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ANALYSIS OF COENZYME BINDING BY HUMAN PLACENTAL 3β-HYDROXY-5-ENE-STEROID DEHYDROGENASE AND STEROID 5→4-ENE-ISOMERASE USING 5′-[p-(FLUOROSULFONYL)BENZOYL]ADENOSINE, AN AFFINITY LABELING COFACTOR ANALOG

JAMES L. THOMAS,* RICHARD P. MYERS and RONALD C. STRICKLER
Department of Obstetrics and Gynecology, Washington University School of Medicine, St Louis, MO 63110, U.S.A.

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Summary—3β-Hydroxy-5-ene-steroid dehydrogenase and steroid 5→4-ene-isomerase co-purify as a single, homogeneous protein from human placental microsomes. Affinity alkylation with 2α-bromoacetoxyprogesterone suggests that the dehydrogenase and isomerase substrate steroids bind at different sites on the same protein. However, the coenzyme, NADH, completely abolishes the alkylation of both enzyme activities by the progestin analog [Thomas J. L., Myers R. P., Rosik L. O. and Strickler R. C., *J. Steroid Biochem.* **36** (1990) 117–123]. Unlike bacterial 3-keto-5-ene-steroid isomerase, the human isomerase reaction is stimulated by diphosphopyridine nucleotides (NADH, NAD^+). The affinity labeling nucleotide analog, 5′-[p-(fluorosulfonyl)benzoyl]adenosine (FSA), inactivates the dehydrogenase and isomerase activities at similar rates in an irreversible manner which follows first order kinetics with respect to both time and alkylator concentration (0.2–0.6 mM). FSA is a cofactor site-directed reagent that binds with similar affinity as a competitive inhibitor of NAD^+ reduction by dehydrogenase ($K_i = 162\ \mu M$) or as a stimulator of isomerase ($K_m = 153\ \mu M$). Parallel plots derived from Kitz and Wilson analysis indicate that FSA inactivates the two enzyme activities with equal alkylation efficiency (k_3/K_i = 1/slope = 0.5 l/mol-s for both). The 3β-hydroxysteroid substrate, pregnenolone, protects isomerase as well as dehydrogenase from inactivation by FSA. These observations are evidence for a single cofactor binding region which services both enzyme activities.

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) catalyze the synthesis of progesterone from pregnenolone and the production of androstenedione from dehydroepiandrosterone [1, 2]. Our laboratory has copurified the dehydrogenase and isomerase activities as a single, homogeneous protein from human placental microsomes and mitochondria [3, 4]. Kinetic analyses of the purified human placental enzyme [4] show that the cofactors, NAD^+ and NADH, dramatically stimulate the isomerase reaction in addition to serving as coenzymes for the 3β-hydroxysteroid oxidoreductase.

Affinity alkylation of the enzyme by 2α-bromoacetoxyprogesterone [5] indicates that the dehydrogenase and isomerase activities occupy separate sites on a single enzyme protein but raises questions about how cofactors bind in relation to the two activities. The affinity labeling cofactor analog, 5′-[p-(fluorosulfonyl)-benzoyl]adenosine (FSA), has been used to study the nucleotide binding sites of human placental 17β,20α-hydroxysteroid dehydrogenase [6] and bacterial 3α,20β-hydroxysteroid dehydrogenase [7]. FSA inhibits both activities of 3β-hydroxysteroid dehydrogenase/isomerase purified from rat adrenal gland [8, 9] or rat testis [9, 10]. We report affinity alkylation with FSA to evaluate how the human placental enzyme binds cofactor to perform the distinctly

*To whom correspondence should be addressed at:
Department of Obstetrics and Gynecology, The Jewish Hospital of St Louis, 216 S. Kingshighway Boulevard, St Louis, MO 63110, U.S.A.

Trival names and abbreviations: pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; 2α-bromoacetoxyprogesterone, 2α-bromoacetoxy-4-pregnene-3,20-dione; FSA, 5′-[p-(fluorosulfonyl)benzoyl]-adenosine; SDS, sodium dodecylsulfate; EDTA, ethylenediaminetetraacetic acid.

different functions required by the dehydrogenase and isomerase reactions.

EXPERIMENTAL

Materials

Steroid hormones, FSA, *p*-(fluorosulfonyl)-benzoyl chloride and pyridine nucleotides were purchased from Sigma Chemical Co. (St Louis, MO); 5-androstene-3,17-dione and 5-pregnene-3,20-dione from Steraloids Inc. (Wilton, NH); reagent grade salts and analytical grade solvents from Fisher Scientific Co (St Louis, MO). Emulgen 913 was a gift from Kao Co. (Tokyo). Glass distilled, deionized water was used for all aqueous solutions.

Purification of the enzyme

The 3β-hydroxy-5-ene-steroid dehydrogenase/steroid 5$\rightarrow$4-ene-isomerase sequence of reactions if referred to as 3-HSD/isomerase activity. The 3β-hydroxy-5-ene-steroid dehydrogenase reaction is abbreviated to 3-HSD activity, and steroid 5$\rightarrow$4-ene-isomerase is shortened to isomerase.

3-HSD/isomerase was purified from human placental microsomes using our previously described method: solubilization with sodium cholate, ion exchange chromatography with elution by non-ionic detergent (Emulgen 913), and hydroxylapatite chromatography [3]. The isolated enzyme 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 [3, 4].

Protein determination

Protein was assayed by the method of Bradford [11] using bovine serum albumin as the standard with modifications noted previously [3] to correct for the presence of Emulgen 913.

Synthesis of p-(fluorosulfonyl)benzoic acid

The non-specific alkylator, *p*-(fluorosulfonyl)benzoic acid, was synthesized from *p*-(fluorosulfonyl)benzoyl chloride as described previously [6]. The physical properties of the pure product agree with literature values.

Stability of FSA

Purity of the commercial preparation of FSA was confirmed by chromatography on silica gel thin layer glass plates developed with methyl ethyl ketone–acetone–water (65:20:15, by vol) [12]. Because the 5′-ester linkage between adenosine and the *p*-(fluorosulfonyl)benzoic acid group is susceptible to hydrolysis [7], we tested the stability of FSA in incubations which mimic the experimental conditions for enzyme inactivation. At 20 min intervals, aliquots of FSA dissolved in 0.2 M potassium phosphate buffer, pH 6.3–7.1, 4% ethylene glycol were chromatographed using the TLC system described above. At pH 6.7, FSA ($R_f = 0.90$, u.v. detection, 254 nm) decomposed to produce a spot of adenosine ($R_f = 0.75$) which became visible at 160 min. Consequently, all affinity alkylation experiments were performed at pH 6.7 using concentrations of FSA which inactivated the enzyme to at least 50% of control activity within 160 min. These conditions avoided the deviations from linearity described for enzyme inactivation plots in earlier studies with FSA [7, 12].

Affinity alkylation and assay of the enzyme

In the inactivation experiments, incubations contained pure enzyme (1.0 μM) and various concentrations of FSA (final solvent content, 4% ethylene glycol) in 0.2 M potassium phosphate buffer, pH 6.7, 20% glycerol, 0.1 mM EDTA (Buffer A) at 22°C. Identical control incubations contained adenosine in place of FSA.

In protection studies, the control and experimental mixtures contained the same concentration of potentially protecting cofactor or steroid with no increase in final solvent content compared to incubations without protector. The concentrations of potential protectors were at least five times the K_m or K_i measured for the 3-HSD or isomerase activity with FSA at subsaturating concentrations to facilitate competition. The protection of pregnenolone against the inactivation of isomerase by 2α-bromoacetoxyprogesterone in the presence of NADH (0.3 μM, $0.12 \times K_m$) used the same conditions previously described for these experiments in the absence of cofactor [5].

Assays which measured the 3-HSD and isomerase activities during enzyme inactivation were performed in duplicate using our published conditions [5]. The slope of the initial linear increase in absorbance at 340 nm (due to NADH production) as a function of time was used to determine the dehydrogenase activity. Isomerase activity was calculated from the slope

of the initial increase in absorbance 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 vs log % control enzyme activity using a Texas Instruments TI-60 calculator.

Inhibition of NAD^+ reduction by FSA (0–0.40 mM) was measured by the 3-HSD assay with NAD^+ (15.0 or 30.0 μM), pregnenolone (0.01 mM), and enzyme (0.26 μM) in 0.02 M potassium phosphate buffer, pH 7.4, at 22°C. Dixon analysis [13] determined the inhibition type and constant (K_i) for FSA as an inhibitor of dehydrogenase activity.

The stimulation of isomerase by NAD^+ (0–0.20 mM), NADH (0–0.10 mM), or FSA (0–0.15 mM) was determined in incubations containing enzyme (0.065 μM) and 5-pregnene-3,20-dione (0.015 mM) in 0.02 M potassium phosphate buffer, pH 7.4, at 22°C. Michaelis constants (K_m, V_{max}) were calculated by the method of Lineweaver and Burk [14]. In mixed cofactor analysis, the same assay measured stimulation of isomerase activity by NAD^+ (3.0 μM), NADH (0.5 μM), or FSA (0.1 mM) alone or in appropriate mixtures. The concentrations of NAD^+ and NADH used in this study were kinetically equivalent: 20% of the K_m for isomerase activation.

The assays were performed with purified enzyme in a Varian Cary 219 recording spectrophotometer. Non-specific or background enzyme activity was determined by blanks which contained either no enzyme or no steroid substrate. Incubation conditions were devised which eliminated this type of background activity from all assays.

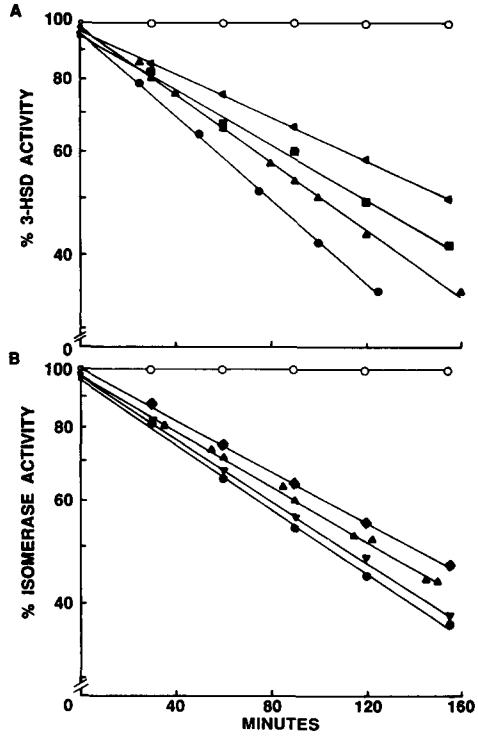

Fig. 1. Inactivation of 3-HSD and steroid 5→4-ene-isomerase by FSA. (A) Four concentrations of FSA (0.60 mM, ●; 0.40 mM, ▲; 0.275 mM, ■; and 0.20 mM, ◀) inactivated the 3-HSD activity of the enzyme (1.0 μM). (B) Isomerase was inactivated by FSA (0.60 mM, ●; 0.50 mM, ▼; 0.40 mM, ▲; and 0.30 mM, ◆) in identical incubations with enzyme. Control mixtures (○) contained adenosine in place of the affinity alkylator. Additional experimental conditions are described in the text. The percent 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 represented on the plots are the means from at least duplicate experiments.

RESULTS

Inactivation of 3-HSD and isomerase by FSA

FSA inactivates the 3-HSD and isomerase activities in an irreversible manner which follows pseudo-first-order kinetics with respect to both time and alkylator concentration (Fig. 1). When 2-mercaptoethanol (2.0 molar excess relative to FSA) is added at 60% enzyme inhibition, no further inactivation or restoration of either activity is observed, showing that the electrophilic fluorosulfonyl group of the nucleotide analog is responsible for the loss of enzyme activity. *p*-(Fluorosulfonyl)benzoic acid (0.6 mM) inactivates 3-HSD or isomerase activity 20-fold slower that the same concentration of FSA. Thus, non-specific alkylation by reagent which lacks the active site-directing adenosine does not contribute significantly to the rate of inactivation observed with the affinity alkylator.

The kinetics of enzyme inactivation fit the model devised by Kitz and Wilson [15] for active site-directed, irreversible covalent inhibitors. Using the inactivation data in Fig. 1, the double reciprocal plots, $1/K_{app}$ vs 1/(FSA), were derived for alkylation of the dehydrogenase and isomerase activities (Fig. 2). An inhibition constant (K_i, $-1/X$ intercept) and a rate constant for covalent binding (k_3, 1/Y intercept) were determined for 3-HSD ($K_i = 0.51$ mM, $k_3 = 2.64 \times 10^{-4}\,s^{-1}$) and isomerase ($K_i = 0.34$ mM, $k_3 = 1.72 \times 10^{-4}\,s^{-1}$). Because k_3/K_i (1/slope) measures the alkylation efficiency of a reagent [16], the parallel relationship between the

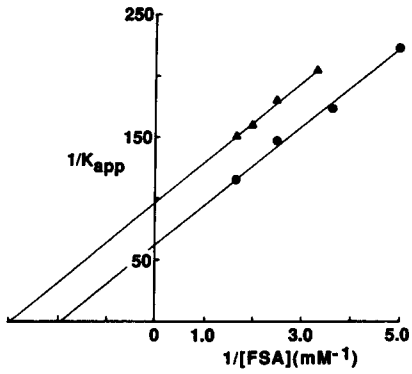

Fig. 2. Kitz and Wilson analysis of the inactivation of 3-HSD and steroid 5 → 4-ene-isomerase by FSA. First order rate constants (K_{app}) were calculated from $K_{app} = 0.693/t_{1/2}$, where $t_{1/2}$ is the time required for FSA to inactivate the enzyme to 50% of control activity. K_{app} values for the loss of 3-HSD (●) and isomerase (▲) activities were derived from the inactivation data described in Fig. 1. The reciprocals of the K_{app} values are plotted along the ordinate, and reciprocals of the FSA concentrations are represented on the abscissa.

Kitz and Wilson plots shows that FSA inactivates the two enzyme activities with equal efficiency but at slightly different rates. 3-HSD is inhibited approx. 20% faster than isomerase over the range of alkylator concentrations studied (0.2–0.6 mM). In terms of the C_{21} and C_{19} enzyme reactions, FSA (0.4 mM) simultaneously inactivates the pregnene and androstene dehydrogenase activities as well as the corresponding isomerase activities (data not shown).

Binding specificity of FSA

According to Dixon analysis (data not shown), FSA competitively inhibits NAD^+ reduction by the dehydrogenase activity ($K_i = 161.5\ \mu M$). Similar to the activation of isomerase seen with NAD^+ and NADH (Fig. 3), FSA stimulates isomerase activity in a concentration-dependent, hyperbolic manner but much less effectively than either cofactor (Table 1). Mixed cofactor analysis (Table 2) shows that FSA competes with both NADH and NAD^+ as a stimulator of isomerase activity.

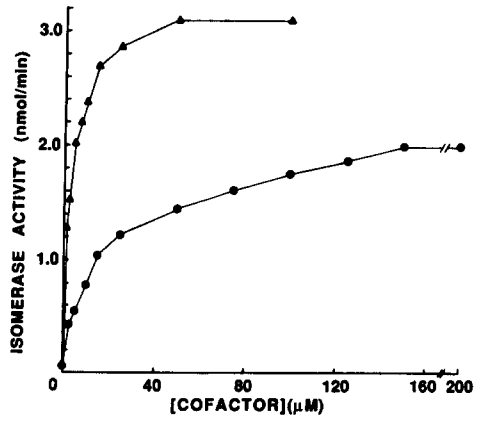

Fig. 3. Activation of steroid 5 → 4-ene-isomerase by NAD^+ and NADH. Isomerase activity was measured with various concentrations of NADH (0–100.0 μM, ▲) or NAD^+ (0–200.0 μM, ●) as described in the text. The enzyme activity (nmol progesterone formed/min) is plotted on the ordinate and the concentration of cofactor (NADH or NAD^+) is represented along the abscissa.

Table 1. Kinetic constants of FSA, NAD^+ and NADH as stimulators of steroid 5 → 4-ene-isomerase activity

Nucleotide analog[a]	K_m (μM)	V_{max} (nmol/min/mg)
FSA	152.6	58.7
NAD^+	14.4	394.3
NADH	2.4	609.5

[a]Isomerase activity (241 nm, pH 7.4, 0.015 mM 5-pregnene-3,20-dione, 5.0 μg enzyme) was measured in duplicate incubations containing FSA (0–0.15 mM), NAD^+ (0–0.20 mM) or NADH (0–0.10 mM).

Protection studies

The inactivation of 3-HSD activity by FSA is slowed more effectively by cofactors than by substrate or product steroids [Fig. 4(A)]. Unlike this anticipated profile of protection for the dehydrogenase activity, the inactivation of isomerase by FSA is completely abolished by either NADH, 5-androstene-3,17-dione, or pregnenolone, the 3-HSD substrate steroid [Fig. 4(B)].

In our previous affinity alkylation study of the human placental enzyme [5], pregnenolone did not protect isomerase from inactivation by 2α-bromoacetoxyprogesterone. However, when NADH is included in a similar incubation, pregnenolone significantly slows the inactivation of isomerase compared to the rate measured with alkylating steroid plus cofactor alone (Fig. 5). This concentration of NADH (0.3 μM) stimulates isomerase to the same

Table 2. Mixed kinetic analysis of the stimulation of steroid 5 → 4-ene-isomerase by FSA and cofactor

Nucleotide analog	Isomerase activity[a] (nmol/min ± range/2)
None	0.01 ± 0.01
FSA	0.11 ± 0.01
NADH	0.37 ± 0.04
FSA + NADH	0.33 ± 0.04[b]
NAD^+	0.22 ± 0.01
FSA + NAD^+	0.18 ± 0.02[b]

[a]Isomerase activity (241 nm, pH 7.4, 0.015 mM 5-pregnene-3,20-dione, 5.0 μg enzyme) was determined in duplicate with FSA (0.10 mM), NADH (0.5 μM) or NAD^+ (3.0 μM) alone and in identical incubations containing mixtures of FSA plus NADH or FSA plus NAD^+.

[b]Total isomerase activity produced by the mixture of nucleotides is less than the sum of the individual rates of isomerization determined for each member of the pair.

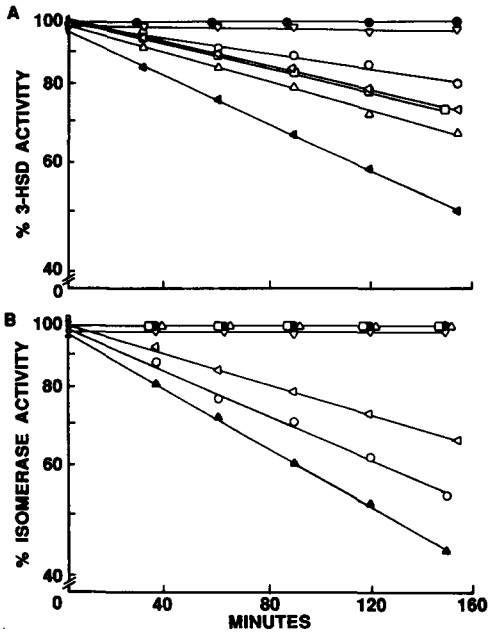

Fig. 4. Protection of cofactors, substrates, and product steroids against inactivation of 3-HSD and steroid 5→4-ene-isomerase by FSA. (A) 3-HSD activity was measured in incubations of enzyme (1.0 μM) with FSA alone (0.20 mM, ◀) and in mixtures with FSA plus NADH (0.05 mM, ▽), NAD^+ (0.15 mM, ○), androstenedione (0.12 mM, △), 5-androstene-3,17-dione (0.12 mM, □) or pregnenolone (0.01 mM, △). (B) Isomerase activity was determined in identical incubations of FSA either alone (0.40 mM, ▲) or in combination with pregnenolone (0.01 mM, △), NADH (0.05 mM, ▽), 5-androstene-3,17-dione (0.12 mM, □), androstenedione (0.12 mM, ◁) or NAD^+ (0.15 mM, ○). Control mixtures (●) contained adenosine in place of FSA and included the protecting steroid or cofactor when appropriate. Additional incubation conditions are described in the text. The percent of control enzyme activity is plotted on a logarithmic scale along the ordinates, and time is represented by the linear scale on the abscissas. Each value is the mean of at least duplicate experiments.

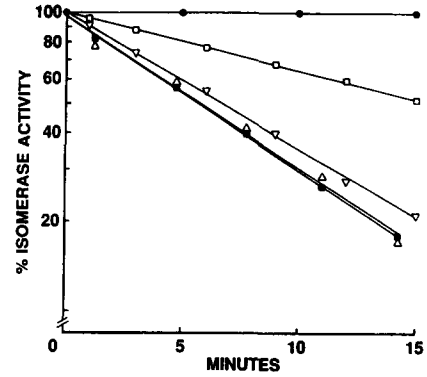

Fig. 5. Protective effects of pregnenolone on the inactivation of steroid 5→4-ene-isomerase by 2α-bromoacetoxyprogesterone (2-BAP) in the presence and absence of NADH. Isomerase activity was measured in incubations of enzyme (1.0 μM) with 2-BAP either alone (40.0 μM, ■) or in combination with pregnenolone (10.0 μM) plus NADH (0.3 μM) (□), with NADH alone (0.3 μM, ▽) or with pregnenolone alone (10.0 μM, △). Control mixtures (●) contained 2α-acetoxyprogesterone instead of 2-BAP and included pregnenolone and/or NADH when appropriate. Experimental conditions are referenced in the text. The percent of control enzyme activity is indicated by the logarithmic scale on the ordinate, and time is plotted as a linear scale along the abscissa. Each plot represents duplicate experiments.

activity (36.0 nmol/min/mg) as the FSA (0.4 mM) used in Fig. 4(B).

DISCUSSION

The 3-HSD and isomerase activities copurify as a single protein from human placenta [3, 4], rat testis [10] and rat adrenal cortex [8] but function as separate enzymes in Pseudomonas testosteroni [17, 18]. Diphosphopyridine nucleotides, NAD^+ and NADH, stimulate the mammalian isomerase activities but not bacterial isomerase. Based on affinity alkylation of the human placental enzyme with 2α-bromoacetoxyprogesterone [5], steroid substrates for the dehydrogenase ad isomerase reactions are bound at different regions on the same protein. However, NADH completely blocked inactivation of both activities by the progestin alkylator, which suggested further exploration of coenzyme binding with an affinity labeling cofactor analog. FSA is structurally similar to NADH and has been employed to characterize a variety of nucleotide-dependent enzymes [19]. Our study with FSA clarifies how human placental 3-HSD/isomerase binds cofactor during both steps of the reaction sequence.

FSA is a competitive inhibitor of NAD^+ reduction by dehydrogenase and competes with NADH and NAD^+ to activate isomerase. The affinity alkylator appears to bind at the cofactor site associated with each of the two enzyme activities.

The greater protection of NADH or NAD^+ than substrate or product steroids against the inactivation of 3-HSD activity is consistent with FSA alkylating the cofactor region of dehydrogenase. Similar to affinity alkylation of the cofactor site of 17β,20α-hydroxysteroid dehydrogenase by FSA [6], the steroids protect to some degree because alignment of substrate and coenzyme facilitates hydride ion transfer.

The protection of isomerase activity from alkylation by FSA is intriguing: inactivation is abolished by the dehydrogenase substrate, pregnenolone, as well as by NADH and the isomerase substrate steroid. Pregnenolone also protects isomerase from inactivation by

2α-bromoacetoxyprogesterone in the presence, but not in the absence, of NADH. Clearly, the enzyme recognizes FSA as NADH with respect to the isomerase activity. Either coenzyme or FSA induces the protein to bind pregnenolone so that isomerase activity is protected from alkylation.

Affinity alkylation with FSA suggests that cofactor binds at a single region on the protein: (i) the enzyme exhibits equal affinity for FSA as either an inhibitor of 3-HSD ($K_i = 162\ \mu M$) or an activator of isomerase ($K_m = 153\ \mu M$); (ii) pregnenolone protects both activities from the alkylating cofactor analog; and (iii) FSA inactivates dehydrogenase and isomerase with equal alkylation efficiency and at similar rates.

FSA does not simultaneously inactivate the two activities of 3-HSD/isomerase as was seen for the single cofactor site associated with human placental 17β,20α-hydroxysteroid dehydrogenase [6] or bacterial 3α,20β-hydroxysteroid dehydrogenase [7]. If the human placental enzyme expresses some isomerase but no dehydrogenase activity after alkylation by FSA, isomerase would appear to be inactivated more slowly than 3-HSD even though the reagent binds at one site. Although our attempts to completely inactivate the placental enzyme were unsuccessful, FSA rapidly inactivates rat adrenal 3-HSD to zero activity, while 10% of the isomerase activity remains after alkylation is complete [9]. This observation complements our binding specificity study in which FSA stimulates isomerase and inhibits 3-HSD activity.

Information about the binding specificity of NAD^+ and NADH with respect to the human placental enzyme has been conflicting. NADH competitively inhibits NAD^+ reduction by 3-HSD and competes with NAD^+ to stimulate isomerase activity [5]. NAD^+ significantly protects dehydrogenase from alkylation by FSA but marginally protects isomerase. NADH abolishes the inactivation of both enzyme reactions by either FSA or 2α-bromoacetoxyprogesterone [5]. Finally, NADH produces a 1.5-fold greater maximal isomerase activity than NAD^+ despite the evidence for competitive binding. A model wherein the two cofactors bind differently within one site reconciles all these observations.

The stimulation of isomerase by cofactor has been called an allosteric effect [5, 9, 20], but the concentration-dependent velocity curves are not signoidal as would be expected for allosterism [21]. Activation of isomerase by both NAD^+ and NADH is described by hyperbolic velocity curves which follow Michaelis–Menton kinetics. Isomerase activity is negligible in the absence of cofactor. Rather than NAD^+, kinetically preferred NADH is the likely activator of isomerase during the reaction sequence *in vivo*. It appears, therefore, that NADH is an essential coenzyme for the isomerase activity and not an allosteric effector.

These studies with FSA suggest that the activity expressed by human placental 3-HSD/isomerase is defined by the oxidation state and binding orientation of cofactor. NAD^+ is reduced during oxidation of the 3β-hydroxy-5-ene-steroid to form NADH which properly aligns the 3-keto-5-ene-steroid and enzyme protein for the isomerase reaction. Further investigation of how NADH participates in the isomerase mechanism may explain why dehydrogenase-linked mammalian isomerase requires a coenzyme and the dehydrogenase-independent bacterial isomerase does not.

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