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AFFINITY ALKYLATION OF HUMAN PLACENTAL 3β-HYDROXY-5-ENE-STEROID DEHYDROGENASE AND STEROID 5→4-ENE-ISOMERASE BY 2α-BROMOACETOXYPROGESTERONE: EVIDENCE FOR SEPARATE DEHYDROGENASE AND ISOMERASE SITES ON ONE PROTEIN

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Summary—We have copurified human placental 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase, which synthesize progesterone from pregnenolone and androstenedione from fetal dehydroepiandrosterone sulfate, from microsomes as a homogeneous protein based on electrophoretic and NH_2-terminal sequencing data. The affinity alkylator, 2α-bromoacetoxyprogesterone, simultaneously inactivates the pregnene and androstene dehydrogenase activities as well as the C_{21} and C_{19} isomerase activities in a time-dependent, irreversible manner following first order kinetics. At four concentrations (50/1–20/1 steroid/enzyme M ratios), the alkylator inactivates the dehydrogenase activity ($t_{1/2}$ = 1.5–3.7 min) 2-fold faster than the isomerase activity. Pregnenolone and dehydroepiandrosterone protect the dehydrogenase activity, while 5-pregnene-3,20-dione, progesterone, and androstenedione protect isomerase activity from inactivation. The protection studies and competitive kinetics of inhibition demonstrate that the affinity alkylator is active site-directed. Kitz and Wilson analyses show that 2α-bromoacetoxyprogesterone inactivates the dehydrogenase activity by a bimolecular mechanism (k'_3 = 160.9 l/mol·s), while the alkylator inactivates isomerase by a unimolecular mechanism (K_i = 0.14 mM, k_3 = 0.013 s^{-1}). Pregnenolone completely protects the dehydrogenase activity but does not slow the rate of isomerase inactivation by 2α-bromoacetoxyprogesterone at all. NADH completely protects both activities from inactivation by the alkylator, while NAD^+ protects neither. From Dixon analysis, NADH competitively inhibits NAD^+ reduction by dehydrogenase activity. Mixed cofactor studies show that isomerase binds NAD^+ and NADH at a common site. Therefore, NADH must not protect either activity by simply binding at the cofactor site. We postulate that NADH binding as an allosteric activator of isomerase protects both the dehydrogenase and isomerase activities from affinity alkylation by inducing a conformational change in the enzyme protein. The human placental enzyme appears to express the pregnene and androstene dehydrogenase activities at one site and the C_{21} and C_{19} isomerase activities at a second site on the same protein.

INTRODUCTION

Human placental 3β-hydroxy-5-ene-steroid dehydrogenase (EC 1.1.1.145) and steroid 5→4-ene-isomerase (EC 5.3.3.1), an enzyme complex localized in microsomes and mitochondria, catalyze the synthesis of progesterone from pregnenolone and the production of androstenedione from fetal dehydroepiandrosterone sulfate [1–3]. Our laboratory copurified the dehydrogenase and isomerase activities as a single protein from human placental microsomes and mitochondria [4, 5]. These two activities have also been copurified from rat adrenal microsomes [6] and rat testicular microsomes [7].

Affinity alkylators have been used successfully to explore the multiple activities of enzymes such as human placental 17β,20α-hydroxysteroid dehydrogenase [8, 9] and bacterial 3α,20β-hydroxysteroid dehydrogenase [10, 11]. We now report studies with the affinity alkylator, 2α-bromoacetoxyprogesterone,

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Trivial names and abbreviations: 2α-bromoacetoxyprogesterone, 2α-bromoacetoxy-4-pregnene-3,20-dione; 2α-acetoxyprogesterone, 2α-acetoxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; progesterone, 4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid.

which suggest that the microsomal 3β-hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase activities reside at separate sites on the single enzyme protein purified from human placenta.

EXPERIMENTAL

Materials

Steroid hormones (chromatographically pure) and pyridine nucleotides were purchased from Sigma Chemical Co.; 5-pregnene-3,20-dione and 5-androstene-3,17-dione from Steraloids Inc.; reagent grade salts, analytical grade solvents, and Eastman thin-layer chromatography sheets from Fisher Scientific Co. Glass distilled, deionized water was used for all aqueous solutions.

Enzyme purification

The 3β-hydroxy-5-ene-steroid dehydrogenase/steroid 5→4-ene-isomerase sequence of reactions is referred to as 3-HSD/isomerase activity. The 3β-hydroxy-5-ene-steroid dehydrogenase reaction is abbreviated 3-HSD activity, and the steroid 5→4-ene-isomerase reaction is termed isomerase activity. These reactions are shown in Fig. 1.

3-HSD/isomerase was purified from human placental microsomes using our previously described method: solubilization with sodium cholate, ion exchange chromatography and hydroxylapatite chromotography [4, 5]. The purified enzyme, which expresses both 3-HSD and isomerase activities, is a single, homogeneous protein according to SDS–polyacrylamide gel electrophoresis, the NH_2-terminal sequence of amino acids, and fractionation of 3-HSD or isomerase activity during gel filtration chromatography [5].

Protein determination

Protein was assayed by the method of Bradford[12] using bovine serum albumin as the standard with modifications noted previously [4].

Synthesis of 2α-bromoacetoxyprogesterone

2α-Hydroxyprogesterone was synthesized by the method of Sondheimer *et al.*[13]. The physical properties of pure product obtained after silica gel column chromatography (chloroform–ethyl acetate, 19:1, v/v) agreed with literature values. The final yield after the three synthetic steps was 5%.

2α-Bromoacetoxyprogesterone was synthesized by the reaction of 2α-hydroxyprogesterone, bromoacetic acid, and dicyclohexylcarbodiimide using our published conditions [14]. The purification procedure was improved by the use of silica gel column chromatography (benzene–ethyl acetate, 92:8, v/v) prior to recrystallization. The pure product (66% yield) had the following physical characteristics: m.p. 170–172°C (Electrothermal apparatus); λ_{max} (ethanol) 240 nm (ε-15,900); silical gel thin-layer chromatography (benzene–ethyl acetate, 92:8, v/v), single spot, R_f = 0.71. Infrared (KBr, V_{max} cm $^{-1}$): 1730 (O—C=O), 1680 (broad, C—20 and C—3 C=O), 1610 (C=C), 1270, 1230, 700 (Br—CH_2). These properties are somewhat different than those previously reported [14], possibly due to greater purity of the current preparation. [^{1}H]NMR (300 MHz, $CDCl_3$, δ, reported relative to tetramethylsilane) 5.77 (s, 1H, H-4), 5.47 (dd, J = 5, 14 Hz, 1H, H-2), 3.97 (s, 2H, $BrCH_2COO$), 2.13 (s, 3H, H-21), 1.34 (s, 3H, H-19), 0.67 (s, 3H, H-18). The doublet of doublets with couplings of 5 and 14 Hz confirms the earlier assignment [14] that the H-2 proton is axial (β). The reactivity of bromoacetoxyprogesterone analogs with nucleophilic L-amino acids, and evidence that the steroid-amino acid conjugates contain a carboxymethyl linkage has been demonstrated [15, 16].

2α-Acetoxyprogesterone was synthesized from 2α-hydroxyprogesterone by acetylation with acetic anhydride [15]. The physical properties of the pure product (90% yield) agreed with literature values [17].

Inactivation studies and enzyme assays

In all inactivation procedures, the experimental incubations contained purified enzyme (1.0 μM) and various concentrations of 2α-bromoacetoxyprogesterone (final solvent concentration, 4% methanol) in 0.2 M potassium phosphate buffer, pH 7.0, 20% glycerol, 0.1 mM EDTA (Buffer A) at 22°C. Identical control incubations contained 2α-acetoxyprogesterone at the same concentration as alkylator in the experimental mixture. In protection studies, both control and experimental mixtures contained the same concentration of the potentially protecting steroid or cofactor with no increase in final solvent concentration compared to the incubations without "protector". Stock solutions of 2α-bromoacetoxyprogesterone were freshly prepared in methanol and used after no more than 24 h of storage at −20°C

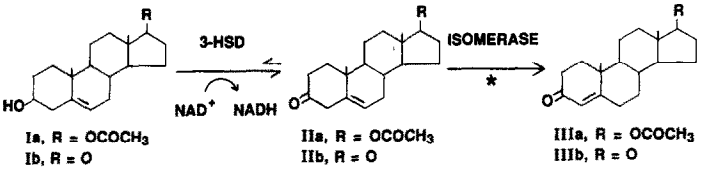

Fig. 1. The 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD)/steroid 5→4-ene-isomerase sequence of reactions in human placenta. Ia, pregnenolone; Ib, dehydroepiandrosterone; IIa, 5-pregnene-3,20-dione; IIb, 5-androstene-3,17-dione; IIIa, progesterone; IIIb, androstenedione. The * represents the positive allosteric effect of cofactors (NADH, NAD^+) on isomerase activity.

(10% hydrolysis was noted by thin-layer chromatography after 48 h at −20°C). The alkylator was completely stable in Buffer A, pH 7.0, for 1 h at 22°C (10% hydrolysis noted at 2 h).

To measure 3-HSD activity, 0.10 ml of the control or experimental mixture was added to incubations (1.0 ml) containing pregnenolone (0.01 mM) or dehydroepiandrosterone (0.014 mM) and NAD^+ (0.10 mM) in 0.05 M glycine–NAOH buffer, pH 9.8, 4% methanol at 22°C. The slope of the initial linear increase in absorbance at 340 nm (due to reduction of NAD^+) as a function of time was used to calculate the 3-HSD activity. To determine isomerase activity, 0.05 ml of the appropriate mixture was incubated with 5-pregnene-3,20-dione (0.015 mM) or 4-androstene-3,17-dione (0.037 mM) and NAD^+ (0.05 mM) in 0.02 M potassium phosphate buffer, pH 7.5, 4% methanol (1.0 ml total volume) at 22°C. Isomerase activity was calculated from the slope of the initial linear increase in absorbance at 241 nm (due to product steroid formation) as a function of time. The inactivation plots were fitted by linear regression analysis of time vs log % control enzyme activity using a Texas Instruments TI-55 III calculator.

The kinetics of enzyme inhibition by 2α-acetoxyprogesterone (0–0.15 mM, five concentrations) used the 3-HSD assay with pregnenolone (1.3 or 2.5 μM) and NAD^+ (0.10 mM) in Buffer A, pH 7.0. Inhibition of 3-HSD activity by NADH (0–0.08 mM, five concentrations) used the same assay with NAD^+ (0.05 or 0.09 mM) and pregnenolone (0.01 mM) in 0.02 M potassium phosphate buffer, pH 7.4. Dixon analysis [18] determined both the type of inhibition and inhibition constants (K_i) for 2α-acetoxyprogesterone and NADH as inhibitors of 3-HSD activity.

The effects of cofactors (NAD^+, NADH, or an equimolar solution of both) on the 3-HSD and isomerase activities were also determined. The 3-HSD incubations contained enzyme (0.10 μM) and pregnenolone (0.01 mM) with or without cofactor(s) (0.10 mM) in 0.05 M glycine–NaOH buffer, pH 9.8, at 22°C. The isomerase mixture included enzyme (0.05 μM) and 5-pregnene-3,20-dione (0.015 mM) with or without confactor(s) (0.05 mM) in 0.02 M potassium phosphate buffer, pH 7.5, at 22°C.

The assays were performed with purified enzyme in a Varian Cary 219 recording spectrophotometer. Nonspecific or "background" enzyme activity was evaluated by "zero-enzyme" and "zero-substrate" blanks. Conditions were chosen which eliminated background activity from all assays.

RESULTS

Inactivation of 3-HSD/isomerase by 2α-bromoacetoxyprogesterone

2α-Bromoacetoxyprogesterone inactivates 3-HSD activity (Fig. 2A) approximately two times faster than isomerase activity (Fig. 2B) at each concentration of alkylator studied (steroid/enzyme molar ratios

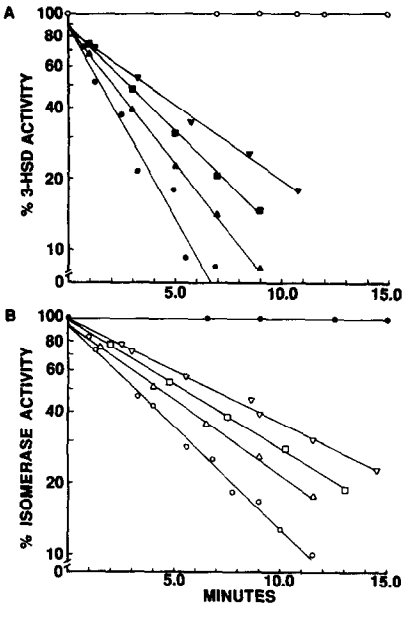

Fig. 2. Inactivation of 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD) and steroid 5→4-ene-isomerase by various concentrations of 2α-bromoacetoxyprogesterone. The experimental conditions are described in the text. (A) Four concentrations of 2α-bromoacetoxyprogesterone (50.0 μM, ●; 36.0 μM, ▲; 26.0 μM, ■; 20.0 μM, ▼) inactivated the 3-HSD activity of the enzyme (1.0 μM). Control incubations (○) contained 2α-acetoxyprogesterone in place of the bromoacetoxy derivative. (B) Isomerase activity was inactivated by the affinity alkylator (50.0 μM, ○; 36.0 μM, △; 26.0 μM, □; 20.0 μM, ▽) in identical incubations with enzyme. Control incubations (●) contained 2α-acetoxyprogesterone in place of the alkylator. The per cent of control 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 duplicate experiments.

of 20/1–50/1). 2α-Bromoacetoxyprogesterone (20/1 steroid/enzyme molar ratio) simultaneously inactivates the pregnene and androstene 3-HSD activities as well as the C_{21} and C_{19} isomerase activities (data not shown). The linear inactivation plots demonstrate that inhibition of both 3-HSD and isomerase activities is an irreversible, time-dependent reaction which follows first-order kinetics. The addition of 2-mercaptoethanol (3.75 molar excess relative to steroid) to the incubation mixture at 70% inhibition prevents further inactivation of both activities and does not restore enzyme activity, as has been shown for other steroids with the affinity alkylating bromoacetate group [15, 16]. Ethyl bromoacetate (60/1 alkylator/enzyme molar ratio) does not inactivate either enzyme activity in the presence of pregnenolone (0.01 mM), showing that bromoacetoxy reagents which are not active site-directed do not inactivate 3-HSD/isomerase by nonspecific alkylation.

Evidence that 2α-bromoacetoxyprogesterone binds at the 3-HSD and isomerase active sites

Dixon analysis (plot not shown) confirmed that 2α-acetoxyprogesterone is a competitive inhibitor

($K_i = 0.06$ mM) of the 3-HSD activity, which indicates that 2α-bromoacetoxyprogesterone binds at the 3-HSD active site. Attempts to perform Dixon analysis for 2α-acetoxyprogesterone and the isomerase activity were unsuccessful because the steroid absorbs ultraviolet light strongly at 241 nm, the wavelength at which progesterone production is measured in the isomerase assay. However, an inhibition constant for 2α-bromoacetoxyprogesterone as an affinity alkylator of isomerase was determined from the model of irreversible, covalent inhibition devised by Kitz and Wilson [19]. K_{app} values were determined from the slopes of ln(% activity) versus time plots in Fig. 2, and the double reciprocal plots, $1/K_{app}$ versus 1/[I], were constructed for the 3-HSD and isomerase activities (Fig. 3). For the isomerase activity, an inhibition constant (K_i, $-1/X$ intercept) and a rate constant for covalent binding (k_3, $1/Y$ intercept) were determined for the alkylator ($K_i = 136.0\ \mu M$, $k_3 = 0.013\ s^{-1}$). The Kitz and Wilson plot for the 3-HSD activity intersects the ordinate and abscissa at the origin, which indicates the kinetics of irreversible inactivation are similar to a bimolecular mechanism. The bimolecular rate constant of covalent binding (k'_3, 1/slope) for inactivation of 3-HSD activity by the alkylator is 160.9 l/mol·s.

Further support for the binding specificity of 2α-bromoacetoxyprogesterone is provided by studies in which substrate or product steroids slow the rate of enzyme inactivation produced by the alkylator. When enzyme is incubated with 2α-bromoacetoxyprogesterone, pregnenolone or dehydroepiandrosterone completely protects the 3-HSD activity from inactivation by the alkylator (Fig. 4A). In identical incubations, 5-pregnene-3,20-dione slows the rate of inactivation of isomerase activity by 1.4-fold, progesterone plus NAD^+ decreases the inactivation rate

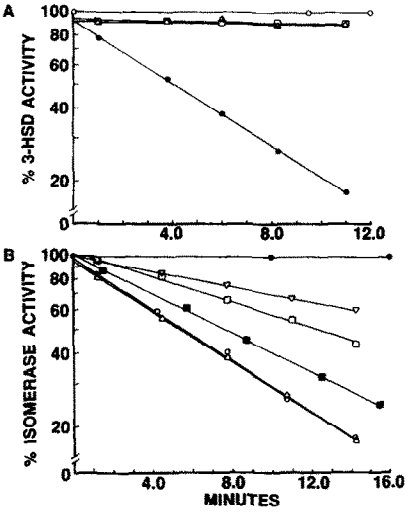

Fig. 4. Protective effects of substrates, product steroids, or NAD^+ on the inactivation of 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD) and steroid 5→4-ene-isomerase by 2α-bromoacetoxyprogesterone. The experimental conditions are described in the text. (A) 3-HSD activity was measured in incubations of enzyme (1.0 μM) with the affinity alkylator alone (●, 40.0 μM) and in identical incubations with alkylator plus pregnenolone (△, 10.0 μM) or dehydroepiandrosterone (□, 14.0 μM). Control mixtures (○) contained 2α-acetoxyprogesterone in place of alkylator as well as the protecting substrate steroid when appropriate. (B) Isomerase activity was followed in incubations of enzyme (1.0 μM) both with 2α-bromoacetoxyprogesterone alone (○, 40.0 μM) and with alkylator (40.0 μM) plus 5-pregnene-3,20-dione (■, 48.5 μM) NAD^+ alone (△, 50.0 μM), progesterone (150.0 μM) and NAD^+ (50.0 μM) (□), or androstenedione (150.0 μM) and NAD^+ (50.0 μM) (▽). Control incubations (●) contained 2α-acetoxyprogesterone in place of the alkylator as well as the potentially protecting steroid and/or cofactor when appropriate. The per cent of control 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.

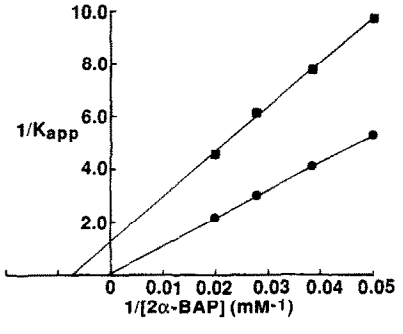

Fig. 3. Kitz and Wilson analyses of the inactivation of 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD) and steroid 5→4-ene-isomerase by 2α-bromoacetoxyprogesterone (2α-BAP). First-order rate constants (K_{app}) were calculated from $K_{app} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time at which the enzyme has lost half of the control activity. K_{app} values for the loss of isomerase (■) and 3-HSD (●) activities were derived from the inactivation plots described in Fig. 2. The reciprocals of the 2α-BAP concentrations are represented on the abscissa, and reciprocals of the K_{app} values are plotted along the ordinate.

by 2.2-fold, and androstenedione plus NAD^+ slows inactivation by 3.4-fold. NAD^+ alone does not decrease the rate of inactivation of isomerase activity by 2α-bromoacetoxyprogesterone (Fig. 4B).

Studies which elucidate enzyme binding site mechanisms

Additional experiments in which substrates, product steroids, and/or cofactors were incubated with 2α-bromoacetoxyprogesterone and enzyme reveal a complex pattern of "protection" against the inactivation of 3-HSD and isomerase activities (Table 1). The 3-HSD substrate, pregnenolone, completely protects the 3-HSD activity but does not slow the rate of isomerase inactivation at all. The other "protectors"—progesterone, progesterone plus NAD^+, NAD^+, or NADH—exhibit the same effect on both activities. Except for the concentration of NAD^+ used in combination with progesterone, the potentially protecting steroids or cofactors were at

saturating concentrations ($5 \times K_m$ or K_i) with respect to the 3-HSD [4, 5] and isomerase [20] activities.

Because NADH fully protects both activities from inactivation while NAD^+ protects neither, kinetic studies were performed to determine if the cofactors share common sites on the enzyme. At equimolar concentrations of cofactor, isomerase activity is less than the sum of the individual rates of isomerization measured with the allosteric activators, NAD^+ or NADH (Table 2). Thus, NAD^+ and NADH compete for a single site associated with the isomerase activity [21]. In similar incubations, 3-HSD activity is inhibited 2.9-fold with an equimolar mixture of NAD^+ and NADH compared to the activity seen with NAD^+ alone (Table 2). Dixon analysis shows that NADH ($K_i = 8.6\ \mu M$) competitively inhibits NAD^+ reduction by the 3-HSD activity (data not shown).

Protein sequence

The complete cDNA-derived sequence of amino acids reported for the enzyme protein [22] confirms our published NH_2-terminal sequence of 29 amino acids determined by Edman degradation of purified 3-HSD/isomerase [5]. The cDNA sequence predicts the amino acid, Met, at position 1, followed by our directly determined sequence. In addition, the cDNA-derived sequence confirms the amino acid, Cys, at position 6. This residue could only be tentatively identified by Edman degradation.

DISCUSSION

3-HSD/isomerase purified from human placenta expresses four enzyme activities: the pregnene and androstene dehydrogenase reactions as well as C_{21} and C_{19} isomerase reactions. pH studies [1] and kinetic analyses [23] of human placental organelle fractions suggest that the 3-HSD and isomerase activities reside either at separate sites on a single enzyme or on separate proteins. Based on the kinetics of inhibition by product or substrate steroids, the C_{21} and C_{19} dehydrogenase activities have been attributed to either separate isozymes [2] or a single protein [24, 25], respectively, in crude human placental microsomes. Bromoacetoxy derivatives of progesterone have been used effectively to analyze enzymes expressing dual activities [8-11]. Our studies with the affinity alkylator, 2α-bromoacetoxyprogesterone, clarify the multiple activities of the purified human placental enzyme in terms of the 3-HSD and isomerase as well as the pregnene and androstene reactions.

Table 1. Protection of 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD) and steroid 5→4-ene-isomerase from inactivation by 2α-bromoacetoxyprogesterone

Protector[a]	Protection of 3-HSD activity	Protection of isomerase activity
Pregnenolone	Complete	None
Progesterone	None	None
Progesterone + NAD^+	Partial[b]	Partial[b]
NAD^+	None	None
NADH	Complete	Complete

[a]Incubations contained enzyme (1.0 μM) and 2α-bromoacetoxyprogesterone (0.04 mM) in 0.2 M potassium phosphate buffer, pH 7.0, 20% glycerol, 0.1 mM EDTA, 4% methanol at 22 C with or without pregnenolone (0.01 mM), progesterone (0.15 mM), progesterone (0.15 mM) plus NAD^+ (0.05 mM), NAD^+ (0.125 mM), or NADH (0.05 mM). 3-HSD activity (340 nm, pH 9.8, 0.01 mM pregnenolone, 0.10 mM NAD^+) was determined using 0.10 ml of the incubation mixture. Isomerase activity (241 nm, pH 7.5, 0.015 mM 5-pregnene-3,20-dione, 0.05 mM NAD^+) was measured using 0.05 ml of the mixture.

[b]2-Fold slower rates of both 3-HSD and isomerase inactivation than by alkylator alone. All experiments were performed in duplicate.

Table 2. Effect of cofactors on the 3β-hydroxy-5-ene-steroid dehydrogenase (3-HSD) and steroid 5→4-ene-isomerase activities

Nucleotide	3-HSD activity[a] nmol/min ± range/2	Isomerase activity[b] nmol/min ± range/2
None	0	0.10 ± 0.01
NAD^+	0.46 ± 0.04	0.57 ± 0.07
NADH	0	1.08 ± 0.02
NAD^+ + NADH	0.16 ± 0.01	1.14 ± 0.02

[a]3-HSD activity (340 nm, pH 9.8, 0.01 mM pregnenolone, 0.10 μM enzyme) was measured in triplicate with or without the addition of nucleotides (each at 0.10 mM).

[b]Isomerase activity (241 nm, pH 7.5, 0.015 mM 5-pregnene-3, 20-dione, 0.05 μM enzyme) was determined in triplicate in the presence or absence of the designated cofactors (each at 0.05 mM).

2α-Bromoacetoxyprogesterone is an active site-directed affinity alkylator of both the 3-HSD and isomerase activities according to the following evidence. 2α-Acetoxyprogesterone competitively inhibits 3-HSD activity. The bromoacetoxy derivative could not be used in this inhibition study because it inactivates too rapidly to perform meaningful Dixon analysis. The kinetics of inactivation of both activities fit the Kitz and Wilson model for active site-directed, irreversible, covalent inhibitors [19]. Appropriate substrates or product steroids protect the 3-HSD or isomerase activity from inactivation by competing with the affinity alkylator for access to the active site. However, the product steroid, progesterone, protects the two activities from inactivation only in the presence of NAD^+ (which does not protect alone), while substrates protect the appropriate activity in the absence of NAD^+. Apparently, the 3-HSD and isomerase activities recognize the affinity alkylator as a substrate analog rather than as a product analog due to the 2α-bromoacetoxy side chain on the steroid molecule.

The observed simultaneous inactivation of the pregnene and androstene 3-HSD activities by 2α-bromoacetoxyprogesterone indicates that the two reactions are catalyzed at a common on site the enzyme. Further, both pregnenolone and dehydroepiandrosterone, the C_{21} and C_{19} dehydrogenase substrates, completely protect 3-HSD activity from inactivation by the alkylator. Similarly, 2α-bromoacetoxyprogesterone inactivates the pregnene and androstene isomerase activities at equal rates, while both C_{21} and C_{19} product steroids, progesterone and androstenedione, slow the rate of isomerase inactivation. These affinity alkylation data are consistent with our previously reported mixed substrate analyses which suggest that each activity uses the

appropriate pregnene and androstene substrates competitively [4, 5]. Thus, our hypothesis that a flood of fetal dehydroepiandrosterone sulfate can both stimulate estrogen production (via androstenedione) and inhibit progesterone synthesis through the human placental 3-HSD/isomerase activities is strongly supported. Such a shift in steroidogenesis may catalyze the onset of labor [26, 27].

Affinity alkylation of the enzyme with 2α-bromoacetoxyprogesterone suggests that the 3-HSD and isomerase activities reside at separate sites. The alkylator inactivates 3-HSD activity at twice the rate of isomerase activity. The 3-HSD substrate, pregnenolone, completely protects dehydrogenase activity but does not slow the rate of inactivation of isomerase activity by the affinity alkylator. Although kinetic and protection studies show that the affinity alkylator binds at the 3-HSD and isomerase active sites, Kitz and Wilson analysis indicates that the alkylator inactivates the 3-HSD and isomerase activities by different mechanisms. 2α-Bromoacetoxyprogesterone inactivates isomerase activity by a unimolecular mechanisms, schematically represented by:

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

The covalent enzyme-alkylator complex (E − I) is formed by the reversible unimolecular species (E · I). The affinity of isomerase activity for the alkylator is estimated by an inhibition constant (K_i), and the rate of covalent binding is described by the rate constant (k_3). In constrast, the affinity alkylator inactivates 3-HSD activity by a bimolecular mechanism, represented as:

$$E + I \xrightarrow{k_3} E - I$$

In this mechanism, E − I is formed at a rate described by a bimolecular rate constant (k_3') from the enzyme and alkylator molecules (E and I), but an inhibition constant cannot be determined. Although reversible enzyme–alkylator complexes are formed because the reaction follows first-order kinetics and the alkylator is active site-directed, they may be ignored in this scheme since they are very weak [19]. Separate sites for the 3-HSD and isomerase activities are supported by this evidence, but the sites could reside on the same or separate proteins.

In *Pseudomonas testosteroni*, the 3-HSD and isomerase activities are separate enzyme proteins [28, 29]. Isomerase activity did not copurify with bovine adrenal 3-HSD activity [30]. In addition to human placenta, the two activities did copurify from rat adrenal [6] and rat testis [7]. Affinity alkylation by 2α-bromoacetoxyprogesterone supports our biophysical data which indicate that human placental 3-HSD/isomerase is a single protein. The purified protein gives a single band (monomeric $M_r = 41{,}000$) in SDS–polyacrylamide gel electrophoresis, is fractionated at constant specific 3-HSD or isomerase activity during gel filtration chromatography, and has a single NH_2-terminal sequence of amino acids according to Edman degradation [4, 5]. The NH_2-terminal sequence and monomeric molecular weight ($M_r = 42{,}000$) have been confirmed by the complete protein sequence determined with cDNA clones [22]. In our affinity alkylation experiments, NADH completely protects both 3-HSD and isomerase activities from inactivation by 2α-bromoacetoxprogesterone, while NAD^+ does not protect either activity at all. Dixon analysis shows that NADH competitively inhibits NAD^+ reduction, indicating that the two cofactors bind at a common site associated with the 3-HSD activity. Mixed cofactor studies show that NAD^+ and NADH bind at a common site [21] as allosteric activators of isomerase. Therefore, NADH must not protect either 3-HSD or isomerase activity by simply binding at the cofactor site, because NAD^+ would have to show some degree of protection as well. We postulate that NADH binding as an allosteric activator of isomerase induces a conformational change in the single enzyme protein which both stimulates isomerase activity and prevents the affinity alkylator from achieving the binding orientation necessary to inactivate the 3-HSD and isomerase activities. Allosteric stimulation of the isomerase activity by structural change could abolish affinity alkylation of the 3-HSD activity only if both catalytic sites are on the same protein. From kinetic studies of NAD^+ and NADH as activators of isomerase in human placental microsomes, Blomquist *et al.*[20] also hypothesized stimulation of activity by allosteric conformational change and suggested that NAD^+ and NADH may induce different structural changes in the enzyme. Our results with purified enzyme support this concept: both cofactors stimulate isomerase activity, but only NADH protects isomerase from affinity alkylation.

Based on these observations using 2α-bromoacetoxyprogesterone, human placental 3-HSD/isomerase appears to express the pregnene and androstene dehydrogenase activities at one site and the C_{21} and C_{19} isomerase activities at a second site on a single enzyme protein. Because pregnenolone protects 3-HSD and not isomerase activity, affinity radioalkylation with 2α-bromo[2′-^{14}C]acetoxyprogesterone can distinguish between amino acids or peptides identified in the two catalytic regions. Affinity alkylation using additional analogs of product steroids, substrates, and cofactors will further elucidate the binding site mechanisms associated with the multiple activities of the enzyme. Armed with knowledge of the complete protein sequence [22], these studies are capable of describing the topography of human placental 3-HSD/isomerase.

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