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THE SIDE-CHAIN CLEAVAGE OF CHOLESTEROL SULFATE—I. THE EFFECT OF ADRENODOXIN ON THE BINDING OF CHOLESTEROL SULFATE TO CYTOCHROME P-450scc

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Summary—Difference spectroscopy was used to measure the binding of cholesterol sulfate (CS) to cytochrome P-450scc. The uncomplexed cytochrome and the complex of the cytochrome with adrenodoxin (ADX) were both titrated with CS in order to test whether ADX increased the affinity of the cytochrome for the sterol sulfate. The addition of ADX to the cytochrome had different effects on the binding of the sterol sulfate depending on several factors including: (1) The method of preparation of the cytochrome P-450scc, (2) The concentration of cytochrome P-450scc, (3) The method by which CS was suspended in aqueous solution, and (4) Whether or not the solutions of cytochrome contained non-ionic detergents. The results of this study suggest that the method of isolation of cytochrome P-450scc, and non-ionic detergents, greatly modulate the apparent affinity of cytochrome P-450scc for CS. In the absence of detergents the addition of adrenodoxin to dilute solutions of cytochrome P-450scc appears to enhance only slightly (1- to 2-fold) the affinity of the cytochrome for the sterol sulfate.

INTRODUCTION

Bovine adrenal tissue contains a substantial amount of cholesterol 3-monosulfate (1.5 mg/kg) [1]. In addition, approx. 9% of the total amount of pregnenolone isolated from bovine adrenal cortex is sulfated at the 3β position [2]. It has been shown that cholesterol sulfate can be converted to pregnenolone sulfate *in vivo* without the loss of the sulfate moiety [3]. Moreover, cholesterol sulfate is a better substrate than unesterified cholesterol for side-chain cleavage when intact mitochondria from rat adrenals are employed as the source of a cholesterol side-chain cleavage system [4]. Although steroid sulfates are major metabolites of human fetal adrenal and testicular tissues [5, 6] little is known about the intimate details of the biosynthesis and metabolism of the steroid sulfates. The interaction of reconstituted side-chain cleavage systems with cholesterol has been well investigated [7–12], however there have been relatively few studies on the interaction of the sterol sulfate with the cholesterol side-chain cleavage system [13].

This report examines further the effects of adrenodoxin addition on the binding of cholesterol sulfate by cytochrome P-450scc. The binding of the sterol sulfate is compared to the binding of cholesterol. There have been conflicting reports as to whether the binding of adrenodoxin enhances the binding of cholesterol to cytochrome P-450scc. Greenfield *et al*[13] found that the addition of adrenodoxin did not appreciably increase the affinity of the cytochrome for cholesterol. However, Seybert *et al.*[12] found that the binding of adrenodoxin enhanced up to 20-fold the affinity of the cytochrome P-450 for cholesterol. In this present study, the cytochrome P-450 was prepared by the two different procedures used by Greenfield *et al.*[13] and Seybert *et al.*[12]. In addition, the two substrates were solubilized in several different fashions in an attempt to elucidate the effects of detergents on the apparent substrate binding behavior. The results of this study suggest that the factors influencing the binding of cholesterol sulfate to cytochrome P-450scc are very complex. The apparent effect of adrenodoxin on the binding affinity of cytochrome P-450scc for cholesterol sulfate depends both upon the method of purification of the cytochrome P-450scc and the method of dispersing the substrate.

The abbreviations and trivial names used are: C, cholesterol, 5-cholesten-3β-ol; CS, cholesterol sulfate, 5-cholesten-3β-yl sulfate; pregnenolone, 3β-hydroxy-5-pregnene-20-one; Tris, tris (hydroxymethyl) methylamine; cytochrome P-450scc, P-450scc, P-450, the cytochrome which catalyzes the side-chain cleavage of cholesterol to yield pregnenolone and isocaproaldehyde; Tween 80, polyethylenesorbitanmonooleate; Tween 20, polyoxyethylenesorbitanmonolaurate; Emulgen 913, polyoxyethyleneonylphenolether; DTT, dithiothreitol; ADX, adrenodoxin.

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EXPERIMENTAL

Cholic acid (Na salt) was obtained from Sigma Chemical Co. (St Louis, Mo) and recrystallized after charcoal treatment in 70% ethanol and water with the addition of acetone. Dithiothreitol (DTT), ethylenediaminetetraacetate disodium salt (EDTA). Tween 80 and Tween 20, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were also obtained from Sigma. Hexyl agarose was obtained from Miles-Yeda (Israel). Emulgen 913 was obtained from Kao-Atlas, Japan. Sephadex G-25 and PD-10 columns prefilled with Sephadex G-25 were obtained from Pharmacia (Piscataway, NJ). NADPH was obtained from Boehringer Mannheim (West Germany). Cholesterol was obtained from Amend Drug and Chemical Corp. (Hillsdale, NJ) and purified via the dibromide according to Fieser[14]. Cholesterol sulfate was prepared as described by Gasparini *et al.*[15] m.p. = 174–175°C. Glycerol was spectroquality grade from Fisher Scientific Co. (Fairlawn, NJ). Ultrapure Tris buffer was obtained from Swartz–Mann (Spring Valley, NY). Buffer A is 50 mM Tris–HCl, 1 mM EDTA, 1 mM DTT in 20% glycerol, pH 7.4.

Preparation of enzymes

Adrenodoxin was isolated by the method of Suhara *et al.*[16] or purchased from Sciogen Inc. Detroit, MI and adrenodoxin reductase was isolated by the method of Lambeth and Kamin[17]. Cytochrome P-450scc (Fraction I) was prepared by a modification of the method of Greenfield *et al.*[13] and corresponds to fraction P1A of that publication. In the modified procedure, the size of the column containing Biobeads SM-2 was increased to 3 × 15 from 1 × 15 cm. The dialyzed eluates from the octyl Sepharose resin, containing Emulgen 913, were percolated through the SM-2 resin at a rate of 5 ml/h (overnight) to remove the detergent in one pass. Cytochrome P-450scc (Fraction II) was prepared by the method of Seybert *et al.*[12]. Proteins were stored at −70°C. Substrate-free cytochrome P-450 preparations were prepared by a modification of the method of Seybert *et al.*[12]. Forty-five nmol of cytochrome P-450 (either Fraction I or II) were incubated with 4.4 nmol of adrenodoxin and 1.5 nmol of adrenodoxin reductase, 0.1 mg of NADP, 1.8 mg of glucose 6-phosphate and 10 μl glucose 6-phosphate dehydrogenase (340 units/ml) in 3 ml of 0.05M potassium phosphate buffer containing 0.1 mM EDTA, 0.1 mM DTT, pH 7.4 for 20 min at 37°C. The solutions were applied to 2 × 10 cm columns of hexyl agarose equilibrated with buffer A. The P-450 fractions were recovered by developing the columns with buffer A, buffer A containing 0.1 or 0.5 M KCl, and buffer A containing 0.5 M KCl + 0.3% sodium cholate. The recovered low spin cytochromes were concentrated via ultrafiltration on PM 30 membranes (Amicon Corp., Lexington, MA). Cholate was removed by chromatography of the P-450 fractions on 2 × 20 cm columns of Sephadex G-25 or on PD-10 columns of Sephadex G-25 which were equilibrated with buffer A. The concentration of cytochrome P-450 was determined using the method of Omura and Sato[18] as described previously [13].

Difference spectral measurements

Detergent-free stock solutions of cholesterol sulfate, 500 μM, were prepared by sonicating suspensions of weighed samples of the sterol sulfate in buffer A. The samples were sonicated for 1 min at 50 watts power with a Branson Sonifier, Model W185D, fitted with the standard tip. Stock solutions of cholesterol sulfate and cholesterol (5.0 mM) were prepared in 0.05 M Tris–HCl containing 5% Tween 80 and 5% sodium cholate (w/v) as described previously [13].

Difference spectral titrations were performed in two fashions. For the titrations with cholesterol sulfate, detergent-free, samples of Fraction I or II in buffer A were placed in the sample cuvette and buffer A was placed in the reference cuvette. Aliquots of stock solutions of CS were added to both the sample and reference cuvettes and the changes in the difference in absorption between 416 and 380 nm caused by each addition of cholesterol sulfate were determined.

Difference spectral titrations of the P-450 fractions with stock solutions of cholesterol, 5 mM, or cholesterol sulfate, 5 mM, dissolved in solutions containing Tween 80, 5% and sodium cholate 5% were performed as described previously [13].

The spectral binding data were analyzed in several fashions.

The method of Engel[19] was used to determine the dissociation constants and maximal absorbance changes when the substrate affinity was apparently high, i.e. when the dissociation constants were less than or equal to the P-450 concentration used for the spectral titrations. When the dissociation constants were large compared to the enzyme concentration the data were fit to Scatchard plots [20] or reciprocal plots [21] via the method of least squares. Data were plotted and calculations were performed on an Apple II + computer.

Characterization of the cytochrome P-450 fractions

Molecular weights were estimated by the method of Laemmli[22] using thin slabs of polyacrylamide gels. Phospholipids were extracted by the method of Folch *et al.*[23] and quantified by the method of Chalvarjian and Rudnicki[24]. Protein contents were estimated by the method of Bradford[25].

RESULTS

Characterization of cytochrome P-450scc prepared by two different protocols

Fraction I was prepared by a modification of the method of Greenfield *et al.*[13] from cholate extracts

of mitochondrial sonicates which were unfractionated. Typically 20–30 nmol were obtained from 250 g of adrenal cortical tissue. The modified method yielded a preparation which had at least 75% of its heme in the low spin configuration. The A(280/416) ratio was 1.1 and the heme content was approx. 14 nmol/mg protein. Fraction II was prepared by the method of Seybert *et al.*[12] from cholate extracts of pellets from mitochondrial sonicates which were collected at 100,000 *g*. Yields were typically 250–300 nmol per 250 g of cortex. The configuration of the heme of this preparation was predominantly high spin. The A(280/390) ratio of duplicate preparations of Fraction II varied between 1.4 and 2.1 and the heme content varied between 4–5 nmol/mg protein. Fraction I and II had the same molecular weight, approx. 53,000 Daltons, and both preparations were greater than 95% homogeneous by the criteria of SDS gel electrophoresis. Neither preparation appeared to be contaminated with either adrenodoxin or adrenodoxin reductase. The phospholipid content of each preparation was low, approx. 2 nmol phosphate/nmol P-450.

Both fractions of cytochrome P-450scc were treated with adrenodoxin, adrenodoxin reductase and NADPH to oxidize endogenous substrates and the cytochrome fractions were reisolated on hexyl agarose as described by Seybert *et al.*[12]. The elution patterns from the hexyl agarose resins of both preparations are illustrated in Fig. 1. Fraction II, substrate depleted, did not bind tightly to the hydrophobic resin. Some of the cytochrome was eluted when the column was washed with the buffer used to apply the cytochrome (50 mM Tris–HCl, 1 mM EDTA, 1 mM DTT in 20% glycerol pH 7.4, Buffer A). Fraction II could be totally eluted from hexyl agarose by developing the column with Buffer A containing 0.1 M or 0.5 M KCl. Contrariwise, Fraction I, substrate depleted, bound tightly to the hexyl agarose resin. None of Fraction I was eluted by the buffer wash. Buffers containing cholate, 0.3%, were required for complete elution of Fraction I from the hexyl agarose resin.

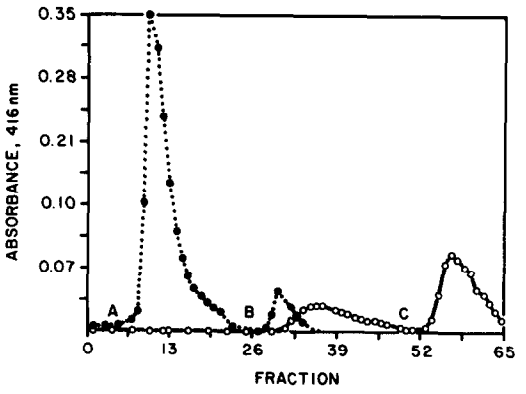

Fig. 1. Chromatography of cytochrome P-450scc, Fraction I and II, on hexyl agarose. Substrate depleted preparations, 45 nmol in 3 ml, were applied to 10 × 2 cm columns of hexyl agarose and eluted with A, 0.05 M potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol in 20% glycerol, pH 7.4; B, same as A + 0.5 M KCl; C, same as B + 0.3% sodium cholate w/v. Two ml fractions were collected. (○ ○ ○) Fraction I (●·●·) Fraction II.

Comparison of the apparent substrate affinities of Fraction I and Fraction II for cholesterol and cholesterol sulfate: the effect of non-ionic detergents

Both substrate-depleted cytochrome P-450scc preparations were titrated with cholesterol and cholesterol sulfate and the titrations were followed by difference spectroscopy. Initially in order to compare the binding of the substrates to Fraction II with the binding of the substrates to Fraction I, reported previously, each sterol was dissolved in mixed micelles of Tween 80 and cholate as described by Greenfield *et al.*[13].

In the absence of added detergents, other than those used to emulsify the substrates, the apparent binding affinities of Fraction I and Fraction II for both substrates were similar. The apparent dissociation constants of the P-450–sterol complexes increased as a function of P-450 concentration. The dependence of the apparent substrate affinity on the P-450 concentration is illustrated in Table 1. A typical titration of Fraction II with mixed micelles of Tween and cholate containing cholesterol is illus-

Table 1. The binding of substrates dissolved in Tween/cholate mixtures to cytochrome P-450scc

P-450 μM	Adrenodoxin 4 μM	Cholesterol			Cholesterol sulfate		
		N	K_d μM	A_{max}	N	K_d μM	A_{max}
0.7	–	0.5 ± 0.1	0.14 ± 0.01	40 ± 11	0.8 ± 0.3	0.35 ± 0.05	30 ± 1
	+	0.5 ± 0.1	0.43 ± 0.17	73 ± 2	0.5 ± 0.1	0.45 ± 0.04	60 ± 17
0.5	–	0.5 ± 0.1	0.19 ± 0.03	43 ± 3	0.7 ± 0.4	0.42 ± 0.36	29 ± 11
	+	0.5 ± 0.1	0.23 ± 0.07	67 ± 6	0.5 ± 0.1	0.30 ± 0.12	51 ± 7
0.2	–	0.4 ± 0.1	0.04 ± 0.02	73 ± 9	0.2 ± 0.1	0.06 ± 0.04	17 ± 11
	+	0.5 ± 0.1	0.006 ± 0.004	101 ± 12	0.5 ± 0.1	0.06 ± 0.04	59 ± 12

Cytochrome P-450scc (Fraction II) was isolated by the method of Seybert *et al.*[12]. Substrates, 5 mM, were dissolved in mixtures of Tween 80, 5%, and sodium cholate, 5% as described by Greenfield *et al.*[13]. The number of apparent substrate binding sites, N, apparent dissociation constants of the P-450–substrate complexes, K_d, and the maximal changes in the differences in absorption between 416 and 380 nm caused by the addition of substrates/mM P-450scc, A_{max}, were calculated by the method of Engel[19].

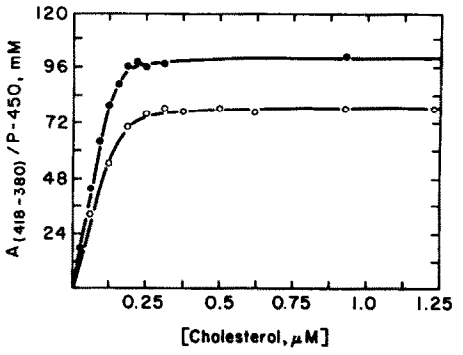

Fig. 2. The difference spectral titration of cytochrome P-450scc, Fraction II, 0.3 μM, in 0.05 M potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, pH 7.4 with cholesterol. The substrate, 5 mM, was dissolved in Tween 80, 5% w/v, sodium cholate, 5% w/v, and Tris–HCl, 0.1 M, pH 7.4. "A" is the change in the difference in absorption between 416 and 380 nm/cm caused by the binding of substrate to the cytochrome. The solid lines represent the best fits of the difference spectral data calculated by the method of Engel[19]. (○ ○ ○) No adrenodoxin: $N = 0.52$ binding sites/mole P-450scc, $K_d = 0.011\ \mu$M, $A_{max} = 79.3$/mM P-450·cm. (● ● ●) Adrenodoxin = 4.0 μM: $N = 0.55$ binding sites/mole P-450scc, $K_d = 0.0055\ \mu$M, $A_{max} = 100.4$ mM P-450scc·cm.

trated in Fig. 2. Each preparation had only one class of high affinity binding sites for each substrate when the sterols were dissolved in Tween and cholate. However, both preparations of cytochrome appeared to be saturated with the substrates at a ratio of substrate to heme of approx. 0.5. Moreover, the addition of "saturating" amounts of the Tween–cholate–substrate mixtures only caused partial conversion of the heme irons of the two cytochrome fractions from the low spin configuration to the high spin configuration. Addition of adrenodoxin to either preparation of cytochrome P-450scc had only a small effect on their apparent affinity for substrates when the substrates were dissolved in the micelles of Tween and cholate in the absence of additional detergents. The addition of adrenodoxin did increase the amount of cytochrome of each preparation whose heme iron was in the high spin configuration.

The effects of the addition of the non-ionic detergents, Emulgen 913, 0.04% and Tween 20, 0.3%, on the difference spectral parameters for the binding of cholesterol and cholesterol sulfate to Fraction I and Fraction II are summarized in Table 2. The two preparations of cytochrome P-450scc showed quite different binding affinities for the two substrates when the solutions of cytochrome contained either of the detergents. Scatchard plots [20] of the difference spectral titration of Fraction I and Fraction II with cholesterol sulfate in the presence of Emulgen 913, 0.04%, are illustrated in Fig. 3 and with cholesterol are illustrated in Fig. 4.

The binding of cholesterol sulfate (detergent-free) to Fraction I and II

The binding cholesterol sulfate, detergent free, to both preparations of cytochrome P-450scc was also monitored by difference spectroscopy. The estimated values of the dissociation constants obtained for the P-450scc–cholesterol sulfate complexes and the estimated extinction coefficients for the changes in spectra associated with the binding of cholesterol sulfate

Table 2. The effect of non-ionic detergents on the binding of substrates to cytochrome P-450scc

			Cholesterol			
			Fraction I		Fraction II	
Addition	P-450 concn μM	Adx 4 μM	K_d μM	A_{max}	K_d μM	A_{max}
None	0.2	–	0.02 ± 0.01	30 ± 3	0.04 ± 0.01	73 ± 9
		+	0.03 ± 0.01	98 ± 9	0.006 ± 0.004	101 ± 12
Emulgen 913	0.4	–	23 ± 3	56 ± 10	90 ± 1	43 ± 2
0.04%		+	9 ± 3	97 ± 17	11 ± 6	87 ± 3
Tween 20	0.3	–	220 ± 120	62 ± 16	340 ± 40	87 ± 11
0.3%		+	35 ± 20	97 ± 12	70 ± 30	114 ± 5
			Cholesterol sulfate			
None	0.2	–	0.09 ± 0.01	17 ± 5	0.06 ± 0.04	17 ± 11
		+	0.06 ± 0.02	39 ± 12	0.06 ± 0.04	59 ± 12
Emulgen 913	0.4	–	22 ± 5	44 ± 6	220 ± 20	115 ± 9
0.04%		+	16 ± 4	79 ± 8	66 ± 20	115 ± 11
Tween 20	0.3	–	230 ± 12	33 ± 6	990 ± 90	120 ± 40
0.3%		+	170 ± 90	69 ± 17	210 ± 90	120 ± 20

Fraction I was isolated by the method of Greenfield *et al.*[13]. Fraction II was isolated by the method of Seybert *et al.*[12]. Substrate stock solutions, 5 mM, were prepared in mixtures of Tween 80, 5% w/v, and sodium cholate, 5% w/v, as described by Greenfield *et al.*[13]. The dissociation constants, K_d, and the maximal changes in the differences in absorption between 416 and 380 nm caused by the addition of substrates to the solutions of cytochrome, A_{max}, were estimated from Scatchard Plots [20]. A_{max} is the extinction coefficient of the spectral change/mM cytochrome P-450scc·cm.

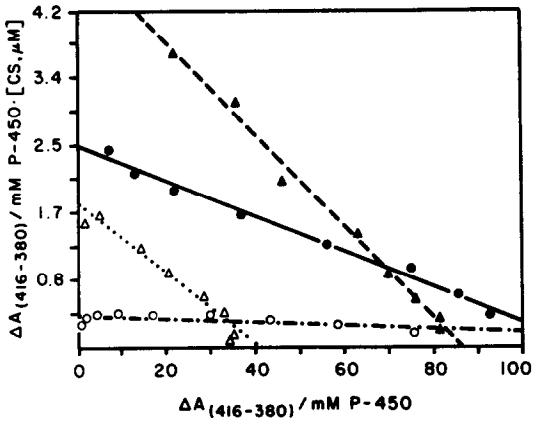

Fig. 3. The difference spectral titration of two preparations of cytochrome P-450scc, 0.4 μM with cholesterol sulfate. The conditions are the same as Fig. 2. However the enzyme and substrate buffers each contain Emulgen 913, 0.04%. (△ △ △) Fraction I; (▲ ▲ ▲) Fraction I + adrenodoxin, 4.0 μM; (○ ○ ○) Fraction II; (● ● ●) Fraction II + adrenodoxin, 4.0 μM. "A" on the y axis is the difference in absorbance between 416 and 380 nm/mM P-450scc·cm.

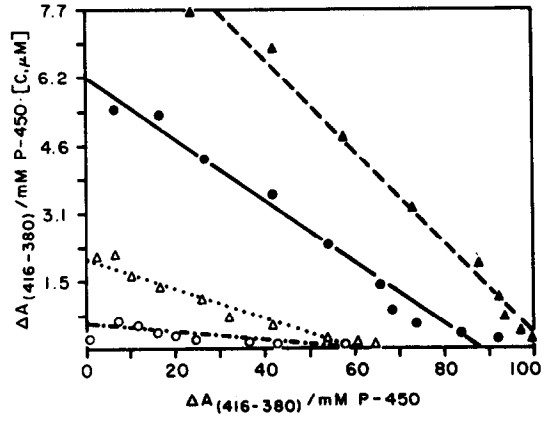

Fig. 4. The difference spectral titration of two preparations of cytochrome P-450scc, 0.4 μM, with cholesterol. The enzyme and substrate buffers each contain Emulgen 913, 0.04%. The legend is the same as that used in Fig. 3.

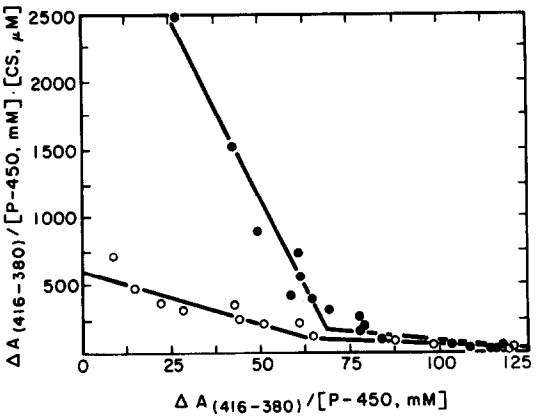

Fig. 5. The difference spectral titration of cytochrome P-450scc, Fraction II, 0.1 μM with detergent-free cholesterol sulfate in 50 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, pH 7.4. (○ ○ ○) no adrenodoxin; (● ● ●) adrenodoxin = 4.0 μM.

are given in Table 3. Both Scatchard plots [20] and Benesi–Hildebrand plots [21] of the difference spectral titration binding data for Fraction II were non-linear, suggesting that this preparation has at least two non-equivalent binding sites for the sterol sulfate. Scatchard plots of the difference spectral data obtained for the titration of Fraction II, 0.1 μM, with cholesterol sulfate in the presence and absence of adrenodoxin are illustrated in Fig. 5. The binding of cholesterol sulfate to Fraction I, in contrast, showed only one class of binding sites; i.e. Scatchard plots of the binding data were linear. As shown in Table 3, the addition of adrenodoxin increased 3 to 10-fold the affinity of Fraction I for cholesterol sulfate. The addition of adrenodoxin to Fraction II raised the extinction coefficient of the change in adsorption between 416 and 390 nm caused by binding of cholesterol sulfate to the high affinity site and decreased 2–3-fold the dissociation constants of both sites of Fraction II for cholesterol sulfate.

Table 3. The binding of cholesterol sulfate (detergent-free) to cytochrome P-450scc

P-450 Concn μM	Cytochrome fraction	Adrenodoxin 4 μM	K_{d1} μM	A_{max1}	K_{d2} μM	A_{max2}
0.1	I	–	1.1	160	—	—
		+	0.7	112	—	—
	II	–	0.1	73	0.6	129
		+	0.02	75	0.3	121
0.3	I	–	1.8	139	—	—
		+	0.2	128	—	—
	II	–	0.6	96	1.5	135
		+	0.3	122	0.7	139
0.5	I	–	3.8	139	—	—
		+	0.7	130	—	—
	II	–	1.4	104	3.6	156
		+	0.8	129	1.8	155

The dissociation constants of the P-450–cholesterol sulfate complexes, K_{d1} and K_{d2}, and the maximal values of the changes in absorption between 416 and 380 nm, A_{max1} and A_{max2}, caused by the addition of cholesterol sulfate to the cytochrome P-450scc fractions were estimated from Scatchard plots [20]. The maximal changes in absorption between 416 and 380 nm are expressed in absorbance units/mM P-450·cm.

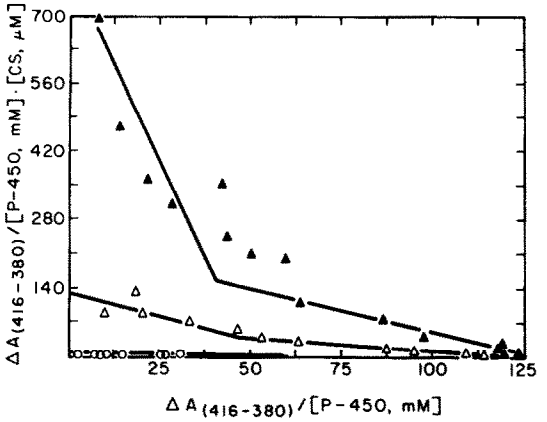

Fig. 6. The effect of mixtures of Tween and cholate on the difference spectral titration of cytochrome P-450scc, Fraction II, 0.2 μM with cholesterol sulfate. The buffer is the same as Fig. 5. (▲ ▲ ▲) no detergents; (△ △ △) Tween 80 = sodium cholate = 0.0005% w/v; (○ ○ ○) Tween 80 = sodium cholate = 0.25% w/v.

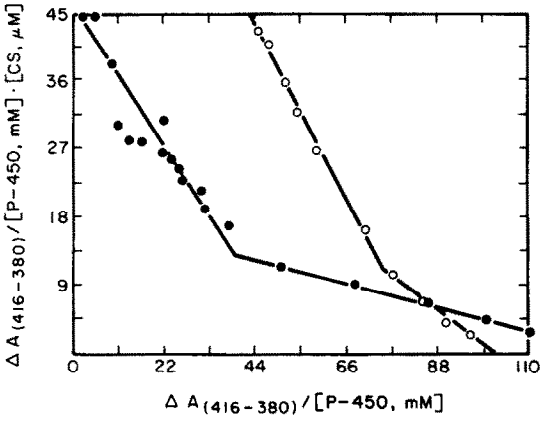

Fig. 7. The effect of adrenodoxin, 4.0 μM, on the difference spectral titration of cytochrome P-450scc, Fraction II, 0.7 μM with cholesterol sulfate. The enzyme and substrate were dissolved in 0.05 M potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol pH 7.4 containing Tween 80, 0.0005% w/v and sodium cholate, 0.0005% w/v (● ● ●) no adrenodoxin; (○ ○ ○) adrenodoxin = 4.0 μM.

Fraction II was also titrated with cholesterol sulfate in the presence of fixed concentrations of 1:1 w/v mixtures of Tween 80 and cholate to examine the effect of these detergents on the substrate binding behavior. In these experiments Tween and cholate were added to the P-450 before the titrations were begun and the sterol sulfate was dissolved in buffers containing the same concentration of the detergent. The results of adding these fixed concentration of detergent to cytochrome P-450scc on the apparent binding affinity for cholesterol sulfate are illustrated in Fig. 6. At low concentrations of Tween and cholate (0.00005–0.0025%) the cytochrome displayed two classes of binding sites for cholesterol sulfate. Addition of saturating concentrations of the sterol converted all of the cytochrome to the high spin form. At higher concentrations of Tween and cholate (0.25%) the binding of substrate was greatly inhibited and only one site was observed. However at this concentration of detergent the binding was so weak that 100 μM CS was not a saturating concentration. The effect of adding adrenodoxin to Fraction II on its binding of CS in the presence of fixed concentrations of Tween and cholate was similar to its effect on the detergent free cytochrome. The effect of the addition of adrenodoxin on the difference spectral titration is illustrated in Figure 7.

DISCUSSION

These studies were undertaken to examine whether the addition of adrenodoxin to cytochrome P-450scc modulates the affinity of the cytochrome for cholesterol sulfate. The effects of adrenodoxin on the binding of the sterol sulfate appeared to vary depending on several factors including: (1) The method of purification of cytochrome P-450scc, (2) Whether or not the solutions of cytochrome P-450scc contain non-ionic detergents, and (3) How the sterol sulfate is dispersed in aqueous solution.

Cytochrome P-450scc was prepared by two different protocols. The method of Greenfield *et al.*[13] yielded Fraction I and the method of Seybert *et al.*[12] yielded Fraction II. Both fractions appeared to have similar degrees of purity and identical mol. wt, 53,000 Daltons, by the criteria of SDS gel electrophoresis. Reports from several laboratories had suggested that the molecular weight of fraction II was 47,000–49,000 and that the protein migrated faster than adrenodoxin reductase [12, 26]. In our gel system both fractions migrated more slowly than adrenodoxin reductase. The differences in the results are probably due to the differences in the gel systems used for the estimation of the molecular weights.

While the two preparations appeared to have similar physical properties, the two migrated quite differently on hexyl agarose even when stripped of endogenous substrates. It is possible that Fraction I and II are isozymes and contain different polypeptide chains. However, the isolation procedure yielding Fraction I employs the non-ionic detergent, Emulgen 913, in the purification scheme while the procedure yielding Fraction II only employs the ionic detergent, sodium cholate. The different detergents could remove different ligands from the two fractions. Small differences in the phospholipid or apoprotein content of the two preparations might modulate their chromatographic and substrate binding behavior. The question of whether the proteins contain isozymes may be resolved by sequencing the two protein fractions.

Regardless of whether or not these cytochrome fractions contain different proteins, it should be emphasized that their apparent affinities for substrates, especially cholesterol sulfate, were quite different from one another. Moreover, the addition of adrenodoxin affected the substrate binding of the two

preparations to different extents. The differences between the two preparations were most apparent when the cytochrome preparations were dissolved in buffers which contained high levels of non-ionic detergents.

When either Fraction I or Fraction II was titrated with a detergent-free solution of cholesterol sulfate, both fractions appeared to bind the sterol sulfate tightly, i.e. the dissociation constants of the P-450–sterol sulfate complexes were similar in magnitude to the concentrations of cytochrome P-450scc. Fraction I appeared to have only one class of binding sites for cholesterol sulfate. Addition of saturating amounts of the sterol sulfate converted all of the heme iron in the cytochrome to the high spin configuration. Fraction II, on the other hand, appeared to have two classes of binding sites for cholesterol sulfate. The apparent biphasic binding of the sterol sulfate could be due to aggregation of the lipophilic cytochrome P-450scc in aqueous solution. This aggregated form may have lower affinity for the sterol substrate. Alternatively, residual phospholipids or other ligands, in Fraction II might perturb the binding of substrates to the cytochrome leading to the observed biphasic binding curves. In addition, Fraction II appears to be contaminated with apoprotein as evident from its low heme:protein ratio. It is possible that the apparent titration curve of Fraction II with the sterol sulfate, as monitored by a spectral change of the heme, would be perturbed if the apoprotein binds the substrate.

The addition of adrenodoxin enhanced the binding of detergent-free cholesterol sulfate to both preparations of cytochrome P-450scc 2- to 9-fold. The exact role of adrenodoxin in the enhancement of the binding of the sterol sulfate is difficult to interpret because the apparent dissociation constants of the cholesterol sulfate–P-450 complexes increased as a function of enzyme concentration. Adrenodoxin addition to cytochrome P-450scc may help to prevent aggregation of the cytochrome. It is possible that the non-aggregated form might have a higher affinity for the sterol sulfate than the aggregated form. Moreover, the addition of adrenodoxin appears to stabilize cytochrome P-450scc. If an active conformation of cytochrome P-450scc is stabilized by adrenodoxin, the stabilization could also result in an apparent increase in the binding affinity of the cytochrome for the sterol sulfate.

When either cholesterol sulfate or cholesterol was suspended in micelles of Tween 80 and cholate and the substrate–Tween 80–cholate mixture was used to titrate the cytochrome P-450scc, both fractions I and II showed a single class of high affinity binding sites for each of the sterols. Moreover, the addition of adrenodoxin appeared to have little effect on the apparent affinity of cytochrome P-450scc for either free cholesterol or its sulfate. However, addition of "saturating" amounts of either substrate (50 μM) resulted in only partial conversion of the heme iron of the P-450 fractions to the high spin configuration when the substrates were suspended in the Tween 80–cholate mixtures. Moreover, when titrated with the Tween 80–cholate micelles containing the substrates, each species of cytochrome P-450 appears to bind only 1 mol of either substrate/2 mol of P-450. It is likely that the apparent saturation of the cytochrome with substrate at less than a 1:1 ratio of substrate to heme is artifactual. In this set of experiments both the concentration of the substrate and of Tween and cholate was increased during the titrations. The addition of substrates raised the amount of cytochrome in the high spin form, but the addition of detergents lowered the amount of cytochrome in the high spin form. The resulting titration curve reflected both phenomena. When the preparations of cytochrome were titrated under conditions where the concentration of Tween and cholate were unchanged, the addition of cholesterol sulfate to cytochrome P-450 completely converted its spectrum to the high spin form.

When either Emulgen 913, 0.04% or Tween 20, 0.3%, were added to the two preparations of cytochrome P-450scc, before they were titrated with the Tween 80–cholate micelles containing cholesterol or cholesterol sulfate, their apparent binding affinities for the substrates were greatly diminished. Moreover, the two fractions exhibited quite different affinities for both of the substrates. In agreement with results reported previously [13], Fraction I showed equal binding affinity for cholesterol and cholesterol sulfate in the absence of adrenodoxin and the addition of adrenodoxin to Fraction I did not increase its binding affinity for cholesterol sulfate. The apparent binding affinity of Fraction I for cholesterol was increased 2-fold by the addition of adrenodoxin when the cytochrome was dissolved in buffers containing Emulgen 913, 0.04% and 7-fold when the cytochrome was dissolved in buffers which contained Tween 20, 0.3%. In contrast to Fraction I which had equal affinity for unesterified and sulfated cholesterol, Fraction II showed a 2–3-fold lower affinity for cholesterol sulfate than for cholesterol when it was dissolved in buffers which contained the non-ionic detergents. Moreover, addition of adrenodoxin to Fraction II increased 3-fold its apparent affinity for cholesterol sulfate and increased 7–8-fold its apparent affinity for cholesterol. The increase in affinity of cytochrome P-450scc for cholesterol, observed upon the addition of adrenodoxin, when the buffers contain Tween 20, 0.3%, is in agreement with the results of Hanukoglu *et al.*[11].

Although the factors influencing the binding of cholesterol sulfate to cytochrome P-450scc are complex, several conclusions can be drawn. First, in agreement with earlier results [13] non-ionic detergents such as Emulgen 913 and Tween 20 inhibit the binding of substrates to cytochrome P-450 by several orders of magnitude. In addition, these detergents appear to inhibit the binding of cholesterol sulfate to

Fraction II, to a much greater extent than they inhibit the binding of cholesterol. These findings suggest that high concentrations of these detergents probably should not be used in experiments designed to mimic the *in vivo* situation.

Second, in the absence of detergents the binding of cholesterol sulfate to cytochrome P-450scc is quite tight. The addition of adrenodoxin to cytochrome P-450scc has only small effects on the affinity of dilute solutions of the cytochrome for the sterol sulfate.

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