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Adrenal Mitochondrial Cytochrome P-450$_{scc}$

CHOLESTEROL AND ADRENODOXIN INTERACTIONS AT EQUILIBRIUM AND DURING TURNOVER*

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Purified cytochrome P-450$_{scc}$ from bovine adrenal cortex mitochondria after treatment with BrCN yielded a core peptide which retains heme. The amino acid composition of this peptide was similar to that of the analogous peptide isolated from cytochrome P-450$_{cam}$ of *Pseudomonas putida*.

Adrenodoxin and cholesterol association with P-450$_{scc}$ was analyzed in nonionic Tween 20 micelles where cholesterol appears to be fully in equilibrium with the cytochrome. Adrenodoxin binding to cholesterol-free P-450$_{scc}$ was observed by a type I spectral shift in the cytochrome ($K_d = 4 \times 10^{-7}$ M). Binding to the cholesterol-P-450$_{scc}$ complex was over 10 times stronger ($K_d = 3 \times 10^{-8}$ M). Binding of adrenodoxin to both free enzyme and cholesterol complex was unaffected by Tween 20, indicating a clear separation of the adrenodoxin binding site from the hydrophobic membrane binding domain. Adrenodoxin binding is driven by a large increase in entropy (ΔS = 30 e.u., ΔH = 0 kcal), while cholesterol activation of this binding is a consequence of a further increase in ΔS (from 30 to 53 e.u.) which more than offsets an increase in ΔH (5.7 kcal). The spin states of the complexes of cytochrome P-450$_{scc}$ with both cholesterol and adrenodoxin and of the ternary complex were insensitive to changes of temperature (5-35°C), but the high spin content of the cholesterol complex in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer was raised by increased ionic strength (200 mM KCl, 83%) and by the binding of adrenodoxin (92%). The K_d for adrenodoxin-P-450$_{scc}$ complex and the apparent K_m for adrenodoxin in cholesterol side chain cleavage reaction both increased exponentially with ionic strength. In contrast, the analogous constants for cholesterol were insensitive to ionic strength.

The initial velocity patterns with varied adrenodoxin and cholesterol intersected below the horizontal axis, the apparent K_m for each reactant increasing with increasing concentrations of the second reactant. The true K_m for each reactant was manyfold greater than the respective K_d for complex formation with P-450$_{scc}$. The results, overall, are consistent with a random non-rapid equilibrium mechanism with positive cooperativity (synergism) for the binding of adrenodoxin and cholesterol to P-450$_{scc}$ during turnover.

The rate-limiting step in steroidogenesis, cholesterol side chain cleavage, is a complex process which consumes 3 NADPH, 3 O_2, and 3 H^+, one each for three consecutive monooxygenase reactions (1, 2). In the adrenal cortex, this process is catalyzed by a mitochondrial cytochrome P-450 which is specific for cholesterol side chain cleavage (P-450$_{scc}$)[1] and distinct from the other mitochondrial cytochrome(s) P-450 that catalyze 11β- and 18-hydroxylation of Δ^4-3-ketosteroids (3-6).

The mitochondrial cytochromes P-450 are in many respects similar to the *Pseudomonas putida* P-450$_{cam}$ which functions in camphor hydroxylation (6). We have recently shown immunological cross-reactivity between cytochromes P-450$_{scc}$ and P-450$_{cam}$ (7). BrCN cleavage of bacterial as well as microsomal cytochromes P-450 yields a heme-binding peptide containing 40-50 amino acids which probably retains certain structural features of the heme and substrate binding sites of the cytochrome (8-10). In this paper, we describe the isolation of a similar peptide from cytochrome P-450$_{scc}$, the first to be isolated from a mitochondrial cytochrome P-450.

Monooxygenation by mitochondrial P-450 hemeproteins requires both a flavoprotein (adrenodoxin reductase) and a ferredoxin-type iron-sulfur oxidation-reduction protein (adrenodoxin) which function in the transport of electrons from NADPH to P-450 (6, 11). Also, in this respect, the mitochondrial monooxygenases resemble the camphor hydroxylation system of *P. putida* (6, 11, 12). However, in contrast to the bacterial enzymes which are soluble, the mitochondrial enzymes are membrane-bound; adrenodoxin reductase and ADX appear to be peripheral membrane proteins and P-450$_{scc}$ is an integral membrane protein (11, 13, 14). Recent evidence suggests that ADX transports electrons from adrenodoxin reductase to P-450 by shuttling between these two enzymes (14-17). According to Kido and Kimura, ADX binding to cytochrome P-450$_{scc}$ requires both cholesterol and a detergent or a phospholipid (18, 19), while in apparent contrast, Lambeth *et al.* (20) report that ADX can bind to cholesterol-free cytochrome P-450$_{scc}$ in phospholipid vesicles.

In this paper, we examine the interactions of ADX and cholesterol with cytochrome P-450$_{scc}$ in the presence and

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[1] The abbreviations used are: P-450$_{scc}$, cytochrome P-450 specific for cholesterol side chain cleavage; P-450$_{cam}$, *Pseudomonas putida* cytochrome P-450 specific for camphor hydroxylation; ADX, adrenodoxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

absence of the detergent Tween 20.[2] This detergent provides optimal activity for cholesterol side chain cleavage (17, 21) and its nonionic nature permits the analysis of ion effects on the enzymes independent of interactions of ions and proteins with charged head groups of phospholipid vesicles. Our results indicate that Tween 20 micelles do not significantly affect the affinity of adrenodoxin to P-450$_{scc}$ and provide an environment where cholesterol appears to be fully in equilibrium with the cytochrome. The binding of ADX to P-450$_{scc}$ is shown to be much more sensitive to changes in metal ion concentrations than is cholesterol binding, and this difference is observed to be maintained in the catalytic constants. Steady state kinetics is used to examine the sequence of cholesterol and ADX binding to cytochrome P-450$_{scc}$ during side chain cleavage, and the results are compared with the monooxygenase scheme for camphor hydroxylation by P-450$_{cam}$.

METHODS[3]

Aniline Sepharose--Aniline-Sepharose was prepared by a modification of the method of Cuatrecasas (22). Washed Sepharose 4B (100 ml) was diluted 1:1 with water. BrCN (2.5 ml, 1 g/ml acetonitrile) was added with rapid stirring and the slurry was maintained at pH 11 ± 0.5 by dropwise addition of 5 N NaOH and at 20°C by addition of ice. After 20 min, Sepharose was rapidly washed with 1.5 liters 0.2 M $NaHCO_3$ (pH 9.5) and then added to an aniline-buffer mixture (10 ml freshly redistilled aniline is mixed with 125 ml water, followed by 100 ml 0.2 M $NaHCO_3$). After stirring overnight at 4°C, the aniline-Sepharose was washed successively with 500 ml of 0.1 N sodium acetate (pH 4.0), 2 N urea, and 0.1 N $NaHCO_3$, each containing 0.5 N NaCl. Finally, the aniline-Sepharose was suspended in 50 mM K phosphate buffer, pH 7.3, and 0.02% sodium azide.

Adrenodoxin-Sepharose--A solution of ADX (5 ml, 1.7 μmol with A_{280}/A_{414} = 1.2) was dialyzed against 0.1 M $NaHCO_3$, pH 7.8, 0.4 M KCl. Sepharose-4B (30 ml packed volume) was activated with BrCN as described by March et al. (23). BrCN-activated Sepharose was washed and equilibrated with 30 ml coupling buffer (0.1 M $NaHCO_3$, pH 7.8, 0.4 M KCl). The ADX solution was added and coupling was performed at 4°C for 40 h. After coupling, the slurry was filtered under vacuum. Only 16% of ADX, as determined by A_{414}, was recovered in the wash, indicating coupling of 84% of the ADX preparation. After filtration, the gel was stirred with 30 ml of coupling buffer containing 0.5 M glycine at 4°C for 3 h to mask unreacted groups.

ADX-Sepharose was kept in 10 mM Tris, pH 7.5, 0.5 M KCl, 0.02% sodium azide. Immidiately after using a column, this buffer was run through the column to prevent bacterial growth. Under these conditions, ADX-Sepharose is stable for several months.

Adrenodoxin and Adrenodoxin Reductase--ADX was prepared according to the method of Orme-Johnson and Beinert except that Sephadex G-100 chromatography was substituted for the gel electrophoresis step (24). A final chromatography on Sephadex G-50 in 10 mM Tris buffer, pH 7.5, containing 0.5 M KCl improved the A_{280}/A_{415} ratio from 2.8 to 1.2. AR was prepared according to the procedure of Hiwatashi et al. (25) to A_{272}/A_{450} = 8.4.

Cytochrome P-450$_{scc}$--Cytochrome P-450$_{scc}$ --was prepared by a modification of the procedure of Takemori et al. (26). Beef adrenocortical scrapings (1 kg from 100 adrenals) were washed with several liters of 0.25 M sucrose, homogenized (Waring blender), and centrifuged at 900 X g for 10 min. The resultant supernatant was centrifuged at 16,000 X g for 10 min. The mitochondrial pellet was suspended to a final concentration of 30-40 mg protein/ml in 100 mM K phosphate, pH 7.3, 200 μM EDTA, left overnight at 4°C and then sonicated (power output, 140 watts; Heat Systems - Ultrasonics) in 50-ml portions in a 50-ml beaker for 5 min. During sonication, the beaker was kept on ice, but the temperature of the suspension rose to 35°C at the end of the sonication. The sonicated suspension was centrifuged at 60,000 X g for 60 min. The supernatant was kept for AR purification. The pellet was suspended in 100 mM K phosphate, pH 7.3, 200 μM EDTA to a final protein concentration of 30-40 mg/ml and stored in 30-60-ml portions at -70°C.

The mitochondrial pellet (1400 mg) was thawed and solubilized in 50 mM K phosphate, pH 7.3, 100 μM EDTA, 100 μM dithiothreitol (buffer A) at 15 mg protein/ml with 0.5 mg cholate/mg protein for 1 1/2 h. The suspension was centrifuged (105,000g X 60 min), diluted 1:1 with water and applied to an aniline-Sepharose column (16 cm X 2.2 cm) equilibrated with buffer A (a column of this size can accommodate the cholate extract of at least up to 6 g of mitochondrial protein). The column was washed with 50 ml of buffer A and then 50 ml of buffer A - 0.3% cholate - 50 mM KCl. The red band in the top quarter of the column was not shifted while the eluate contained hemoprotein with λ_{max} 420 nm. Cytochrome P-450 was then eluted from the column with 200 ml of buffer A - 0.3% cholate - 1 M KCl. The red band slowly moved through the column preceded by a yellow band. When the spectra of eluting fractions were examined, a fraction (30 ml) with slight turbidity and low specific activity preceded a fraction in which A_{390}/A_{415} = 1.5 and A_{280}/A_{390} = 3.0. The second fraction was pooled (61 ml, 490 nmol) and fractionated with ammonium sulfate (25-45%) (pH was maintained at 7.2 by dropwise addition of 2 M NH_4OH). The 25-45 fraction was gently resuspended in buffer A - 10% glycerol (15 ml) and dialyzed against 1 liter of the same buffer overnight (210 nmol). This solution remained completely clear and was applied to an aniline-Sepharose column (10 X 1.5 cm) equilibrated with the dialysis buffer. P-450$_{scc}$ bound as a tight red band which was eluted with 0.2 M KCl, 0.2% cholate. The fractions with A_{280}/A_{393} = 1.7 or less were combined (22 ml, 100 nmol), dialyzed against 50 volumes or more of buffer A - 10% glycerol and stored at -20°C. When P-450$_{scc}$ was needed, one aliquot of 30-60 nmol was thawed and applied onto an ADX-Sepharose (0.9 X 10 cm) column equilibrated with buffer A - 10% glycerol. The column was washed with 50 ml of the same buffer and P-450$_{scc}$ eluted with buffer A - 10% glycerol - 400 mM KCl collecting 1.2-ml fractions. Fractions with A_{280}/A_{393} = 1.2 were combined and dialyzed 3 to 4 h against 100 volumes of buffer A - 10% glycerol. P-450$_{scc}$ was stable in this buffer at 4°C for at least a few weeks. The absorbance ratio of 1.2 corresponds to 13 nmol P-450$_{scc}$/mg protein as determined by the Lowry assay (27) and the reduced-CO difference spectrum = 91 (28).

Preparation of Cholesterol-Free P-450$_{scc}$--The purified P-450$_{scc}$ in buffer A - 10% glycerol, was diluted with an equal volume of H_2O and placed in a 3-ml cuvette. ADX, AR and NADPH were added in that order to both the reference and sample cuvette (final concentrations of enzymes were 5.5, 1.2 and 0.04 μM, respectively, NADPH was 0.13 mM) and the progress of the reaction was monitored by scanning the spectra at 360-660 nm at 10 nm/s. Under these conditions, the P-450$_{scc}$ spectra became fully low spin within 5 to 10 min at room temperature. This solution of P-450 was applied on ADX-Sepharose (0.9 X 7 cm), the column was washed with 15 ml buffer A - 10% glycerol, and P-450$_{scc}$ eluted with buffer A - 10% glycerol and 0.4 M KCl. The eluted enzyme contained no significant amount of P-420 (less than 5%), and in the solvent extracts of the enzyme solution, no cholesterol or pregnenolone could be detected.

Hemepeptide--Core peptides containing heme (hemepeptides) were prepared by BrCN cleavage (48 h) of purified P-450$_{scc}$ and subsequent column chromatography on Sephadex G-75 equilibrated with 20% acetic acid in the dark as described for photoaffinity labeled P-450$_{cam}$ by Swanson and Dus (29). Heme content of the hemepeptide was measured using the pyridine hemochrome procedure (30). The hemepeptide was submitted to SDS-PAGE in highly cross-linked gels (15%) (31).

Amino Acid Analyses--Prior to amino acid analysis, the P-450$_{scc}$ preparation was passed over a column of Sephadex G-150 (1.5 X 75 cm) equilibrated with 20% acetic acid to remove high molecular weight trace contaminants which were sometimes noted in heavily overloaded SDS-PAGE gels.

Amino acid analyses were carried out in duplicate with a Beckman, Model 120C amino acid analyzer on aliquots of the hemeprotein and the hemepeptide, hydrolyzed in 5.7 N HCl for 24 and 48 h without prior removal of the heme.

Spectra—All optical spectra were carried out on a DW-2 spectrophotometer (Aminco) operating either in the dual wavelength or split beam modes. ADX binding curves were determined from the ΔA (390–420 nm) responses upon addition of concentrated solutions of ADX in 10 mM Tris buffer (500 μM) to P-450$_{scc}$. Cholesterol binding to P-450$_{scc}$ was determined by measuring the changes at 420 and 390 nm in direct spectra of solutions made up in the appropriate concentration of cholesterol. For ADX binding, it was necessary to calculate the concentrations of bound and free proteins. ΔA_{max} and hence $\Delta\epsilon$ for complex formation was determined by measuring the response induced by saturating concentrations of ADX. The small direct contribution of the ADX absorption was measured independently and subtracted from the type I response. The concentration of P-450$_{scc}$ was quantitated by the reduced CO difference spectrum (28). ADX and adrenodoxin reductase concentrations were determined using ϵ = 10 and 11 $mM^{-1}\ cm^{-1}$ at 414 and 450 nm, respectively (32, 33).

Analysis of Kinetic Data—The steady state kinetic nomenclature used in this paper is that of Cleland (34). The initial velocity pattern presented in Fig. 11 was analyzed by SEQUEN and PINGPONG programs in Fortran (35). These programs fit the data to the intersecting and parallel patterns described by sequential and ping-pong mechanisms, respectively, using a nonlinear least squares method. The lines in Fig. 10 are based on the SEQUEN fit. The lines in Fig. 8 were fit to the data using the Fortran program HYPER (35). The concentration of ADX_{free} in Figs. 8 and 10 was calculated as previously described (17). The K_d values for the adrenodoxin reductase-ADX complex (2.5–40 × 10^{-8} M) were taken from Fig. 2 of Lambeth *et al.* (16), and K_d values for the ADX P-450$_{scc}$ complex (1.5–58 × 10^{-8} M) were estimated by binding experiments (Fig. 8, *inset*).

Other Procedures—Lipid content was measured by analysis of organic phosphate after Folch extraction (36, 37) and cholesterol content by gas-liquid chromatography using [^{3}H]cholesterol to quantitate recoveries after extraction by methanol and hot ethyl acetate. Unless otherwise specified, all protein determinations were done using the biuret procedure (38). Cholesterol side chain cleavage was assayed in 0.3% Tween 20 at 37°C. The preparation of Tween 20-cholesterol solution and the assays of [^{3}H]cholesterol conversion to [^{3}H]pregnenolone were carried out as previously described (39).

RESULTS

In the present studies, cytochrome P-450$_{scc}$ has been purified to high specific activity (13 nmol of P-450/mg of protein,

[2] These studies were presented in part at the American Society of Biological Chemists Minisymposium on Cytochrome P-450 in New Orleans, Louisiana, June 5–8, 1980, by I. Hanukoglu, C. T. Privalle, and C. R. Jefcoate.

[3] Portions of this section are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-1559, cite author(s), and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

A_{280}/A_{393} = 1.2, > 10 preparations) using a modification of the procedure of Takemori *et al.* (26) in which the cytochrome is eluted from an ADX-Sepharose column in concentrated form and free of detergent. The preparations typically contain 0.8 to 1.0 nmol of cholesterol and 2 nmol of phospholipid/nmol of P-450. The amino acid composition of the protein (Table I) is essentially identical with that reported by Katagiri *et al.* (40) and similar to that of Akhrem *et al.* (41); however, it deviates in certain values significantly from a composition reported by Tilley *et al.* (42) even when adjusted to the same weight of 47 × 10^3.

Characterization of the "Hemepeptide"—A core peptide containing 20% of the heme of P-450$_{scc}$ was readily generated from cytochrome P-450$_{scc}$ by degradation with BrCN and resolved from other peptides (29). The amino acid composition of this peptide is shown in Table I. The Soret maximum (ϵ_{mM} = 62 in 5% acetic acid) at 360 nm (Fig. 1*B*) is clearly distinct from that of free heme (Fig. 1*C*). The 390 nm shoulder of the hemepeptide spectrum probably, in part, reflects the absorbance of dissociated heme, which under these conditions, has a Soret maximum at 393 nm.

The reduced-CO complex of the hemepeptide has a Soret maximum at 406 nm while that of hemin is at 410 nm. The extinction coefficient of the complex is increased by decreased polarity in the solvent, but even in 5% acetic acid/acetone (1:1), the extinction coefficient of the heme-CO complex (ϵ_{mM} = 45) is substantially less than in the peptide (ϵ_{mM} = 68).

Binding of Adrenodoxin and Cholesterol to P-450$_{scc}$—Detergent-free P-450$_{scc}$, as isolated, was mostly in a high spin form, probably due to complex formation with the endogenous cholesterol. In the presence of Tween 20, the P-450$_{scc}$ preparation became fully low spin. Restoration of the high spin state by addition of cholesterol to Tween 20, indicated that the Tween 20-induced shift to low spin derived from dissociation of endogenously bound cholesterol into the detergent. ADX bound to detergent-free P-450$_{scc}$ with high affinity and with a type I difference change (Fig. 2). ADX also bound to

TABLE I

Amino acid composition of cytochrome P-450$_{scc}$ and its cyanogen bromide-derived hemepeptide, as compared to those of P-450$_{cam}$

These values are based on the occurrence of 11 residues of methionine/molecule of P-450$_{scc}$ hemeprotein and 1 residue of histidine/molecule of P-450$_{scc}$ hemepeptide.

Amino Acids	Hemeproteins		BrCN-Derived Hemepeptides	
	P-450$_{cam}$	P-450$_{scc}$	P-450$_{cam}$	P-450$_{scc}$
$CySo_3H$	6	4	1	1
Asx	36	37	3	3
$MetSo_2$	9	11	-	-
Thr	19	20	2	2
Ser	21	22	3	2
Glx	55	48	3	5
Pro	27	26	3	2
Gly	26	22	8	3
Ala	34	20	6	3
Val	24	22	4	3
Ile	24	22	2	2
Leu	40	40	3	4
Tyr	9	14	1	2
Phe	17	23	2	2
His	12	12	1	1
Lys	15	27	2	3
Arg	24	21	2	2
Trp	1	5	-	-
HSeLac	-	-	(~1)	(~1)
Total	397	396	47	41

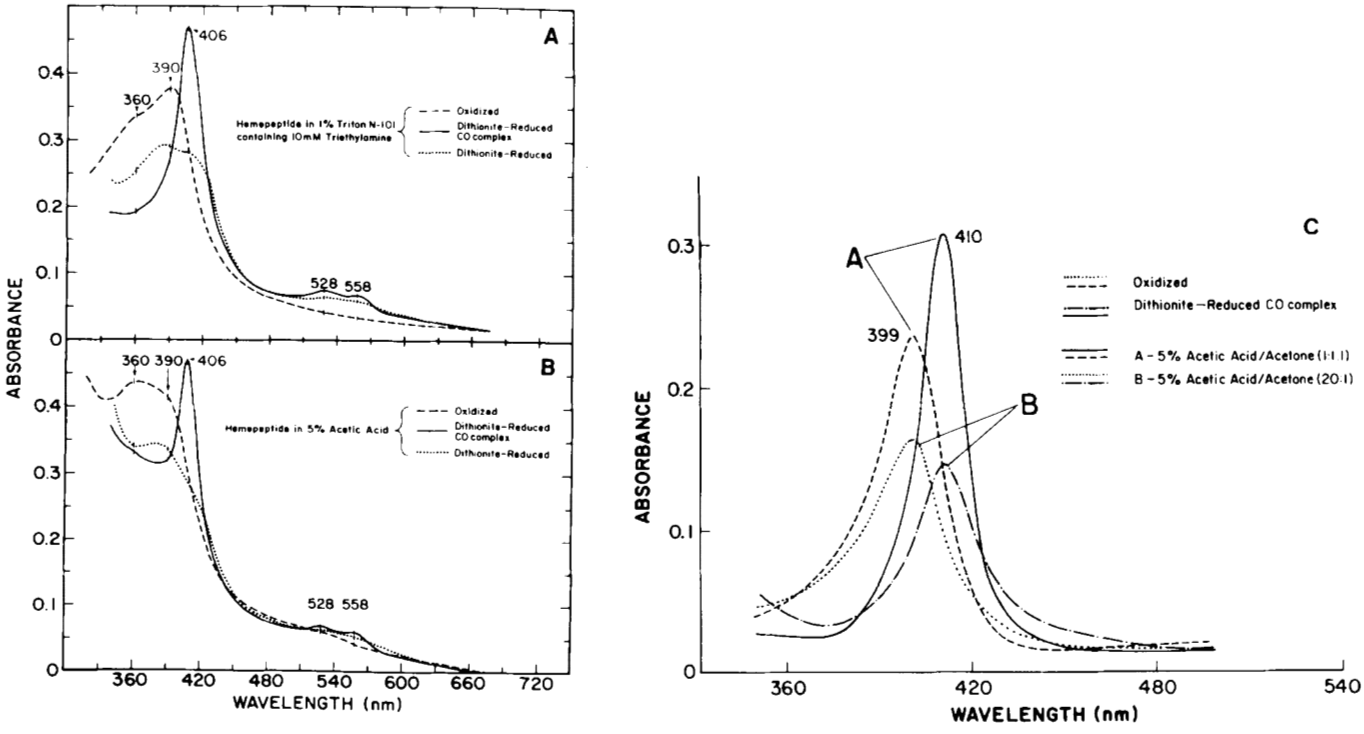

FIG. 1. **Absorption spectra of P-450$_{scc}$-hemepeptide and free heme.** *A*, oxidized, dithionite-reduced, and dithionite-reduced CO complex in 10 mM triethylamine containing 1% triton, or *B*, in 5% acetic acid. *C*, oxidized heme (6.5 μM) and dithionite-reduced CO complex in 5% acetic acid/acetone; (*A*) 1:1.1, (*B*) 20:1.

P-450$_{scc}$ which was fully depleted of endogenously bound cholesterol (see "Methods"), as observed by a type I difference spectrum (λ_{max} 385, λ_{min} 425 nm) which was shifted by the presence of 0.3% Tween 20 (λ_{max} 383, λ_{min} 417 nm) (Fig. 2).

The affinity of cholesterol-free P-450 for ADX was not significantly affected by 0.3% Tween 20 (K_d = 0.4 μM in both cases). The affinity for ADX was greatly enhanced by the presence of cholesterol (*e.g.* 6 times by 90 μM cholesterol, Fig. 3). The K_d for ADX binding at saturation of cholesterol, calculated from the plot in Fig. 3 (*inset*) was enhanced 13-fold over binding in absence of cholesterol. Similarly, 0.5 μM ADX enhanced the affinity of P-450$_{scc}$ for cholesterol by 6 to 7 times (data not shown). Affinity of ADX to P-450$_{scc}$ in Tween 20 with 200 μM cholesterol was 2 times stronger than to P-450$_{scc}$ formed in a complex with endogenous cholesterol in absence of Tween 20 (the percentage of P-450$_{scc}$ formed in a complex with cholesterol was similar under the two conditions), suggesting a small stabilizing effect of Tween 20 on the ternary complex of ADX-cholesterol-P-450$_{scc}$ (Fig. 2).

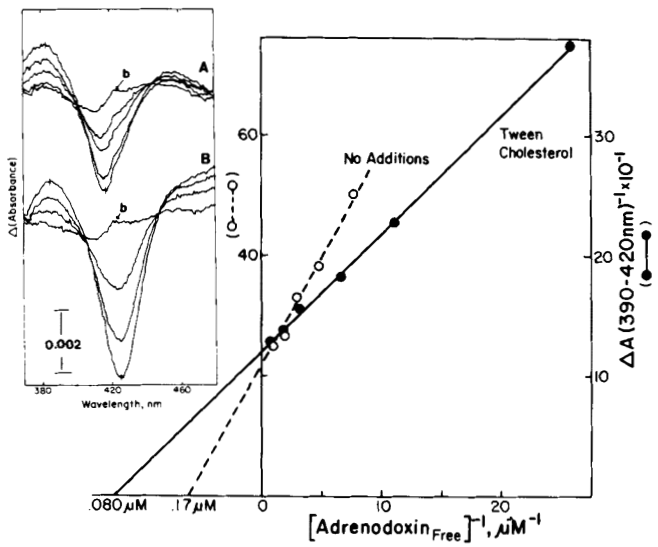

FIG. 2. **Adrenodoxin binding to cytochrome P-450$_{scc}$ in the presence and absence of Tween 20.** ADX was added to P-450$_{scc}$ (0.16 μM) at 35°C in 25 mM K phosphate (○- - -○) and with 0.3% Tween 20 and 200 μM cholesterol (●——●). Difference spectra (*inset*) show addition of ADX (0.5 μM increments) to cholesterol-depleted P-450$_{scc}$ (0.82 μM) with (*A*) and without 0.3% Tween 20 (*B*). The baseline prior to addition of ADX is indicated (*b*).

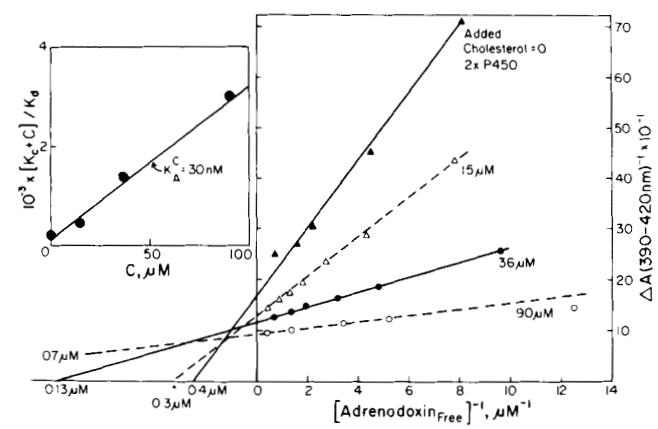

FIG. 3. **Effect of cholesterol on adrenodoxin binding to cytochrome P-450$_{scc}$.** Type I spectral responses were measured at 35°C for addition of ADX to cytochrome P-450$_{scc}$ (0.15 μM) in 0.3% Tween 20, 5 mM K phosphate, 50 mM KCl, and 0, 15, 36, or 90 μM cholesterol. [ADX$_{free}$] was calculated as described under "Methods." The values at the *x* axis intercept represent the K_d for the ADX-P-450 complex at each cholesterol concentration. *Inset*, replot of K_d *versus* cholesterol concentration (*C*) based on the relationship (modified from Ref. 53)

$$[K_C + C]/K_d = C/K_{CA} + K_{AC}/K_{CA}$$

where K_{CA} is the constant for dissociation of ADX from the P-450-cholesterol complex and K_{AC} is the constant for dissociation of cholesterol from the P-450-ADX complex. The slope of the graph is $1/K_{CA}$.

The spin states of the various P-450$_{scc}$ complexes have been calculated from changes in the Soret absorption bands relative to the spectrum of a fully low spin cholesterol-free cytochrome (Table II). The proportions of high spin state induced at saturation with only ADX or only cholesterol were 20% and 67%, respectively; whereas at saturation of both, it was 92%, suggesting an additive effect of the two ligands on this parameter (Table II).

Effect of Temperature on Complex Formation—The binding of ADX to cytochrome P-450$_{scc}$ in the absence of cholesterol was insensitive to temperature changes from 22–35°C. However, below 22°C, complex formation increased with decrease of temperature. The differences shown in Fig. 4 between complex formation at 6°C and 22°C were measured by changing the temperature on a single sample and were therefore directly observable. Spectral changes produced by warming and cooling the sample in the range 6–35°C were fully reversible.

Elevated temperature decreased the affinity of P-450$_{scc}$ for cholesterol (K_d (36°C) = 100 μM, K_d (5°C) = 35 μM) (Fig. 5). Expression of this data as a Van't Hoff plot provided ΔH =

TABLE II

Spin states of cytochrome P-450$_{scc}$ complexes

Complex	Temperature	[KCl]	High spin state[a]
	°C	mM	%
ADX-P-450	5–30	0–50	20
Chol-P-450[b]	5	50	73
	30	50	67
	30	0	60
	30	200	83
Chol-ADX-P-450[b, c]	5	50	90
	30	50	92

[a] High spin state is calculated from absolute absorption spectra in 0.3% Tween, 5 mM Hepes (pH 7.2) from the change (ΔA) in *A* (390–420 nm) relative to the fully low spin spectrum of P-450$_{scc}$ without added cholesterol or ADX. The increase in percentage high spin state is ΔA/nmol P-450/0.11 × 100, which is based on $\Delta\epsilon$ = 110 cm^{-1} mM^{-1} for a complete spin state change.

[b] Calculated from the extrapolation of ΔA to saturation with cholesterol.

[c] 2.0 μM ADX.

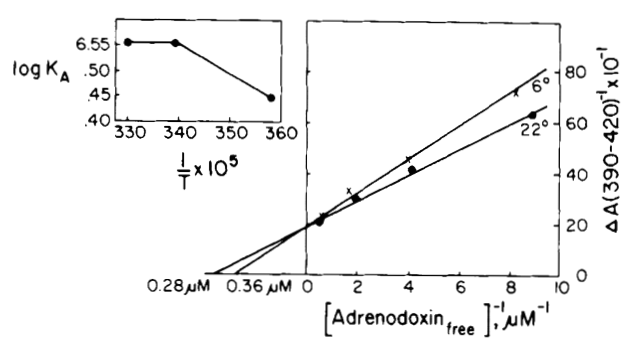

FIG. 4. **Temperature dependence of adrenodoxin binding to cytochrome P-450$_{scc}$.** Cytochrome (0.55 μM) was in 13 mM Hepes (pH 7.2), 0.3% Tween 20, 0.5 mM dithiothreitol, and 50 mM KCl. Measurements at 6°C, 22°C, and 30°C were all carried out on the same solution.

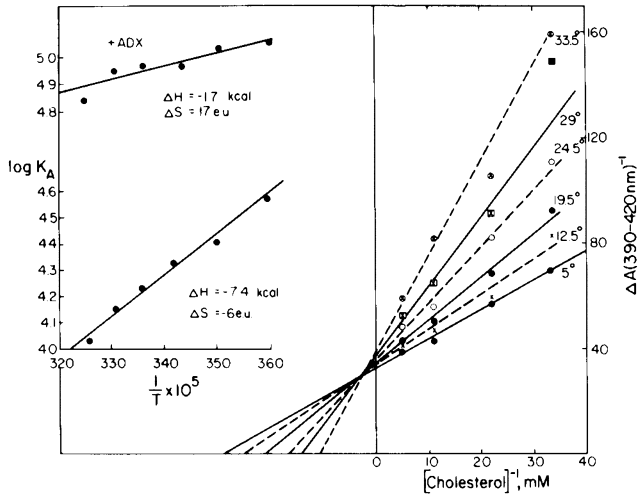

FIG. 5. **Temperature dependence of cholesterol binding to cytochrome P-450$_{scc}$.** Cytochrome (0.37 μM) was in the buffer described in Fig. 4. The *inset* shows Van't Hoff plots of these data and for a parallel experiment with 0.5 μM ADX.

-7.4 kcal mol^{-1} and $\Delta S = -5.9$ e.u. In the presence of ADX (0.5 μM), K_d was less temperature-sensitive with $\Delta H = -1.7$ kcal mol^{-1} and $\Delta S = 17$ e.u. (Fig. 5). The increase in temperature induced only a slight decrease in the proportion of high spin state in the cholesterol-P-450$_{scc}$ complex formed by saturating levels of cholesterol (73% at 5°C to 67% at 30°C). The spin state of the ternary cholesterol-ADX-P-450$_{scc}$ complex was also relatively insensitive to temperature (Table II).

Effect of Ions on Complex Formation—Increasing concentrations of NaCl increased the K_d for ADX both in the absence and presence of 45 μM cholesterol (Fig. 6). Effects of NaCl on the optical change at saturation of ADX, both with and without cholesterol, were negligible (Fig. 6). In contrast, although NaCl progressively increased the proportion of high spin cytochrome at saturation with cholesterol (60–85%), it did not significantly change the K_d for cholesterol (Fig. 7). NaCl also increased the proportion of high spin cytochrome P-450 when added to the cholesterol complex in the absence of Tween 20. $CaCl_2$ (1–5 mM) had no effect on either the affinity of the cytochrome for cholesterol or the spectrum of the complex.

Effect of Ionic Strength on Adrenodoxin and Cholesterol Dependence of Side Chain Cleavage Activity—When cholesterol side chain cleavage was reconstituted from purified adrenodoxin reductase, ADX, and cytochrome P-450$_{scc}$ in the presence of 0.3% Tween 20, activity in 5 mM Hepes buffer was greatly enhanced by the addition of univalent ions. At 5 μM ADX, we observed a similar bell-shaped dependence on the concentration of added NaCl to that previously reported by Takikawa *et al.* (21) with peak activity between 80 and 150 mM KCl. However, the fall-off in activity at high salt was caused by an increase in the K_m for ADX which paralleled the increase in the K_d for ADX (Fig. 8, *inset*). Indeed, at 200 mM NaCl and 200 μM cholesterol, the V_{max} in terms of saturation with ADX was at the highest value of 30 nmol of pregnenolone/nmol P-450/min. The same dependence of activity on ionic strength was obtained when Hepes/NaCl was replaced by increasing concentrations of K phosphate buffer. In this buffer, salt had only a slight effect on the K_m for cholesterol up to 42 mM K phosphate (ionic strength equivalent to 100 mM NaCl) (Fig. 9). However, at 82 mM K phosphate (equivalent to 200 mM NaCl), there was a decrease in the apparent K_m for cholesterol. Thus, a 2-fold increase in ionic strength (100–200) produces a 15-fold increase in K_m for ADX and, at most, a 2-fold decrease in K_m for cholesterol.

Side Chain Cleavage Activity: Initial Velocity Patterns—In order to determine the kinetic mechanism of cholesterol and ADX additions during each cycle of cholesterol side chain cleavage, initial velocity pattern studies were carried out. Plots of v^{-1} *versus* $[ADX_{free}]^{-1}$ exhibited a decreasing slope

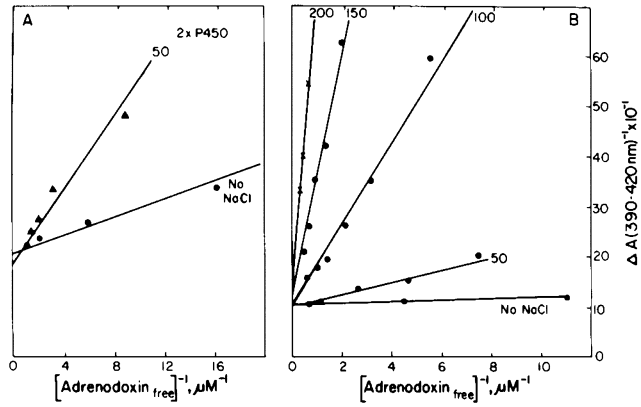

FIG. 6. **Effect of NaCl on adrenodoxin binding to cytochrome P-450$_{scc}$.** *A*, in absence of cholesterol; *B*, in presence of 45 μM cholesterol. Type I spectral changes were measured with cytochrome P-450$_{scc}$ (*A* = 0.24 μM; *B* = 0.12 μM) present in 0.3% Tween 20, 5 mM Hepes (pH 7.2), and 0.5 mM dithiothreitol at 30°C.

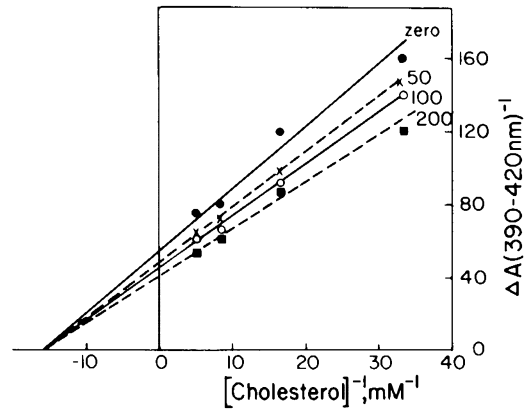

FIG. 7. **Effect of NaCl on cholesterol-binding to cytochrome P-450$_{scc}$.** Type I spectral changes were measured with cytochrome P-450$_{scc}$ (0.28 μM) present in 13 mM Hepes (pH 7.2), 0.3% Tween 20 at 30°C.

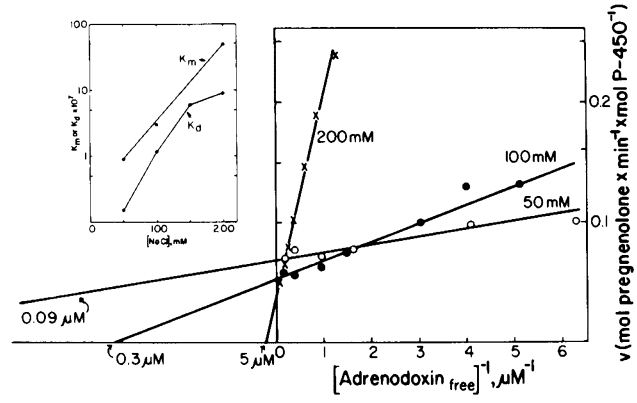

FIG. 8. **Effect of NaCl on adrenodoxin dependence of cholesterol side chain cleavage activity.** The assays were carried out in 10 mM Hepes buffer (pH 7.2) with 0.3% Tween 20, 200 μM cholesterol, 0.3 μM adrenodoxin reductase, and 0.2 μM P-450$_{scc}$. The concentrations of NaCl are indicated next to each reciprocal plot. *Inset* shows the K_m values determined by these reciprocal plots and the K_d values determined in the same buffer with 200 μM cholesterol and 0.125 μM P-450$_{scc}$.

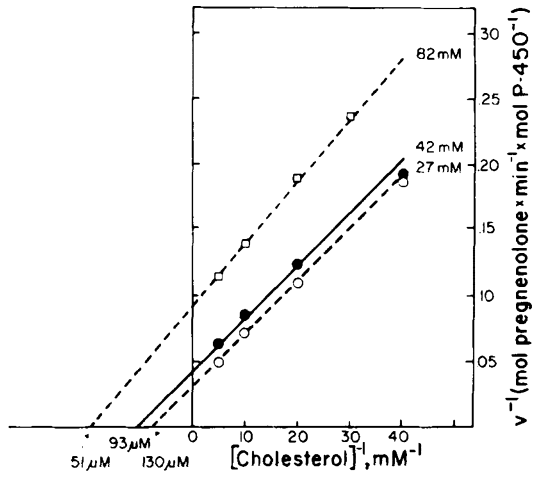

FIG. 9. **Effect of ionic strength on cholesterol dependence of cholesterol side chain cleavage activity.** The assays were carried out in 27, 42, or 82 mM K phosphate buffer (pH 7.2) with 10 mM KCl, 0.3% Tween 20, 200 μM cholesterol, 0.25 μM adrenodoxin reductase, 4.5 μM ADX, and 0.22 μM P-450$_{scc}$.

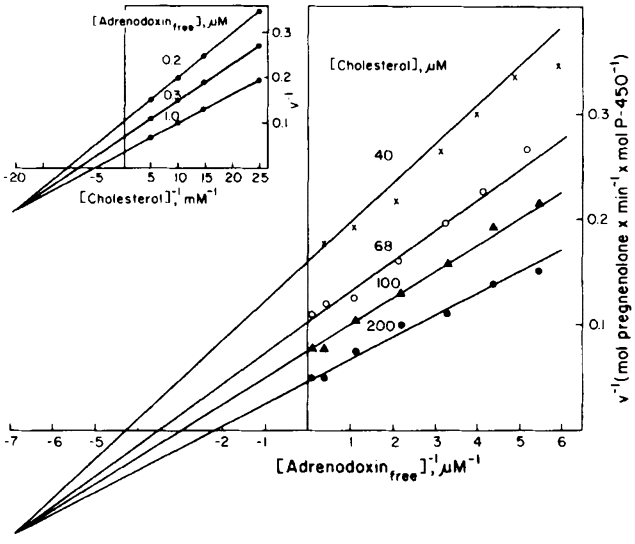

FIG. 10. **Initial velocity pattern analysis of cholesterol side chain cleavage reaction with varying adrenodoxin at constant cholesterol concentrations.** The *inset* shows some of the same data plotted with varying cholesterol at constant ADX$_{free}$ concentrations. The assays were carried out in 10 mM Hepes buffer (pH 7.2) with 100 mM NaCl, 0.3% Tween 20, 0.3 μM adrenodoxin reductase, and 0.2 μM P-450$_{scc}$.

with increasing cholesterol, intersecting at a single point (Fig. 10). The same data replotted as v^{-1} *versus* [cholesterol]$^{-1}$ at various [ADX$_{free}$] also provides a set of intersecting lines (Fig. 10, *inset*). A notable feature of these plots is that the apparent K_m increases in both cases as the concentration of the fixed component is increased. The true K_m values corresponding to saturation of the second component were computed to be approximately 390 μM for cholesterol and 1.2 μM for ADX. The V_{max} at saturation of both cholesterol and ADX was 68 nmol of pregnenolone/min/nmol of P-450.

DISCUSSION

Cytochrome P-450$_{scc}$ and the proteins mediating transfer of electrons from NADPH to P-450$_{scc}$, adrenodoxin reductase and ADX are located on the matrix side of the inner membranes of adrenal cortical mitochondria (6, 14, 43). P-450$_{scc}$ is an integral membrane protein, whereas adrenodoxin reductase and ADX appear to be peripheral membrane proteins (14). Cholesterol, the substrate, is very insoluble in water (CMC ≈ 30 nm, Ref. 44) and more readily gains access to the cytochrome within detergent micelles or phospholipid bilayers (14).

The extensive studies on *P. putida* ferredoxin-cytochrome P-450$_{cam}$-catalyzed camphor monooxygenation provide the basis for our current understanding of the catalytic cycle for this class of reactions (12). Many similarities are apparent between cytochromes P-450$_{cam}$ and P-450$_{scc}$, including immunological cross-reactivity (7) and inter-relationship of substrate and ferredoxin binding to the cytochromes. Cytochromes P-450$_{scc}$ and P-450$_{cam}$ have nearly identical sizes while the amino acid composition of P-450$_{scc}$ differs most noticeably from that of P-450$_{cam}$ in the large increases in aromatic amino acids and lysine as compared to a substantial decrease in alanine (Table I).

Hemepeptide—P-450$_{scc}$ also shares with P-450$_{cam}$ and P-450$_{LM-2}$ a structural feature which permits release with BrCN of a very hydrophobic, heme-binding core peptide which in each case comprises about 10% of the total proteins (7, 8, 29). The peculiar spacing of methionine residues in the surrounding sequences possibly makes this domain fortuitously accessible via BrCN cleavage. The purified hemepeptide of P-450$_{scc}$ was found to contain 20% of the heme of the native hemeprotein. However, recoveries of up to 80% have been observed in hemepeptides released from P-450$_{scc}$ photocovalently labeled at a site close to the heme binding site by an azido derivative of the inhibitor, aminoglutethimide. For other P-450 cytochromes, a substrate-based photoaffinity probe also greatly increases this recovery, thus further relating the peptide to the functional center of the cytochrome (8, 29).

Adrenodoxin and Cholesterol Binding to P-450$_{scc}$—There has been a conflict in previous reports (18–20) concerning the binding of ADX to substrate depleted P-450$_{scc}$. This study clearly shows that ADX binds to P-450$_{scc}$ metabolically depleted of endogenous cholesterol in the presence or absence of detergent Tween 20 (Fig. 2). The kinetics of cholesterol association with P-450$_{scc}$ in Tween 20 (Fig. 5) indicate that in Tween 20 micelles cholesterol is fully in equilibrium with the cytochrome. The positive cooperativity we observe between the interactions of cholesterol and ADX with P-450$_{scc}$ in Tween 20 is quantitatively similar to that reported by Lambeth *et al.* for P-450$_{scc}$ in lecithin-cholesterol vesicles (20). In the present studies, we also show that Tween slightly activates rather than inhibits the binding of ADX to the P-450$_{scc}$-cholesterol complex and that it has no effect on binding to the substrate-free cytochrome. This insensitivity to Tween 20 implies that the hydrophobic domain which anchors the cytochrome to membranes is clearly separated from the ADX binding site. The small perturbation of the Soret band of the substrate-free cytochrome by Tween 20 does, however, suggest a communication between the membrane environment and the active center (Fig. 2, *inset*). Seybert *et al.* have established that cholesterol gains access to the substrate binding site through the membrane (14).

ADX binding to P-450$_{scc}$ is driven by a large increase in entropy. Consideration of the full cycle (Fig. 11) indicates that cholesterol activation of this binding is a consequence of a further increase in ΔS (from 30 to 53 e.u.) which more than offsets an increase in ΔH (5.7 kcal). The high negative charge on the surface of ADX and the observed competition between ions and ADX for the cytochrome suggest that the entropy increase may derive from a release of water and ions during the interaction of complementary charged domains on the two proteins.

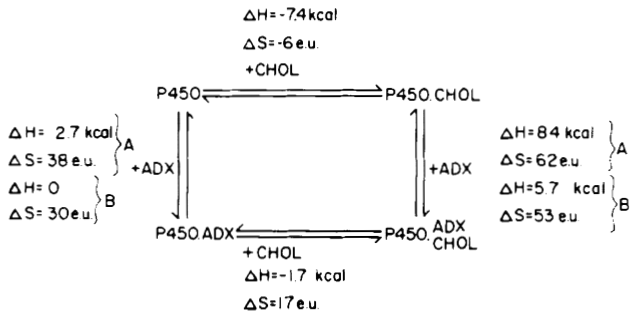

FIG. 11. **Entropy and enthalpy changes for binding of cholesterol (*CHOL*) and adrenodoxin to cytochrome P-450$_{scc}$.** The values were calculated from data represented in Figs. 4 and 5. *A* at 22°C, *B* at 35°C.

The spin state change from low to high spin is associated with an increase in entropy (14–30 e.u.) and a decrease in enthalpy (2.5–10 kcal) (46, 53).[4] Subtraction of the contribution from the spin state change (60%) indicates that cholesterol binding is associated with large opposing changes in enthalpy and entropy. The transference of cholesterol from detergent to binding site therefore seems to be associated with restricted movement, possibly of both cholesterol and protein, which is partially relieved by prior binding of ADX.

The spin state of cytochrome P-450 is an indicator of the configuration of ligands around the heme (12). Increases in the proportion of the high spin state are also frequently associated with facilitated reduction of the cytochrome (45). Both ADX and cholesterol shift the spin state equilibrium of P-450$_{scc}$ to high spin form (17–20, 51). This provides a difference from P-450$_{cam}$ where camphor stabilizes the high spin form but putidaredoxin causes a shift to the low spin form (12, 52).

The high spin state induced by a fixed concentration of cholesterol declines with rising temperature. In Tween 20, this change derives from an increased dissociation of cholesterol rather than from an intrinsic change in the spin state of the substrate complex, as has been reported for P-450$_{cam}$ (46). The similar temperature-dependent change to low spin observed for the P-450$_{scc}$ complex with endogenous cholesterol in absence of Tween 20 (51) presumably also derives from movement of cholesterol out of the substrate site. However, cholesterol remains bound to the protein under these conditions, suggesting that cholesterol moves to a secondary site on the protein associated with a low spin complex. ADX and ions increase the proportion of high spin state in the cholesterol-P-450$_{scc}$ complex by comparable amounts (Table II) suggesting that an ionic effect on the ADX site may alone be sufficient to communicate a change in heme configuration from the surface of the cytochrome. However, changes in spin state in absence of cholesterol and activation of cholesterol binding are induced by ADX but not by elevation of ionic strength.

Ionic Effects on Adrenodoxin and Cholesterol Dependence of P-450$_{scc}$ Activity—Previous studies by Takikawa *et al.* (21) have indicated an unusual "bell-shaped" dependence of cholesterol side chain cleavage activity on univalent cation concentration. However, we have recently shown that although, on the basis of ionic strength, activation by Mg^{2+} follows a pattern similar to univalent metal ions, Ca^{2+} causes very little activation and inhibits side chain cleavage activity optimally activated by 100 mM NaCl (54). Activation at low ionic strength can in part be ascribed to the activation of ADX reduction (16). Activation of ADX reduction also results in decreased levels of oxidized ADX which we have previously shown to inhibit monooxygenase activity (17, 47). Here, we show that the inactivation at high concentrations of univalent ions is only apparent and is due entirely to an over 50-fold increase in the K_m for ADX which parallels the increase in the K_d for ADX (Fig. 8). Indeed, V_{max} at 200 μM cholesterol continues to increase up to the highest salt concentration examined. In contrast, the apparent K_m and the K_d for cholesterol are both relatively insensitive to changes in salt concentration (Figs. 7 and 9). The shift in the apparent K_m for cholesterol at the highest salt concentration examined is in the same direction as that caused by decreased [ADX] (Fig. 10). Thus, this shift is probably due to an increase in the K_m for ADX (Fig. 8) (which would render the [ADX] used in this experiment less than saturating), rather than a true decrease in the K_m for cholesterol.

Complex Formation during Enzyme Turnover—The catalytic cycle for camphor monooxygenation at P-450$_{cam}$ has been presented as an ordered sequence of substrate binding followed by putidaredoxin binding and first electron transfer (12, 48). However, our results, as well as those of Lambeth *et al.* (20), clearly indicate that ADX can bind to P-450$_{scc}$ that is free of substrate cholesterol. Since on the basis of these results there appears to be no obligatory order for the binding of ADX and cholesterol to P-450$_{scc}$, the sequence of additions of these two reactants would have to be considered as random rather than ordered (34, 49). Superficially, the intersecting initial velocity pattern and the linearity of the plots (Fig. 10) are consistent with a fixed order of binding of cholesterol and ADX (Ref. 49, p. 564). However, apparent order can be reconciled with random binding if, at these relative concentrations of reactants under these experimental conditions, the addition of one of the reactants occurs first with much higher frequency. These reaction conditions are far removed from those found in the adrenal cortex mitochondria where high local concentrations of both ADX and P-450$_{scc}$ on the surface of the inner membrane and a low steady state level of cholesterol may result in frequent binding of ADX to cholesterol-free P-450$_{scc}$.

Given the substantial synergism in the binding of cholesterol and ADX to P-450$_{scc}$, the observed initial velocity patterns in which cholesterol increases the K_m for ADX and *vice versa* (point of intersection below the abscissa, Fig. 10) are atypical for a random rapid equilibrium mechanism (Ref. 49, p. 274). Indeed, there is strong evidence that, unlike camphor hydroxylation (48), rapid equilibration of these reactants does not occur during cholesterol side chain cleavage. The true K_m values for cholesterol and ADX are manyfold larger than the respective K_d values for either binary or ternary complex formation. This is normally observed when the rate-limiting step is faster than the dissociation of the substrates by similar factors. Simulation of kinetic patterns corresponding to such nonrapid equilibrium conditions shows that synergism in the binding of reactants can be consistent with an intersection point below the abscissa (50).

The apparent slow dissociation of reduced ADX from P-450$_{scc}$ raises the important question of whether oxidized ADX dissociates from the cytochrome during the catalytic cycle. Possibly, dissociation may be accelerated by either reduction of P-450$_{scc}$ or oxidation of cholesterol. In view of the evidence that adrenodoxin reductase, ADX, and P-450$_{scc}$ do not readily form a ternary complex (14–17), the dissociation of oxidized ADX from P-450$_{scc}$ must be considered a potential rate-limiting factor in the cholesterol side chain cleavage mechanism.

[4] These authors use the sign convention opposite to that used here. Our data derive from the conventional relationship $\Delta G = -RT \ln K_{eq} = \Delta H - T\Delta S$.

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