Studies of the Active Site of Cytochrome P-450$_{scc}$ with a High-Affinity Spin-Labeled Inhibitor†

Daniel Seeley,‡ Heinz Schleyer,§ Kiyoko Kashiwagi,‡,∥ David Cooper,§ and Hilton A. Salhanick*,‡

Department of Population Sciences, Harvard School of Public Health, and Department of Obstetrics and Gynecology, Harvard Medical School, Boston, Massachusetts 02115, and Harrison Department of Surgical Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Received September 18, 1986

ABSTRACT: The intramolecular site of P-450$_{scc}$ for conversion of cholesterol to pregnenolone involves a substrate site, an active site, and a site for transmission of electrons. The substrate site was studied with a high-affinity, high-potency nitroxide spin-labeled inhibitor of cholesterol side-chain cleavage. This substance, 17α-hydroxy-11-deoxycorticosterone nitroxide (SL-V), has an affinity comparable to that of the most active substrate inhibitors ever reported and 2–50 times greater than that of the natural substrate cholesterol. Competition experiments with cholesterol and its analogues confirmed that SL-V binds reversibly to the substrate site. Titration experiments showed a single binding site on the P-450 molecule. The substrate site is on the apoprotein and has little or no direct interaction with the heme. Spin–spin interactions between the Fe^{3+} and side-chain or A-ring spin-labeled groups could not be demonstrated, which is consistent with carbons 22 and 20 being closest to the heme iron. We postulate that substrate disrupts a histidine nitrogen coordination with the heme iron and induces conformational changes in the apoprotein. These changes lead to increased affinity for iron–sulfur protein.

Cytochrome P-450$_{scc}$[1] is the terminal enzyme of the oxygenase system that converts cholesterol to pregnenolone in mitochondria of the corpus luteum, testis, adrenal cortex, and placenta. At the active site, cholesterol, oxygen, and the heme prosthetic group of the enzyme interact to effect cleavage of the substrate in three oxygenation steps: carbon 22 (22*R*) hydroxylation, carbon 20 (20*R*) hydroxylation, and the oxidative cleavage of the resulting 20,22-diol to pregnenolone (Bursten et al., 1975).

The P-450$_{scc}$ molecule has at least three sites involved in the cholesterol cleavage process. (1) The substrate binding site is required for orientation of cholesterol and sterol "intermediates" for enzymatic reaction. (2) The iron sulfur protein binding site facilitates transfer of electrons for heme reduction. (3) The heme environment is the active site where oxygenation and cleavage of the cholesterol molecule occur.

The events associated with these sites have commonly relied on interpretation of changes in optical absorption of the iron–porphyrin system. These changes fall generally into three groups: (1) Substrates bound to ferric P-450$_{scc}$ yield type I optical difference spectra with maxima at about 390 nm and minima at about 420 nm. (2) Reverse type I spectral changes are induced by certain steroidal inhibitors and have spectral characteristic opposite those of type I ligands. (3) Type II optical difference spectra elicit maxima at about 425–435 nm and minima at about 390 nm (Schenkman et al., 1972; Whysner et al., 1970). These spectral changes have been attributed to the redistribution of the electron density in the iron–porphyrin structure. With type I ligands there is a blue shift of the Soret band with a concomitant change in electron spin state from mainly low spin ($S = {}^1/_2$) to mainly high spin ($S = {}^5/_2$). With reverse type I and type II ligands the shift is from predominantly high spin to low spin. While reverse type I spectra induced by inhibitors probably result from displacement of cholesterol, type II changes probably result from effects on the electronic structure of the iron–porphyrin system.

The magnitude of the diverse types of spectral changes caused by these agents is, with few exceptions, concentration-dependent, and the amount of enzymatic inhibition correlates with the amount of spectral change (Uzgiris et al., 1977).

Electron paramagnetic resonance (EPR) spectroscopy, which allows direct determination of the prevailing spin states of the Fe^{3+} heme moiety, has been used to understand the underlying mechanisms of the spectral changes.

In addition to conventional EPR spectroscopy of the heme environment of the enzyme, four spin-labeled compounds were used as probes of the substrate binding site of P-450$_{scc}$: androstane, cholestane and cholesterol with stable nitroxide-containing groups attached to carbon 3, and a nitroxide derivative esterified to carbon 21 of 17α-hydroxy-11-deoxycorticosterone (Figure 1). The latter substance, labeled SL-V, has affinity for P-450$_{scc}$ comparable to that of the most avid ligands ever reported (Uzgiris et al., 1977; Hochberg et al., 1974; Vickery & Kellis, 1982). This probe was used to study

† This work was supported in part by U.S. Public Health Service Grant AM-10081 from the National Institutes of Health.

‡ Harvard School of Public Health and Harvard Medical School.

§ University of Pennsylvania School of Medicine.

∥ Present address: 137 Tanazawa, Atsugi-City, Kanagawa Ken, Japan.

[1] Abbreviations: P-450$_{scc}$, cholesterol side-chain cleavage cytochrome P-450; 17α-hydroxy-11-deoxycorticosterone, 17α,21-dihydroxy-4-pregnene-3,20-dione; SL-I or androstane nitroxide, 17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxy; SL-II or cholestane nitroxide, 4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxy; SL-III or piperidine nitroxide, 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl phosphate; SL-IV or cholesterol nitroxide, 5-cholesten-3β-yl 2,2,5,5-tetramethyl-1-oxypyrrolidine-3-carboxylate; SL-V or 17α-hydroxy-11-deoxycorticosterone nitroxide, 17α-hydroxy-3,20-dioxo-4-pregnen-21-yl 2,2,5,5-tetramethyl-1-oxy-pyrrolidine-3-carboxylate; 17OH-DOC-BZ or 17α-hydroxy-11-deoxycorticosterone 21-benzoate, 17α-hydroxy-3,20-dioxo-4-pregnen-21-yl benzoate; cholesterol, 5-cholesten-3β-ol; 25-hydroxycholesterol, 5-cholestene-3β,25-diol; 22-azacholesterol, 22-aza-5-cholesten-3β-ol; SDS, sodium dodecyl sulfate; ISP, iron–sulfur protein; EDTA, ethylenediaminetetraacetic acid; CPK, Corey–Pauling–Koltun.

0006-2960/87/0426-1270$01.50/0

Table I: Nomenclature of Spin-Labeled and Other Compounds

designation	abbreviated name (solvent)	chemical name (source)
SL-I	androstane nitroxide (ethanol)	17β-hydroxy-4′,4′-dimethylspiro[5α-androstane-3,2′-oxazolidin]-3′-yloxy (Syva Research Chemicals)
SL-II	cholestane nitroxide (propylene glycol/ethanol, 1:1)	4′,4′-dimethylspiro[5α-cholestane-3,2′-oxazolidin]-3′-yloxy (Syva Research Chemicals)
SL-III	piperidine nitroxide (buffer A)	2,2,6,6-tetramethyl-1-oxypiperidin-4-yl phosphate (Syva Research Chemicals)
SL-IV	cholesterol nitroxide (propylene glycol/ethanol, 1:1)	5-cholesten-3β-yl 2,2,5,5-tetramethyl-1-oxypyrrolidine-3-carboxylate (gift of W. R. Benson, FDA, Washington, DC)
SL-V	17α-hydroxy-11-deoxycorticosterone nitroxide (ethanol/dimethylformamide, 9:1)	17α-hydroxy-3,20-dioxo-4-pregnen-21-yl 2,2,5,5-tetramethyl-1-oxypyrrolidine-3-carboxylate (gift of W. R. Benzon, FDA, Washington, DC)
17OH-DOC-BZ	17α-hydroxy-11-deoxycorticosterone 21-benzoate (dimethylformamide)	17α-hydroxy-3,20-dioxo-4-pregnen-21-yl benzoate (gift of Kaj Arends, Steraloids, Inc.)

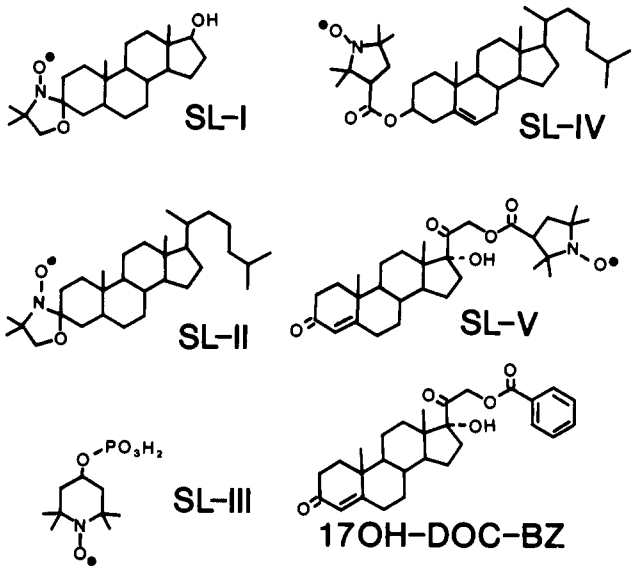

FIGURE 1: Chemical structures and designations. See Table I for nomenclature.

the orientation of the substrate with respect to the heme.

Materials and Methods

Spin-Labeled and Other Compounds. The proper nomenclature, abbreviated names, sources, and structures of the nitroxide spin-labeled (SL) compounds and the control substances study are presented in Table I and Figure 1. The compounds were dissolved in various solvents as indicated in the text and Table I. 17α-Hydroxy-11-deoxycorticosterone was purchased from Steraloids, Inc.; its benzoate ester was prepared for us through the generosity of Dr. Kaj Arends of Steraloids, Inc.

Cytochrome P-450$_{scc}$ Preparations. Cytochrome P-450$_{scc}$ was purified from mitochondria of bovine corpora lutea as described previously (Kashiwagi et al., 1980). This cytochrome P-450 is the only one that has been demonstrated either spectroscopically or enzymatically to be present in bovine corpora lutea (McIntosh et al., 1971). Measurements of P-450$_{scc}$ content and protein analysis were performed as described previously (Uzgiris et al., 1977). The specific contents of the various P-450 preparations ranged from 1.5 to 15.4 nmol of P-450/mg of protein. The specific contents of various preparations differed mainly in heme content rather than in amount of contaminating proteins. The preparation containing 4.0 nmol of P-450/mg of protein was 70% homogeneous by SDS–polyacrylamide gel electrophoresis and contained about 75% heme-less apoprotein. Other preparations used in individual experiments are described appropriately in the text and figure legends.

Reagents. In most of these experiments, the buffer consisted of 20 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, and 0.1 mM dithiothreitol (buffer A). All other chemicals were of reagent grade and were purchased from standard sources.

Optical Spectroscopy. Absorption difference spectra were recorded at room temperature in a modified Fisher FRL-1000 dual-beam scanning spectrophotometer. Small volumes of 0.5–1 mM stock solutions of spin-labeled compounds were added to 1-cm path length sample cuvettes, which contained 2 mL of a P-450$_{scc}$ preparation in buffer A. The same amount of solvent was added to the reference cuvettes, which also contained 2 mL of the same P-450$_{scc}$ preparation. Solvent concentration never exceeded 10% to avoid conversion to enzymatically inactive P-420. The samples were equilibrated for 15 min before difference spectra were recorded. The changes in absorbance (ΔA) were measured between the peaks (at about 420 nm) and troughs (at about 390 nm) of the difference spectra. Typical reverse type I spectra were obtained at various concentrations of each of the compounds tested. The apparent dissociation constants were estimated graphically from plots of the absorbance differences vs. concentration (Figure 2) by the method of Rodbard (1973).

EPR Spectroscopy. EPR spectroscopy was performed at room temperature in 100-μL flat cells or at 93 K in 350-μL 3-mm-i.d. cylindrical cells by using a Varian E-9 spectrometer operating at X-band (approximately 9.1 GHz). The spin-labeled compounds were incubated with the P-450$_{scc}$ preparation for 20 min at room temperature prior to the measurements. The concentrations of the spin-labeled substances in solution were determined from the amplitudes of the unbroadened N-hyperfine lines or by double integration (Benson et al., 1977; Basset et al., 1975). Concentrations of the spin-labeled compounds, which were "immobilized" to various degrees by binding to a macromolecule, i.e., P-450$_{scc}$, or in large self-aggregates of the steroid spin-labeled compound, were calculated from the difference between total spin-label added and free spin-label in solution (Benson et al., 1977; Basset et al., 1975).

Analysis of EPR Data. Titration experiments of the heme protein and spin-labeled compound SL-V were carried out over a 50-fold range of concentrations. To correlate the spectroscopic variable with spin-label concentration, the results were initially plotted as amplitudes of the N-hyperfine lines vs. analytical concentrations. To correct for the dilution factor D at each titration point, a modified Woolf transformation

$$F/D(T - F) = (F + K_D)/Q_0$$

was used, where F is the concentration of free SL-V, T is the total concentration (free plus bound) of SL-V, Q_0 is the concentration of binding sites, and K_D is the equilibrium dissociation constant (Haldane & Stern, 1932).

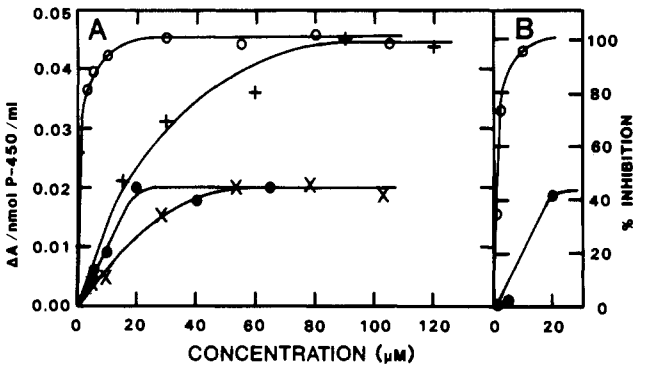

FIGURE 2: Concentration-dependent curves of the induction of reverse type I spectra and of the inhibition of cholesterol side-chain cleavage activity by spin-labeled and other compounds. (A) Each cuvette contained 0.6 μM P-450$_{scc}$ with a specific content of 2.4 nmol of P-450/mg of protein. Various concentrations of SL-I (×), SL-V (○), 17α-hydroxy-11-deoxycorticosterone (+), and 17OH-DOC-BZ (●) were added to the sample cuvette, and an equal amount of solvent was added to the reference cuvette. After 15-min incubations, spectra were recorded between 460 and 360 nm. Absorbances were measured between the peaks at about 420 nm and the troughs at about 390 nm. (B) The decrease in enzymatic activity is expressed as percent inhibition of cholesterol side-chain cleavage activity. Various concentrations of SL-V (○) and 17OH-DOC-BZ (●) were preincubated with solubilized mitochondria (0.5 μM P-450; specific content, 0.5 nmol of P-450/mg of protein) for 10 min. After addition of the NADPH-generating system to start the reaction, duplicate samples were removed at 5-min intervals to determine the rate of cholesterol side-chain cleavage.

Assay for Aromatase Activity. The rate of aromatization was measured by the method of Thompson and Siiteri (1974) as modified by Graves and Salhanick (1979).

RESULTS

Affinity of Spin-Labeled Substances for P-450$_{scc}$ by Difference Spectroscopy. Interaction of P-450$_{scc}$ with the androstane nitroxide (SL-I) and the pregnane nitroxide ester (SL-V) resulted in typical reverse type I difference spectra with maxima at 418–422 nm and minima at about 390 nm. Substances SL-II, SL-III, and SL-IV elicited no spectral change; this lack of effect is attributed to the low solubilities of SL-II and SL-IV as well as their low affinities. SL-III is a nonsteroidal compound that was included to detect nonspecific effects. Even with various organic solvents such as propylene glycol, ethanol, and dimethylformamide in maximum concentrations of 10%, SL-II and SL-IV could not be sufficiently solubilized to elicit any spectral effect. Consequently, only SL-I and SL-V were used for further work.

Spectroscopic equilibrium dissociation constants (K_s) were obtained from the analysis of the concentration dependence of optical difference spectra for the spin-labeled compounds SL-I and SL-V, as well as for the nonlabeled compounds 17α-hydroxy-11-deoxycorticosterone and 17α-hydroxy-11-deoxycorticosterone 21-benzoate (Figure 2A). The apparent K_s value for SL-I was about 20 μM, a value that may be underestimated due to the limited solubility of this compound in the buffer–ethanol mixture. SL-V had an apparent K_s of about 0.5 μM, which is comparable to that of 20-tolyl-pregnenediol, one of the most potent inhibitors of cholesterol side-chain cleavage that can induce a reverse type I spectral change (Uzgiris et al., 1977; Vickery & Kellis, 1982). Furthermore, the affinity of corpus luteum P-450$_{scc}$ for SL-V is 2–50 times greater than published affinities for cholesterol (Hanukoglu et al., 1981; Greenfeld et al., 1981). The nonparamagnetic analogues of SL-V, 17α-hydroxy-11-deoxycorticosterone and 17α-hydroxy-11-deoxycorticosterone 21-benzoate, had apparent K_s's of about 20 and 10 μM, respectively.

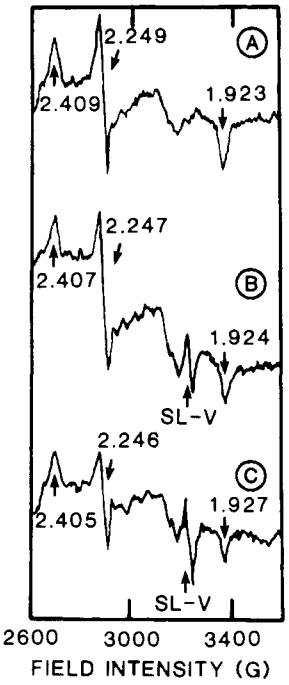

FIGURE 3: Effect of SL-V on EPR spectra of cytochrome P-450$_{scc}$. (A) EPR spectrum of 8.8 μM P-450 with a specific content of 15.4 nmol of P-450/mg of protein. (B) EPR spectrum of 6.7 μM P-450 with a specific content of 15.4 nmol of P-450/mg of protein in the presence of 5.5 μM SL-V. (C) EPR spectrum of 7.1 μM P-450 with a specific content of 3.7 nmol of P-450/mg of protein in the presence of 5.5 μM SL-V. The spectra were recorded between 2600 and 3800 G at 9.099 GHz. Sample temperatures were 93 K. Microwave power was 200 mW, and the receiver gain was 8 × 10^3 for all spectra.

Enzymatic Inhibition of Cholesterol Side-Chain Cleavage. SL-V inhibited cholesterol side-chain cleavage with an apparent K_i of about 1 μM, a value which is in reasonable conformity with its affinity by difference spectroscopy as well as with the inhibitory effects of analogues such as tolyl-pregnenediol (Uzgiris et al., 1977). At a concentration of 20 μM, which is its limit of solubility in nondenaturing solutions, 17α-hydroxy-11-deoxycorticosterone 21-benzoate inhibited cholesterol side-chain cleavage by 40% (Figure 2B). This agrees with the difference spectra data that show saturation with this compound at about 20 μM (Figure 2A).

Modification of EPR Spectra of Cytochrome P-450 by SL-V. The various P-450 preparations obtained from corpus luteum mitochondria had similar EPR low-spin characteristics regardless of the specific content of P-450 (Figure 3). The addition of SL-V had little effect on the calculated g values of the P-450 even for the preparation that had a specific content of 15.4 nmol of P-450/mg of protein. All the spectra have EPR spectral features with principal components of the g tensor within 0.002 of 1.925, 2.247, and 2.407. EPR spectra of samples that contain SL-V or other spin-labeled compounds have, as expected, an additional narrow signal at $g = 2.0$ due to the nitroxide free radical.

Modification of Nitroxide EPR Spectra by Binding to Cytochrome P-450$_{scc}$ and to Its Apoprotein. The free, rapidly tumbling nitroxide spin-label in dilute solution has a sharp, narrow "triplet" signal (Figure 4A). Binding of SL-I and SL-V to P-450$_{scc}$ markedly broadened the N-hyperfine resonance. This is illustrated in the EPR spectrum of Figure 4B where the sharp narrow triplet signal of the free, rapidly tumbling spin-label is seen superimposed on the broadened EPR resonance absorption of the bound spin-labeled species.

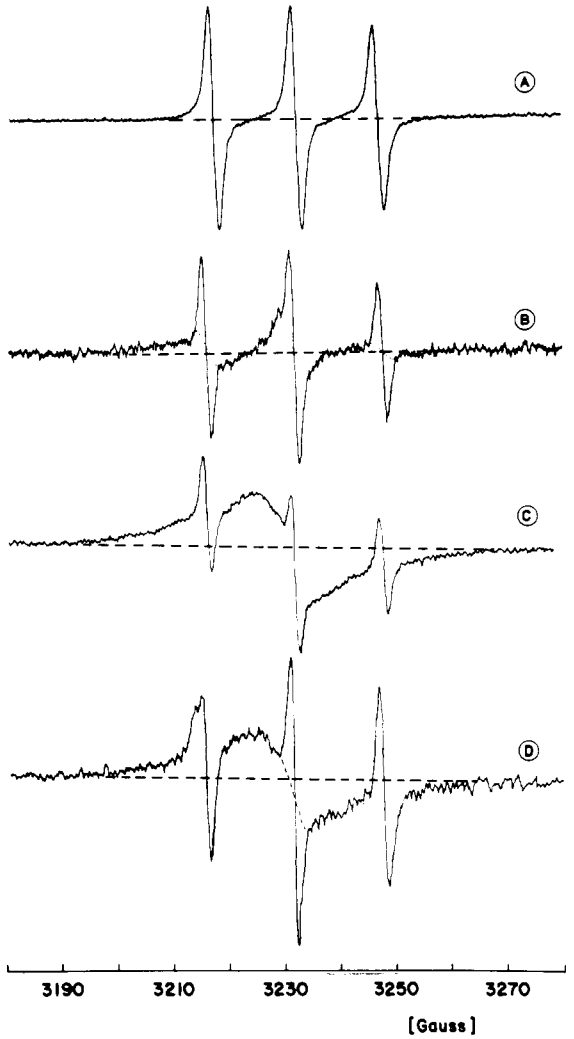

FIGURE 4: EPR spectrum of SL-V in the presence and absence of cytochrome P-450. (A) Hyperfine splitting of 93 μM SL-V in ethanol. Receiver gain was 2×10^2. (B) SL-V (4.6 μM) equilibrated with 8.0 μM P-450$_{scc}$ in buffer A. Receiver gain was 4×10^4. (C) Excess SL-V (93 μM) equilibrated with 6.6 μM P-450$_{scc}$ in buffer A. Receiver gain was 2×10^4. (D) Aggregation of 93 μM SL-V after 30 min in buffer A. Receiver gain was 4×10^4. Specific content of P-450$_{scc}$ was 4.8 nmol of P-450/mg of protein. The spectra were recorded between 3180 and 3280 G at 9.099 GHz. Microwave power was 20 mW. Sample temperatures were 20 °C.

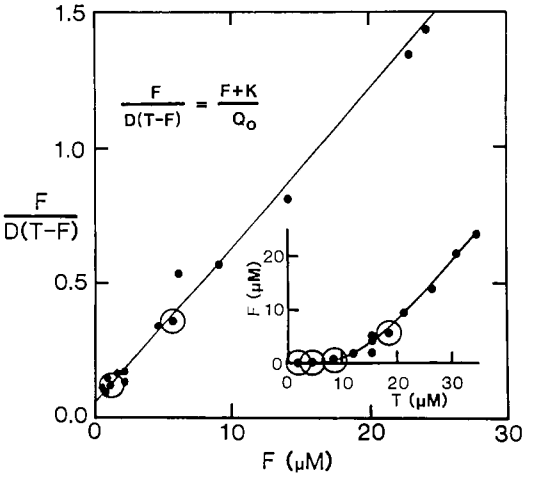

FIGURE 5: Binding of SL-V to cytochrome P-450$_{scc}$ as determined by EPR. Various concentrations of SL-V were added to a solution of P-450$_{scc}$ (specific content, 4.0 nmol of P-450/mg of protein; initial protein concentration, 1 mg/mL). The concentration F of unbound SL-V was calculated from the unbroadened hyperfine lines at a g value of about 2. T is the concentration of the total SL-V present, and D is the amount of dilution incurred upon addition of SL-V. The insert is a direct plot of F vs. T. The curve is the insert was calculated from the values of the equilibrium dissociation constant K and the initial concentration of binding sites Q_0 indicated by the Woolf plot and included the dilution factor D. The open circles indicate second, superimposing data points.

Table II: Equilibrium Dissociation Constants of SL-V and Binding Site Concentrations of P-450$_{scc}$ by Several Methods

method	equilibrium dissociation constant of SL-V (μM)	concentration of binding sites (μM)
optical absorbance	0.5	4[a]
EPR	1.1	17
enzymatic inhibition	0.8	
protein concentration		15[b]

[a] Based on a difference in absorbance of 91 mM^{-1} cm^{-1} between 450 and 490 nm for the P-450–CO complex. [b] Based on a molecular weight of 48 000 for P-450.

At high concentrations, on the order of 100 μM, self-aggregation of the spin-labeled compounds became the dominating feature of these spectra even in the absence of protein (Figure 4C,D). At low concentration of SL-V, however, the effects of aggregation and nonspecific binding to other proteins, such as albumin, are negligible. Thus, problems associated with self-aggregation or nonspecific binding were avoided by using SL-V at concentrations less than 30 μM. In control experiments with bovine serum albumin (5 mg/mL) comparable concentrations of SL-V showed neither self-aggregation nor significant nonspecific binding.

Affinity of SL-V for P-450$_{scc}$. Concentrations of free SL-V were measured at various total concentrations of the spin-labeled compound (Benson et al., 1977; Basset et al., 1975) in the presence of a P-450 preparation that had a specific content of 4.0 nmol of P-450/mg of protein. From the Woolf plot analysis, the dissociation constant, K_D, of the P-450$_{scc}$–SL-V complex was calculated to be 1.1 μM (Figure 5).

Concentration of SL-V Binding Sites on P-450$_{scc}$. The concentration of binding sites for SL-V, estimated from the Woolf plot, is 17 μM. This plot was derived from measurements of a preparation that had a P-450 concentration of 4.0 μM when the P-450 content was estimated from the absorbance of the P-450–CO complex (Omura & Sato, 1964) (Table II). By SDS–polyacrylamide gel electrophoresis, the protein was about 70% homogeneous. By gravimetric analysis based on a molecular weight of 48 000 (Kashiwagi et al., 1980) and a protein concentration of 1.0 mg/mL, the concentration of P-450 apoprotein of this preparation is 15 μM.

Displacement of SL-V from Cytochrome P-450 by Substrates and Analogues. Although SL-V is an ester of 17α-hydroxy-11-deoxycorticosterone, the substance resembles an analogue of cholesterol in this system. Thus, cholesterol was more effective than either 11-deoxycorticosterone or 17α-hydroxy-11-deoxycorticosterone in displacing SL-V from the P-450 (Table III). At a much lower ratio of steroids, 25-hydroxycholesterol was even more effective than cholesterol in displacing SL-V, presumably because of its greater solubility. Preincubation of P-450$_{scc}$ with 25-hydroxycholesterol before the addition of SL-V doubled the relative increase of free SL-V. Since equilibrium had not been reached in 15 min, the rate exchange between the SL-V bound to P-450$_{scc}$ and 25-hydroxycholesterol is quite slow. These data were confirmed by repeating the study with a preparation of P-450$_{scc}$ that had a specific content of 4.8 nmol of P-450/mg of protein. Cholesterol, 17α-hydroxy-11-deoxycorticosterone, and 11-deoxycorticosterone displaced 4.5 μM SL-V from 7.6 μM P-450$_{scc}$ comparably.

Table III: Displacement of Bound SL-V from P-450$_{scc}$ by Competing Compounds

compd	[compd] (μM)	[P-450] (μM)	[SL-V] (μM)	increase in free SL-V (%)	rel increase in free SL-V (%/μM)[a]
cholesterol	85	1.3[b]	4.4	180	7.2
17α-hydroxy-11-deoxy-corticosterone	94	1.3[b]	4.4	80	2.9
11-deoxy-corticosterone	138	1.3[b]	4.4	120	2.9
25-hydroxy-cholesterol	24	2.8[c]	9.9	30	4.4
25-hydroxy-cholesterol[d]	24	2.8[c]	9.9	60	8.8

[a] Relative increase was calculated according to the formula:

$$\text{rel increase} = \frac{\text{total increase (\%)}}{\text{[P-450] (}\mu\text{M)}} \frac{\text{[SL-V] (}\mu\text{M)}}{\text{[displacing compd] (}\mu\text{M)}}$$

[b] Specific content = 7.2 nmol of P-450/mg of protein. [c] Specific content = 1.5 nmol of P-450/mg of protein. [d] P-450 was incubated for 15 min with the competing compound before addition of SL-V.

Discussion

Of the spin-labeled steroids tested, two compounds, SL-I and SL-V, had significant affinity for cytochrome P-450$_{scc}$ (Figure 2A). SL-I, labeled with the nitroxide affixed to the 3-position of androstanediol, had an apparent K_s of about 20 μM. However, the maximal absorbance difference obtained by spectrophotometric methods depends, in part, on the solubility of the affinant. Since the maximum absorbance difference for SL-I was only 0.02 (nmol of P-450)$^{-1}$ mL^{-1}, it is evident that saturation of the P-450$_{scc}$ did not occur. By comparison, the spectral changes induced by 17α-hydroxy-11-deoxycorticosterone, an equally weak affinant with an apparent K_s about 20 μM, showed a maximum absorbance difference of 0.045 (nmol of P-450)$^{-1}$ mL^{-1} at a concentration of about 90 μM. On the other hand, SL-V also has low solubility, but its affinity is so great that it achieves maximal optical difference absorbance at less than 30 μM. As stated above, this affinity is the same order of magnitude as that of other high-affinity substances, including 20-*p*-tolyl and 20-phenyl analogues of cholesterol (Uzgiris et al., 1977; Hochberg et al., 1974; Vickery & Kellis, 1983) and 22-azacholesterol analogues with aryl-substituted side chains (Delaney & Lu, 1981), among others.

Substance SL-V was equally effective as an inhibitor of cholesterol side-chain cleavage (Figure 2B) and an inducer of reverse type I spectral changes. However, SL-V has some notable structural differences from the reported analogues of cholesterol. It is unexpected that a steroid with the 4-en-3-one configuration in the A–B rings and a side chain with three oxygen-containing functional groups at 17, 20, and 21, in addition to the oxygen of the ester link, would have high affinity for P-450$_{scc}$ (Graves et al., 1980). Furthermore, the tetramethyl-1-oxypyrrolidine substituent differs significantly from the hydrophobic phenyl derivatives at carbon 22 or elsewhere on the steroid side chain of other potent inhibitors. In spite of these nonconformities with other model compounds, SL-V does have high affinity for the enzyme and high inhibitory potency and is, therefore, a unique tool for study of the substrate site.

One goal of this study was to probe the location and orientation of cholesterol substrate in the cytochrome P-450$_{scc}$ molecule. By labeling both "ends" of the molecule, it might be possible to ascertain the distance of each end from the heme Fe^{3+} of the active center. Carbon 21 of SL-V may be considered to be analogous to carbon 22 of cholesterol because of the intervening oxygen. The distance of the nitroxide group from carbon 21 of SL-V is from 0.7 to 0.8 nm on CPK space filling and Dreiding skeletal models and more than 1 nm from the heme iron if allowance is made for a molecule of oxygen between carbon 21 of SL-V and the heme. If the heme iron were a few tenths of a nanometer from carbon 21, the nitroxide radical would be expected to influence the EPR spectrum of the heme. However, no changes could be detected in the low-temperature EPR spectra of the P-450 within the technical limits of the study (Figure 3).

SL-I has moderate affinity for P-450$_{scc}$ but low solubility. The location of its spin-labeled group at carbon 3 of the A ring is more than twice as far from the side chain as was the nitroxide radical on SL-V. As expected, the nitroxide radical on SL-I also did not modify the EPR spectrum of the cytochrome P-450.

From these data, it appears that neither the A ring nor the terminal carbons of the side chain are close to the heme iron. This conforms to the reasonable hypothesis that the most proximate carbon to the heme iron is carbon 22, which is the first carbon to be hydroxylated (Burstein et al., 1975). Carbon 22 is also the site where modification of steroids has the greatest effect on inhibition of cholesterol side-chain cleavage (Burstein et al., 1976; Delaney & Lu, 1981; Kellis et al., 1984). Whether the side chain is oriented in extended configuration from carbon 23 as proposed by Teicher et al. (1978) or is folded back toward the cholesterol nucleus, as we believe, is not demonstrable from these data and, in fact, may not be important for understanding the side-chain cleavage.

Specific content of cytochrome P-450 is determined conventionally by computing the molarity from the optical difference spectrum of the reduced P-450–CO spectrum between 450 and 490 nm by using a coefficient of absorption of 91 mmol^{-1} cm^{-1} (Omura & Sato, 1964). On the basis of a molecular weight of 48 000 (Kashiwagi et al., 1980), the theoretical maximum specific content for absolutely pure P-450 without heme loss would be 20.8 nmol/mg of protein. The highest specific content reported so far for corpus luteum P-450$_{scc}$ is 15.4 nmol/mg of protein; this P-450 was greater than 99% pure as judged by equilibrium ultracentrifugation and SDS–polyacrylamide gel electrophoresis (Kashiwagi et al., 1980). The spectroscopically determined specific content of that cytochrome P-450 agreed reasonably well with the specific content for iron of 16.8 nmol/mg of protein as determined by atomic absorption and the specific content for heme of 12.0 nmol/mg of protein as determined by pyridine hemochromogen (Kashiwagi et al., 1980). Thus, heme loss occurred during purification. In our experience, a variable amount of heme loss is unavoidable in the preparation of cytochrome P-450$_{scc}$ and probably other P-450's as well (Smith et al., 1983).

One binding site for SL-V per P-450 apoprotein is indicated by the 1:1 ratio of the concentration of SL-V binding sites (17 μM) by Woolf plot analysis and the concentration of P-450 apoprotein (15 μM) by gravimetric analysis (Figure 5, Table II). Comparison of these values with the concentration of heme determined by optical absorbance of the P-450–CO complex (4 μM) indicates that this preparation had lost about 75% of its heme. Titration experiments of Orme-Johnson et al. (1979) also detected binding site to heme ratios of greater than 1. Their data for 20α-hydroxycholesterol, for example, indicated about 1.3 sites per heme. This is consistent with a loss of about 25% of the heme from the cytochrome. Many

authors have recognized the inevitability of some heme loss (Smith et al., 1983); spin-labeled substrates provide a method of direct quantification of the amount of loss.

The relative contribution of the apoprotein and heme to the energy of substrate binding can be calculated from equilibrium dissociation constants K_D ($\Delta G° = -RT \ln K_D$) for 293 K. The equilibrium dissociation constant of SL-V in the 75% heme-less preparation of P-450 was 1.1 μM (Figure 5, Table II), which yields $\Delta G° = -8.0$ kcal/mol. The K_D of SL-V for P-450 with intact heme was 0.5 μM (Figure 2A, Table II), which yields $\Delta G° = -8.5$ kcal/mol. Therefore, -8.0 kcal/mol out of -8.5 kcal/mol can be accounted for by the affinity of SL-V for the apoprotein. Thus, the heme makes little contribution to substrate binding.

These findings permit some speculation on the microenvironment of the cytochrome P-450$_{scc}$ active site and on changes occurring at the active site during the process of side-chain cleavage. Substrate-free heme protein exists predominantly in a hexacoordinated low-spin ferric form. The sixth ligand is believed to be a histidine nitrogen from the protein chain (Dus et al., 1973, 1974). This proposal is supported by the high-spin to low-spin shifts that occur with increasing pH in the range 7.0–8.0 (Mitani & Horie, 1969). Substrate displaces the histidine ligand to form the pentacoordinate high-spin form, and substrate analogue inhibitors act to maintain the hexacoordinate form. Rupture of this ligand leads to a conformational change in the apoprotein that "opens" the iron–sulfur protein binding site. Thus, the presence of substrate (high spin, pentacoordinated) enhances iron–sulfur protein binding, and iron–sulfur protein binding enhances the affinity of cholesterol (Hanukoglu et al., 1981). These accommodations must occur as a result of changes in the histidine ligand.

Acknowledgments

We thank Dr. W. R. Benson, FDA, Washington, DC, for the gift of SL-IV and SL-V and Dr. K. Arends, Steraloids, Inc., for synthesizing 17α-hydroxy-11-deoxycorticosterone 21-benzoate.

Registry No. P-450$_{scc}$, 9035-51-2; SL-V, 63793-95-3; cholesterol, 57-88-5.

References

Basset, M., DeFaye, G., & Chambaz, E. M. (1975) *FEBS Lett. 60*, 364–367.

Benson, W. R., Maienthan, M., Yang, G. C., Sheinin, E. B., & Chung, C. W. (1977) *J. Med. Chem. 20*, 1308–1312.

Burstein, S., Middleditch, B. S., & Gut, M. (1975) *J. Biol. Chem. 250*, 9028–9037.

Burstein, S., Letourneux, Y., Kimball, H. L., & Gut, M. (1976) *Steroids 27*, 361–382.

Delaney, N. G., & Lu, M. C. (1981) *J. Med. Chem. 24*, 1034–1037.

Dus, K., Miguel, A. G., Smith, P. C., Litchfield, W. J., & Harrison, J. R. (1973) Abstracts of the 9th International Congress of Biochemistry, Stockholm, p 341.

Dus, K., Litchfield, W. J., Miguel, A. G., van der Hoever, T. A., Dean, W. L., & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 60*, 15–21.

Graves, P. E., & Salhanick, H. A. (1979) *Endocrinology (Baltimore) 105*, 53–57.

Graves, P. E., Uzgiris, V. I., & Salhanick, H. A. (1980) *Steroids 35*, 543–559.

Greenfeld, N. J., Gerolimatos, B., Szwergold, B. S., Wolfson, A. J., Prasad, V. V. K., & Lieberman, S. (1981) *J. Biol. Chem. 256*, 4407–4417.

Haldane, J. B. S., & Stern, K. (1932) *Algemeine Chemie der Enzyme*, pp 119–120, Steinkopf, Leipzig and Berlin.

Hanukoglu, I., Spitsberg, V., Bumpus, J. A., Dus, K. M., & Jefcoate, C. R. (1981) *J. Biol. Chem. 256*, 4321–4328.

Hochberg, R. B., McDonald, P. D., Feldman, M., & Lieberman, S. (1974) *J. Biol. Chem. 249*, 1279–1285.

Kashiwagi, K., Dafeldecker, W. P., & Salhanick, H. A. (1980) *J. Biol. Chem. 255*, 2606–2611.

Kellis, J. T., Jr., Sheets, J. L., & Vickery, L. E. (1984) *J. Steroid Biochem. 20*, 671–676.

Mitani, F., & Horie, S. (1969) *J. Biochem. (Tokyo) 66*, 139–149.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2379–2385.

Orme-Johnson, N. R., Light, D. R., White-Stevens, R. W., & Orme-Johnson, W. H. (1979) *J. Biol. Chem. 254*, 2103–2111.

Rodbard, D. (1973) in *Receptors for Reproductive Hormones* (O'Malley, B. W., & Means, A. R., Eds.) pp 289–326, Plenum, New York.

Schenkman, J. B., Cinti, D. L., Orrenius, S., Moldeus, P., & Kraschnitz, R. (1972) *Biochemistry 11*, 4243–4251.

Smith, M. L., Hjortsberg, K., Ohlsson, P.-I., & Paul, K. G. (1983) *Biomed. Biochim. Acta 42*, 805–811.

Teicher, B. A., Koizumi, N. K., Koreeda, M., Shikita, M., & Talalay, P. (1978) *Eur. J. Biochem. 91*, 11–19.

Thompson, E. A., & Siiteri, P. K. (1974) *J. Biol. Chem. 249*, 5373–5378.

Uzgiris, V. I., Graves, P. E., & Salhanick, H. A. (1977) *Biochemistry 16*, 593–600.

Vickery, L. E., & Kellis, J. T. (1983) *J. Biol. Chem. 258*, 3832–3836.

Whysner, J. A., Ramseyer, J., & Harding, B. W. (1970) *J. Biol. Chem. 245*, 5441–5449.