Cytochrome P-450$_{scc}$-Mediated Oxidation of (20*S*)-22-Thiacholesterol: Characterization of Mechanism-Based Inhibition†

Emily Miao,‡,§ Subhendu Joardar,‡,∥ Chunsong Zuo,‡,⊥ Normand J. Cloutier,‡ Atsushi Nagahisa,‡,Δ Chang Byon,‡,# Stephen R. Wilson,*,▽ and William H. Orme-Johnson*,‡

Contribution from the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the Department of Chemistry, New York University, New York, New York 10003

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ABSTRACT: (20*S*)-22-thiacholesterol (**1**) is found to be a potent competitive inhibitor of pregnenolone biosynthesis from cholesterol by purified reconstituted bovine adrenal cytochrome P-450$_{scc}$. The apparent dissociation constant K_d, determined from difference spectra, is 0.6 μM, close to the value from kinetic studies for the apparent inhibition constant, K_i, of 0.8 μM. Studies of the time course of pregnenolone production indicate that under turnover conditions the competitive inhibitor (**1**) is converted to a tighter binding inhibitor, shown to be (20*S*,22*R*)-22-thiacholesterol *S*-oxide (**4**), with high diastereoselectivity and in a time-dependent manner. Both the diastereomeric sulfoxides, (20*S*,22*S*)-22-thiacholesterol *S*-oxide (**3**) and (20*S*,22*R*)-22-thiacholesterol *S*-oxide (**4**), exhibit properties consistent with their being competitive *versus* cholesterol, but the (22*R*)-sulfoxide (**4**) binds approximately 10 times more tightly than the (22*S*) diastereomer (**3**). The apparent K_d values of sulfoxides **4** and **3** are 0.1 and 1.14 μM, respectively. EPR and absorption spectroscopic studies of enzyme−inhibitor complexes suggest direct coordination of the oxygen atom of the (22*R*)-sulfoxide (**4**) with the catalytic heme center. This implies that the inhibitor operates by directly blocking further reaction at the active site heme group, with a substantial lifetime of the enzyme−inhibitor complex.

Cytochrome P-450$_{scc}$[1] catalyzes three successive oxygenations of cholesterol, eventuating in the scission of the C-20−C-22 bond and the formation of pregnenolone and isocapraldehyde as the first step in mammalian steroidogenesis (Burstein & Gut, 1971, 1976; Koritz & Kumar, 1970; Sato & Omura, 1978). One major issue in the study of P-450$_{scc}$ centers on the definition of the steric and electronic properties of the steroid binding pocket. All three oxidation steps are believed to occur at the same heme active site, and each step in the transformation from cholesterol to pregnenolone is both regioselective and stereospecific. The origins of the regio- and stereoselectivity are the specific orientations in which cholesterol and the two hydroxylated sterol intermediates are bound in the enzyme active site.

In the present studies, the inhibitory, kinetic, and spectral properties of a mechanism-based sulfur-containing cholesterol analog, (20*S*)-22-thiacholesterol (**1**) (S. R. Wilson, E. Miao, P. A. Caldera, M. Guzewska, A. M. Venkatesan, J. Dewan, A. Nagahisa, S. Joardar, & W. H. Orme-Johnson, manuscript in preparation), on purified bovine adrenal mitochondrial P-450$_{scc}$ were investigated. Under turnover conditions, this inhibitor is converted enzymatically to a more potent inhibitor (a sulfoxide) with a preferred configuration at the 22 position, thus further demonstrating the stereoselective nature of a steroidogenic oxygen-transfer reaction catalyzed by cytochrome P-450$_{scc}$. This is an example of a special case of mechanism-based inhibition, the formation of a slow, tight-binding inhibitor, i.e., a "quasi-irreversible inhibitor", during turnover.

MATERIALS AND METHODS

Cytochrome P-450$_{scc}$ (Orme-Johnson *et al.*, 1979), adrenodoxin (Orme-Johnson & Beinert, 1969), and adrenodoxin reductase (Sugiyama & Yamano, 1978) were purified using previously published procedures. The P-450 content was determined from the carbon monoxide binding difference spectra for the dithionite-reduced enzyme plus carbon monoxide *versus* reduced enzyme alone, using a difference extinction coefficient ($A_{450} - A_{490}$) of 91 mM^{-1} cm^{-1} according to the method of Omura and Sato (1963, 1964). Protein was determined by a modified Lowry method (Peterson, 1977). The heme content of the P-450 preparations used for the studies ranged from 5 to 12 nmol/mg of protein. Syntheses and characterization of (20*S*)-22-thiacholesterol (**1**), (20*S*,22*S*)-22-thiacholesterol *S*-oxide (**3**), and (20*S*,22*R*)-22-thiacholesterol *S*-oxide (**4**) will be detailed in another paper (S. R. Wilson, E. Miao, P. A. Caldera, M. Guzewska, A. M. Venkatesan, J. Dewan, A. Nagahisa, S. Joardar, & W. H. Orme-Johnson, manuscript in preparation). (20*S*)-22-[21-^{3}H]thiacholesterol was prepared by following

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* Author to whom correspondence should be addressed.

‡ Massachusetts Institute of Technology.

§ Present address: Allegretti & Witcoff Ltd., 10 South Wacker Dr., Chicago, IL 60606.

∥ Present address: 4B, Evergreen Plaza; 117, B. T. Road; Calcutta, India 700 035.

⊥ Present address: NMR Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129

Δ Present address: Pfizer Central Research Nagoya, 5-2 Thaetoyo, Aichi 470-23, Japan.

Present address: New England Nuclear, Boston, MA.

▽ New York University.

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[1] Abbreviations: P-450$_{scc}$, cytochrome P-450 cholesterol side-chain cleavage enzyme; EPR, electron paramagnetic resonance; LAH, lithium aluminum hydride; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tween 20, polyoxyethylene sorbitan monolaurate, EDTA, ethylenediaminetetraacetic acid; GPED buffer, glycerol (20%, v/v), potassium phosphate (0.1 M, pH 7.4), 1.0 mM EDTA, and 1 mM dithiothreitol.

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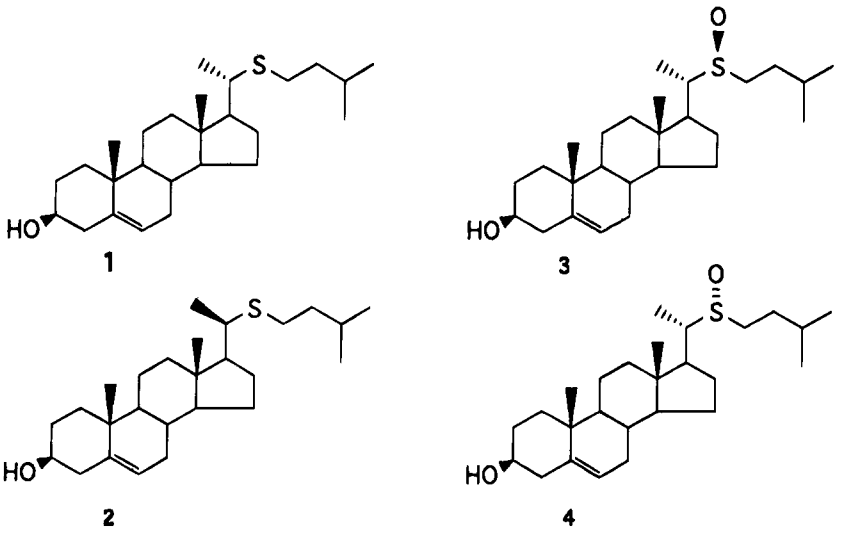

FIGURE 1: Chemical structures of sulfur-containing cholesterol analogs used in the present studies.

the same method as used for **1**, except that lithium aluminum tritide was used instead of LAH for the reduction of the C-21 ethyl ester of (20*S*)-22-thiacholesterol synthetic intermediate (S. R. Wilson, E. Miao, P. A. Caldera, M. Guzewska, A. M. Venkatesan, J. Dewan, A. Nagahisa, S. Joardar, & W. H. Orme-Johnson, manuscript in preparation). NADPH, MOPS, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, catalase, cholesterol, and pregnenolone were purchased from Sigma, and carbon monoxide was from Matheson Gas Products. [1,2-$^3H_2$]Cholesterol and [4-^{14}C]pregnenolone were purchased from New England Nuclear. All other materials were of reagent grade.

Enzyme Incubations and Kinetic Studies. The enzyme activity was assayed at 37 °C essentially according to the method of Takikawa *et al.* (1978), as modified by Nagahisa *et al.* (1985a). Incubations were carried out in 50 mM MOPS (pH 7.2), 10 mM $MgCl_2$, 0.2% Tween 20, and 1 unit/mL catalase in the presence of an NADPH-regenerating system consisting of 32 μM NADPH, 3 mM glucose 6-phosphate, 1 unit/mL glucose-6-phosphate dehydrogenase, and 100 μM cholesterol. A typical reconstituted system was 0.25 μM in P-450$_{scc}$, 2 μM in adrenodoxin reductase, and 0.1 μM in adrenodoxin. The reaction was initiated by the addition of the NADPH-regenerating system. The addition of catalase in the assay system protects P-450$_{scc}$ from self-catalyzed inactivation via oxidation of the active site heme (Nagahisa *et al.*, 1985a).

Spectrophotometric Studies. Absorption spectra were obtained on a Perkin-Elmer 557 double-wavelength double-beam spectrophotometer equipped with a Haake F-3 circulating water bath. The spectra were taken at 25 °C using 10-mm path length quartz cuvettes. Complexes of P-450$_{scc}$ with steroid were prepared by adding steroid (10 mM stock solution in ethanol) to 5 μM P-450$_{scc}$ in 0.1 M potassium phosphate (pH 7.4) containing 1.0 mM EDTA, 1 mM dithiothreitol, and 20% glycerol ("GPED" buffer). After visible spectra were recorded, the samples were transferred to 4-mm (outer diameter) quartz EPR tubes and frozen in liquid nitrogen.

EPR Studies. EPR spectroscopy was carried out using a Bruker ESP 300 CW spectrometer at 9.29 GHz. Other conditions of the EPR spectra were as given in the captions for Figures 5 and 6.

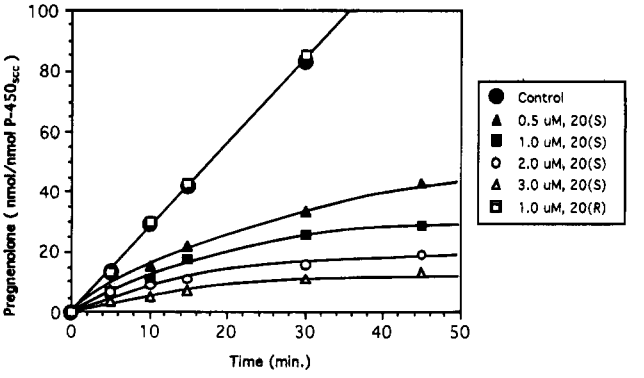

FIGURE 2: Inhibition of cholesterol side-chain cleavage reaction. All samples were 0.25 μM in P-450$_{scc}$ and 100 μM in cholesterol. Control solutions had no inhibitor.

Binding Studies. P-450$_{scc}$ in 0.1 M potassium phosphate buffer was titrated with steroid inhibitor stock solution, and the change in the difference in absorptions at 420 and 390 nm ($\Delta A_{420-390}$) was followed spectrophotometrically. The dissociation constants (K_d) were determined from double-reciprocal plots ($1/\Delta A_{420-390}$ *vs* $1/[\text{Inhibitor}]_{free}$) as described previously (Orme-Johnson *et al.*, 1979; Light 1978; Sheets & Vickery, 1982, 1983).

RESULTS

Enzymatic Incubations. The time course of the cholesterol side-chain cleavage reaction catalyzed by the reconstituted cytochrome P-450$_{scc}$ system is shown in Figure 2. Both adrenodoxin and adrenodoxin reductase were present in catalytic excess with P-450$_{scc}$ as the rate-limiting component in the reconstituted system. In the absence of inhibitor, pregnenolone production is linear for at least 15 min with a turnover number of 3 min^{-1}.

In the presence of (20*S*)-22-thiacholesterol (**1**), the rate of pregnenolone production progressively decreases with time, in contrast to the constant rate exhibited by the control (no inhibitor) as shown in Figure 2. In the presence of 1 μM (20*S*)-22-thiacholesterol (**1**), the reaction is inhibited by approximately 75%; in contrast, P-450$_{scc}$ activity was not diminished by (20*R*)-22-thiacholesterol (**2**) at comparable concentrations (see Figure 2) and under the time scales of the measured kinetics.

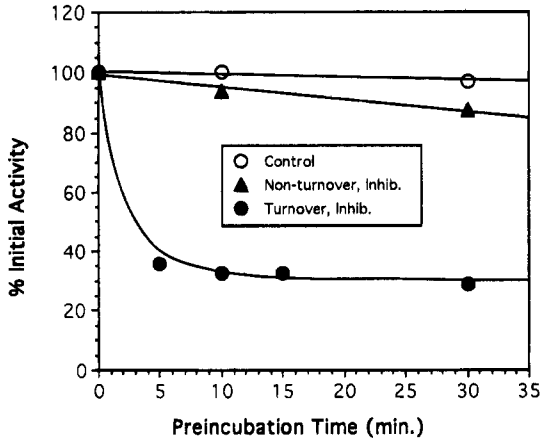

FIGURE 3: Activity of P-450$_{scc}$ after incubation with 22-thiacholesterol (1). 2.5 μM P-450$_{scc}$ was preincubated with 10 μM thiacholesterol, and at several time points, 100-μL aliquots were assayed for enzyme activity. Control assay solution contained NADPH but no inhibitor, non-turnover inhibitor solution had no NADPH, and turnover inhibitor solution had NADPH.

To determine the nature of inhibition by (20*S*)-22-thiacholesterol (**1**), the rate of pregnenolone formation was measured using several concentrations of cholesterol and the thiacholesterol (**1**). The initial velocities of the formation of pregnenolone in the presence of (20*S*)-22-thiacholesterol (**1**) were measured at inhibitor concentrations of 0.5, 1.0, and 2.0. With this data, the classical double-reciprocal plot and the corresponding slope replot were obtained (Segel, 1975), establishing that (20*S*)-22-thiacholesterol (**1**) is a competitive-type inhibitor with an apparent K_i value of 0.8 μM (Table 3). The nonlinear plots (Figure 2) obtained from the time course of pregnenolone production, however, suggest that simple competitive inhibition by (20*S*)-22-thiacholesterol (**1**) was not the only factor in the observed inhibition of pregnenolone formation (see Discussion).

In preincubation studies with (20*S*)-22-thiacholesterol (**1**), as shown in Figure 3, a loss of enzyme activity ($t_{1/2}$ = 2.5 min), dependent on the length of prior incubation with the inhibitor (**1**) in the presence of NADPH and O_2, was observed. In the absence of NADPH and O_2, however, essentially no inactivation of the enzyme on preincubation with (**1**) was observed.

An independent incubation of (20*S*)-22-[21-^{3}H]thiacholesterol with P-450$_{scc}$ in the presence of NADPH and O_2 produced a mixture of steroidal sulfoxide derivatives. Comparison of the enzymatic products with chemically synthesized steroidal sulfoxides revealed that the (22*R*)-sulfoxide (**4**) is preferentially formed in the enzymatic process by a factor of 4.2 to 1 over the (22*S*)-sulfoxide (**3**) (Table 1). X-ray crystallography and NMR studies (S. R. Wilson, E. Miao, P. A. Caldera, M. Guzewska, A. M. Venkatesan, J. Dewan, A. Nagahisa, S. Joardar, and W. H. Orme-Johnson, manuscript in preparation) allowed the assignment of the absolute stereochemistry of the two 22-sulfoxides, which supports our view that the more tightly binding sulfoxide is produced by the stereoselective enzymatic oxidation at the 22-sulfur atom of the (20*S*)-22-thiacholesterol inhibitor (**1**).

The rate of the cholesterol side-chain cleavage reaction was measured at (22*R*)-sulfoxide (**4**) concentrations of 0.0, 0.05, 0.1, and 1.0 μM. The degrees of inhibition measured were 25% and 44% for (22*R*)-sulfoxide concentrations of 0.05 and 0.1 μM, respectively. The reaction was virtually completely inhibited by the presence of 1.0 μM (22*R*)-sulfoxide (**4**); in contrast, the P-450$_{scc}$ activity was not inhibited by equivalent concentrations of the (22*S*)-sulfoxide (**3**) (data not shown).

Separate incubations were performed to determine whether the two sulfoxides were substrates of P-450$_{scc}$. Incubations of the (22*R*)- and (22*S*)-sulfoxides under normal turnover conditions yielded no product detectable on either TLC or gas chromatographic analysis.

The dissociation constants (K_d) were determined spectrophotometrically from absorption difference spectra for the 22-thiacholesterol (**1**) and the (22*R*)- (**4**) and (22*S*)-sulfoxides (**3**), by titration of the low-spin ferric P-450$_{scc}$ (Orme-Johnson *et al.*, 1979; Light, 1978; Sheets & Vickery, 1982, 1983). The titration experiments were carried out in 0.1 M potassium phosphate buffer (pH 7.4) at 20 °C. The K_d values obtained for (22*R*)-(**4**) and (22*S*)-sulfoxide (**3**) and that of (20*S*)-22-thiacholesterol (**1**) are 0.1, 1.14, and 0.6 μM, respectively (Table 3). The on and off rates of binding of sulfoxides **3** and **4** were not measured. In addition, the kinetic K_i

Table 1: Relative Yields of Steroidal Sulfoxides Produced by Enzymatic and Chemical Oxidation of (20*S*)-22-Thiacholesterol (**1**)

(20S)-22-Thiacholestrol (1) + [O] → A + B + C

oxidation condition	thiasteroid[a] (equiv)	% oxidation	% abundance of products[b] A	B	C
P-450$_{scc}$-catalyzed	5	55	61	29	10
	2	82	61	25	14
	1.5	92	75	18	7
H_2O_2/acetone		38	41	54	5

[a] Stoichiometry with respect to P-450$_{scc}$. [b] A = (20*S*,22*R*)-22-thiacholesterol *S*-oxide (**4**); B = (20*S*,22*S*)-22-thiacholesterol *S*-oxide (**3**); C = unidentified product.

Table 2: EPR *g*-Values and Soret Absorption Maxima of Cytochrome P-450 complexes

complex (ligand-type)	g_x	g_y	g_z	λ_{max} (nm)	ref
P-450$_{cam}$					
oxygen donors	2.43−2.48	2.27	1.91−1.93	415−420	17
sulfur donors	2.50	2.27	1.89	424	17
P-450$_{scc}$					
native	2.44	2.25	1.91	387	*a*
1	2.44	2.24	1.91	418	*a*
2	2.44	2.25	1.91	395	*a*
3	2.45	2.24	1.90	414	*a*
4	2.48	2.25	1.90	420	*a*

[a] Present work.

Table 3: Summary of Inhibitory and Binding Studies of Some Sulfur Analogs of Cholesterol

compound	inhibition/ binding pattern	K_d (μM)	K_i (mM)
(20*S*)-22-thiacholesterol	competitive[a]	0.67	0.8
(20*R*)-22-thiacholesterol	no inhibition[b]	not measured	N/A
(20*S*,22*R*) sulfoxide	competitive[c]	0.1	not measured
(20*S*,22*S*) sulfoxide	competitive[c]	1.1	not measured

[a] Determined by enzyme inhibition kinetic studies. [b] No inhibition was observed for this compound, on comparable time scales and with the concentrations that were used in the study of (20*S*)-22-thiacholesterol. [c] Determined by absorption difference and EPR binding studies.

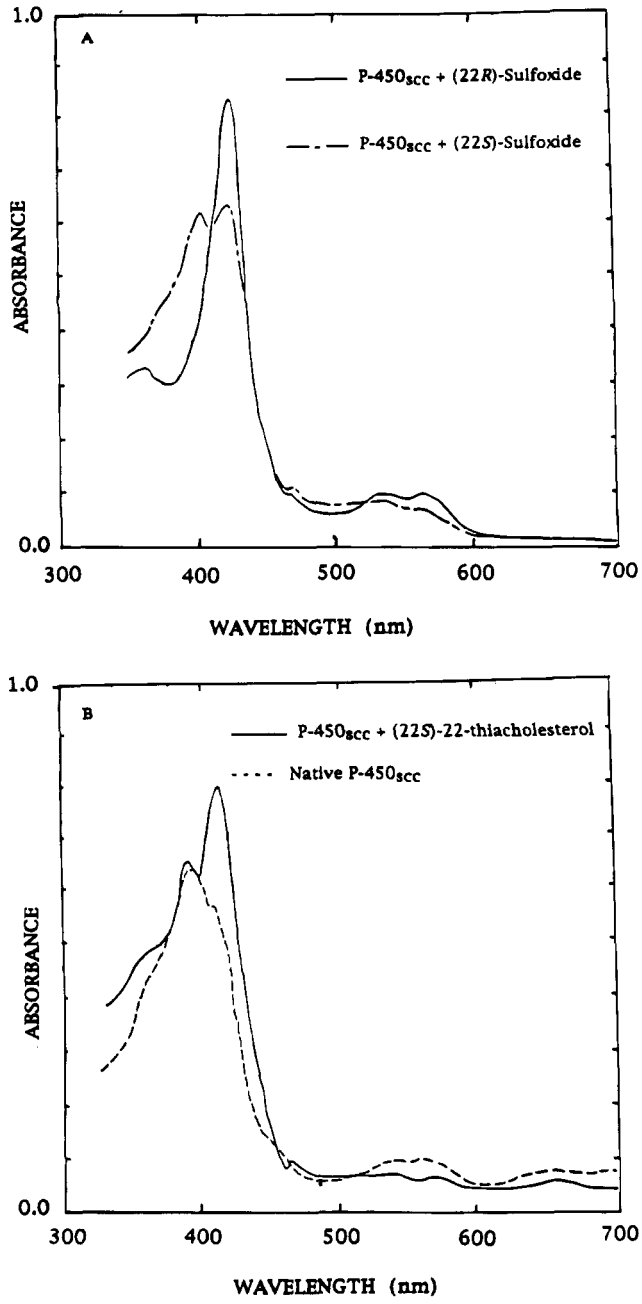

FIGURE 4: Optical absorbance spectra of P-450$_{scc}$ thiasterol complexes. The P-450$_{scc}$ concentration was 5 μM; 1-mm cuvette. A: —, 25 μM in (22*R*)-sulfoxide (**4**); — - —, 25 μM in (22*S*)-sulfoxide (**3**). B: —, 25 μM in (20*S*)-22-thiacholesterol; - - -, native P-450$_{scc}$ as isolated.

determinations were not obtained for either sulfoxide **3** or **4**, nor was their inhibition pattern verified as competitive by the usual intersecting double-reciprocal lines, obtainable for kinetic experiments at different concentrations of inhibitor (Segel, 1975). However, the dissociation constants obtained from spectroscopic titration and the formation of a low-spin form of the heme (Figure 5) as well as the shift of the absorbance spectra (Figure 4) are consistent with coordination to the heme as a mode of binding. Subsequent structural studies of the active site (with bound C-20 deuterated sulfoxide (**4**)) also place the sulfoxide in a coordinating position, with respect to the heme iron. From this, we judge these diastereomeric sulfoxides to be competitive inhibitors *versus* cholesterol in cytochrome P-450$_{scc}$.

Because the absorption spectra of P-450$_{scc}$ are sensitive to pH, buffer, and temperature, all spectra were taken at 25 °C in GPED buffer. The absorption spectra of ferric P-450$_{scc}$ complexes of (22*R*)- (**4**) and (22*S*)-sulfoxide (**3**) and (20*S*)-22-thiacholesterol (**1**) are shown in Figure 4. EPR spectra of native cytochrome P-450$_{scc}$ and its complexes with (22*R*)-(**4**) and (22*S*)-sulfoxide (**3**) as well as with (20*S*)-22-thiacholesterol (**1**) and (20*R*)-22-thiacholesterol are compared in Figure 5. Figure 6 is an EPR spectrum for a more concentrated sample of a P-450$_{scc}$ complex of the (22*R*)-sulfoxide (**4**), which proves that the complex of (**4**) is essentially low-spin (see Discussion). The *g*-values obtained for the present P-450$_{scc}$ complexes are within the range reported for known P-450$_{cam}$ complexes with sulfur and oxygen ligands (Dawson *et al.*, 1982).

DISCUSSION

Preliminary *in vitro* studies (Krueger *et al.*, 1985; Nagahisa *et al.*, 1985b) of (20*S*)-22-thiacholesterol (**1**) with rat adrenocortical cells indicated that the thiasteroid (**1**) strongly inhibits both cortisol and corticosterone production. When pregnenolone was supplemented into the media, the inhibitory effects of (**1**) were overcome. On the basis of these studies, it was postulated that the sulfur-containing cholesterol analog (**1**) exerts its effect by direct inhibition of the side-chain cleavage enzyme, P-450$_{scc}$. The present studies were undertaken not only to establish the nature of the inhibition of P-450$_{scc}$ by **1** but also to characterize the complex interaction of **1** and its metabolite, **4**, with the enzyme.

The kinetics and binding studies presented herein with purified reconstituted bovine adrenocortical mitochondrial cytochrome P-450$_{scc}$ show that (20*S*)-22-thiacholesterol (**1**) competitively inhibits the side-chain cleavage, evidently by occupying the cholesterol binding pocket. The binding constant (K_d) and the apparent inhibition constant (K_i) determined from the spectral titration and kinetic experiments, respectively, are nearly identical for (20*S*)-22-thiacholesterol (**1**), confirming the competitive nature of this inhibitor against cholesterol substrate.

The nonlinear appearance of the time course plot of pregnenolone production (Figure 2), however, suggests that simple competitive inhibition by (20*S*)-22-thiacholesterol (**1**) was not the only factor in the observed inhibition of pregnenolone formation, since the inhibition becomes increasingly stronger as the reaction progresses. In fact, this indicates that compound **1** is most likely enzymatically oxidized to form a more potent (sulfoxide) inhibitor. Its C-20-epimer, (20*R*)-22-thiacholesterol (**2**), on the other hand, did not inhibit P-450$_{scc}$ at the concentrations tested. Furthermore, preincubation studies with (20*S*)-22-thiacholesterol (**1**) demonstrated an NADPH requirement for inhibition, which is most easily explained as a mechanism-based inhibition.

Independent turnover incubations with C-21-tritiated 22-thiacholesterol indicate that (22*R*)- and (22*S*)-sulfoxides were produced in a ratio as high as 4.2:1 (Table 1), demonstrating at least the minimum diastereoselectivity of enzymatic oxidation. H_2O_2 oxidations of the 22-thiacholesterol (**1**) produced a nearly equimolar mixture of the 22*R*- and 22*S*-diastereomeric sulfoxides (1:1.3 molar ratio), precluding neighboring group effects as any significant source of diastereoselectivity in the enzyme-catalyzed reaction. Suc-

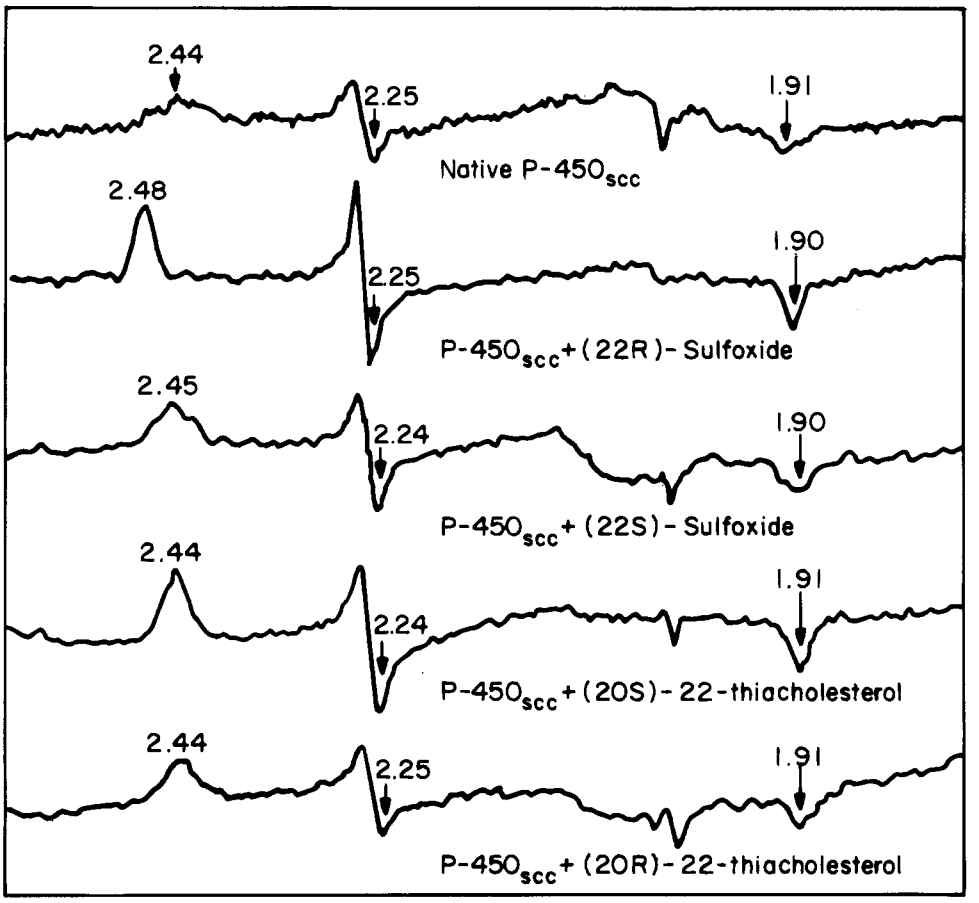

FIGURE 5: EPR spectra of P-450$_{scc}$ thiasterol complexes between 200 and 5200 G at 9.29 GHz and a field modulation frequency of 100 kHz. Temperature = 13 K; microwave power = 0.30 mW; field modulation amplitude = 10 G; time constant = 0.33 s; scanning rate = 336 G/min. Samples contained 5 μM P-450$_{scc}$ with 5 equivs of thiasteroid. Samples (300 μL) used in Figure 9 were transferred into quartz EPR tubes and frozen in liquid nitrogen for recording these EPR spectra.

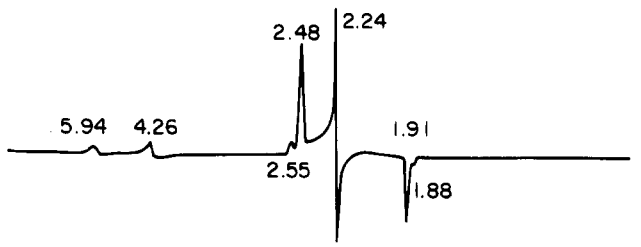

FIGURE 6: EPR spectrum of P-450$_{scc}$ complex of (22*R*)-sulfoxide (**4**) between 200 and 5200 G at 9.29 GHz. Temperature = 12 K; microwave power = 2 mW; field modulation amplitude = 5 G; receiver gain = 5.0 × 10^4. A 500 μM P-450$_{scc}$ sample (300 μL) containing 5 equivs of inhibitor (**4**) was transferred into a quartz tube and frozen in liquid nitrogen for recording this EPR spectrum.

cessful separation of the two chemically synthesized diastereomeric sulfoxides by fractional crystallization and subsequent NMR analysis and X-ray crystallography allowed the assignment of the absolute stereochemistry of the (22*S*)-sulfoxide (S. R. Wilson, E. Miao, P. A. Caldera, M. Guzewska, A. M. Venkatesan, J. Dewan, A. Nagahisa, S. Joardar, & W. H. Orme-Johnson, manuscript in preparation). This was chromatographically identical to the less potent inhibitor. The diastereomeric and more potent inhibitor was therefore assigned the 22*R*-configuration **4**.

Previous studies have shown a difference in binding constants of a factor of 10 between the natural (22*R*)-22-hydroxycholesterol (K_d = 4.9 ± 0.5 mM; pH 7.4) and its epimer, (22*S*)-22-hydroxycholesterol (K_d = 46 ± 6 nM; pH 7.4) (Orme-Johnson *et al.*, 1979) for P-450$_{scc}$. That supports the notion that in, addition to a steroid nucleus recognition site in P-450$_{scc}$, there is also a sterically demanding side-chain binding pocket in the enzyme. The results from the present study lend further credence to this hypothesis, since enzymatic oxidation at the 22-sulfur of steroid **1** led to a strong preponderance of one diastereomeric sulfoxide (22*R*) over the other (22*S*). This implies that the hydrocarbon side chain is held in some semi-rigid manner in a specific binding pocket of the active site prior to enzymatic oxidation. This notion is consistent with the respective dissociation constants of the two isomeric steroidal sulfoxides, which indicate that the binding affinity of the (22*R*)-sulfoxide (**4**) toward P-450$_{scc}$ (K_d = 0.1 μM) is at least 1 order of magnitude greater than that for the (22*S*)-sulfoxide (**3**) (K_d = 1.14 μM).

The previously reported spectral and kinetic properties of competitive inhibitors (22*R*)-amino- and (22*S*)-aminocholesterols (Nagahisa *et al.*, 1985a), which differ only in their configuration at the C-22 atom, have shown that P-450$_{scc}$ binds (22*R*)-22-aminocholesterol (K_d = 25 nM) 1000 times more tighly than (22*S*)-22-aminocholesterol (K_d = 13 μM). The preferential binding of (22*R*)-22-aminocholesterol (over its C-22 epimer) is consistent with the 22*R* and 22*S* hydroxycholesterols and sulfoxides; however, the question that remains is why is the (22*S*)-22-aminocholesterol such a poor binder. This can be answered by the possibility that the amino group of the (22*S*)-22-aminocholesterol projects into a region of the enzyme which requires an uncharged group. The extra 2 orders of magnitude of weaker binding

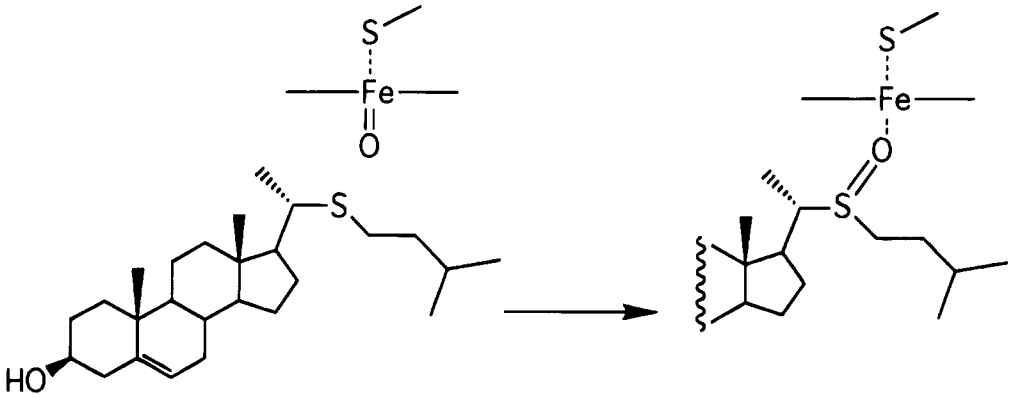

FIGURE 7: Proposed (22*R*)-sulfoxide oxygen coordination with P-450$_{scc}$ heme center.

(compared to the binding ratios within each pair of diastereomeric hydroxycholesterols and sulfoxides) may be the result of a necessary deprotonation of the alkylammonium ion of the bound aminocholesterol, which is normally protonated at neutral pH (see also Nagahisa *et al.*, 1985a).

The foregoing results also indicate that the behaviors of the (22*R*)- and (22*S*)-sulfoxides toward P-450$_{scc}$ closely parallel the behaviors of (22*R*)- and (22*S*)-22-hydroxycholesterols, respectively (Heyl *et al.*, 1986). The (22*R*)-sulfoxide may be thought of as being formed in place by transfer of the ownership of the oxygen atom from the heme iron to the sulfur atom in the substrate. This picture, in conjunction with the spectral evidence presented in Figures 4−6 and discussed below, suggests that the (22*R*)-sulfoxide product (**4**) directly coordinates with the heme, presumably as an axial oxygen donor ligand in the manner depicted in Figure 7 . On the other hand, similar coordination of the heme iron by the 22*S*-diastereomer (**3**), through the sulfoxide oxygen, would require an altogether different and most probably unfavorable orientation of the steroid side chain (crowding with some amino acid side chains within the active site) because the sulfoxide group has an "unnatural" configuration. This would severely restrict such coordination, which accounts for the relatively weak low-spin-inducing capability of the (22*S*)-diastereomer (**3**) observed. Since at least some 22*S*-sulfoxide (**3**) is formed by the enzymatic oxidation of **1**, despite the presumed relative instability of the postulated transition state (involving transfer of an oxygen atom from heme Fe to the sulfur atom) in its case, some transient coordination of the heme Fe by the (22*S*)-sulfoxide oxygen probably does occur when it is initially formed, but it is definitely not stable enough to allow a large population of the low-spin form to exist (Figure 5).

The foregoing postulates are consistent with both optical absorption (Figure 4) and EPR (Figures 5 and 6) spectroscopic properties of P-450$_{scc}$ complexed with (20*S*)-22-thiacholesterol (**1**) and its sulfoxide derivatives (**3** and **4**). P-450$_{scc}$ in the high-spin state is characterized by a Soret absorption maximum around 390 nm and a strong EPR signal at $g \approx 8.0$, whereas in the low-spin state the enzyme shows a Soret absorption around 420 nm and characteristic EPR signals at $g \sim 2.48$ and 1.9, but none at all near $g = 8.0$ (Light, 1978; Orme-Johnson *et al.*, 1979). The native ferric P-450$_{scc}$ gave a λ_{max} at 387 nm and shoulders at 410 and 370 nm. The enzyme under these conditions is estimated to be ~55% in the high-spin state (Light, 1978; Orme-Johnson *et al.*, 1979). The P-450$_{scc}$−(22*S*)-sulfoxide complex gave a λ_{max} at 414 nm and shoulders at 395 and 370 nm. The (22*R*)-sulfoxide (**4**) complex gave a λ_{max} at 420 nm and a separate peak at 365 nm, but no peak or shoulder near 390 nm. The (22*R*)-sulfoxide (**4**) is, therefore, a powerful low-spin-forming compound, whereas the (22*S*)-sulfoxide (**3**) is a relatively weak low-spin inducer. The visible spectrum of (20*S*)-22-thiacholesterol−enzyme complex shows a λ_{max} at 418 nm and a separate peak at 390 nm, which shows that (20*S*)-22-thiacholesterol is also a low-spin inducer, but a relatively weak one. The EPR *g*-values of low-spin species of ferric cytochromes P-450 for mitochondrial, microsomal, and bacterial (P-450$_{cam}$) enzymes are dependent on the structure and type of ligands bound to the cytochromes (Light, 1978; Orme-Johnson *et al.*, 1979; Dawson *et al.*, 1982). Dawson et al. (1982) reported spectroscopic data for P-450$_{cam}$−ligand complexes which demonstrated that empirically derived *g*-values and Soret absorption maxima can be used to identify the atom-type involved as the sixth ligand of heme. Table 2 summarizes the range of *g*-values and Soret absorption maxima obtained for oxygen and sulfur ligands of P-450$_{cam}$ and the present results for P-450$_{scc}$ complexes. Table 2 shows that all the steroid analogs considered here yield low-spin complexes with P-450$_{scc}$, with EPR and electronic transitions similar to those of the complexes of P-450$_{cam}$ with similar donor ligands. It is noteworthy, however, that of the four compounds, only the actual major product (**4**) of the action of P-450$_{scc}$ on 22-thiacholesterol (**1**) shows a single low-spin species in the EPR and UV−vis spectra (Figures 6 and 4A). This feature is strongly reminiscent of the behavior of (22*R*)-22-hydroxycholesterol (Orme-Johnson *et al.*, 1979), a natural intermediate of the cholesterol side-chain cleavage reaction. The other three steroid analogs yield evidence of two species when complexed with P-450$_{scc}$ (Figure 4; EPR data not shown). Both EPR and UV−vis spectroscopic properties of P-450$_{scc}$−steroidal sulfoxide complexes are consistent with the hypothesis that the sulfoxide oxygen of the 22*R* compound (**4**) acts as the axial sixth ligand for heme. Together, this information strongly supports our hypothesis of a mechanism-based inhibition of P-450$_{scc}$ by (20*S*)-22-thiacholesterol (**1**) as depicted in Figure 7.

We infer from these studies that substantial interaction energies between P-450$_{scc}$ and both the steroid nucleus and the aliphatic side chain account for the highly regioselective and stereospecific nature of the oxidative cleavage sequence. The sulfur steroid molecule (**1**), derived by replacing C-22 in the cholesterol molecule with sulfur but maintaining the side-chain structure, closely resembles the natural substrate. Enzymatic turnover of this heteroatom-substituted steroid

derivative qualitatively parallels the stereochemical outcome of the initial oxidation step in the natural side-chain cleavage reaction. The resulting metabolite (**4**) does not undergo any further reaction and, we believe, consequently traps the active site as a hexacoordinated complex. This is, therefore, an example of mechanism-based enzyme inhibition by an intermediate or transition-state analog generated from a precursor substrate analog (Silverman, 1988).

(20*S*)-22-Thiacholesterol (**1**) and its more potent metabolite, (20*S*,22*R*)-22-thiacholesterol *S*-oxide (**4**), may be expected to serve as prototypes for future, rationally designed, specific inhibitors for steroidogenic cytochromes P-450, which may be useful in the treatment of certain hormone-related pathologies (Labrie *et al.*, 1983; Brodie, 1985; Mantero *et al.*, 1991). Such tight-binding steroid analogs should also prove useful as molecular probes for P-450 active site studies.

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