C-22-substituted Steroid Derivatives as Substrate Analogues and Inhibitors of Cytochrome P-450<sub>sec</sub>*

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Joel J. Sheets§ and Larry E. Vickery§

From the Department of Physiology and Biophysics, University of California, Irvine, California 92717

Spectral and kinetic studies are reported for the effects of C-22-substituted steroids on purified bovine adrenocortical cytochrome P-450<sub>sec</sub>. The results are consistent with the recent proposal that the potency of 22-amino-23,24-bisnor-5-cholen-3β-ol as an inhibitor of the enzyme arises from a dual interaction, the binding of the steroid ring to the cholesterol site and bonding of the amine to the heme iron (Sheets, J. J., and Vickery, L. E., (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5773-5777). An analogue of the inhibitor with the 5,6 double bond reduced, 22-amino-23,24-bisnor-5α-cholan-3β-ol, was synthesized by a similar procedure. A complex of this form with P-450<sub>sec</sub> produced a 412 nm Soret absorption maximum as found for the parent compound, indicating nitrogen coordination to the heme iron. A decrease in the spectral dissociation constant and inhibitory potency was also observed and is consistent with binding of the steroid ring to the cholesterol site on the enzyme. The 22-hydroxy analogue, 23,24-bisnor-5-cholene-3β,22-diol, was also prepared. This derivative produced a complex with P-450<sub>sec</sub> having a Soret peak at 417 nm as in the substrate-free form of the enzyme; the diol was also a competitive inhibitor, but exhibited decreased potency relative to the amine form. These results provide additional support for the role of amine coordination in the 422 nm species and in contributing to tight binding.

Cytochrome P-450<sub>sec</sub> catalyzes the initial step in the biosynthesis of steroid hormones, the side chain cleavage of cholesterol to produce pregnenolone. The reaction is the rate-determining step in steriodogenesis and is regulated by adrenocorticotropic hormone in the adrenal cortex and luteinizing hormone in the gonads (1-3). Several laboratories have succeeded in partially purifying the enzyme (4-10), and it appears to consist of a single peptide of M<sub>s</sub> = 50,000 with one P-450-type heme group. While the molecular details of the reaction catalyzed have not been fully elucidated, it appears that three mixed function oxidation cycles occur at the single heme center (11-15). Cholesterol is first hydroxylated at C-22, then at C-20, followed by oxidative scission of the glycol (16, 17). Small amounts of the intermediates, (22R)-22-hydroxysterol and (20R,22R)-20,22-dihydroxysterol, can be detected in preparations of the isolated enzyme (18), but these do not accumulate during catalysis (11, 19). This is in accord with observations of enhanced binding of the hydroxycholesterols relative to cholesterol (cf. Ref. 20) and the assumption that there is only a single active site on the enzyme.

What is not understood is how a single active site can catalyze three different reactions or by what mechanism the regio- and stereospecific sequence of attack on the substrate is achieved. Studies on other cytochromes P-450 have provided evidence for the involvement of a higher valent iron-oxo intermediate equivalent to [Fe-O]<sup>2+</sup> (21), and such an activated form of oxygen could be common to all three reactions in the side chain cleavage sequence. Specificity could thus arise from the positioning of the substrate relative to the heme-bound oxidant. Little, however, is known about how this positioning occurs. The fact that cholesterol and hydroxycholesterols induce changes in the spin state (7, 22) and the redox potential (23) of P-450<sub>sec</sub> suggests that the substrate binding site must be near enough to the heme to cause conformational changes which result in changes in the axial coordination of the iron, but it is not certain over what distances these effects can be propagated. More direct evidence for the proximity of the steroid binding site and the heme comes from the results of van Lier and co-workers (24-26). They showed that hydroperoxycholesterols can react with cytochrome P-450<sub>sec</sub> to yield specific, hydroxylated products, and that the oxygen atom in the hydroxyl group of the products originates from the hydroperoxy group of the substrate. Since both 20- and 25-hydroperoxy derivatives were reactive, it is inferred that close approach of these regions of the substrate to the heme to yield a ferryl-oxygen intermediate must be possible.

With the aim of providing additional insight into the structure of the active site of cytochrome P-450<sub>sec</sub> and its mechanism of catalysis, we have begun studies on the interaction of different steroid derivatives with the enzyme. In a recent study directed toward determining the distance between the substrate and catalytic sites, we presented evidence for the formation of a stable, enzymatically inactive complex between the cholesterol analogue, 22-amino-23,24-bisnor-5-cholen-3β-ol and the enzyme (27). Kinetic inhibition results suggested that the 5-androsten-3β-ol ring bound to the substrate site and spectral studies suggested that the 22-amine formed a bond with the heme iron. If this interpretation is correct, it limits the distance between the D ring of the steroid substrate and the iron of the heme to about 5 Å. This model also predicts that alterations in the steroid ring structure of 22-ABC<sup>1</sup> of the type which affect the affinity of the enzyme for cholesterol

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1 The abbreviations used are: 22-ABC, 22-amino-23,24-bisnor-5-cholen-3β-ol; 5α-22-ABC, 22-amino-23,24-bisnor-5α-cholan-3β-ol; 22-Diol, 23,24-bisnor-5-cholene-3β,22-diol.
Substrate Analogue Interaction with P-450sec

should similarly affect the affinity for 22-ABC, this would not, however, be expected to alter the coordination of the amine to the iron. In order to test this hypothesis, we have synthesized a derivative of 22-ABC with the 5,6 double bond reduced, 5α-22-ABC (Fig. 1). On the other hand, substitution of a different atom for the amine nitrogen should affect the type of spectral complex formed and would at the same time allow us to assess the importance of the iron-nitrogen bond in stabilizing the complex. For this purpose, we have prepared the 22-hydroxy analogue of 22-ABC, 22-Diol (Fig. 1). The effects of these derivatives on the spectral and kinetic properties of purified cytochrome P-450sec support the model proposed for the 22-ABC enzyme complex.

EXPERIMENTAL PROCEDURES

Isolation of Enzymes

Cytochrome P-450sec was isolated from bovine adrenocortical mitochondria as previously described (28). The final preparations exhibited a single major band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis but contained only 9-16 nmol of heme/mg of protein, indicating 45-80% purity; no difference in enzymatic or spectrosopic behavior between preparations, however, was observed. Cytochrome P-450 concentrations were calculated according to the method of Omura and Sato (29), and the absolute spectrum of the enzyme complex. For each sample were determined several times over a 30-min period to verify equilibrium.

Spectral Studies

Highly purified cytochrome P-450sec was used in 100 mM potassium phosphate, pH 7.2, at ambient temperature. Steroids were added from a stock solution in ethanol such that the final alcohol content did not exceed 5%; for difference spectra ethanol was also added to the solution, causing precipitation of the white salt. Filtration and crystallization from boiling ethanol gave a single peak on high performance liquid chromatography: m.p. 257-260°C; primary amine salt.

Assay Procedures

Cholesterol side chain cleavage was measured in reconstituted assay systems containing cytochrome P-450sec, together with excess electron carriers, adrenodoxin and adrenodoxin reductase, were purified from the same tissue by published procedures (30-32).

Experimental Procedures

Materials

Cholesterol, pregnenolone, 23,24-bisnor-5-cholenic acid-3β-ol, and 23,24-bisnor-5-cholenic acid-3β-ol acetate, and 23,24-bisnor-5-cholenic acid-3β-ol acetate were obtained from Steroids.

RESULTS

Effects of 22-ABC—Our initial studies with 22-ABC indicated that it was a potent inhibitor of cytochrome P-450sec.
catalyzed conversion of cholesterol to pregnenolone (27). Fig. 2 shows the time course of a reaction using a reconstituted cholesterol side chain cleavage assay system which incorporates purified bovine adrenocortical cytochrome P-450_{sec} as the rate-limiting component. In the absence of inhibitor, the rates are linear for up to 12 min, and under these conditions give a specific activity of 6 mol of pregnenolone/mol of P-450/min. In the presence of 0.15 μM 22-ABC, the rate is approximately 40% that of the control. In addition, the rate remains linear through the experiment. If 22-ABC were required to be metabolized to cause inhibition, either as a “suicide-type” of inhibitor or to a more active form, we would expect to observe increased inhibition concurrent with the metabolism and a decreasing slope during the time course. If, on the other hand, 22-ABC were metabolized to an inactive form, an upward curvature toward a slope approaching that of the control would be expected. Since no lag or change in slope was observed, we conclude that no substantial metabolism of 22-ABC occurs under these conditions and that 22-ABC need not be metabolically activated in order to inhibit the enzyme. In the same experiment, 22-ABC was also incubated with the enzyme system at concentrations of 10 and 100 μM in the absence of added cholesterol, but no metabolism to pregnenolone could be detected (data not shown).

We also reported that the binding of 22-ABC to purified cytochrome P-450_{sec} causes a shift in the Soret absorption maximum to 422 nm and that this occurs whether 22-ABC is added to the high spin cholesterol complex or to the low spin substrate-free form (27). This was interpreted as evidence of coordination of the 22-amine to the heme iron since the same spectrum is produced by addition of high concentrations of isobutyl amine and since a similar shift is observed when a number of nitrogen bases bind to liver microsomal P-450 (36) and bacterial P-450_{sec} (37). Fig. 3 shows the spectra observed for the different forms of P-450_{sec}. The visible region spectrum of the 22-ABC complex is characteristic of a low spin (S = 1/2) ferric heme complex, lacking the charge transfer band near 645 nm which is typical of the high spin (S = 5/2) form of the enzyme. The visible spectrum is also similar to complexes of nitrogen bases with other P-450 enzymes, having a weaker α-band and a red-shifted β-band when compared to the substrate-free low spin form. Unfortunately, the spectra do not allow one to distinguish between the different types of nitrogen-containing ligands. Fig. 4 shows that, for example, addition of 4-phenylimidazole to P-450_{sec} can produce a complex with a near UV and visible spectrum like that caused by 22-ABC. In this sample, in which the imidazole nitrogen probably forms a bond with the heme iron, the Soret peak is also shifted to near 422 nm and the α-band is of lower intensity than the β-band. Thus, caution must be used in weighing the spectral evidence for 22-ABC in favor of amine-nitrogen coordination.

**Effects of 5α-reduction**—In order to further determine the importance of the structural features of 22-ABC required for its specific interaction with P-450_{sec}, two new derivatives were prepared. 5α-22-ABC differs in having the double bond between carbons 5 and 6 reduced, which alters the conformation of both the A and B rings of the steroid. This allows us to assess the importance of the steroid ring system in inducing the 422 nm spectral form and in promoting tight binding. If coordination of the 22-amine is responsible for the shifted spectrum, we would expect that 5α-22-ABC might also induce the 422 nm form; if a specific, steroid-induced conformational change was required to form the 422 nm species, 5α-22-ABC might prove ineffective. In addition, since reduction of the 5,6 double bond in cholesterol decreases its ability to serve as a substrate (38, 39), we would expect 5α-22-ABC to bind less well than 22-ABC and to be a less effective inhibitor if both compounds bind to the cholesterol site.

A dose-response study for inhibition by 22-ABC and 5α-22-ABC of cholesterol conversion to pregnenolone is presented in Fig. 5. Under these conditions, in the presence of 70 μM cholesterol, 22-ABC exhibits an IC50 value near 0.1 μM, while 5α-22-ABC has an IC50 value of approximately 3 μM. This 30-fold decrease in affinity for 5α-22-ABC is consistent with a mechanism of inhibition dependent upon binding of the steroid moiety to the substrate site on the enzyme.

The spectral properties of cytochrome P-450_{sec} treated with 5α-22-ABC were also investigated, and 5α-22-ABC was found to produce the same spectral form as that caused by 22-ABC.
binding with a Soret absorption maximum at 422 nm (cf. Fig. 3). Difference spectra obtained during a titration of the high spin form of P-450scc are shown in Fig. 6A. These curves are very similar to those reported for 22-ABC binding (27), and exhibit an isosbestic point at 411 nm. This rules out the possibility that binding of the amino-steroid produces the 417 nm form at lower concentrations and is then converted to the 422 nm form only at high, excess levels of the amine.

Titrations with 5α-22-ABC were also carried out in the presence of 0.3% Tween 20. This detergent was used to solubilize cholesterol in the enzyme assay system and converts P-450scc to the low spin 417 nm form. Binding of 5α-22-ABC was found to shift the Soret absorption peak to 422 nm, and the difference spectra produced during the course of a titration are shown in Fig. 6B. These are again similar to those produced by 22-ABC in Tween 20, providing further evidence for a similar coordination of the heme iron in both complexes. An analysis of the titration is shown in Fig. 6D (curve I), and the observed spectral dissociation constant is approximately 0.9 μM. This value is surprisingly high considering the I50 value of 3 μM observed in the enzymatic studies (Fig. 5) and could indicate that the complex formation observed spectrally is not that which gives rise to the inhibition. The linearity of the titration, however, argues against this interpretation, the same apparent affinity being observed over the concentration range tested of 0.75 to 4.5 μM. An additional possible source of different apparent affinities is that adrenodoxin is present in the enzymatic assays, and binding of adrenodoxin to P-450scc has been shown to increase the affinity of the enzyme for cholesterol (40). For this reason, a similar titration with 5α-22-ABC was carried out in the presence of added adrenodoxin. In this case, 7.5 μM adrenodoxin was used, compared to 1.0 μM adrenodoxin in the assays, in an attempt to saturate the effect. As shown in Fig. 6C, significantly larger absorbance changes are observed in the presence of adrenodoxin; in addition, the spectra are slightly blue-shifted, the zero crossings occurring at 422 and 377 nm in the presence of the iron-sulfur protein compared to 423 and 379 nm in its absence, indicating a slight shift toward the high spin state in the reference sample. The graph shown in Fig. 6D yields an apparent spectral dissociation constant of 3.8 μM, a value closer to that expected based on the I50 value. Since adrenodoxin promotes cholesterol binding (40), its effect of enhancing the affinity of 5α-22-ABC provides additional evidence for binding of the amino-steroid to the substrate site of P-450scc.

Effects of the 22-Hydroxy Analogue—While the 5α-reduced derivative of 22-ABC yielded results consistent with our proposed model for the interaction of 22-ABC with cytochrome P-450scc, it remained possible that the essential feature(s) for inducing the 422 nm spectral form was in other parts of the steroid ring system or was simply the presence of a shortened side chain. For this reason, we also prepared the 22-hydroxy analogue of 22-ABC, changing only the identity of the polar substituent on C-22.

Because the 22-Diol resembles the 22-hydroxycholesterol intermediate in the side chain cleavage reaction, we first tested it for conversion to pregnenolone. Following incubations using 10 and 100 μM 22-Diol for up to 12 min, however, no significant amounts of pregnenolone (less than 2% of that produced from cholesterol or 22-hydroxycholesterol) could be detected by the radioimmunoassay procedure used. In fact, the 22-Diol was found to be an effective inhibitor of the cholesterol side chain cleavage reaction. In an experiment similar to that for 22-ABC shown in Fig. 2, 10 μM 22-Diol gave 40% inhibition when 70 μM cholesterol was used. Thus, the 22-Diol appears to bind to the P-450scc form of cholesterol. As with the 22-amine, no deviation from linearity in time course which might indicate significant metabolism was observed.

A dose-response curve for the inhibition of cholesterol side chain cleavage by 22-Diol is also shown in Fig. 6. The I50 value (15 μM) is 50-fold higher than that for 22-ABC but more tightly than cholesterol. As with the 22-amine, no deviation from linearity in time course which might indicate significant metabolism was observed.

Fig. 5. Inhibition of cholesterol side chain cleavage by 22-substituted substrate analogues. Standard reconstituted assay system containing 70 μM cholesterol; control rates in the absence of inhibitor were 10–15 nmol of pregnenolone/mg of P-450scc/min. The Ki, found for 22-Diol, 4.6 μM, is 115 times higher.
Substrate analogue interaction with \( P-450_{\text{cyc}} \)

Binding of 22-Diol to high spin \( P-450_{\text{cyc}} \) gives a zero crossing at 405 nm, while binding of the amines to produce the red-shifted spectrum gives a zero crossing at 411 nm. The visible absorption spectrum of the complex formed with 22-Diol is also different from that of the amines, exhibiting a sharp \( \alpha \)-band having greater intensity than the \( \beta \)-band (data not shown). These results indicate a different coordination of the heme in the 22-diol and 22-amine complexes and provide further evidence for a specific role of the 22-amine group of 22-ABC in the formation of the 422 nm species.

**Discussion**

In a previous paper, we reported that 22-ABC was a potent inhibitor of cytochrome \( P-450_{\text{cyc}} \), and studies on the mechanism of inhibition suggested that it acted by binding to both the substrate and heme site on the enzyme (27). In addition to explaining the high affinity of 22-ABC, this dual interaction has important structural and mechanistic implications for cytochrome \( P-450_{\text{cyc}} \) since it limits the distance between the substrate binding site and the heme and allows for direct attack of heme-bound oxidants on the cholesterol side chain.

In that study, the evidence for the interaction of 22-ABC with the substrate site was the finding that it was a competitive inhibitor with respect to cholesterol. The evidence for bonding of the 22-amine to the heme was based on the finding that complex formation with ferri-\( P-450_{\text{cyc}} \) produced a new spectral species having a Soret absorption maximum at 422 nm. We showed that a similar spectrum could be produced by high concentrations of isobutyl amine. Since the \( K_s \) for the spectral shift and the \( K_i \) for inhibition were found to be nearly identical, we concluded that binding of 22-ABC to the ferric form of the enzyme caused the inhibition and that the inhibitor interacted with both the substrate and heme catalytic sites at the same time. The finding that 22-amide analogue, 23,24-bisnor-5-cholestan-3\( \beta \)-ol-22-carboxamide, did not produce the 422 nm species and was ineffective as an inhibitor also supported the proposed role of the 22-amine group.

The results presented here, in which we prepared and tested analogues of 22-ABC modified in the steroid ring or at C-22, provide further evidence for this model. The 5\( \alpha \)-reduced form of 22-ABC exhibits weaker binding to \( P-450_{\text{cyc}} \) and decreased potency as an inhibitor; this and the stimulatory effect of adrenodoxin are consistent with the proposed binding of the steroid ring of 22-ABC to the 5-androstene binding domain on the enzyme. Despite the lower affinity, however, 5\( \alpha \)-22-ABC binding produces a new spectral species having a maximum at 422 nm, and the 5\( \alpha \)-reduced form of 22-ABC gives a spectral peak with a Soret absorption maximum at 417 nm. The difference spectra obtained during the titration with 22-Diol can also be contrasted with those of 22-ABC (27) and 5\( \alpha \)-22-ABC (Fig.6A).

The finding that 22-Diol acts as an inhibitor rather than serving as a substrate is also of interest. Since most evidence indicates that cholesterol is hydroxylated at C-22 during side chain cleavage, we had anticipated that 22-Diol might resemble the intermediate 22-hydroxycholesterol and be converted to pregnenolone. The lack of significant metabolism suggests that in cholesterol the side chain carbons 23–27 may play some role in positioning the substrate for hydroxylation. The origin of the inhibitory effect is not clear, but this could simply result from competitive binding of 22-Diol to the cholesterol.

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**Fig. 7.** Kinetic analysis of inhibition of cholesterol side chain cleavage by 22-Diol. \( \Theta \), no inhibitor; \( \Delta \), 5 \( \mu M \) 22-Diol; \( \Xi \), 15 \( \mu M \) 22-Diol.

**Fig. 8.** Spectral titration of cytochrome \( P-450_{\text{cyc}} \) with 22-Diol. \( P-450_{\text{cyc}} \) concentration, 2.0 \( \mu M \) in 50 mM potassium phosphate, pH 7.0, 22 °C. Upper panel shows difference spectra obtained following addition of 0.5, 1.0, 1.5, and 2.0 \( \mu M \) 22-Diol to the sample cuvette. Lower panel shows absolute spectra of the reference and sample cuvettes at the end of the titration in which the final concentration of 22-Diol was 4.0 \( \mu M \).

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2 No systematic study comparing the affinity of ligands containing different donor atoms has been carried out with a P-450-type heme complex, i.e. containing a thiolate anion as the fixed, trans ligand. White and Coon (36) reported that liver microsomal P-450 exhibited an apparent \( K_i \) < 1 \( \mu M \) for primary alcohols while alkyl amines typically have binding constants in the micromolar to millimolar range. We have found that with P-450, primary alcohol concentrations of up to 1 \( \mu M \) convert only a small fraction of the high spin enzyme to the low spin form, while primary alkyl amines can cause complete conversion in the millimolar range.
site as suggested by the kinetic study. In addition, because it appears that the 22-amine of 22-ABC can coordinate the heme iron, the possibility exists that the 22-hydroxyl group of 22-Diol may similarly form a bond to the heme. This would further stabilize the complex and might inhibit electron transfer from adrenodoxin. The spectral properties of the complex of 22-Diol with the enzyme are consistent with this type of interaction since they resemble those reported for other oxygen-donor ligand complexes of P-450 enzymes (36, 37); however, the spectrum of the diol complex is also similar to the substrate-free form of P-450, and hence it is not possible to distinguish diol bonding to the heme iron from coordination of an endogenous oxygen ligand.

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