresponding decrease in that of the slow phase but correspondingly increasing the total concentration of P-450 reduced. This result, as well as the dilution effect on only the slow phase, indicates that there is apparently no interaction between P-450 molecules reduced in the two phases. The reason for the lack of effect of the substrate on the extent of the slow phase in the washed microsomes is not clear. However, when one considers that the low-spin P-450 in the adrenal microsomes is not enzymatically reducible, the lack of effect of the substrate would be consistent if the P-450 reduced in the slow phase is already in the high-spin state due to unknown reasons. In any case, the results indicate that the slow phase does not represent reduction of the substrate-free P-450 in the manner suggested by Gigon et al. (1969) for the liver microsomes. Second, the P-450 in the adrenal microsomes apparently does not exist as an equilibrium mixture of the low- and high-spin states, unlike that in the case of liver microsomes (Backes et al., 1982) as indicated by the partial reduction of P-450 in the absence of the added type I steroid.

Considering that P-450 is the substrate for the reductase enzyme and assuming that the reduced product formed in the two phases has the same absorption spectrum and extinction coefficient, the two phases are interpretable as being due to one of the following: (1) two functionally different P-450's; (2) reduction of a single population of $P\text{-}450_{C\text{-}21}$ by a mechanism involving two sequential reactions in which the first one is reversible; (3) two different populations of $P\text{-}450_{C\text{-}21}$, which may arise if the P-450 is situated in two different environments in the membrane; (4) two different populations of the reductase in addition to two different populations of $P\text{-}450_{C\text{-}21}$.

Although the extents of the two phases can be altered independently and adrenal microsomes contain three hydroxylation activities known to be catalyzed by specific P-450 enzymes, it is highly unlikely that the two phases represent functionally different P-450's for the following reasons: The three known activities are C-21, C-17α, and lyase. The lyase activity in bovine adrenocortical microsomes is negligible (Peter Hall, personal communication). The 17α-hydroxylase activity, although quite low, amounting to about 10% of the C-21 hydroxylase activity (Hiwatashi et al., 1979), is not negligible. Therefore, in bovine adrenocortical microsomes, C-21 and C-17α hydroxylases are of main concern. Progesterone serves as a substrate for both C-21 and C-17α hydroxylases whereas 17α-hydroxyprogesterone serves as a substrate for C-21 hydroxylase. Accordingly, in the present experiments, in the presence of a saturating concentration of progesterone, the total P-450 reduced is slightly higher (12%) than in the presence of 17α-hydroxyprogesterone. The 12% higher value is similar to the 17α-hydroxylase activity being 10% of the C-21 hydroxylase activity (Hiwatashi et al., 1979), implying that the turnover rates of $P\text{-}450_{C\text{-}21}$ and $P\text{-}450_{17\alpha}$ are the same. In the simultaneous presence of the two steroids, at the saturating concentrations used which may have been somewhat inhibitory,

the total P-450 reduced was slightly lower than that in the presence of progesterone alone. In contrast, the relative (A_2/A_1) extents of the two phases of P-450 reduction were 1.0–1.3 in the absence of the dissociable components and as high as 2.0 in their presence. If the turnover rate is the same for the two hydroxylases, this high a ratio can never be reached if the slow phase (the one in question) is due to P-$450_{17\alpha}$. In addition, biphasic reduction has also been demonstrated (Kominami & Tekemori, 1982) by using the highly purified P-$450_{C\text{-}21}$ and the reductase from bovine adrenocortical microsomes. Furthermore, these authors have reported that in the purified system the total P-450 reduced in the presence of 17α-hydroxyprogesterone is not significantly different from that in the presence of progesterone. These results in the case of the purified enzymes are very similar to the present results obtained with the microsomal suspensions. These considerations indicate that P-450 reduced in the presence of 17α-hydroxyprogesterone in the microsomes is essentially all P-$450_{C\text{-}21}$.

The biphasicity is not likely to be due to two sequential reactions because the extents of the two phases can be altered independently. The sequential reaction mechanism requires that an increase in the extent of one phase be compensated by a corresponding decrease in the extent of the other. The observed results would be consistent with an assumption of two different populations of the enzymatically reducible form of P-$450_{C\text{-}21}$, although the present results cannot distinguish between cases 3 and 4. Since the results reported (Kominami & Takemori, 1982) in the case of purified P-$450_{C\text{-}21}$ are essentially similar to the present results obtained with the microsomal suspensions, membrane effects are probably not involved in producing the two different populations of P-$450_{C\text{-}21}$. Under these circumstances, any attempts to explain the biphasicity of reduction of P-$450_{C\text{-}21}$ must take into account the components involved in producing the biphasicity and the independence of the two phases. From the independence of the two phases, we suggest that two pools of the enzymatically reducible P-$450_{C\text{-}21}$ are formed during the course of reduction under anaerobic conditions.

Registry No. Cytochrome P-450, 9035-51-2; NADH–cytochrome P-450 reductase, 9039-06-9; 17α-hydroxyprogesterone, 68-96-2; progesterone, 57-83-0.

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