Acetylenic Mechanism-based Inhibitors of Cholesterol Side Chain Cleavage by Cytochrome P-450ssc

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The following acetylenic steroids appear to be the first reported mechanism-based inhibitors of cytochrome P-450ssc: 20-(1-propynyl)-5-pregnen-3β,20α-diol, 20-(1-hexynyl)-5-pregnen-3β,20α-diol, and 20-(1,5-hexadiynyl)-5-pregnen-3β,20α-diol. Oxygen and NADPH are required for enzymatic oxidation and all three steroids yield pregnenolone as a major product. Incubation of P-450ssc with 20-(1,5-hexadiynyl)-5-pregnen-3β,20α-diol under turnover conditions completely inactivates the enzyme with a half-time of 11 min. The partition ratio for inactivation by the steroid was determined to be about 6 molecules of the steroid processed per molecule of P-450ssc inactivated.

Compounds containing acetylenic, olefinic, or allylic functional groups seem to act as mechanism-based inhibitors of the phenobarbital-induced liver microsomal P-450 isozymes (1-6). We were encouraged by the findings of Ortiz de Montellano and his co-workers, who showed that acetylenes and olefins yield covalent heme adducts when acted on by the microsomal P-450 oxygenation system (4, 7-9), to design mechanism-based inhibitors of P-450ssc which oxidatively cleaves cholesterol to pregnenolone (10). In the present study, steroid derivatives with acetylenic side chains were prepared in an effort to devise a substrate of P-450ssc which would generate a reactive species in the active site (1, 11), thus leading to mechanism-based or suicide inhibition of the enzyme (Scheme 1). These are potentially valuable for mechanistic studies of the enzyme as well as for physiological and clinical investigations in which specific chemical ablation of steroidogenesis could be of interest (12). Here, we report that several acetylenic steroids are excellent mechanism-based inhibitors of P-450ssc, although they appear to inactivate the enzyme in a manner distinct from the action of acetylenes on the microsomal enzyme.

EXPERIMENTAL PROCEDURES

Materials

The cytochrome P-450ssc (9 nmol of P-450/mg of protein), adrenodoxin, and adrenodoxin reductase were isolated and assayed as previously published (13-16). 20α-hydroxycholesterol was a gift from Dr. M. Gut (Worcester Foundation for Experimental Biology, MA). 7-3H-pregnenolone was purchased from New England Nuclear. All materials were the best reagent grade available.

Methods

Steroid Syntheses—20-(Ethynyl)-5-pregnen-3β,20α-diol (Scheme 1, 1), 20-(1-propynyl)-5-pregnen-3β,20α-diol (Scheme 1, 2), 20-(1-hexynyl)-5-pregnen-3β,20α-diol (Scheme 2, 3), and 20-(1,5-hexadiynyl)-5-pregnen-3β,20α-diol (Scheme 1, 4) were synthesized from pregnenolone and the corresponding lithium acetylide prepared by the method of Midland (17).

Chromatography and Mass Spectroscopy—Reverse-phase high performance liquid chromatography was performed on a Waters Associates μBondapak C18 column. The applied sample was 1 ml of a saturated methanol solution. Elution was performed with methanol/water (80:20, v/v) and the flow rate was 2 ml/min. Analytical GC was performed on an instrument equipped with flame ionization detectors and a glass column (2 mm, inner diameter, × 3 feet) packed with 2% SP-2100 on Supelcoport (80-100 mesh). The temperature gradient was from 210 to 300 °C at 30 °C/min, and nitrogen gas was used as the carrier at 20 pounds-inch⁻². Mass spectroscopy was performed on a Hewlett-Packard gas chromatograph-quadrupole mass spectrometer model 5992A equipped with 2% SP-2100 column (2 mm, inner diameter, × 4 feet), operated similarly to the analytical GC, with helium as carrier gas at 30 ml/min.

Enzyme Incubations and Steroid Isolation—The reconstitution system was typically 0.7 μM in P-450ssc, 4 μM in adrenodoxin, 0.2 μM in adrenodoxin reductase, 16 μM in NADPH, 10 mM in MOPS, pH 7.4, 3 mM in glucose-6-P and contained 1 unit each of catalase and glucose-6-phosphate dehydrogenase/ml. Incubations were at 20 °C and reactions were started by addition of either adrenodoxin reductase or the steroid. For TLC, GC, and GC-MS analyses, the steroid products were extracted from the reaction mixture with a small column containing C₈ reverse-phase gel which had been washed with methanol and equilibrated with water. The reaction mixtures were passed through the columns, washed with water, and the steroids were eluted with methanol into small test tubes. The methanol was evaporated to dryness in a vacuum desiccator. The recovery was determined in trial cases with added [7-3H]pregnenolone standards carried through the same process. These recoveries were all in excess of 90%.

RESULTS

The addition of 20-(ethynyl)-5-pregnen-3β,20α-diol (1), 20-(1-propynyl)-5-pregnen-3β,20α-diol (2), 20-(1-hexynyl)-5-pregnen-3β,20α-diol (3), and 20-(1,5-hexadiynyl)-5-pregnen-

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Fig. 1. Optical absorbance spectra of P-450 in the presence of steroid 4. A, 1.8 μM P-450 in the turnover buffer without steroid 4 (---) and with 6 μM steroid 4 (-----) and after 1-h incubation (----). B, Kinetics of Soret absorbance (418–500 nm) decrease during turnover. 0.7 μM in P-450, 10.5 μM is steroid 4 without NADPH (---), without O2 (-----), cuvette was opened at the time indicated by the arrow and with NADPH and O2 (-----).

Fig. 2. Kinetics of Soret decrease during turnover with steroid 4. The P-450 concentration was 0.7 μM (0.7 nmol). The reaction was initiated with addition of the steroid. a, control, no steroid; b, 0.7 nmol added twice; c, 1.4 nmol twice; d, 4.2 nmol; and e, 7 or 10.5 nmol. The inset shows the final ΔA (in the limit t → ∞) as a function of the initial ratio of steroid 4 to P-450.

Fig. 3 (left). Time course for inactivation of P-450 by steroid 4. The P-450 concentration was 4.5 μM and the steroid was 45 μM. The control was incubated without the steroid; the subsequent assay for activity was done with 20α-hydroxycholesterol as a substrate.

Fig. 4 (center). Time course for reduced P-450+CO complex absorbance decrease by the acetylenic steroids. The P-450 concentration was 1.3 μM and the steroids were at 18 μM. O, data for steroid 4; □, for steroid 3; △, for steroid 2; O, for no steroid; □, for cholesterol; △, for steroid 1.

Fig. 5 (right). Time course for GC detectable steroids during turnover of steroid 3 and steroid 4. The P-450 concentration was 2.0 μM and steroid 3 (□) and steroid 4 (○) were 30 μM each. Isolation and quantification of the steroids are described in the text. O, data for pregnenolone from 4; □, pregnenolone from 3.

3α,20α-diol (4) to cytochrome P-450scc, produced predominately type II (18) low spin absorbance changes. This is, in fact, characteristic of all 20α-monohydroxylated 5-pregnen-3β-ol derivatives we have tried. The dissociation constant, Kd, estimated as earlier described (19), was 2.4 μM for 1 and less than 0.2 μM for 2, 3, and 4.

Incubations of 2, 3, and 4 with P-450scc in the presence of electron donors and oxygen led to a time-dependent absorbance decrease in the Soret region. This absorbance decrease was found to be dependent on the presence of adrenodoxin, adrenodoxin reductase, NADPH, and oxygen, suggesting a direct association with turnover of the acetylenic steroids. The time course of the decrease in the Soret region, in the presence of steroid 4, is shown in Fig. 1. The requirements for NADPH and oxygen are clearly seen. The change in the Soret absorbance during action of P-450 on the steroid could be titrated, with a 10-fold molar excess of steroid 4 being sufficient to titrate the system (Fig. 2). The partition ratio, calculated from the final ΔA at each value of [steroid]/[P-450], is 6. The decrease followed first order kinetics with a rate constant of 0.059 ± 0.003 min⁻¹ (t0 = 11.8 ± 0.6 min) at the final concentration. In an attempt to intercept potential electrophilic intermediates, incubations were carried out in the presence of 10 mM dithiothreitol or β-mercaptoethanol, but no inhibition of the Soret absorbance decline was observed. On the other hand, the presence of 20 μM pregnenolone reduced the rate of the Soret decrease by 50% (data not shown).

The time-dependent inactivation of P-450scc was monitored by incubating the complete reconstitution system with steroid 4, separating the enzyme and inhibitor by gel filtration on a Bio-Gel P-6 column, and assaying for the production of pregnenolone from 20α-hydroxycholesterol (21) in the presence of the treated P-450scc as described under “Experimental Pro-

While the number of steroid molecules present is the number of inactivation events (Partition Ratio = Keq/Kcat).

A simple derivation from the algebra of Tatsunami et al. (20) shows that the plot of the final ΔA versus [steroid]₀/[P-450]₀ exhibits a break in the plot with a horizontal coordinate equal to the partition ratio plus one (r + 1).
is 5.9 ± 0.5 min with 45 μM (10-fold excess) steroid. We also measured the integrity of the chromophore in the enzyme by testing its ability to form the CO complex (in the presence of excess dithionite) which has a maximal absorbance at 448 nm. A first order time-dependent decay of complex-forming ability was observed with a half-life (t 1/2 ) of 10.3 ± 0.5 min for steroid 4 (Fig. 4). For steroids 2 and 3, the rate of decrease in the absorbance result at 448 nm returned to that of the control level before the ability of the enzyme to form the Fe(II)-CO complex was completely lost. Upon further addition of 2 or 3, the rate of absorbance loss at 448 nm was not altered.

Silica gel thin layer chromatography and GC-MS revealed that steroid 7 does not yield pregnenolone or hydroxylated steroids when acted on by P-450 but that action of the enzyme on steroids 2, 3, and 4 yields pregnenolone as the major product. Oxygen and NADPH were required for turnover of the steroid when acted on by P-450, but that action of the enzyme was completely lost upon further addition of 2 or 3. Upon further addition of 2 or 3, the rate of absorbance loss at 448 nm was not altered.

We thus propose that alkylation of an amino acid residue at the active site of P-450, rather than derivatization of the porphyrin, is likely to be responsible for the inactivation we have documented by the kinetic experiments presented here.

In conclusion, steroid 4 (and, preliminarily, steroids 2 and 3) has been characterized as a potent mechanism-based inhibitor of cytochrome P-450. From measurements of the amount of steroid 4 remaining after enzyme inactivation and the Soret absorbance decrease, the partition ratio has been estimated to be 6. This is in agreement with an estimate of the partition ratio derived from a ratio of the turnover number of P-450, with cholesterol (k 1 = 1 min -1) to the inactivation rate constant with 4 (k 2 = 0.1 min -1). This value is an order of magnitude lower than the ratio of 200 determined for microsomal P-450 inactivation by allylisopropyl acetamide (24). Thus, the mechanism and the relative effectiveness of the suicide inhibition of hepatic microsomal and adrenal mitochondrial P-450s by alkynes appear to be interestingly diverse.

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