INACTIVATION OF Δ^5-3-KETOSTEROID ISOMERASE(S) FROM BEEF ADRENAL CORTEX BY β,γ-ACETYLENIC KETOSTEROIDS

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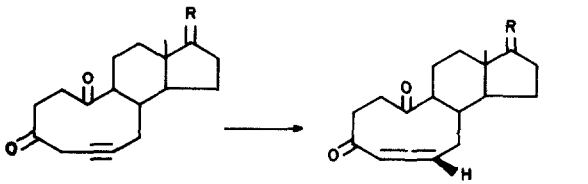
ABSTRACT

The β,γ-acetylenic ketosteroids, 5,10-seco-19-norpregn-5-yne-3,-10,20-trione 1 and 5,10-secoestr-5-yne-3,10,17-trione 2 irreversibly inactivate both the C_{19}- and the C_{21}-Δ^5-3-ketosteroid isomerase activities of beef adrenal cortex microsomes. At saturating concentrations of inhibitor half-lives of these enzyme activities vary from 45 to 240 s. It is uncertain whether the enzyme generates its own alkylating agent by isomerizing compounds 3 and 4 to the corresponding allenic ketones, namely (4R)-5,10-seco-19-norpregn-4,5-diene-3,10,20-trione 3 and (4R)-5,10-secoestra-4,5-diene-3,10,17-trione 4 since these are formed spontaneously in the buffer used to stabilize enzyme activity. In the presence of catalytic quantities of adrenal enzyme compound 4 is a powerful competitive inhibitor for both 5-androstene-3,17-dione (K_i 8.0 μM) and 5-pregnene-3,20-dione (K_i 3.5 μM) indicating that the eventual alkylation event is active site-directed. The differences in K_i values and half-lives for inactivation support the view that the C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities do not reside at the same catalytic site in beef adrenal cortex microsomes.

INTRODUCTION

Batzold and Robinson (1) have synthesized the β,γ-acetylenic ketosteroids, 5,10-seco-19-norpregn-5-yne-3,10,20-trione 1 and 5,10-secoestr-5-yne-3,10,17-trione 2. These compounds are suicide substrates for the Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) from Pseudomonas testosteroni (2-4), since they are isomerized by the normal catalytic mechanism of the enzyme to give predominantly the corresponding (4R)-allenic ketones 3 and 4 (Fig. 1), which then alkylate the active site (3-5). Recently, compound 2 has been shown to alkylate asparagine 57

at the active site of the bacterial isomerase (6). Since the Δ^5-3-ketosteroid isomerase from adrenal cortex microsomes is of central importance for the biogenesis of androgens, gestagens and corticosteroids, it was of interest to determine whether compounds 1 and 2 were effective inactivators of this enzyme activity.



R; α = H; β = CH_3CO-; 1
R = O ; 2

R ; α = H; β = CH_3CO-; 3
R = O ; 4

Fig. 1. Structures of the β,γ-acetylenic ketosteroids and their corresponding (4R)-allenic ketones. 5,10-Seco-19-norpregn-5-yne-3,10,20-trione 1; 5,10-secoestr-5-yne-3,10,17-trione 2; (4R)-5,10-seco-19-norpregna-4,5-diene-3,10,20-trione 3; and (4R)-5,10-seco-estra-4,5-diene-3,10,17-trione 4.

MATERIALS AND METHODS

Chemicals. Spectroscopic quality acetonitrile was obtained from Burdick and Jackson, Muskegon, MI, U.S.A., and crystalline bovine serum albumin was a product of Reheis Pharmaceuticals, Phoenix, AZ, U.S.A.

5-Androstene-3,17-dione and 5-pregnene-3,20-dione were synthesized from 3β-hydroxy-5-androsten-17-one and 3β-hydroxy-5-pregnen-20-one, respectively (7). 5,10-Seco-19-norpregn-5-yne-3,10,20-trione 1, 5,10-secoestr-5-yne-3,10,17-trione 2 and (4R)-5,10-secoestra-4,5-diene-3,10,17-trione 4 were synthesized as previously described (1,3) and were gifts from Dr. C. H. Robinson, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.

Preparation of beef adrenal microsomes. Beef adrenal cortex was obtained from fresh defatted and demedullated adrenals. The cortex was homogenized in a Waring Blendor with 3 volumes of 50 mM Tris-HCl, 250 mM sucrose, 5 mM $MgCl_2$, 25 mM KCl, pH 7.4 at 4°C. Differential centrifugation yielded the microsomal pellets which were washed once and then resuspended in the same buffer to give a final protein concentration of 10 mg/ml. Specific activities of the crude enzyme preparation were found to be 660 nmol of 5-androstene-3,20-dione isomerized min^{-1} per mg of protein and 1704 nmol of 5-pregnene-3,20-dione isomerized min^{-1} per mg of protein under standard assay conditions described below. Aliquots (1 ml) were frozen rapidly and stored at -80°C for subsequent use. Protein concentrations were determined by the method of Lowry et al. (8).

Enzyme assays. The standard spectrophotometric assay system for adrenal Δ^5-3-ketosteroid isomerase contained in a final volume of 3.0 ml: 100 mM potassium phosphate at pH 7.0, 1.0 µM neutralized bovine serum albumin and 1.67% (v/v) methanol containing either 5-androstene-3,17-dione or 5-pregnene-3,20-dione to give final concentrations of 68 µM and 10 µM of these steroids, respectively. The reaction was initiated by addition of the microsomal suspension. The initial velocity of formation of the Δ^4-3-ketosteroid (a_m = 16,300 M^{-1} cm^{-1}) was measured at 248 nm and at 25°C against a blank containing all components except the steroid. Corrections were made, when necessary, for nonenzymatic isomerization rates.

It should be emphasized that the concentration of albumin in the assay system is a critical factor since Bertolino et al. (9) discovered that isomerase activity from beef or calf adrenals is stimulated 10- to 20-fold by micromolar concentrations of various albumins but not other proteins. Under the assay conditions described, a concentration of 1.0 µM bovine serum albumin is adequate to promote near maximum stimulation of beef adrenal C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities.

Inactivation of beef adrenal cortex isomerase. Microsomal suspension (0.25 ml) in Tris-HCl sucrose buffer was incubated with a saturating concentration of steroid inhibitor (200 µM) dissolved in 4% (v/v) acetonitrile at 25°C. At suitable time intervals, portions (1 µl) were removed and diluted directly into the standard assay system containing 5-androstene-3,17-dione as substrate, and enzyme activity was measured. This represented a final 3000-fold dilution of the enzyme. Alternatively, portions (10 µl) were removed at time intervals and diluted 100-fold with buffer. Aliquots (10 µl) were then further diluted into the standard assay system containing 5-pregnene-3,20-dione as substrate and enzyme activity was measured. This represented a final 30,000-fold dilution of the enzyme. In every instance, semilogarithmic plots of the percentage of activity remaining with respect to time were constructed.

Competitive inhibition of beef adrenal cortex isomerase(s). Initial velocities of isomerization of either 5-pregnene-3,20-dione or 5-androstene-3,17-dione were measured in the standard assay system, except that the steroids were dissolved in 1.67% (v/v) acetonitrile. In every experiment the K_m and V_{max} values for the substrates were determined over the concentration range 0 - 12.0 μM for 5-pregnene-3,20-dione or 0 - 120 μM for 5-androstene-3,17-dione. The effect of a range of inhibitor concentrations was examined for each of three to five substrate concentrations. Dixon (10) plots (1/v against [I]) were constructed and K_i values determined.

RESULTS AND DISCUSSION

In the first set of experiments, high concentrations of adrenal microsomal protein (10 mg/ml) were incubated with saturating concentrations of compounds 1 and 2, aliquots were removed at various time intervals, and diluted for assay of C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities. The dilutions were considerable so that the final concentration of inhibitor in the assay system were negligible. Semi-logarithmic plots of the percentage of residual enzyme activity remaining with respect to time are shown in Fig. 2. Compounds 1 and 2 irreversibly inactivate both C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities in a pseudo-first-order fashion. The half-life values ($t_{1/2}$) range from 45 to 240 s corresponding to apparent pseudo-first-order-rate constants ($k_{app} = 0.693/t_{1/2}$) of 13.1×10^{-3} s^{-1} and 2.87×10^{-3} s^{-1}, respectively.

The irreversible inactivation of an enzyme by an inhibitor can be explained by the following kinetic scheme (11);

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \xrightarrow{k_{+2}} E^*$$

where E^* is the inactivated enzyme, k_{-1}/k_{+1} is K_i (the dissociation constant of the reversible enzyme-inhibitor complex) and k_{+2} is the

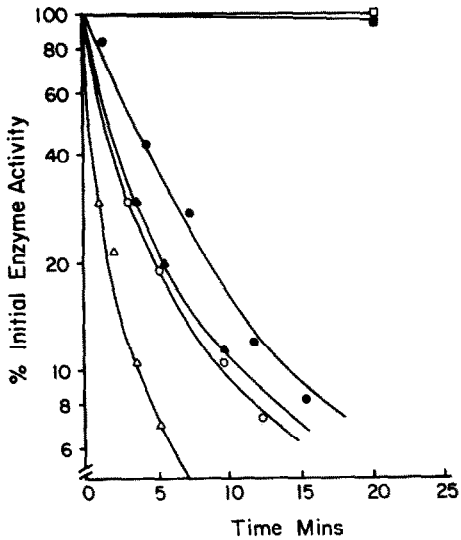


Fig. 2. Time course of inactivation of C_{19}-Δ^5-3-ketosteroid isomerase activity of beef adrenal cortex microsomes by 200 μM 5,10-seco-19-norpregn-5-yne-3,10,20-trione 1 (○) and 200 μM 5,10-secoestr-5-yne-3,10,17-trione 2 (●). In addition, the time course of inactivation of C_{21}-Δ^5-3-ketosteroid isomerase activity of beef adrenal cortex microsomes by 200 μM 1 (Δ) and 200 μM 2 (▲), are also presented. The final concentration of acetonitrile was 4% (v/v). Activities of the adrenal C_{21}-Δ^5-3-ketosteroid isomerase (□) and C_{21}-Δ^5-3-ketosteroid isomerase (■) are unaffected by this concentration of acetonitrile over the time course of the experiment.

Table 1. Comparison of the maximum rates of inactivation of Δ^5-3-ketosteroid isomerase by β,γ-acetylenic ketosteroids. Values shown for the inactivation of Δ^5-3-ketosteroid isomerase from P. testosteroni were taken from Penning, et al. (Biochem. J. 193, (1981), 217-227). Values shown for the inactivation of the adrenal isomerase activities were obtained from the experiments presented in Fig. 1. The $t_{1/2}$ lives for inactivation yield a pseudo-first-order rate constant (k_{app}) which is equivalent to the limiting rate constant for inactivation k_{+2}, since under these conditions the enzyme approaches saturation with inhibitor (See Text).

Inhibitor	Δ^5-3-ketosteroid isomerase from P. testosteroni			C_{19}-Δ^5-3-ketosteroid isomerase activity from beef adrenal cortex microsomes			C_{21}-Δ^5-3-ketosteroid isomerase activity from beef adrenal cortex microsomes		
	Final Inact. (%)	$t_{1/2}$ (s)	k_{+2} (s^{-1})	Final Inact. (%)	$t_{1/2}$ (s)	k_{+2} (s^{-1})	Final Inact. (%)	$t_{1/2}$ (s)	k_{+2} (s^{-1})
1	90	170	4.1×10^{-3}	90	120	5.75×10^{-3}	95	45	15.3×10^{-3}
2	90	300	2.3×10^{-3}	92	240	2.87×10^{-3}	97	105	6.6×10^{-3}

limiting or maximum rate constant for inactivation. It is assumed that the concentration of inhibitor [I] is much larger than the concentration of enzyme [E], that the reversible enzyme-inhibitor complex (EI) is at all times in equilibrium with enzyme and inhibitor, and that $k_{+2} \ll k_{-1}$. Then $-d(\epsilon)/dt = k_{+2}[EI]$ where $(\epsilon) = [E] + [EI]$. Thus, when all the enzyme is saturated with inhibitor, k_{app} approaches k_{+2}. (This conclusion is also valid for inactivation by suicide substrates provided that the formation of E^* is the rate limiting step.) As the inhibitor concentrations used in the present studies were sufficient to saturate bovine adrenal isomerase, the calculated k_{app} values are also a measure of the k_{+2} for the reaction (Table 1). The k_{+2} values indicate that compound 1 inactivates both the C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities from bovine adrenal cortex microsomes at more than twice the rate of compound 2. Thus, compound 1 is the superior inhibitor. Furthermore, both compounds 1 and 2 inactivate the adrenal C_{21}-Δ^5-3-ketosteroid isomerase twice as rapidly as the corresponding activity for C_{19}-Δ^5-3-ketosteroids, suggesting that in bovine adrenal cortex microsomes, these two isomerase activities reside at least partially at distinct active sites. This is in accord with our previous findings which showed that steroid analogues containing α,β-acetylenic ketones preferentially alkylate one isomerase activity (12).

For comparison, the k_{+2} values for the suicide inactivation of the Δ^5-3-ketosteroid isomerase from Pseudomonas testosteroni are

included in Table 1. It can be readily seen that the maximum rates of alkylation of both the bacterial and the adrenal C_{19}-Δ^5-3-ketosteroid activities are very similar, while compounds 1 and 2 are somewhat more potent inhibitors of the adrenal C_{21}-Δ^5-3-ketosteroid isomerase activity.

According to Kitz and Wilson (11) a plot of the reciprocal of the apparent pseudo-first-order-rate constant for inactivation ($1/k_{app}$) with respect to the reciprocal of the inhibitor concentrations (1/[I]) for a series of values of [I], gives a linear plot from which both K_i and k_{+2} can be determined. However, the Kitz-Wilson method of analysis was not employed in the present study for a number of reasons. First, it has been our experience that the microsomal suspensions contain endogenous nucleophiles that can scavenge acetylenic inhibitors, and at low inhibitor concentrations especially, this leads to curved pseudo-first-order plots. Second, at the high buffer concentration (50 mM Tris-HCl, pH 7.4) necessary to promote full enzyme activity, there is a large spontaneous rate of formation of the allenic ketones. Since these factors would be expected to complicate the kinetics, K_i values were determined for the allenic ketone 4 by competitive inhibition studies. In these experiments, catalytic quantities of adrenal cortex enzyme were used to isomerize either 5-androstene-3,17-dione or 5-pregnene-3,20-dione in the presence of varying concentrations of allenic ketone 4 (Fig. 3). The allenic ketone 4 displayed pure competitive inhibition on Dixon plots (10) against either the C_{19}- or C_{21}-

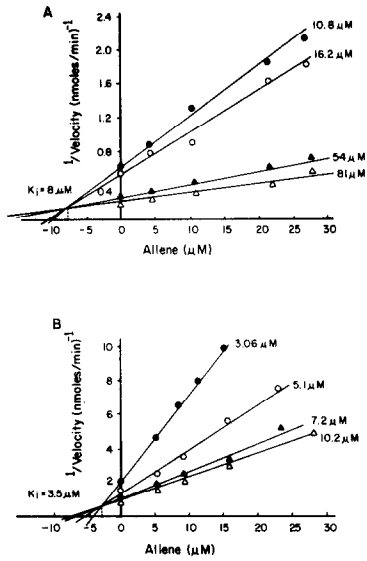


Fig. 3. Dixon plots showing the competitive inhibition of initial rates of isomerization of (A) 5-androstene-3,17-dione and (B) 5-pregnene-3,20-dione by the allenic ketone, (4R)-5,10-secoestra-4,5-diene-3,10,17-trione 4 (allene). For (A) initial rates of isomerization of fixed concentrations of 5-androstene-3,17-dione (10.8, 16.2, 54.0 and 81.0 μM) were measured in the presence of variable concentrations of the allene (0-30 μM). In each case, the assay curvette contained 10 μg of microsomal protein. For B, initial rates of isomerization of fixed concentrations of 5-pregnene-3,20-dione (3.06, 5.1, 7.2 and 10.2 μM) were measured in the presence of variable concentrations of allene (0-30 μM). In each case the assay cuvette contained 1.0 μg of microsomal protein. The assay system is described in "Materials and Methods." Under the conditions of these experiments the K_m and V_{max} for 5-androstene-3,17-dione were 30.1 ± 4.0 μM and 614 ± 38 nmoles isomerized min^{-1} per mg of protein, respectively, while the K_m and V_{max} for 5-pregnene-3,20-dione were 9.4 ± 2.3 μM and 1984 ± 288 nmoles isomerized min^{-1} per mg of protein, respectively. The K_i values for the allenic ketone 4 were found to be 8.0 and 3.5 μM for the enzyme activity toward either C_{19}- or C_{21}-Δ^5-3-ketosteroid, respectively. All lines were drawn by linear regression analysis (R values varied from 0.969 to 0.999).

substrates suggesting that the inhibitor is an active-site-directed alkylating agent for both the C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase activities of beef adrenal. Moreover, the allenic ketone 4 is bound quite tightly with K_i values of 8.0 μM and 3.5 μM for the enzyme activity toward C_{19}- and C_{21}-Δ^5-3-ketosteroids, respectively. Indeed the K_i values for the allenic ketone 4 are much lower than those obtained for estradiol-17β which has been reported to be a good competitive inhibitor of adrenal isomerase (13). In addition, the different K_i values for the binding of the allenic ketone 4 to the C_{19}- and C_{21}-Δ^5-3-ketosteroid isomerase adds further support to the view that these two enzyme activities reside at different catalytic sites in the adrenal cortex microsomes (12).

In summary, the Δ^5-3-ketosteroid isomerase activities of bovine adrenal cortex microsomes are potently and irreversibly inhibited by the β,γ-acetylenic ketosteroids 1 and 2. The final alkylating species in all probability are the corresponding (4R)-allenic ketones, which based on the findings that compound 4 can behave as a competitive inhibitor, would appear to label the active-site. It has not been possible to demonstrate directly that compounds 1 and 2 are substrates for the adrenal isomerase activities, because of the relatively rapid spontaneous rate of allene information in the buffer system employed. Thus, although compounds 1 and 2 are suicide substrates of the Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni*, we cannot as yet conclude the same for the isofunctional mammalian enzyme(s).

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