Affinity Labeling of Human Placental 3β-Hydroxy-Δ⁵-steroid Dehydrogenase and Steroid Δ-Isomerase: Evidence for Bifunctional Catalysis by a Different Conformation of the Same Protein for Each Enzyme Activity†

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ABSTRACT: 3β-Hydroxy-Δ⁵-steroid dehydrogenase and steroid Δ-isomerase copurify from human placental microsomes as a single enzyme protein. The affinity-alkylating secosteroid, 5,10-secoestr-4-yne-3,10,17-trione, inactivates the dehydrogenase and isomerase reactions in a time-dependent manner, but which of the two activities is targeted depends on the concentration of secosteroid. At 2–5 μM secosteroid, the dehydrogenase activity is alkylated in a site-specific manner (pregnenolone slows inactivation) that follows first-order inactivation kinetics (K_I = 4.2 μM, k_3 = 1.31 × 10^{-2} min^{-1}). As the secosteroid level increases from 11 to 30 μM, dehydrogenase is paradoxically inactivated at progressively slower rates, and pregnenolone no longer protects against the alkylator. The inactivation of isomerase exhibits the expected first-order kinetics (K_I = 31.3 μM, k_3 = 6.42 × 10^{-2} min^{-1}) at 11–30 μM secosteroid. 5-Androstene-3,17-dione protects isomerase from inactivation by 15 μM secosteroid, but the substrate steroid unexpectedly fails to slow the inactivation of isomerase by a lower concentration of alkylator (5 μM). A shift from a dehydrogenase to an isomerase conformation in response to rising secosteroid levels explains these results. Analysis of the ligand-induced conformational change along with cofactor protection data suggests that the enzyme expresses both activities at a bifunctional catalytic site. According to this model, the protein begins the reaction sequence as 3β-hydroxysteroid dehydrogenase. The products of the first step (principally NADH) promote a change in protein conformation that triggers the isomerase reaction.

3β-Hydroxy-Δ⁵-steroid dehydrogenase (EC 1.1.1.145) and steroid Δ-isomerase (EC 5.3.3.1) function sequentially to convert 3β-hydroxy-5-ene steroids to 3-keto-4-ene steroids during steroidogenesis in placental, adrenal, testicular, and ovarian tissues. The dehydrogenase and isomerase activities have been copurified from the microsomes of human placenta (Thomas et al., 1988), rat adrenals (Ishii-Ohba et al., 1986), rat testis (Ishii-Ohba & Tamaoki, 1987), and bovine adrenals (Rutherfurd et al., 1991). Although the dehydrogenase and isomerase reactions are performed by separate enzymes in *Pseudomonas* bacteria (Kawahara et al., 1962; Shikita & Talalay, 1979), the two activities are catalyzed by the same protein in mammalian tissues.

Affinity alkylation of the human placental enzyme by 2α-(bromoacetoxy)progesterone[1] (Thomas et al., 1990) and 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Thomas et al., 1991) has suggested that a single cofactor site services two different binding domains responsible for substrate oxidation and isomerization. To further explore the bifunctional enzyme, the affinity-labeling secosteroid, 5,10-secoestr-4-yne-3,10,17-trione, was synthesized. This secosteroid has been shown to be a site-directed alkylator of steroid Δ-isomerase activity from *Pseudomonas testosteroni* (Penning et al., 1981) and bovine adrenals (Penning & Covey, 1982). The present study with the alkylating secosteroid evaluates the relationship between the human placental dehydrogenase and isomerase activities and questions whether the two enzyme reactions are catalyzed at coexisting separate sites on the purified protein.

Materials and Methods

Materials. Steroid hormones and pyridine nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO), 5-pregnene-3,20-dione and 5-androstene-3,17-dione from Steraloids Inc. (Wilton, NH), and reagent-grade salts and analytical-grade solvents from Fisher Scientific Co. (St. Louis, MO). Glass-distilled, deionized water was used in all aqueous solutions.

Enzyme Purification. 3β-Hydroxy-Δ⁵-steroid dehydrogenase/steroid Δ-isomerase (3β-HSD/isomerase) was purified from human placental microsomes by our previously described method: solubilization with sodium cholate, ion-exchange chromatography, and hydroxylapatite chromatography (Thomas et al., 1988). The purified enzyme, which expresses both 3β-HSD (V_{max} = 70 nmol min^{-1} mg^{-1}) and isomerase (V_{max} = 900 nmol min^{-1} mg^{-1}) activities, is a homogeneous protein according to SDS–polyacrylamide gel electrophoresis, the NH_2-terminal sequence of amino acids, and fractionation of each activity during gel filtration chromatography (Thomas et al., 1988, 1989). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard with modifications noted previously (Thomas et al., 1988).

Synthesis of the Secosteroid. 5,10-Secoestr-4-yne-3,10,17-trione was synthesized according to the method of

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[1] Abbreviations and trivial names: secosteroid, 5,10-secoestr-4-yne-3,10,17-trione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; 2α-(bromoacetoxy)progesterone, 2α-(bromoacetoxy)-4-pregnene-3,20-dione; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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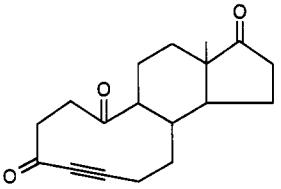

FIGURE 1: Structure of 5,10-secoestr-4-yne-3,10,17-trione.

Covey and Parikh (1982). The physical properties of the pure product agreed with literature values. The structure of the secosteroid is illustrated in Figure 1. Michael addition of nucleophilic amino acids to similar secosteroids that contain conjugated acetylenic ketones has been documented (Auchus & Covey, 1987; Auchus et al., 1988).

Inactivation and Assay of the Enzyme. Inactivation of pure enzyme (1.0 μM) was carried out in incubations containing secosteroid (added in methanol to yield 4% solvent) dissolved in 0.2 M potassium phosphate buffer, pH 7.0, 20% glycerol, and 0.1 mM EDTA at 22 °C. Identical control incubations contained 17α,21-dihydroxy-4-pregnene-3,11,20-trione (cortisone) in place of the secosteroid. Cortisone did not inhibit or stimulate either enzyme activity compared to similar control mixtures containing 4% methanol without steroid.

In protection studies, the control and experimental mixtures contained the same concentration of the potentially protecting steroid or cofactor with no increase in final solvent content compared to incubations without protector. The concentrations of these ligands were at least three times the K_m or K_I measured for 3β-HSD or isomerase activity to facilitate competition with the secosteroid.

Mixed substrate analyses (Thomas et al., 1988, 1989) have shown that pregnenolone and dehydroepiandrosterone are competitive substrates for 3β-HSD and that 5-pregnene-3,20-dione and 5-androstene-3,17-dione compete as substrates for isomerase. For each of the two activities, the pregnene and androstene substrates have similar K_m values and produce equivalent maximal velocities. Therefore, either of the appropriate C_{21} or C_{19} steroids could be used as a potential protector against the inactivation of 3β-HSD or isomerase. 5-Androstene-3,17-dione was selected as the protecting substrate during isomerase inactivation because 5-pregnene-3,20-dione was insoluble at the desired concentration ($4 \times K_m$).

Assays that monitored the loss of 3β-HSD or isomerase activity during enzyme inactivation were performed in duplicate according to our published conditions (Thomas et al., 1990). The slope of the initial linear increase in absorbance at 340 nm (due to NADH production) per unit time was used to determine 3β-HSD activity. Isomerase activity was measured by the initial absorbance increase at 241 nm (due to progesterone formation from 5-pregnene-3,20-dione) as a function of time. The inactivation plots were fitted by linear regression analysis of time versus log % of initial enzyme activity. Changes in absorbance were measured with a Varian Cary 219 recording spectrophotometer. Nonspecific or spontaneous enzyme activity were determined using blanks that contained either no enzyme or no steroid substrate. The incubation conditions cited in this paper were devised to minimize spontaneous activity in the enzyme assays. All measurements of enzyme activity were corrected for nonspecific conversions of substrate.

The half-times ($t_{1/2}$) of enzyme inactivation determined with various secosteroid concentrations [I] were employed to analyze the kinetics of affinity alkylation. To correct for the expected (Kitz & Wilson, 1962) deviations from linear inactivation in experiments using low secosteroid concentrations (2/1–5/1 alkylator/enzyme molar ratios), the initial linear portions of these plots were extrapolated to 50% enzyme activity to obtain the $t_{1/2}$ values. First-order rate constants ($k_{app} = 0.693/t_{1/2}$) were used to construct the double-reciprocal plot, $1/k_{app}$ versus $1/[I]$, according to the method of Kitz and Wilson (1962). An inhibition constant ($K_I = -1/X$ intercept) and a rate constant for covalent binding ($k_3 = 1/Y$ intercept) were calculated from the plot.

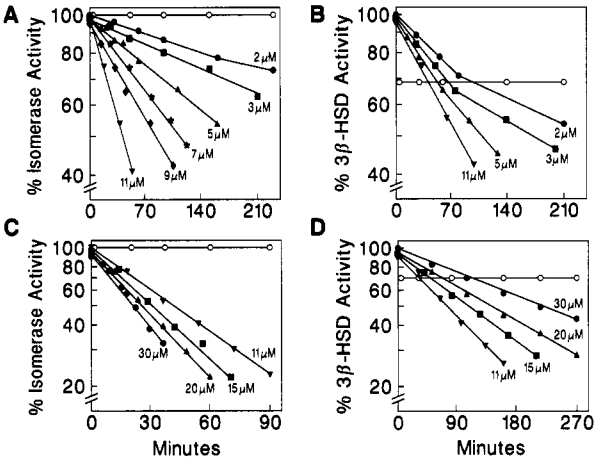

FIGURE 2: Inactivation of 3β-HSD and isomerase by 5,10-secoestr-4-yne-3,10,17-trione. Experimental conditions are described in the text. The isomerase activity of the enzyme (1.0 μM) was inactivated by 2.0–11.0 μM secosteroid (A) or by 11.0–30.0 μM secosteroid (C). In identical incubations, 3β-HSD was inactivated by 2.0–11.0 μM alkylating steroid (B) or by 11.0–30.0 μM secosteroid (D). Each inactivation plot is labeled with the concentration of alkylator used so that the "reverse" kinetics of dehydrogenase inactivation (panel D) are clearly indicated. Identical control incubations (O, unlabeled plots) contained cortisone in place of each of the secosteroid concentrations and were assayed for 3β-HSD or isomerase activity. The precent of initial enzyme activity is plotted on a logarithmic scale along each ordinate, and time is represented by the linear scale on each abscissa. Each plot is the result of at least duplicate experiments.

Results

Inactivation of 3β-HSD and Isomerase by 5,10-Secoestr-4-yne-3,10,17-trione. The secosteroid inactivates both isomerase and dehydrogenase activities in an irreversible, time-dependent manner at alkylator/enzyme molar ratios of 2/1–30/1 (Figure 2). When 2-mercaptoethanol (5.0 molar excess relative to alkylator) is added at 60% enzyme inhibition, no further inactivation or restoration of either activity is observed (data not shown). Isomerase inactivation occurs at progressively faster rates as the level of secosteroid increases both from 2.0–11.0 μM (Figure 2A) and from 11.0–30.0 μM (Figure 2C). The inactivation of 3β-HSD activity also follows pseudo-first-order kinetics with respect to alkylator concentrations between 2.0 and 11.0 μM (Figure 2B), but the rate of dehydrogenase inactivation paradoxically decreases as the secosteroid concentration increases from 11.0 to 30.0 μM (Figure 2D).

To further test the unprecedented kinetics of 3β-HSD inactivation, the secosteroid concentration was increased 3-fold above the highest level shown in Figure 2, and inactivation of both dehydrogenase and isomerase was measured (Figure 3). Because 90.0 μM secosteroid inactivates 3β-HSD 2.5 times more slowly than the rate observed at 30.0 μM in Figure 2D, the "reverse" profile of dehydrogenase inactivation persists over a broad range of alkylator concentrations above 11.0 μM. At the 90/1 alkylator/enzyme molar ratio, isomerase is rapidly inactivated to 30% of initial activity. Between 30% and 10% activity, the rate of isomerase inactivation is reduced until it is similar to the inactivation rate of dehydrogenase. Over the same time frame, 3β-HSD has been slowly inactivated at a

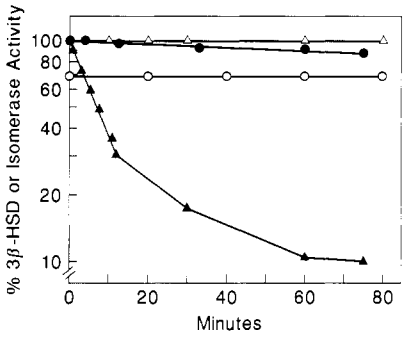

FIGURE 3: Inactivation of 3β-HSD and isomerase with a 90/1 molar ratio of 5,10-secoestr-4-yne-3,10,17-trione/enzyme. Loss of the isomerase (▲) and dehydrogenase (●) activities was followed in both the same and separate incubations containing 90 μM secosteroid and 1.0 μM enzyme. Identical control mixtures containing cortisone in place of secosteroid were also assayed for the isomerase (△) and 3β-HSD (○) activities. The percent of initial 3β-HSD or isomerase activity is plotted on the logarithmic scale alone the ordinate, and time is represented by the linear scale on the abcissa. Each plot is a combination of time points from experiments using the same or separate incubations for the two enzyme activities.

constant rate to 90% of the initial dehydrogenase activity.

Although the control and experimental enzyme mixtures have equivalent isomerase activities at zero time (Figures 2A,C and 3), the presence of secosteroid stimulates initial dehydrogenase activity relative to control incubations without alkylating steroid. The effect is concentration-dependent below 2.0 μM secosteroid (data not shown) and at a maximal constant level throughout the 2.0–90.0 μM range of alkylator concentrations used in this study. The control plots at 69% of initial 3β-HSD activity in Figures 2B,D and 3 reflect this observation.

Kinetic Analysis of 3β-HSD and Isomerase Inactivation. An inhibition constant (K_I = 31.3 μM) and a rate constant for covalent binding ($k_3 = 6.42 \times 10^{-2}$ min^{-1}) were determined for the alkylation of isomerase by applying the method of Kitz and Wilson (1962) to inactivation data obtained with 11.0–30.0 μM secosteroid (plots not shown). Similar treatment of data derived from isomerase inactivation by 2.0–5.0 μM alkylating steroid produced a double-reciprocal plot which intersects the origin. Kitz and Wilson analysis of the inactivation of 3β-HSD by 2.0–5.0 μM secosteroid yields an inhibition constant (K_I = 4.2 μM) and a maximal rate of covalent binding ($k_3 = 1.31 \times 10^{-2}$ min^{-1}). The reverse kinetics of dehydrogenase inactivation obtained with 11.0–30.0 μM secosteroid could not be analyzed by the Kitz and Wilson model.

Using the kinetic values (K_I, k_3) calculated for 3β-HSD, a velocity curve for the inactivation of dehydrogenase was constructed (Plapp, 1982):

$$k_{app} = k_3[I]/(K_I + [I])$$

Plotting the actual inactivation data ($k_{app} = 0.693/t_{1/2}$) for both dehydrogenase and isomerase along with the 3β-HSD velocity curve clarifies how the secosteroid alkylates the bifunctional enzyme (Figure 4). As the rate of 3β-HSD alkylation approaches the theoretical maximum, inactivation of dehydrogenase progressively slows below the expected rate at secosteroid concentrations above 11.0 μM. Concurrently, isomerase inactivation doubles in rate between 9.0 and 11.0 μM secosteroid. There is clearly a dramatic change in the kinetics of alkylation of both activities at approximately 10.0 μM secosteroid.

Protection Studies. When the enzyme is incubated with 5.0 μM secosteroid, the isomerase substrate, 5-androstene-3,17-dione, fails to slow the inactivation of isomerase (Figure

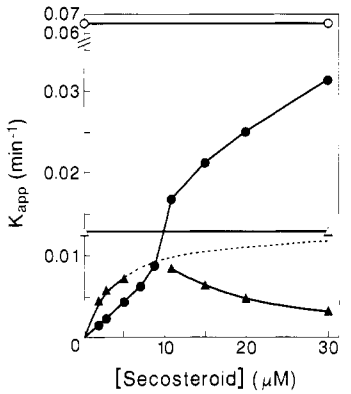

FIGURE 4: Kinetic analysis of the inactivation of 3β-HSD and isomerase by 5,10-secoestr-4-yne-3,10,17-trione. Plots of secosteroid concentrations versus rates of inactivation ($k_{app} = 0.693/t_{1/2}$ of enzyme inhibition) were constructed from the data shown in Figure 2 for the 3β-HSD (▲) and isomerase (●) activities. A theoretical velocity curve (broken line) for 3β-HSD alkylation was calculated from inactivation data obtained with 2.0–5.0 μM secosteroid according to the rate equation shown in the text. Maximal velocities are illustrated for the inactivation of the 3β-HSD (△) and isomerase (○) activities. These maximal values represent rate constants of covalent binding (k_3) determined from the Kitz and Wilson plots described in the text.

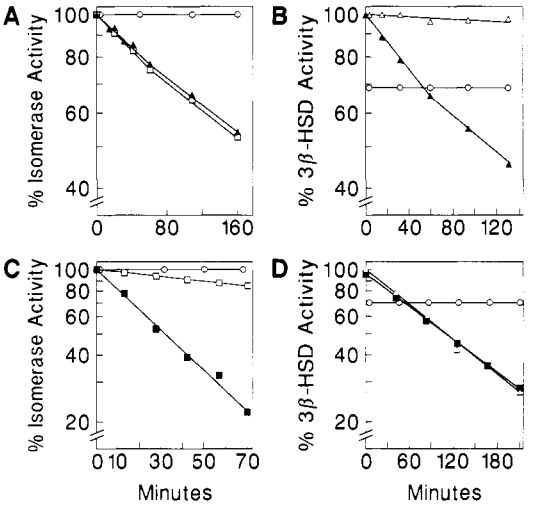

FIGURE 5: Protective effects of substrate steroids on the inactivation of 3β-HSD and isomerase by 5,10-secoestr-4-yne-3,10,17-trione. The experimental conditions are stated in the text. Isomerase activity was measured in incubations of enzyme (1.0 μM) with (A) secosteroid alone (▲, 5.0 μM) or the same concentration of secosteroid plus 5-androstene-3,17-dione (□, 150.0 μM) and with (C) secosteroid alone (■, 15.0 μM) or secosteroid plus 5-androstene-3,17-dione (□, 150.0 μM). 3β-HSD inactivation was followed in identical incubations containing (B) secosteroid alone (▲, 5.0 μM) or secosteroid plus pregnenolone (△, 10.0 μM) and (D) secosteroid alone (■, 15.0 μM) or secosteroid plus pregnenolone (△, 10.0 μM). The 3β-HSD and isomerase activities were also measured in identical control incubations (○) that contained cortisone in place of the alkylating steroid as well as the protecting substrate when appropriate. The percent of initial enzyme activity is plotted on a logarithmic scale along each ordinate, and time is represented by the linear scale on each abscissa. The values are the means of at least duplicate experiments.

5A), and the 3β-HSD substrate, pregnenolone, protects dehydrogenase activity from affinity alkylation (Figure 5B). At 15.0 μM secosteroid, 5-androstene-3,17-dione significantly protects against loss of isomerase activity (Figure 5C), but pregnenolone does not diminish the rate of dehydrogenase inactivation (Figure 5D).

The isomerase protection data are paradoxical because inactivation is slowed greatly by the isomerase substrate at the higher secosteroid concentration (15.0 μM) and is not hindered at the lower level of secosteroid (5.0 μM). Ordinarily, with

Table I: Protective Effects of Cofactors and Product Steroid on Inactivation of 3β-Hydroxy-Δ⁵-steroid Dehydrogenase (3β-HSD) and Steroid Δ-Isomerase by the Secosteroid

protector[a]	effect on 3β-HSD inactivation	effect on isomerase inactivation
NADH	complete protection	complete protection
NAD^+	2.0-fold slower[b]	no protection
androstenedione + NAD^+	2.0-fold slower	3.0-fold slower
androstenedione	no protection	no protection

[a] Incubations contained enzyme (1.0 μM) and secosteroid (30.0 μM) in 0.2 M potassium phosphate buffer, pH 7.0, 20% glycerol, 0.1 mM EDTA, and 4% methanol at 22 °C in the presence or absence of NADH (50.0 μM), NAD^+ (50.0 μM), androstenedione (150.0 μM) plus NAD^+ (50.0 μM), or androstenedione alone (150.0 μM). At appropriate time intervals, aliquots were removed to assay 3β-HSD or isomerase activity spectrophotometrically. [b] The x-fold slower rate is based on the half-time of enzyme inactivation measured in the presence versus the absence of protector(s). All experiments were performed in duplicate.

a site-saturating substrate concentration (5-androstene-3,17-dione, 90 μM, 4 × K_m; Thomas et al., 1988) and subsaturating alkylator levels (5.0 and 15.0 μM secosteroid < isomerase K_I = 31.3 μM), the greater protection is expected against the lower, not the higher, concentration of affinity alkylator (Plapp, 1982). Conversely, the protection by pregnenolone of dehydrogenase inactivation follows a pattern that is consistent with the kinetic constants involved. Pregnenolone (10.0 μM, 5 × K_m; Thomas et al., 1988) protects against inactivation with 5.0 μM but not 15.0 μM secosteroid (3β-HSD K_I = 4.2 μM).

In protection studies of isomerase using substrate steroid, 40% of the initial 5-androstene-3,17-dione (150 μM) is converted to 4-androstene-3,17-dione over 70 min (time course in Figure 5C) according to the change in spectrophotometric absorbance measured at 241 nm. This level of 5-androstene-3,17-dione remains constant from 70 to 160 min (final time point in Figure 5A). The postconversion of concentration of 5-androstene-3,17-dione (90 μM) is at a site-saturating level (4 × K_m; Thomas et al., 1988), and the resulting product steroid, androstenedione, protects neither isomerase nor dehydrogenase from inactivation in the absence of cofactor (Table I). Therefore, the paradoxical protection data obtained with the isomerase substrate cannot be explained by conversion to product steroid in the incubation mixture.

When a combination of androstenedione and NAD^+ is incubated with the enzyme, the two activities are partially protected to different degrees from the secosteroid. Table I also shows that NADH abolishes the inactivation of both activities, whereas NAD^+ only partially protects 3β-HSD and has no effect on the rate of isomerase inactivation by the secosteroid.

Discussion

The inactivation of human placental 3β-HSD and isomerase by 5,10-secoestr-4-yne-3,10,17-trione sets a number of precedents for the affinity alkylation of steroid-metabolizing enzymes. The relative rates of dehydrogenase versus isomerase alkylation reverse as the concentration of secosteroid increases. Isomerase substrate protects the isomerase activity against high, but not low, levels of alkylating steroid. Finally, the rate of dehydrogenase inactivation varies inversely with the alkylator concentration above a certain level of secosteroid.

Affinity alkylation of an enzyme consists of a reversible binding step followed by covalent modification (Kitz & Wilson, 1962):

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E{-}I \qquad K_I = k_2/k_1$$

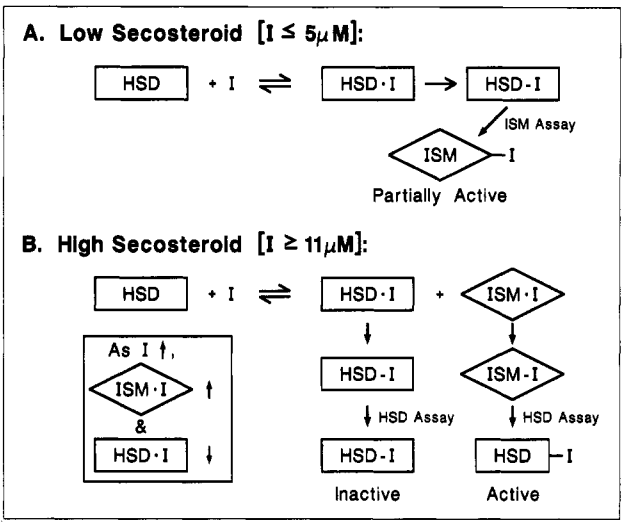

FIGURE 6: Schematic model of the affinity alkylation of enzyme in the 3β-HSD and secosteroid-induced isomerase conformations. The model is discussed in detail in the accompanying text. Enzyme in the 3β-HSD conformation is illustrated by HSD within a rectangle. The isomerase form is represented by ISM within a diamond. The reversible binding of secosteroid (I) is displayed as HSD·I or ISM·I. HSD–I or ISM–I represents enzyme in each conformation covalently bound to secosteroid. Illustration of the covalently bound inhibitor within the rectangle or diamond indicates that the enzyme was alkylated while in the same conformation. When the covalent modification (–I) is illustrated outside of the HSD rectangle or ISM diamond, the enzyme was alkylated while in the opposite conformation. Alkylation incubation conditions: pH 7.0, 1.0 μM enzyme, and 2.0–30.0 μM secosteroid. HSD assay conditions: 340 nm, pH 9.8, 0.01 mM pregnenolone, 0.10 mM NAD^+, 0.10 mL of alkylation mixture, and 1.0-mL total volume. ISM assay conditions: 241 nm, pH 7.5, 0.015 nM 5-pregnene-3,20-dione, 0.05 mM NAD^+, 0.05 mL of alkylation mixture, and 1.0-mL total volume.

A maximal rate of covalent alkylation (k_3) is approached as the concentration of the enzyme/inhibitor complex (E·I) increases with rising levels of affinity alkylator (I). Inactivation of 3β-HSD by 2.0–5.0 μM secosteroid and of isomerase by 11.0–30.0 μM secosteroid obeys Kitz and Wilson kinetics. In both cases, protection by the appropriate substrate steroid verifies that enzyme inactivation occurs by established principles of site-directed affinity alkylation (Sweet & Murdock, 1987). However, the inactivation of 3β-HSD by 11.0–30.0 μM secosteroid as well as the protection of substrate against isomerase inactivation by 5.0 and 15.0 μM secosteroid violates these principles unless the enzyme shifts from a dehydrogenase to an isomerase conformation in response to rising secosteroid levels.

We postulate (Figure 6A) that the isomerase conformation does not exist when enzyme is incubated with secosteroid levels of 5.0 μM or less. Accordingly, enzyme is alkylated in the dehydrogenase form and undergoes a conformational change in the isomerase assay mixture to produce covalently modified isomerase with partial activity. Thus, isomerase appears to be inactivated in a time-dependent manner by 2.0–5.0 μM alkylator. Isomerase substrate steroid does not protect against isomerase inactivation by 5.0 μM secosteroid because the enzyme is not in the isomerase conformation when affinity alkylation occurs. Moreover, the Kitz and Wilson plot derived from isomerase inactivation by 2.0–5.0 μM secosteroid intersects the origin, indicating that site-saturation kinetics are not followed (Plapp, 1982). Both observations are consistent with the absence of an isomerase binding site at low alkylator concentrations.

As the secosteroid concentration increases from 11.0 to 30.0 μM (Figure 6B), an increasing proportion of enzyme molecules

assume the isomerase conformation, and progressively fewer remain in the dehydrogenase form. Although enzyme in each conformation is alkylated over time according to Kitz and Wilson kinetics, dilution of aliquots into the 3β-HSD assay mixture reduces the secosteroid concentration 10-fold and introduces a high concentration of pregnenolone at the 3β-HSD pH optimum so that enzyme in the secosteroid-induced isomerase form converts back to the dehydrogenase conformation. Enzyme alkylated in the isomerase form is postulated to exhibit significant dehydrogenase activity in the 3β-HSD assay system, whereas enzyme alkylated in the dehydrogenase conformation expresses no activity during the 3β-HSD assay. Because less and less of the enzyme can be alkylated in the dehydrogenase form as the level of secosteroid increases, 3β-HSD is inactivated more rapidly by 11.0 μM than by 30.0 μM alkylating secosteroid.

This analysis of the inactivation and protection data suggests that different protein conformations are responsible for the two enzyme activities, as opposed to the dehydrogenase and isomerase reactions being catalyzed at coexisting separate sites on the protein.

Experimental evidence supports our model. If the percent of initial 3β-HSD activity is determined at the half-time ($t_{1/2}$) of isomerase inactivation at each secosteroid concentration between 11.0 and 30.0 μM, the portion of enzyme alkylated in the dehydrogenase form can be calculated: 100% − % of initial 3β-HSD activity at each isomerase $t_{1/2}$. There is a linear relationship ($r^2 = 0.999$) between the percent of enzyme alkylated in the dehydrogenase conformation and the observed rate ($k_{app} = 0.693/3\beta\text{-HSD } t_{1/2}$) of dehydrogenase inactivation at 11.0–30.0 μM secosteroid (data not shown). In fact, the rate of 3β-HSD alkylation parallels the proportion of total enzyme in the dehydrogenase form at any given percent of isomerase inactivation over this range of secosteroid concentrations.

The conversion of enzyme alkylated in the isomerase conformation to yield active dehydrogenase is supported by the inactivation data obtained with 90 μM secosteroid (3 × isomerase K_I). At this high alkylator concentration, most molecules of enzyme should be in the isomerase conformation according to our model. When that activity is measured, 90% of initial isomerase has been inactivated by the secosteroid at 75 min (Figure 3). If dehydrogenase activity is followed in the same incubation, only 10% of the enzyme appears to have been inactivated after 75 min. Since covalent modification is not reversible, enzyme alkylated while in the isomerase conformation must be converted to a form with significant, possible full, dehydrogenase activity after it is added to the 3β-HSD assay mixture. Moreover, the nonlinear isomerase inactivation plot below 30% activity suggests that unmodified enzyme in the isomerase conformation is approaching depletion, leaving a complex mixture of enzyme species in the incubation between 30% and 10% of initial isomerase activity. These species include the alkylated and nonalkylated isomerase forms, both with full potential 3β-HSD activity, in addition to minor contributions by the alkylated and nonalkylated dehydrogenase forms of the enzyme.

The idea that a conformational change may be associated with the sequential catalysis of the 3β-HSD and isomerase reactions was first proposed in our study with 2α-(bromoacetoxy)progesterone (Thomas et al., 1990). Although NADH and NAD^+ exhibited competitive binding kinetics, NADH completely abolished inactivation of both 3β-HSD and isomerase by the progestin alkylator, and NAD^+ had no effect on the inactivation of either activity. In addition, NADH stimulated isomerase to a 50% higher maximal level than NAD^+, and isomerase activity was negligible in the absence of either coenzyme (Thomas et al., 1991). A cofactor-induced conformational change that promotes isomerase activity was suggested by these observations, and NADH seemed to promote the change more effectively than NAD^+.

The protection of cofactors against enzyme inactivation described in this paper (Table I) suggests that the secosteroid, NAD^+, and NADH are each capable of inducing the conformational change, but with an efficiency that increases in that order. In incubations without cofactor, a portion of the total enzyme exists as isomerase due to the presence of 30.0 μM secosteroid. When NAD^+ is also included in these mixtures, a greater percentage of enzyme assumes the isomerase conformation and less remains in the 3β-HSD form. According to our model, when the proportion of enzyme molecules in the isomerase conformation increases, the rate of dehydrogenase inactivation decreases. Hence, NAD^+ appears to protect 3β-HSD but not isomerase from affinity alkylation. It is unlikely that NAD^+ actually interferes with the binding of secosteroid, since this coenzyme does not protect either activity from inactivation by 2α-(bromoacetoxy)progesterone (Thomas et al., 1990).

The partial protection of 3β-HSD by NAD^+ indicates that a portion of the enzyme remains in the dehydrogenase form when incubated with secosteroid and NAD^+, but when exposed to the alkylator plus NADH, virtually all molecules of the enzyme are converted to the isomerase conformation. Since NADH abolishes inactivation of isomerase by the secosteroid (Table I), full isomerase or dehydrogenase activity is measured over time in the respective assay systems. Both activities appear to be completely protected, but NADH has only blocked alkylation of the form of the enzyme present during the actual inactivation, i.e., the isomerase form.

The model also explains how dehydrogenase is protected to a lesser extent than isomerase by androstenedione plus NAD^+. As shown in Table I, the isomerase product steroid protects the enzyme from inactivation only when cofactor is present to form a ternary complex. Because the enzyme is exposed to both secosteroid and NAD^+ in these incubations, most protein molecules are alkylated while in the isomerase conformation. Nonalkylated as well as alkylated isomerase converts to the active dehydrogenase form during the subsequent 3β-HSD assay. Therefore, the 3-fold slower inactivation of isomerase produced by androstenedione (plus NAD^+) is not translated into an equally decreased inactivation rate of the dehydrogenase. As discussed above for "protection" of dehydrogenase by NAD^+, the decreased rate of 3β-HSD inactivation that is observed with androstenedione plus cofactor is most likely caused by NAD^+ forcing more enzyme into the isomerase conformation so that less is alkylated per minute in the dehydrogenase form compared to incubations with secosteroid alone. This interpretation is supported by the identical degrees of dehydrogenase protection that are measured with both NAD^+ alone and NAD^+ plus product steroid (Table I).

Biophysical characterization has suggested that the 3β-HSD and isomerase activities copurify as a single protein from human placenta (Thomas et al., 1988, 1989). The unprecedented inverse relationship between concentrations of 5,10-secoestr-4-yne-3,10,17-trione and rates of dehydrogenase inactivation provides strong evidence that the same protein expresses the two enzyme activities. Moreover, the model which explains these unique inactivation kinetics enhances our understanding of how the enzyme exhibits dual activity. Affinity

alkylation and protection data obtained with the secosteroid suggest that the enzyme is a dynamic protein which begins the reaction sequence as a dehydrogenase. The products of the first step (principally NADH) then convert the enzyme to an isomerase which catalyzes formation of the 3-keto-4-ene steroid without transferring intermediate steroid between spatially separated active sites.

In support of this concept, our study with the affinity-alkylating cofactor analog, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine, indicated that the 3β-HSD and isomerase activities share a single cofactor binding region (Thomas et al., 1991). Affinity alkylation (Thomas et al., 1990) and inhibition (Luu-The et al., 1991) experiments that have suggested separate 3β-HSD and isomerase sites are also consistent with a different enzyme conformation for each activity. Our model is analogous to the idea of a "bifunctional catalytic site" proposed for the cytochrome P450XVII-dependent testicular steroid 17α-hydroxylase/17,20-lyase system (Kuhn-Velten et al., 1991). In both placental 3β-HSD/isomerase and the testicular enzyme system, the two catalytic activities do not coexist at a given time. Changes in protein conformation relative to bound intermediate steroid trigger the second reaction in the sequence.

To further investigate the conformational change associated with the 3β-HSD/isomerase sequence of reactions, it will be necessary to determine the stoichiometry of coenzyme, substrate, and product steroid binding using fluorescence spectroscopy as well as affinity-labeling techniques. Because the synthesis of radiolabeled 5,10-secoestr-4-yne-3,10,17-trione is not feasible, affinity radioalkylation of the human placental enzyme with other dehydrogenase and isomerase site-directed reagents will be used to identify peptides associated with substrate binding for both activities and thereby test the model of a "bifunctional catalytic site".

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