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Biochemistry

Proximity of the substrate binding site and the heme-iron catalytic site in cytochrome P-450scc

(steroid hydroxylation mechanism/cholesterol side-chain cleavage)

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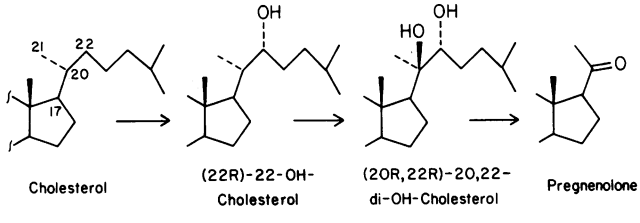
ABSTRACT **As an approach to "mapping" the active site of the cytochrome P-450 that catalyzes cholesterol side-chain cleavage, designated cytochrome P-450scc, we have synthesized steroid derivatives with the potential to interact with both the substrate binding site and the heme-iron catalytic site of the enzyme. The effects of these substrate analogs were studied with cytochrome P-450scc purified from bovine adrenal cortex. One derivative, 22-amino-23,24-bisnor-5-cholen-3β-ol, was found to be a potent inhibitor of pregnenolone formation in a reconstituted enzyme system, and a kinetic analysis of the inhibition showed that binding of the derivative is competitive with respect to cholesterol. The spectral properties of a stable complex formed between the steroidal amine and the purified cytochrome suggest that the 22-amine group coordinates directly to the heme-iron. A model for the structure of this inhibitor–enzyme complex is proposed in which the 5-androstene ring system of the steroid occupies the substrate binding site, and the amine group of the side chain occupies an axial coordination position of the Fe(III) center. This places limits on the distance between these two domains in the enzyme and offers support for proposed mechanisms of cytochrome P-450-catalyzed oxygen-insertion reactions in which an iron-bound oxidant directly attacks the substrate.**

Cytochrome P-450-type enzymes catalyze the monooxygenation of a variety of substrates and play important roles both in physiological pathways and in the metabolism of numerous xenobiotic compounds (1). Although the mechanism of these reactions is not completely understood, considerable progress has been made in elucidating the initial events in the reaction cycle—the substrate-binding, heme-reduction, and oxygen-binding steps (2). The details of the subsequent steps, which lead to cleavage of dioxygen and introduction of one oxygen atom into the substrate, however, have not been defined. Several types of evidence suggest the involvement of a higher-valent iron-oxo intermediate equivalent to $[FeO]^{3+}$ (3–7). It is believed that it is this "activated oxygen" which is introduced into the substrate and provides cytochrome P-450 with the ability to hydroxylate an alkane at an unactivated carbon-hydrogen bond. A further aspect of the reaction is that it is regio- and stereoselective. Studies with Fenton's reagent systems as a model have shown that an iron-bound oxidant can catalyze specific aliphatic hydroxylation of cyclohexanol and 7-hydroxynorborane with retention of configuration at the functionalized carbon (8–10); a stepwise process involving hydrogen abstraction by the oxidant followed by ligand transfer was proposed to occur. In these nonenzymic systems, regio- and stereospecificity is possible because the hydroxy functional group of these substrates can bind to an open coordination site on the iron catalyst. In a cytochrome P-450-catalyzed reaction (11), this presumably does not occur because the heme-iron is already hexacoordinate, being bound to the porphyrin, a protein ligand, and the oxygen atom. For this "oxygen rebound" mechanism of oxygen transfer to occur in cytochrome P-450, the protein moiety of the enzyme must specifically bind and orient the substrate to favor selective hydrogen abstraction and oxygen recombination. This mechanism also predicts that in the enzyme–substrate complex, the carbon center attacked must lie close to the axial heme coordination site for oxygen.

The cytochrome P-450 that catalyzes the side-chain cleavage of cholesterol to pregnenolone is designated cytochrome P-450scc (12). This reaction, which is the rate-determining step in steroid hormone biosynthesis (13), is unique in that it involves three separate, sequential monooxygenations in effecting the carbon-carbon bond scission (14, 15). Cholesterol is first hydroxylated at C-22, then it is hydroxylated at C-20, and the resulting glycol is cleaved to yield the 20-ketone and 4-methylpentanal (16, 17) (Scheme I).



Scheme I

The origin of the regioselectivity that leads to the initial attack at C-22 before C-20 is not clear. Evidence also has been presented that, in the course of 22-hydroxylation, a direct replacement of hydrogen from the hydroxylated position occurs (18), and this finding is consistent with an oxygen-rebound mechanism for the formation of (22*R*)-22-hydroxycholesterol. It is not known, however, whether cholesterol is bound sufficiently close to the heme-iron and with the proper positioning of the alkyl side chain to allow for this mechanism to occur.

With the aim of providing insight into the mechanism and specificity of the cholesterol side-chain cleavage reaction, we have begun studies to probe the active site region of cytochrome P-450scc. We view this region as composed of two domains, one encompassing the substrate binding site and a second including the heme-iron catalytic site. In the approach described here, we have prepared a steroid derivative having the potential to bind to both of these sites and have investigated its interaction with the enzyme. The formation and characterization of a stable complex between the steroid derivative and the enzyme enable

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Abbreviation: 22-ABC, 22-amino-23,24-bisnor-5-cholen-3β-ol.

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us to place limits on the distance between the substrate and catalytic sites.

EXPERIMENTAL PROCEDURES

Cytochrome P-450scc, adrenodoxin, and adrenodoxin reductase were isolated from bovine adrenocortical mitochondria as described (19–22). Cholesterol side-chain cleavage was measured in reconstituted assay systems at 37°C in 33 mM potassium phosphate (pH 7.2), which contained 0.3% Tween-20 to solubilize cholesterol (19). Absorption spectra were recorded in a Cary model 17D.

Synthetic steroids were characterized by IR and NMR spectra, mp, and elemental analysis.† 23,24-Bisnor-5-cholenic acid 3β-ol acetate was converted to the acid chloride (23) and treated with ammonia to yield 23,24-bisnor-5-cholen-3β-ol-22-carboxamide acetate; a portion was saponified to the free alcohol. The amide was reduced to the amine and isolated as 22-amino-23,24-bisnor-5-cholen-3β-ol (22-ABC) hydrochloride (24, 25). A portion was crystallized from aqueous methanol containing sodium carbonate to yield the free base: mp, 165–167°C; NMR, 0.99 ppm (3 H, *s*, C-19 methyl), 0.97 ppm (3 H, *d*, C-21 methyl; J = 7 Hz), and 0.68 ppm (3 H, *s*, C-18 methyl) in ($CDCl_3$).

RESULTS AND DISCUSSION

In developing a steroid derivative capable of interacting with both the substrate site and the heme, we have linked a series of primary aliphatic amines to 5-androsten-3β-ol. The androstene moiety could be expected to bind to the same site as the identical ring system present in cholesterol. Because primary amines are known to weakly coordinate heme-iron, attachment of an alkyl amine of the right length to a proper site on 5-androsten-3β-ol might allow the nitrogen to bind to the heme-iron in the steroid–cytochrome P-450scc complex. The affinity of the steroidal amine derivative for the enzyme would be expected to be enhanced relative to that of simple aliphatic amines because of the specific binding stabilization at the androstene-substrate site.

Enzymatic Studies. We have begun by preparing compounds with amines projecting from C-17 of the *D* ring of 5-androsten-3β-ol. These were initially assayed for inhibition of cytochrome P-450scc-catalyzed cholesterol side-chain cleavage in a system reconstituted from purified bovine adrenocortical enzymes. Derivatives in which a primary amine was positioned close to the *D* ring of the steroid, 17-amino-5-androsten-3β-ol and the (20*R*)- and (20*S*)-epimers of 20-amino-5-pregnen-3β-ol, were found to be without effect at concentrations up to 100 μM.† When the primary amine was positioned farther from the steroid ring on C-22, however, dramatic effects on the properties of the enzyme were observed. Fig. 1 shows the dose–response data for inhibition of pregnenolone formation by 22-ABC. With 70 μM cholesterol, 22-ABC caused significant inhibition at concentrations as low as 30 nM and caused almost complete inhibition at >1 μM. In all incubations, the reaction progress curves were linear, indicating that 22-ABC does not cause irreversible inactivation of the enzyme and is not itself metabolized to a less active form. The curve drawn shows the dose–response behavior expected for noncooperative, reversible binding (26) of an inhibitor to cytochrome P-450scc with a K_d = 0.1 μM. The importance of the amine nitrogen was assessed by assaying the amide analog, 23,24-bisnor-5-cholen-3β-ol-22-carboxamide. Fig. 1 shows that this compound causes only slight

† A detailed description of the results obtained with these and related derivatives will be published elsewhere.

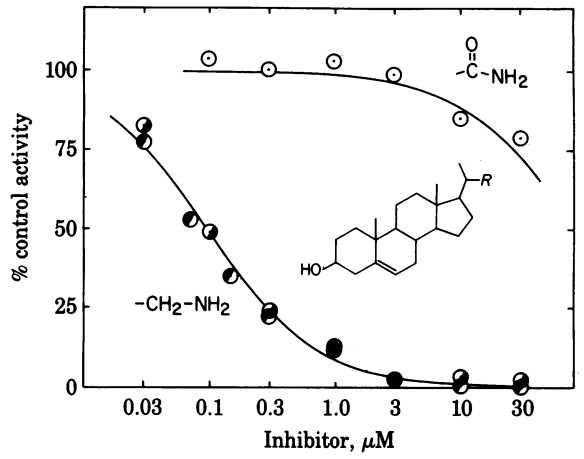


FIG. 1. Dose–response curves for inhibition of cholesterol side-chain cleavage. Activities were assayed in a reconstituted system of enzymes from bovine adrenal cortex and 70 μM cholesterol. Two experiments with 22-ABC (◐ and ◕) and one with 23,24-bisnor-5-cholen-3β-ol-22-carboxamide (⊙) are shown. The curves were calculated by assuming inhibitor binding constants of 0.1 and 100 μM. The reaction rate in the absence of inhibitor, defined as 100% control, was 3.0 mol of pregnenolone per mol of cytochrome P-450scc per min.

inhibition, requiring *ca.* 1,000-fold higher concentrations than the amine.

The question of whether 22-ABC causes inhibition by interacting with the substrate site was investigated by a kinetic analysis of inhibition of the cholesterol side-chain cleavage reaction. We measured the rate of pregnenolone formation as a function of cholesterol concentration in the absence and presence of the inhibitor. Cholesterol and 22-ABC binding were competitive (Fig. 2*A*). Because of the structural identity of regions of the two compounds, we interpret this competition to mean that 22-ABC binds to the enzyme at the same site as cholesterol. Fig. 2*B* shows that the inhibition is linearly competitive and that the apparent K_i for 22-ABC is approximately 40 nM.

Spectral Studies. The properties of the complex of 22-ABC and purified bovine adrenocortical cytochrome P-450scc were investigated by absorption spectroscopy (Fig. 3). In the absorption spectrum of the "resting" enzyme (Fig. 3*A*, curve 1), which was isolated as the cholesterol complex, the Soret absorption maximum occurs at 393 nm, indicative of the predom-

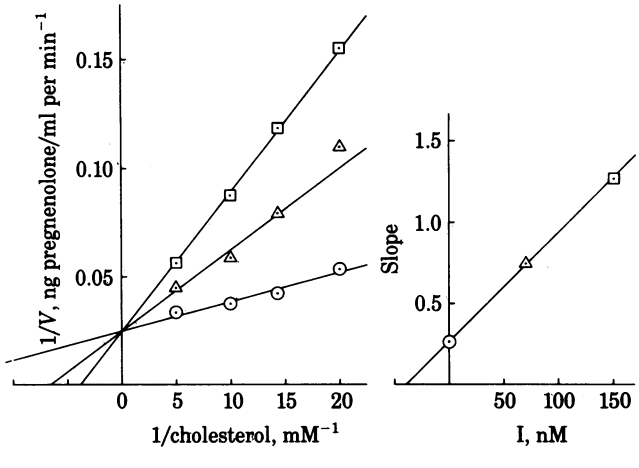


FIG. 2. Kinetic analysis of the mechanism of inhibition of cholesterol side-chain cleavage by 22-ABC. ⊙, No inhibitor; △, 70 nM 22-ABC; and ⊡, 150 nM 22-ABC. (*Left*) Double reciprocal plot. (*Right*) Slope of lines in *Left* vs. inhibitor (I) concentration. *V*, Velocity of reaction.

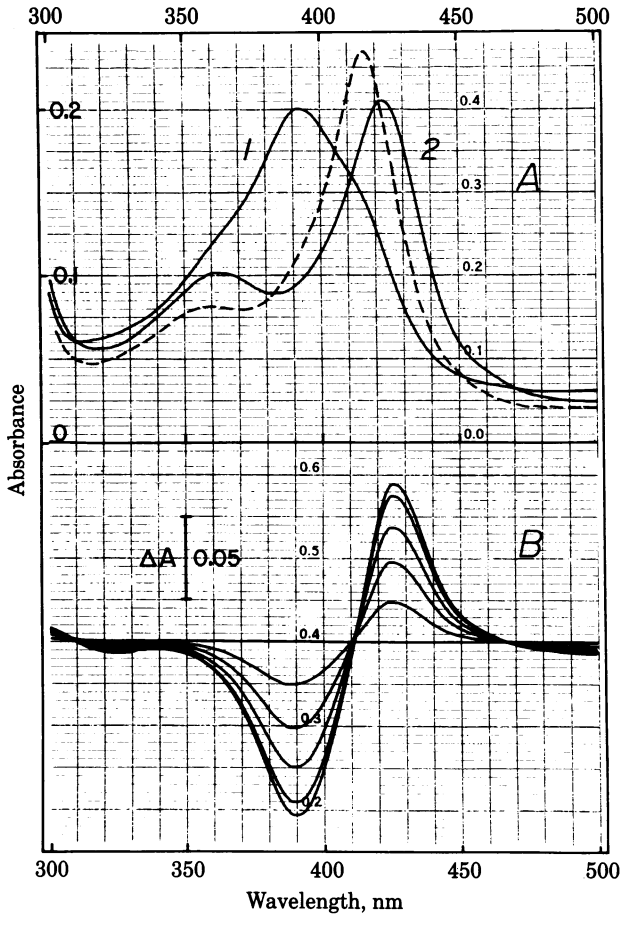


FIG. 3. Effect of 22-ABC on the spectral properties of cytochrome P-450scc. (*A*) Absolute spectra of the purified bovine adrenocortical enzyme in 0.1 M potassium phosphate (pH 7.1) at 22°C (1-cm path). Curves: 1, 1.9 μM cytochrome P-450scc alone; 2, with 4 μM 22-ABC; and ---, with 0.3% Tween 20. (*B*) Difference spectra obtained during a titration of sample 1 from *A*. Curves show spectra recorded after the successive addition of 0.4, 0.8, 1.2, 1.8, and 2.5 μM 22-ABC.

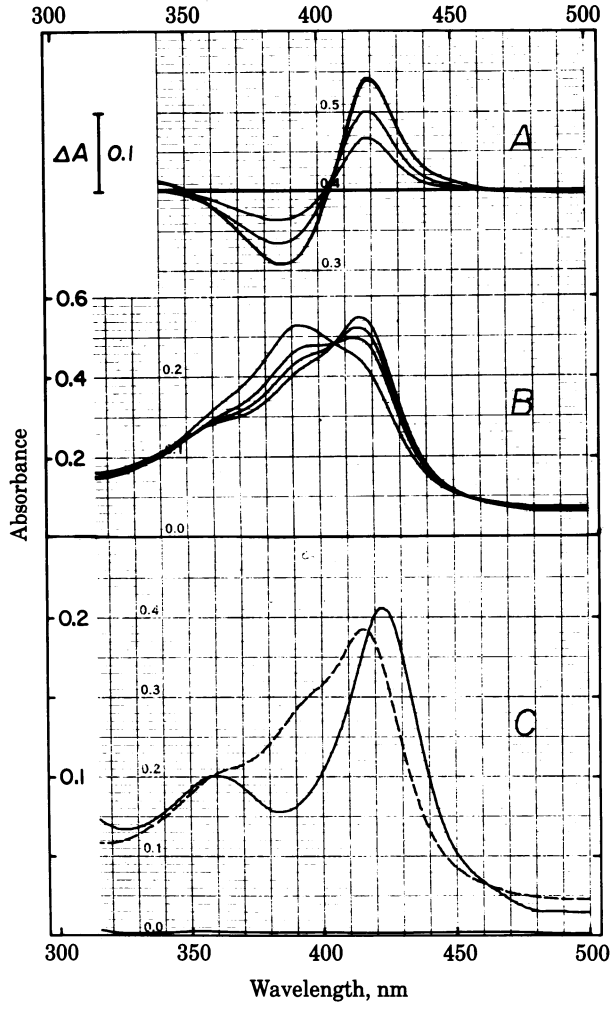


FIG. 4. (*A* and *B*) Effect of 23,24-bisnor-5-cholen-3β-ol-22-carboxamide on cytochrome P-450scc. A 4.9 μM solution of the purified enzyme was treated with 10, 20, and 30 μM amide. In *A*, difference spectra vs. enzyme treated with an equal volume of ethanol were recorded; in *B*, absolute spectra vs. buffer are shown. (*C*) Effect of isobutylamine. The cytochrome P-450scc concentration was 1.9 μM in 0.1 M potassium phosphate (pH 8.0) with (——) and without (---) 0.95 M isobutylamine.

inantly high-spin ($S = 5/2$) nature of the preparation. In the spectrum obtained after addition of 4 μM 22-ABC (Fig. 3*A*, curve 2), the Soret maximum in the steroidal amine complex occurs at 422 nm. This peak position is typical of a low-spin ($S = 1/2$) heme but is significantly red-shifted from the low-spin spectrum produced when cholesterol is removed from the enzyme or when steroids lacking this amino group bind to the enzyme. The broken curve shows an example of a spectrum obtained in the presence of 0.3% Tween-20, a detergent that removes cholesterol from the enzyme, resulting in the formation of a low-spin species with an absorption maximum at 417 nm. A low-spin species having a 417-nm Soret maximum is also formed when pregnenolone, (20*R*)-20-hydroxycholesterol, or (22*R*)-22-hydroxycholesterol bind to cytochrome P-450scc (ref. 27; unpublished observations); these steroids are assumed to displace endogenous cholesterol from the enzyme. The shift in the spin state and the absorption spectrum caused by the detergent or by steroid binding is believed to reflect a conformational change in the protein that leads to the binding of a strong-field amino acid side chain to an axial position of the heme-iron; similar spin and spectral shifts are observed with other types of cytochrome P-450 (28). The results with 22-ABC indicate formation of a complex in which the heme has a different electronic structure and suggest that the ligand that occupies one axial coordination site of the heme-iron in this complex is different from that in the other steroid–cytochrome P-450scc complexes.

Difference spectra obtained during a titration of the high-spin form with 22-ABC are shown in Fig. 3*B*. The tight binding of the inhibitor is evidenced by the nearly linear change in the spectrum after the initial additions.‡ In addition, the curves show isosbestic points at 467 and 411 nm, indicating that only two forms are spectrally detectable. Thus, the high-spin cholesterol complex is converted directly to the new low-spin 422-nm species without intermediate formation of a 417-nm species.

The role of the amine group at C-22 in inducing formation of the new low-spin species was investigated by two approaches. We first determined the effect of a related steroid lacking the primary amine moiety and, for this purpose, used 23,24-bisnor-5-cholen-3β-ol-22-carboxamide. Fig. 4*B* shows the absorption spectra of cytochrome P-450scc before and after addition of the amide derivative. Unlike the amine, this derivative causes for-

‡ A quantitative analysis of the titration is complicated by the preexisting equilibrium between the high-spin cholesterol form and a small amount of a low-spin form assumed to represent a substrate-free species of the enzyme (27).

mation of a 417-nm species; complete saturation of the enzyme with the amide was not possible because of its weak affinity and limited solubility. Fig. 4A shows the difference spectra obtained; these also can be contrasted with those for the amine in Fig. 3B. We also have prepared the oxygen analog of 22-ABC, 23,24-bisnor-5-cholene-3β,22-diol, and, like the amide, binding of this derivative leads to formation of the 417-nm species (data not shown). These results establish that the presence of a shortened side chain is not sufficient to produce the 422-nm species and underscore the role of the amine nitrogen.

In a second approach, we added a nonsteroidal aliphatic amine to cytochrome P-450scc to determine whether the new low-spin species could be formed simply by coordination of a primary aliphatic amine to the heme-iron. Previous studies with preparations of liver microsomal cytochrome P-450 have shown that *n*-alkylamines elicit difference spectra similar to those we observed with 22-ABC (29, 30) and that *n*-octylamine binding produces a low-spin species with a Soret maximum at 423 nm (31). We used isobutylamine because the structure is equivalent to the side chain of 22-ABC. In initial experiments carried out at pH 7.4, the affinity of isobutylamine for cytochrome P-450scc was found to be low; at more alkaline pH where the free base exists in higher concentration, however, the enzyme could be saturated with the amine. Fig. 4C shows the spectrum of purified cytochrome P-450scc at pH 8.0, with and without 0.95 M isobutylamine. At this pH, the spin equilibrium of the resting enzyme favors the low-spin form, having a Soret maximum near 417 nm. In the presence of isobutylamine, the Soret peak is shifted to 422 nm—the same wavelength maximum as observed with 22-ABC (the spectrum of the 22-ABC–cytochrome P-450 complex was unchanged from pH 6.8 to 8.0). Moreover, the absorbance ratio of the Soret band to the δ band at 360 nm is the same for both complexes. This suggests that the heme-iron axial coordination is the same in the two complexes and that the unique spectral properties of the 22-ABC complex arise from binding of its 22-amino group to the heme-iron.

To determine whether 22-ABC amine coordination of ferricytochrome P-450scc is responsible for the observed enzymatic inhibition, we carried out spectral titrations of P-450scc with 22-ABC in the detergent-containing assay buffer. Upon addition of the steroidal amine, the low-spin form with a Soret maximum at 417 nm is converted to a form with a maximum at 422 similar to that formed in the absence of detergent (cf. Fig. 3A). Difference spectra obtained in the course of a titration are shown in Fig. 5. Maxima at 432 nm, minima at 412 nm, and isosbestic points at 423 and 377 were observed for the difference between the two low-spin forms; these spectra are red-shifted relative to the difference spectra of the low-spin vs. high-spin forms shown in Fig. 3B. Analysis of the absorbance changes as a function of the concentration of unbound ligand gives a spectral dissociation constant, K_s = 42 nM. This is close to the K_i value of 40 nM for 22-ABC, and we conclude that complex formation of 22-ABC with ferri-P-450scc to yield an amine-coordinated hemichrome is responsible for inhibition of catalysis.

Structural and Mechanistic Implications. The results of these spectral studies, taken together with the results of the inhibition studies, suggest that 22-ABC is capable of binding both to the substrate binding site and to the heme-iron catalytic site to yield an inactive form of the enzyme. The structure of such a complex is given in Fig. 6. The heme group is assumed to be bound to the protein through noncovalent interactions of the porphyrin with nonpolar amino acid side chains and through coordination of the iron in an axial position by another residue. Although not specified by our studies, this group must be a sufficiently strong-field ligand to yield a low-spin complex and may be a cysteinate residue (32). The amine group of 22-ABC is shown coordinated to the heme-iron in the sixth position, *trans* to the endogenous fifth ligand. This bonding of the nitrogen to the iron places restrictions on the position of the steroid ring system in the complex. Using Kendrew skeletal molecular models and varying the conformation of the side chain, we have estimated limits of the distances between different regions of the steroid binding site and the iron catalytic site. C-17 of the steroid can approach to within ≈3.5 Å of the iron and can be positioned no farther than 5.5 Å away. The oxygen atom of the 3β-hydroxyl group lies 11–16 Å from the iron, depending upon the side-chain orientation. If the torsion angle defined by bonds C(13)—C(17)—C(20)—C(22) is fixed at −180°, which is in the midrange observed in crystal structures of 5-pregnene derivatives (33), this further restricts C-17 to within 4–5 Å of the heme-iron and places the 3β-hydroxyl within a distance of 14–16 Å. Whereas a number of combinations of side-chain torsion angles are not allowed because of steric interactions of the porphyrin and the steroid, a unique structural solution is not possible based only on these results. Additional compounds should be tested to refine the distance measurements and de-

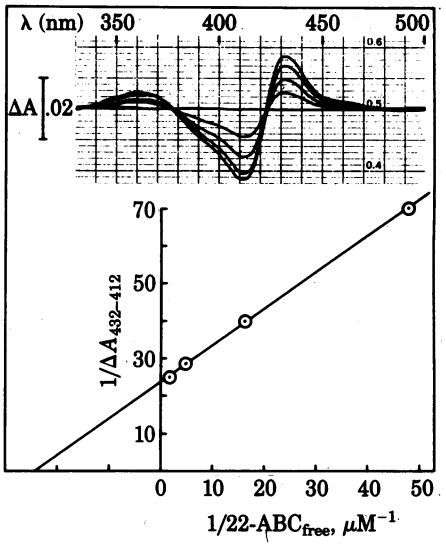


FIG. 5. Titration of low-spin cytochrome P-450scc with 22-ABC. (*Upper*) Difference spectra recorded during a titration of 0.23 μM P-450scc in 0.033 M phosphate, pH 7.2/0.3% Tween 20. Final total concentrations of 22-ABC were 0.1, 0.2, 0.4, and 0.8 μM. (Path = 4 cm.) (*Lower*) Double reciprocal plot of titration data. The concentration of free ligand was calculated by the equation

$$L_f = L_{tot} - \frac{\Delta A \cdot E}{\Delta A_{max}},$$

where L_f is the free ligand concentration, L_{tot} is the total concentration of ligand added, ΔA is the observed absorbance change at L_{tot}, E is the enzyme concentration, and ΔA_{max} is the absorbance change extrapolated to infinite ligand concentration. For 22-ABC binding to low-spin cytochrome P-450scc, we found $\Delta\varepsilon_{432-412}$ = 45 $(mM \cdot cm)^{-1}$.

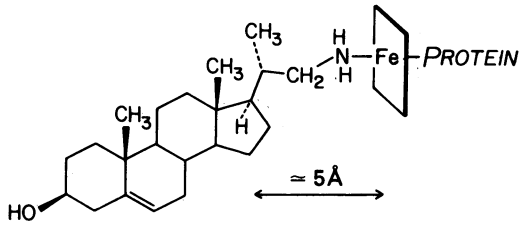
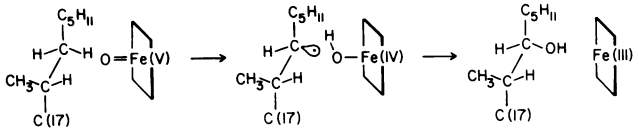


FIG. 6. Proposed structure of the complex of 22-ABC with the heme of cytochrome P-450scc.

termine the orientation of the bound steroid relative to the heme group.

In addition to providing structural information, these results have implications with respect to the mechanism of the cholesterol side-chain cleavage reaction. In the complex shown in Fig. 5, C-22 is immediately adjacent to the sixth coordination site of the heme-iron. Thus, during the course of a cytochrome P-450scc reaction cycle, C-22 could be positioned sufficiently close to an iron-bound, activated form of oxygen to allow for direct transfer of an atom of oxygen. Scheme II illustrates how hydroxylation at C-22 of the cholesterol side chain might occur according to the "oxygen rebound" mechanism proposed by Groves and co-workers (8–11).



Scheme II

The side chain is located so that C-22 lies close to an iron-oxo intermediate, the latter formed by loss of a molecule of water from the $2e^-$-reduced cytochrome P-450–dioxygen complex. In the first step shown, the electrophilic oxygen abstracts a hydrogen atom from C-22. The initial and regioselective attack at this site over that at C-20 and the stereoselectivity of the hydrogen abstraction are proposed to result from restricted positioning of the steroid nucleus and side chain through interactions with the protein and porphyrin in the cholesterol binding site. In the second step, the resulting carbon radical combines with the iron-bound oxidant to yield (22*R*)-22-hydroxy-cholesterol and ferricytochrome P-450scc. Retention of configuration requires that the recombination process occur more rapidly than epimerization of the carbon radical. For both steps, proximity of the substrate site of attack to the iron sixth coordination site is proposed to favor regio- and stereoselective hydroxylation. The closeness of the two reactants also obviates the need to invoke participation of amino acid side chains in the transfer of oxidant from the heme-iron to the substrate, although certain residues may be involved in the formation or stabilization of the iron-bound oxidant.

Once formed, (22*R*)-22-hydroxycholesterol is itself hydroxylated at C-20 in a second mixed-function oxidation reaction cycle catalyzed by the same enzyme. Our finding that neither (20*R*)- nor (20*S*)-20-amino-5-pregnen-3β-ol is able to bond to the heme-iron, however, places C-20 at a greater distance than C-22 is from the iron. Whether the oxygen-rebound mechanism can take place over that distance or whether a rearrangement of the steroid–enzyme complex occurs first is not clear. Further investigations with new steroid derivatives will address these questions.

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