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Adrenal Microsomal Hydroxylating System: Purification and Substrate Binding Properties of Cytochrome P-450$_{C\text{-}21}$[†]

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ABSTRACT: The substrate–cytochrome P-450$_{C\text{-}21}$ binding reaction has been investigated in detail by using the purified cytochrome. The apparent substrate dissociation constant (K_D^{app}) depended on the enzyme concentration, indicating that the binding reaction does not follow simple two-component mass action equilibrium. However, the binding data fit reasonably well to a model in which the P-450$_{C\text{-}21}$ exists in a monomer–dimer equilibrium and the substrate does not bind to the dimer. The intrinsic dissociation constant (K_1) and the dissociation constant for the dimerization reaction (K_2) were calculated from the titration data by a pattern search procedure. K_1 and K_2 were found to be essentially independent of the enzyme concentration, indicating the appropriateness of the assumed model. In the present study, all factors that increased the dissociation of the dimer, as indicated by an increase in K_2, decreased K_D^{app} so that it approached the intrinsic constant K_1. These results suggest that there is mutual interaction of the substrate binding and self-association reactions of cytochrome P-450$_{C\text{-}21}$ in the purified preparation.

Several hydroxylation reactions are involved in the transformation of cholesterol to steroids with hormonal activity. These reactions are catalyzed by highly specific cytochrome P-450 enzymes present in the adrenal cortex. The enzymes which catalyze 11β-hydroxylation [P-450$_{11_\beta}$ (Wilson et al., 1965)] and side-chain cleavage [P-450$_{SCC}$ (Simpson & Boyd, 1966)] reactions are localized in the mitochondria. The enzymes which catalyze C-21 and C-17α hydroxylations (P-450$_{C\text{-}21}$ and P-450$_{C\text{-}17\alpha}$) and side-chain cleavage (P-450$_{lyase}$) are localized in the endoplasmic reticulum (Inano et al., 1969).

All of the adrenal cytochrome P-450 enzymes have been purified from one source or the other, and various aspects of ligand–P-450 interactions have been studied (Whysner & Harding, 1968; Cheung & Harding, 1973; Lambeth et al., 1979; Jefcoate, 1982). The cytochrome P-450$_{C\text{-}21}$ has been purified from bovine adrenocortical microsomes (Kominami et al., 1980; Bumpus & Dus, 1982) as well as pig adrenocortical microsomes (Yuan et al., 1983). Using the purified P-450$_{C\text{-}21}$ and the reductase, Kominami et al. (1984) have investigated one of the important aspects of electron transport, that is, interaction of reductase and P-450. However, interaction of substrates with purified P-450$_{C\text{-}21}$ has not yet been investigated. This is important for reasons indicated below.

Cytochrome P-450$_{C\text{-}21}$ as isolated is in the low-spin state (Kominami et al., 1980). It is generally agreed that binding of substrates to low-spin cytochrome P-450 enzymes transforming these to the high-spin state is obligatory for electron transfer (White & Coon, 1980). The low- to high-spin-state transition is reflected as the characteristic substrate-induced blue shift of the Soret absorption band (Whysner et al., 1970) designated as type I spectral change (Remmer et al., 1966). Studies (Narasimhulu, 1971a,b; Narasimhulu et al., 1966) using bovine adrenocortical microsomes had indicated that binding of type I substrates to the cytochrome P-450$_{C\text{-}21}$ is essential for electron transfer. As a result, the addition of type I substrates to bovine adrenocortical microsomes strikingly increases the extent of P-450 reduced (Narasimhulu & Eddy, 1984). Therefore, especially at concentrations below the saturation level, any changes in the substrate dissociation constant would be expected to alter the parameters of the reduction. Therefore, knowledge of factors which can alter the substrate–P-450 binding reaction is important.

Factors such as temperature and detergents were found to have a striking effect on the apparent substrate dissociation constant for P-450$_{C\text{-}21}$ in the adrenocortical microsomes

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(Narasimuhulu, 1977, 1979). The nonionic detergent Triton N-101 increased the apparent substrate dissociation constant in lipid-depleted microsomes (Narasimhulu, 1975). The mechanisms whereby these factors influence the substrate binding reaction are not known. Many problems concerning the interaction of steroids with P-450$_{C-21}$ can only be unraveled with purified preparations of the enzyme.

In the present paper, we report a modification of the purification procedure of Kominami et al. (1980) which decreased the time required for purification and increased the yield. In addition, using the purified P-450, we have investigated certain aspects of the substrate–P-450 binding reaction.

Materials and Methods

Preparation of Microsomes. The adrenals transported from a slaughterhouse on ice were bisected and demedullated, and the cortex tissue was scraped off the capsule. A 20% homogenate of the scraped tissue in 0.3 M ribonuclease (RNase)-free sucrose containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.005 M *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethylenesulfonic acid (Hepes) buffer, pH 7.4, was prepared by using a loose pestle Potter–Elvehjem-type homogenizer at moderate speed. The homogenate was subjected to differential centrifugation essentially as previously described (Rosenthal & Narasimhulu, 1969). The homogenate was centrifuged 3 times at increasing *g* values (9000*g*, 10500*g*, and 12000*g*) for 15 min, and sediment at the end of each run was discarded. The supernatant was centrifuged at 78000*g* for 1 h. The microsomes sedimented were washed with 0.15 M KCl. The washed microsomes were suspended in 0.3 M RNase-free sucrose containing 0.005 M Hepes buffer, pH 7.4, to a protein concentration of about 20 mg/mL and stored at −70 °C until use.

Purification of P-450$_{C-21}$. The microsomes were thawed and freed of sucrose by diluting with cold water and centrifuging at 100000*g* for 1 h. The sediment was suspended in 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol (this mixture will be referred to as "basal buffer"). Then 20% cholate was added dropwise, slowly during the course of 30–40 min, to give a final cholate concentration of 0.7% and a cholate/protein (mg/mg) ratio of 2.9. This mixture was stirred for an additional 1 h and centrifuged at 100000*g* for 1 h. The supernatant (the 0.7% cholate extract) was subjected to hydrophobic column chromatography as follows: The extract (~300 mL) was loaded on an aminooctylamine–sepharose (AOA–Sepharose) column (2.5 × 30 cm) equilibrated with the basal buffer containing 0.7% cholate. The column was then washed with the basal buffer medium (800 mL) containing 0.4% cholate. The elution was started with the basal buffer (liter) containing 0.4% cholate in which the Emulgen 913 concentration was increased from 0 to 0.16% (v/v) in a linear gradient using a liter gradient maker. Fractions were collected, and then the absorption at 417 nm was measured. Fractions containing P-450$_{C-21}$, as indicated by the 17α-hydroxyprogesterone-induced type I spectral change, were pooled. The pooled fractions which contained 0.8–0.9 μM P-450$_{C-21}$ were stored at −70 °C.

Detergent Removal. The desired amount of frozen P-450 was thawed and dialyzed against 10 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol (DTT). The dialyzed material (250 μL) was loaded on a CM-Sephadex-50 column (1.5 × 22 cm) which had been equilibrated with the dialysis buffer (5 × volume of column). The column was then washed with the same buffer until the washings were just free from Emulgen 913 as indicated by the absence of the UV absorption at 275 nm. The P-450$_{C-21}$ was eluted with 0.25 M potassium phosphate buffer containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis. A 5–20% gradient polyacrylamide gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate. The samples were boiled prior to electrophoresis with 10% SDS and 0.1 M β-mercaptoethanol.

Assay of C-21 Hydroxylation Activity. The assay system consisted of 0.05 M Hepes buffer, pH 7.4, 3.8 mmol of $MgCl_2$, 60 nmol of 17α-hydoxyprogesterone, 0.085 nmol of P-450$_{C-21}$, 0.085 nmol of highly purified rabbit liver microsomal NADPH–cytochrome P-450 reductase, 0.032% Emulgen 913, and 0.04% cholate in a total volume of 1.0 mL. The steroid was introduced as a methanolic solution in the incubation flask, and methanol was removed under nitrogen. Then the remaining components of the assay system with the exception of the NADPH-generating system were added and preincubated for 3 min at 26 °C. The reaction was started by adding 0.025 mL of the NADPH-generating system (3.3 mM glucose 6-phosphate, 0.6 unit/mL glucose-6-phosphate dehydrogenase, and 0.6 mM NADP) and incubated for 30 min at 26 °C. The reaction was stopped, and the steroids were extracted with 7.0 mL of dichloromethane. The product cortexolone formed was separated from 17α-hydroxyprogesterone and quantitated as follows.

A 5.0-mL aliquot of the extract was evaporated under N_2. The residue was redissolved in 50 μL of methanol and subjected to thin-layer chromatography using 20% acetone in isopropyl ether (Touchstone, 1968) as the solvent. This solvent is capable of clearly separating 17α-hydroxyprogesterone (R_f 51), cortexolone (R_f 28), progesterone (R_f 65), and deoxycorticosterone (R_f 35) (Levin et al., 1969). The separated steroids were quantitated with a Kontes Model 800 densitometer.[1]

Spectrophotometry. Absorption spectra of the purified P-450 were obtained with a Hitachi Model 577 split-beam spectrophotometer. All other spectra were obtained with a Perkin-Elmer Model 571 split-beam spectrophotometer. The type I spectral change was titrated with a dual-wavelength filter instrument.

Analytical Procedures. Protein concentration was determined by the modified method of Lowry applicable in the presence of interfering substances, in this case dithiothreitol and detergents (Bensadoum & Weinstein, 1976). The method involves addition of deoxycholate followed by precipitation of the protein with trichloroacetic acid, redissolving of the precipitated protein in Lowry reagent C containing SDS, and measurement of the absorption at 660 nm.

Determination of the Apparent Substrate Dissociation Constant (K_D^{app}). The substrate-produced type I difference spectrum of cytochrome P-450 characterized by a minimum at 421 nm, a maximum at 388–390 nm, and an isosbestic point at 407 nm was used as the criterion for the binding of the substrate to the cytochrome. The details of the procedure for titration by a semimicro technique have been previously described (Narasimhulu, 1977). A cuvette of 12-mm light path containing 3.0 mL of the assay system was placed in a dual-wavelength filter photometer fitted with interference filters of 421 nm (λ_1) and 407 nm (λ_2) of 1-nm half-bandwidth. The assay system was constantly stirred during the titration with a magnetic stirring attachment. The temperature was regulated by a thermostated circulator and measured with a

[1] Work in progress in collaboration with Dr. J. C. Touchstone.

Table I: Purification of P-450$_{C-21}$

fraction	total protein (mg)	total P-450 (nmol)	P-450$_{C-21}$ (nmol) not treated[a]	P-450$_{C-21}$ (nmol) NADPH treated[b]	P-450$_{C-21}$ specific content[a]	% yield
microsomes	468	275	137	211	0.47	100
cholate extract		230	106	ND[c]		90
AOA column	8.0	105	103	ND	13	49[b]
CM-Sephadex column	2.6	50	48	ND	18–19	24[b]

[a] Calculated from the 17α-hydroxyprogesterone-induced type I spectral change using E = 64 mM^{-1} cm^{-1} for ΔA(407–421 nm). [b] Based on the 17α-hydroxyprogesterone-induced type I spectral change in microsomes treated with a NADPH-generating system. [c] ND, not determined.

thermocouple. An appropriate concentration of a methanolic solution of the steroid was added in 0.1-μL aliquots using a 10-μL gas-tight syringe attached to a programmable Hamilton precision dispensor. After each addition, the absorbance difference, ΔA(407–421 nm), was recorded with a strip-chart recorder. The effects of adding methanol alone to the enzyme-containing buffer systems and steroid alone to the buffer were tested and were found to be without effect within the range of concentrations used for the titration.

Data Analysis. Assuming one binding site on the P-450, the bound substrate was calculated by using 64 mM^{-1} cm^{-1} for ΔA(407–421 nm), derived from the type I spectral change, and 113 mM^{-1} cm^{-1} for ΔA(389–422 nm) (Kominami et al., 1980). By subtracting the bound substrate from the total, the free substrate concentration was determined. The K_D^{app} and maximum substrate–P-450 complex formed were calculated by using the equation $[S]/[ES] = K_D^{app}/[E_t] + [S]/[E_t]$ with a computer-assisted weighted linear regression analysis, unless otherwise indicated. In this equation, S is the free substrate, ES is the complex, and E_t is the total enzyme. The intrinsic substrate dissociation constant, K_1, and the dissociation constant for enzyme dimerization, K_2, were determined by a modified pattern search procedure of Hooke & Jeeves (1961). The concentration [ES] is taken from the experimental data point. The resulting calculated $[S_t]$ value is compared with the experimental value of $[S_t]$ to obtain a residual. The sum of squares of the residuals is made a minimum by trial values of $[E_t]$, K_1, and K_2 by the search procedure. The residual sum of squares for $[S_t]$ is chosen as the quantity to be minimized because the major source of error is in adding the substrate under conditions where optical artifacts (due to factors such as changes in turbidity, stirring, and instrumental instabilities) are minimized.

The precision of the values for the different parameters obtained by the present technique is indicated by the results of five titrations with 17 α-hydroxyprogesterone, performed under identical conditions using microsomal suspensions. The 95% confidence intervals obtained from five values for each of the parameters K_D^{app}, $[E_t]$, K_1, and K_2, are 6.7, 9.7, 12.1, and 22.2%, respectively.

It should be pointed out that in earlier experiments (Narasimhulu, 1977, 1978, 1979) total substrate concentration was used instead of free substrate concentration. When tested with the data obtained under the present experimental conditions, the Lineweaver–Burk plots were linear in both cases. However, the K_D^{app} was about 20% overestimated when the total substrate concentration was used. The maximum bound was the same.

Results

Cytochrome P-450$_{C-21}$ Content of the Microsomes Used for Purification. The microsomes contained about 0.56 nmol of P-450/mg of protein as determined from the carbon monoxide difference spectrum of dithionite-reduced enzyme, using 91 mM^{-1} cm^{-1} for P-450·CO. About 55% of the P-450 could bind to exogenous 17α-hydroxyprogesterone as determined from the magnitude of the type I spectral response using 64 mM^{-1} cm^{-1} for ΔA(407–421 nm). However, after treatment with a NADPH-generating system which would be expected to metabolize the endogenous steroids, close to 80% of the total P-450 formed type I complex with 17α-hydroxyprogesterone. Upon further addition of progesterone (which is hydroxylatable by P-450$_{17\alpha}$ as well as by P-450$_{C-21}$), the type I complex could account for about 90% of the total P-450 present in the microsomes. The total dithionite-reducible P-450 per milligram of protein was not affected by treatment with the NADPH-generating system.

Apparent Steroid Dissociation Constant in Microsomes. Due to the presence of more than one hydroxylase in the microsomes and the possibility of overlapping substrate specificities, it was important to find out if the type I spectral change observed at saturating concentrations of a given steroid represents one or more than one affinity for a given substrate. Therefore, the microsomal suspension was titrated with 17α-hydroxyprogesterone and progesterone. Lineweaver–Burk plots were linear within the concentration range of steroids tested. In the case of 17α-hydroxyprogesterone, the observed ΔA_{max} was about 96% of the value calculated from Lineweaver–Burk equation. Thus, the entire spectral change could be titrated with a single dissociation constant. At 26.5 °C, 17α-hydroxyprogesterone and progesterone had K_D^{app}'s of 0.104 and 0.455 μM. In the case of progesterone, the maximum spectral change was only slightly higher, which is consistent with progesterone being the substrate for P-450$_{C-21}$ as well as P-450$_{C-17\alpha}$ and C-17α hydroxylase activity being only a small fraction (10–15%) of the C-21 hydroxylase activity (Hiwatashi et al., 1981).

Purification of P-450$_{C-21}$. The elution profile of the 417-nm-absorbing material which resulted from AOA column chromatography of the cholate extract of the microsomes is shown in Figure 1. The P-450$_{C-21}$ was eluted as a major band (peak 3) with a 5-fold greater P-450 content than the earlier band (peak 1). When we used a blender instead of a homogenizer for the preparation of the microsomes, the P-450$_{C-21}$ was eluted as a minor band. The peak 3 material, when tested after the detergent removal treatment described under Materials and Methods, showed a single band on SDS gel electrophoresis (Figure 2), had a molecular weight of 52 000–53 000, and had a specific content of 18–19 nmol of P-450/mg of protein when tested after the detergent removal treatment. The yield was 24% (Table I), which is 4-fold higher than those reported (Kominami et al., 1980; Bumpus & Dus, 1982). The higher yield was reproducible between the two preparations tested. The yield was calculated similarly to Kominami et al. (1980), on the basis of the 17α-hydroxyprogesterone-induced type I spectral change in the starting material. However, the spectral change in the microsomes after treatment with the NADPH-generating system was used to calculate the starting concentration of P-450$_{C-21}$. The extinction coefficient of 64 mM^{-1} cm^{-1} for ΔA(407–421 nm) was used for calculation.

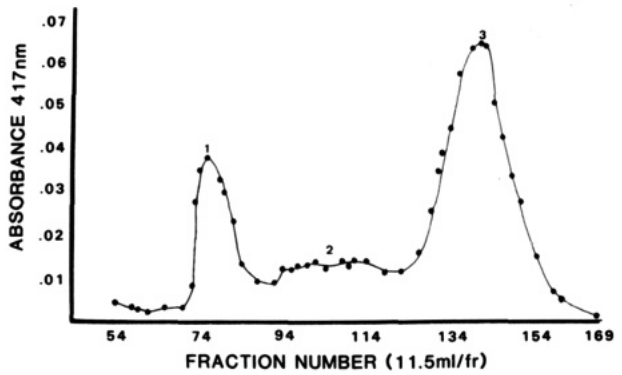

FIGURE 1: Aminooctylamine–Sepharose column elution profile of the cholate extract of the microsomes. Procedure described under Materials and Methods.

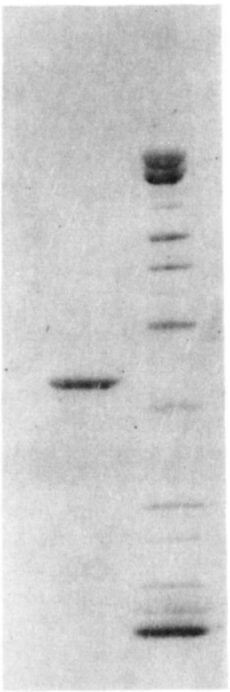

FIGURE 2: SDS gel electrophoresis. (A) P-450$_{C-21}$ (~30 μg of protein); (B) standards.

The absolute spectra of purified P-450$_{C-21}$, substrate-bound P-450$_{C-21}$, and the CO complex with P-450$_{C-21}$ were very similar to those reported by Kominami et al. (1980). In the absence of substrate, the P-450$_{C-21}$ was completely in the low-spin state as indicated by the absence of the 650-nm band which is characteristic of high-spin P-450 (Whysner et al., 1970).

Hydroxylase Activity of Purified P-450$_{C-21}$. When tested with rabbit liver NADPH–cytochrome P-450 reductase, 17α-hydroxyprogesterone and progesterone were C-21 hydroxylated at the rate of 3.8 and 3.0 nmol min^{-1} (nmol of P-450)$^{-1}$ at 26 °C, respectively. There were no detectable 17α-hydroxylated products formed.

Substrate Binding Reaction in Purified P-450$_{C-21}$. Similar to that in the microsomes (Narasimhulu, 1971a), the substrate-produced type I difference spectrum has a maximum around 390 nm, a minimum at 421 nm, and an isobestic point at 407 nm as also reported by Kominami et al. (1980). In addition, similar to that in the microsomes, 17α-hydroxyprogesterone has considerably higher affinity to P-450$_{C-21}$ than progesterone. However, other characteristics of the binding reaction, such as dependence on protein concentration, temperature, and the type and concentration of detergent, were qualitatively identical for the two steroids.

Table II: Effect of the Concentration of P-450$_{C-21}$ on the Parameters of Binding of 17α-Hydroxyprogesterone[a]

[E$_t$] (μM)	K_1 (μM)	K_2 (μM)	SEE, % [S$_t$]	K_D^{app} (μM)
0.020	0.069	0.011	1.74	0.249
0.051	0.073	0.0086	0.77	0.468
0.076	0.082	0.0091	0.96	0.598
0.141	0.063	0.0085	0.31	0.832
$\bar{X}$	0.072	0.0093		0.537
σ	0.008	0.0012		0.244

[a] Purified cytochrome P-450$_{C-21}$ in 3.0 mL of the basal buffer (described in the text) containing 0.008% Emulgen 913 was titrated with 17α-hydroxyprogesterone as described under Materials and Methods. Temperature, 26.5 °C. Procedures for determining the parameters and their precisions are given under Materials and Methods. $\bar{X}$ and σ are mean and standard deviations of the tabulated values.

Effect of P-450$_{C-21}$ Concentration. The purified P-450$_{C-21}$ after the detergent removal treatment contained small amounts of the residual detergent Emulgen. However, in these experiments, taking the residual amount into account, the detergent concentration was kept essentially constant at 0.008% (needed for enzyme stability) while the P-450 concentration was varied. To achieve this, the following points were considered: The extinction coefficients, in the Soret region, of the oxidized, reduced, substrate-bound, and CO-bound P-450$_{C-21}$ are very similar to those reported (Gunsalus & Wagner, 1978; Kominami et al., 1980) for P-450$_{cam}$, for the purification of which no detergents are required. Therefore, it was assumed that the extinction coefficient of the protein absorption peak of P-450 is also similar to that of P-450$_{cam}$. Then the extinction coefficient of the protein at 278 nm (major peak of Emulgen) relative to the Soret band of the oxidized P-450$_{cam}$ and the absorption of the oxidized Soret band of P-450$_{cam}$ were used to calculate the protein absorption at 278 nm for P-450$_{C-21}$. By subtracting this value from the observed absorption at 278 nm, the absorption due to the detergent was calculated. The detergent concentration was estimated by means of a standard curve of detergent vs. absorption at 278 nm. Another assumption made in these calculations is that the protein absorption and detergent absorption are additive. The estimated detergent concentration of 2.62 μM P-450 was 0.01%. In all of the experiments, this was at least 1 to 10 diluted and adjusted to 0.008%.

About an 8-fold increase in the P-450 concentration resulted in an approximately 4-fold increase (from 0.249 to 0.83 μM) in the K_D^{app} of 17α-hydroxyprogesterone (Table II). This dependence of K_D^{app} on enzyme concentration indicates that the substrate–P-450 binding reaction (in the purified P-450$_{C-21}$ preparation in which most of the detergent had been removed) does not follow a simple two-component mass action equilibrium. Therefore, other models were sought to describe the titration data. Many purified enzymes (Neat, 1979) including P-450 enzymes (Autor et al., 1973; Shikita & Hall, 1973; Guerugerich & Halladay, 1979) have been found to exist in oligomeric forms. In addition, in many instances (Neat, 1979) in which the strength of intermolecular interactions is not sufficient to maintain the oligomer state, dilution of the protein favors dissociation into the smallest stable form. In view of these properties of many purified enzymes, a model in which purified P-450$_{C-21}$ exists in a polymerizing equilibrium was considered to explain the protein concentration dependence of K_D^{app}. A simple case in which the P-450 exists in a dimerizing equilibrium was tested. The assumptions made in the mathematical formulation of this model are as follows: (1)

Table III: Effect of Detergents on the Parameters of the Steroid–P-450$_{C-21}$ Binding Reaction[a]

steroid detergent (%)	K_D^{app} (μM)	[E_t] (μM)	K_1 (μM)	K_2 (μM)	SEE, % [S_t]
17α-hydroxyprogesterone cholate					
0	0.468	0.053	0.065	0.009	0.76
0.04	0.066	0.068	0.051	>600	0.89
progesterone cholate					
0	2.73	0.04	1.06	0.09	1.9
0.04	0.37	0.03	0.37	>700	1.7
Emulgen					
0.008	2.73	0.04	1.06	0.09	1.9
0.33	6.34	0.04	4.47	0.14	0.86
0.62	15.41	0.05	9.35	0.14	1.7

[a] The assay system and temperature were as described for Table II. The concentration of P-450$_{C-21}$ was 0.074 μM as determined from the type I spectral change produced by a saturating concentration of 17α-hydroxyprogesterone.

Monomeric P-450 exists in equilibrium with a dimer. (2) At low substrate concentrations, its binding to the dimer is negligible. (3) Under the conditions of the titration experiments, the P-450 does not exist as aggregates of higher molecular weight than a dimer.

The equilibria involved are as follows:

$$K_1 = \frac{[E][S]}{[ES]} \quad (1)$$

$$K_2 = \frac{[E][E]}{[E_2]} = \frac{[E]^2}{[E_2]} \quad (2)$$

$$[S_t] = [S] + [ES] \quad (3)$$

$$[E_t] = [E] + [ES] + 2[E_2] \quad (4)$$

K_1 = the intrinsic dissociation constant of the enzyme–substrate complex, K_2 = the dissociation constant for the dimerization reaction, E = free enzyme, E_t = total enzyme, S = free substrate, and S_t = total substrate.

The four equations are combined algebraically to give

$$2[E]^2 + K_2[E] + (K_2[ES] - K_2[E_t]) = 0$$

$$[E] = \frac{-K_2 \pm \sqrt{K_2^2 + 8K_2[E_t] - 8K_2[ES]}}{4}$$

From eq 1

$$[S] = \frac{K_1[ES]}{[E]}$$

Only the positive solution has physical meaning. The substrate concentrations are then combined according to equ 3. In these equations, the value of [ES] is taken from the experimental data point. The data were analyzed by the curve-fitting procedure described under Materials and Methods. The goodness of fit of the model to the data is indicated by the reasonably low sum of squares of the residuals and standard error of estimate (SEE) expressed as the percent of [S_t] (Table II). As would be expected, the substrate-bound P-450 (ES) increases and the dimer (E_2) decreases with increase in the concentration of the substrate (Figure 3). The parameters of the equilibria involved are shown in Table II. Although there is some scatter in the K_1 and K_2 values, their standard deviations are resonably low. Therefore, K_1 and K_2 are considered essentially independent of enzyme concentration, which is a requirement for the appropriateness of the assumed model to describe the data within the substrate concentration range studied. The concentration ranges studied were 0.01–0.2 μM for 17 α-hydroxyprogesterone and 0.04–2 μM for progesterone.

Although the dimer model fits rather well to the data, it was important to consider models involving oligomers higher than dimer. This is especially because of the reports

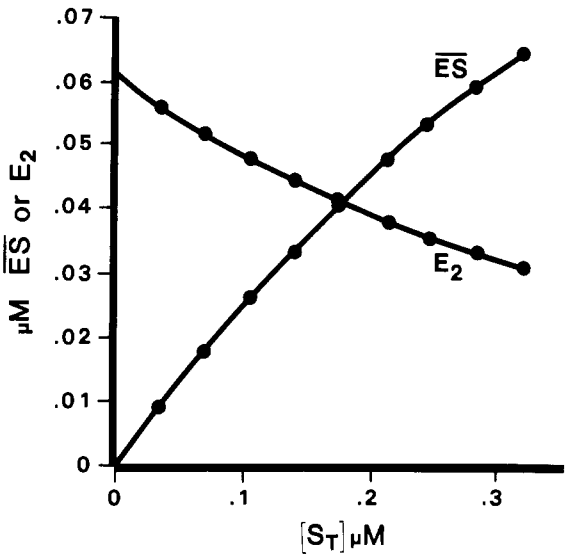

FIGURE 3: Relationship of E_2 and ES. Curves constructed from the theoretical points obtained in the case of the highest P-450 concentration ([E] = 0.141 μM) shown in Table II.

(Gueugerich & Holladay, 1979; Wagner et al., 1984) on the existence of trimers and pentamers in the case of other P-450 enzymes. Models up to pentamer in which the substrate binds only to the monomers were tried with this set of data. The standard errors of estimate for trimer, tetramer, and pentamer were 1–2 times higher than that of dimer. However, these differences are insignificant according to the "F" test.

Effects of Detergents on the Substrate–P-450 Binding Reaction. The ionic detergent cholate strikingly decreased the K_D^{app} of 17α-hydroxyprogesterone as well as progesterone (Table III). In the case of the high-affinity steroid 17α-hydroxyprogesterone, the concentration of the type I complex was considerably higher, and the total P-450 (measured as type I complex) could be titrated with a single K_D^{app} of 0.066 μM. However, in the case of the low-affinity steroid progesterone, only 60% of the total complex was titrated with a decresed K_D^{app} of 0.37 μM, and the remaining 40% had a K_D^{app} of about 4 μM (estimated from the reciprocal plot) as shown in Figure 4. The reason for this difference between the two steroids is not known. By use of the search procedure, further analysis of 17α-hydroxyprogesterone titration data and the high-affinity portion of the progesterone titration (Figure 4) gave the intrinsic parameters shown in Table III. In the absence of cholate, the dissociation constant for the dimerization of P-450 (K_2) is considerably lower than the substrate dissociation constant (K_1) in the case of both steroids. The two steroids differed in their K_1 values. In addition, their K_2 values were also different, indicating that the steroids may influence the dimerization in some manner. In the absence of cholate, K_1

Table IV: Effect of Temperature on the Parameters of the Substrate–P-450$_{C-21}$ Binding Reaction[a]

steroid	temp (°C)	K_D^{app} (μM)	[E_t] (μM)	K_1 (μM)	K_2 (μM)	SEE, % [S_t]
progesterone	15.8	0.96	0.044	0.92	>100	0.26
	26.5	2.7	0.046	1.66	0.253	0.95
17α-hydroxyprogesterone	15.8	0.204	0.052	0.081	0.038	0.55
	26.5	0.468	0.053	0.065	0.006	0.77

[a] The assay conditions were as described under Table III.

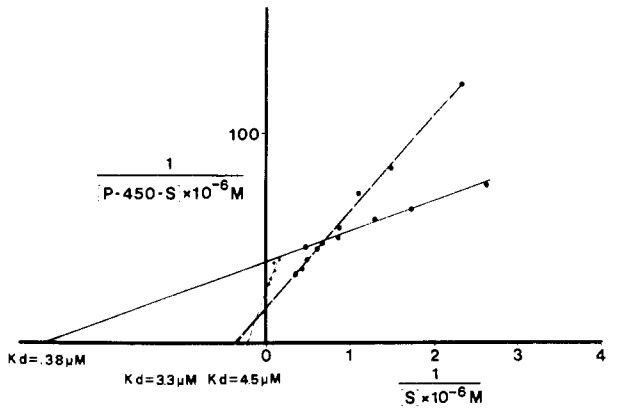

FIGURE 4: Effect of cholate on progesterone binding (Lineweaver–Burk plots). A 3.0-mL aliquot of the basal buffer containing 0.07 μM (measured as 17α-hydroxyprogesterone–cytochrome P-450 complex) P-450$_{C-21}$ and 0.008% Emulgen was titrated with progesterone. Curves are with (—) and without (– –) cholate, respectively. Temperature, 26.5 °C.

is very much less than K_D^{app}. While the ionic detergent decreased K_D^{app} (in the case of progesterone, K_1 also), it increased K_2 strikingly. Under these conditions, the concentration of the dimer was negligible. It should be noted that in the presence of cholate, the K_D^{app} values are equal or nearly equal to K_1 values.

While the ionic detergent decreased the K_D^{app} the nonionic detergent Emulgen 913 increased the apparent dissociation constant (Table III). Increase in the concentration of the nonionic detergent from 0.008% to 0.016% (not shown in the table) had no significant effect on K_D^{app}. About a 40-fold increase in the detergent concentration was required for only a 2-fold increase in the K_D^{app}. At the higher concentrations, however, doubling of the dtergent concentration (from 0.33% to 0.63%) resulted in greater than a 2-fold increase in the K_D^{app}. This result indicates that the detergent has a biphasic effect on the binding reaction. The intrinsic substrate dissociation constant K_1, as well as K_2, increased with increase in Emulgen concentration. The total substrate-P-450 complex formed is either unaltered or somewhat increased at the highest detergent concentration, indicating that the detergent concentrations tested have no detectable denaturation effects on P-450 with respect to the substrate binding reaction.

Effect of Temperature. At 26.5 °C, the K_D^{app} values of progesterone and 17α-hydroxyprogesterone were 2.7 and 0.468 μM, respectively (Table IV). A 10 °C drop in temperature decreased the K_D^{app}'s of the two steroids to 0.96 and 0.20 μM, respectively. Unlike the K_D^{app}'s, the observed as well as the calculated [ES] maxima in the case of the two steroids were independent of temperature as seen in Table IV. However, when tested with progesterone, at concentrations higher than saturation, the type I spectral change exhibited anomalous behavior. The spectral change decreased to a considerable extent.

The effect of temperature on the substrate dissociation constant K_1 and the dissociation constant for the dimerization reaction is shown in Table IV. In the case of both steroids, the decrease in temperature resulted in a considerable increase in K_2 and a decrease in K_D^{app} approaching K_1. The reason for the striking differences in the K_2 values in the case of the two steroids is not known. It is possible that different steroids may influence the self-association of P-450 differently as indicated earlier.

Discussion

The basic procedure which has been used to purify many mammalian cytochrome P-450 enzymes is cholate solubilization of the membrane-bound system in a glycerol-containing buffer medium and hydrophobic column chromatography. By a similar procedure and using an aminooctylamine–Sepharose column, Kominami et al. (1980) have purified cytochrome P-450$_{C-21}$ from bovine adrenocortical microsomes. In the present study, their procedure has been modified, which decreased the time required for purification and increased the yield by 4-fold. It is possible the higher yield is due to the P-450 content of the microsomes being higher (0.50 nmol/mg of protein), and 80% of this is accountable for by P-450$_{C-21}$. The microsomes used by Kominami et al. (1980) and Bumpus & Dus (1982) contained 0.1 nmol of P-450$_{C-21}$/mg and 0.46 nmol of P-450/mg of protein, respectively. In the latter case, the P-450$_{C-21}$ content is not known. Since P-450$_{C-21}$ was eluted as a minor band in their elution profile, it is likely that the P-450$_{C-21}$ content of their microsomes is also low. We believe that the higher P-450$_{C-21}$ content of the microsomes is due to the homogenization procedure we used instead of the blending procedure to prepare the microsomes.

The spectroscopic properties of the purified protein were very similar to those reported by Kominami et al. (1980). However, unlike the experiments of Kominami et al. (1980) in the temperature range studied (15–26.5 °C), temperature dependency of the spin state was not observed in the case of either substrate-free or substrate-bound P-450 at substrate concentrations up to the saturation point. This is indicated by the maximum substrate-bound P-450$_{C-21}$ being the same at the two temperatures. The maximum bound was temperature independent in the case of progesterone as well as 17α-hydroxyprogesterone. However, when tested with progesterone as the substrate, at concentrations 10–12× higher than saturation, there was a considerable decrease in the spectral change. This decrease would be consistent if the high-spin P-450 is converted back to the low-spin state at high substrate concentrations as in the experiments of Kominami et al. (1980). A similar decrease in the type I spectral change at high substrate concentrations has also been observed in the case of P-450$_{11_\beta}$ (Whysner et al., 1968).

As indicated earlier, the increase in the apparent substrate dissociation constant with an increase in the concentration of the purified P-450$_{C-21}$ indicates that the substrate–P-450 binding reaction in this preparation does not follow a simple two-component mass action equilibrium. However, it appears that the data can be adequately described by a model in which an enzyme exists in a monomer–dimer equilibrium and the substrate does not bind to the dimer. The intrinsic substrate dissociation constant K_1 and the dissociation constant for the dimerization reaction K_2 are found to be independent of the P-450 concentration. This indicates the appropriateness of

the assumptions made in the mathematical formulation of the model. The value of K_2 being considerably less than K_1 indicates that the dimerization reaction is much more effective than the substrate binding reaction. That K_1 may be the intrinsic dissociation constant, of the substrate–monomer complex, is indicated by the effects of the ionic detergent cholate. This detergent, like other ionic detergents, may be capable of masking hydrophobic sites which are normally involved in self-association of hydrophobic proteins. Cholate strikingly increased the substrate affinity as indicated by the decrease in K_D^{app} (and K_1 also in the case of the low-affinity steroid progesterone). However, this detergent strikingly increased K_2. The high value for K_2 is consistent with the dimer concentration being negligible. In the absence of the dimer, as would be expected, the substrate dissociation constant K_1 and K_D^{app} are both nearly equal. Accordingly, a decrease in temperature which increased K_2 also descreased K_D^{app}. The nonionic detergent, however, inhibited the substrate binding and the self-association reactions of P-450, as indicated by an increase in K_1 and K_2. Since K_2 is still very much lower than K_1, self-association remains more effective than substrate binding, and a considerable amount of the dimer may be present even at the highest Emulgen concentration.

The observed effects of the two detergents and temperature are consistent with the results reported in the case of other P-450 enzymes. Sedimentation equilibrium analysis studies of Guengerich & Holladay (1979) indicate that an increase in temperature increases the state of aggregation of P-450_{LM_2}. Second, their results indicate that P-450_{LM_2} cannot be dissociated to the monomer state by the nonionic detergent Triton N-101 which is very similar to Emulgen. In the presence of this detergent, the lowest molecular weight of P-450_{LM_2} was a trimer. In the absence of detergents, P-450_{LM_2} existed as a higher molecular weight aggregate. In the presence of an ionic detergent, the P-450_{LM_2} existed as a monomer. Their sedimentation equilibrium analysis results are very similar to ours (S. Narasimhulu and T. F. Kumosinski, unpublished results). However, the concentrations of P-450 used in all sedimentation equilibrium studies are greater than 10 times the highest concentration used in the present titration experiments. Second, concentration dependence of average molecular weight has been observed (Guengerish & Holladay, 1979) in the case of P-450_{LM_2}. Therefore, considering the present results, it is possible that at the low concentration used in the present experiments, P-$450_{C\text{-}21}$ may exist as dimers. However, it should be emphasized that the possibility of oligomers higher than dimer cannot be eliminated.

The functional significance of the state of aggregation of cytochrome P-450 enzymes has been controversial. Autor et al. (1973) suggested that hepatic P-450 exists as an aggregate in the active state complexed with NADPH–P-450 reductase. Ingelman-Sundberg (1977) suggested that it is the monomeric state that is active. Guengrich & Holladay (1979) imply that the state of aggregation has no functional significance. In the present study, since factors which can alter aggregation of P-450 have a profound influence on the substrate dissociation constant, it is possible that self-association of P-$450_{C\text{-}21}$ may be involved in modulating the substrate binding reaction. Whether or not such interactions occur within the membrane is not known. However, since the effects of the detergents and temperature on the apparent substrate dissociation (K_D^{app}) of the purified P-$450_{C\text{-}21}$ are very similar to those in the microsomes (Narasimhulu, 1977, 1978, 1979), the possibility that self-association of P-450 can modulate the substrate binding reaction within the membrane must also be considered. This, however, is not inconsistent with the proposed (Narasimhulu, 1977, 1978, 1979) role of lipid "fluidity" in increasing the K_D^{app} in the microsomal membrnaes with an increase in temperature. This is because increased fluidity may be required for an increase in protein–protein interactions.

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Purification of Human Factor VIII:C and Its Characterization by Western Blotting Using Monoclonal Antibodies†

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ABSTRACT: Human factor VIII:C has been purified over 300 000-fold from cryoprecipitate by polyelectrolyte purification followed by affinity chromatography on Sepharose linked to antibody to factor VIIIR:Ag (monoclonal or polyclonal) and Sepharose linked to monoclonal antibody to factor VIII:C. The purified material has been analyzed by polyacrylamide gel electrophoresis (PAGE) and Western blotting using monoclonal antibodies. PAGE shows predominant bands at 360K (unreduced), 210K, and 90K and an 80K/79K doublet; Western blotting showed all the monoclonal antibodies used bound the 360K form. In a small-scale purification, plasma from blood taken directly into thrombin inhibitor Kabi S-2581 was applied directly to the monoclonal anti-factor VIII:C column. Western blot analysis of this material showed the 360K band on reduction. The purified factor VIII:C could be activated 13-fold by human thrombin. Gel analysis of the activated material showed intensification followed by fading of the band at 90K and generation of bands at 70K/69K, 55K, and 40K. Western blotting shows that the 70K/69K doublet derives from the 80K/79K moiety and the 40K peptide derives from the 90K and is presumed to contain the active site. From these studies an epitope map of the factor VIII:C molecule has been constructed.

The commonest severe congenital bleeding disorder in all races is haemophilia A. This condition is inherited as a sex-linked recessive trait, affecting approximately 1 in 5000 males (Rizza, 1972). The characteristic defect is lack of coagulation factor VIII:C. Factor VIII:C is a glycoprotein (Tuddenham et al., 1979) that functions as a cofactor for the activation of factor X by activated factor IX in the intrinsic coagulation cascade (Mertens & Bertina, 1980) in the presence of phospholipid and calcium. Factor VIII:C is highly susceptible to proteolysis by thrombin, plasmin, and other serine proteases (Atichartakarn et al., 1978). In plasma it is noncovalently linked to von Willebrand factor (Hoyer, 1981). The latter is a high molecular weight protein designated factor VIII related antigen (VIIIR:Ag) consisting of a series of oligomers ranging in size from 10^6 to 20×10^6 through covalent linkage of a 200K protomer (Ruggieri & Zimmerman, 1981; Hoyer & Shainoff, 1982). VIIIR:Ag probably stabilizes factor VIII in vivo by protecting it from proteolysis (Weiss et al., 1977) but is also necessary for platelet adhesion (Weiss et al., 1978). The plasma concentration of factor VIII:C is extremely low, and this combined with its marked tendency to proteolytic degradation has frustrated many attempts to purify it to homogeneity. These problems may be overcome by using very large quantities of starting material as was demonstrated first by Vehar & Davie (1980) for bovine factor VIII. From 125 L of bovine plasma these authors obtained 400 μg of protein that appeared as a triplet of bands of M_r 93K, 88K, and 85K on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

A new approach to purification of factor VIII using specific polyclonal and monoclonal antibodies was reported by Fass et al. (1982) using porcine plasma as starting material. The end product appeared on SDS–PAGE to consist of subunits with M_r 160K, 130K, and 76K. Fulcher & Zimmerman (1982) modified the method of Tuddenham et al. (1978) by substituting a monoclonal antibody to factor VIIIR:Ag for polyclonal antibody and further purified the material on (Aminohexyl)agarose as earlier described by Austin (1979). The starting material for this purification was commercial factor VIII concentrate, and the end product consistently contained high molecular weight contaminants. Also there were strong indications of partial proteolysis. The majority of their material was shown to be factor VIII by binding of specific antibody.

In this paper we describe a method for purifying human factor VIII:C from cryoprecipitate, in which potent proteolytic inhibitors were used throughout. The polyelectrolyte procedure of Johnson et al. (1978) was used as a preliminary step before immunoaffinity chromatography with antibody to factor VIIIR:Ag followed by adsorption to a monoclonal antibody specific to factor VIII:C (Rotblat et al., 1983).

Materials and Methods

Protein was estimated by a modification of the method of Lowry (Hartree, 1972). Factor VIII:C was assayed by a one-stage technique using plasma from a patient with severe haemophilia A (factor VIII:C 0%) as substrate (Breckenridge & Ratnoff, 1962). Factor VIIIR:Ag was measured by a one-stage immunoradiometric assay using rabbit antibody to

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