An NADH-induced Conformational Change That Mediates the Sequential 3β-Hydroxysteroid Dehydrogenase/Isomerase Activities Is Supported by Affinity Labeling and the Time-dependent Activation of Isomerase*

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3β-Hydroxy-Δ5-steroid dehydrogenase (3β-HSD) and steroid Δ-isomerase were copurified as a single protein from human placental microsomes. Because NADH is an essential activator of isomerase (K_{act} = 2.4 μM, V_{max} = 0.6 μmol/min/mg), the affinity alkylating nucleotide, 8-[4-(bromo-2,3-dioxobutyl)thio]adenosine 5'-diphosphate (8-BDB-TADP), was synthesized. 8-BDB-TADP activates isomerase (K_{act} = 338 μM, V_{max} = 2.1 μmol/min/mg) prior to inactivating the enzyme. The inactivation kinetics for isomerase fit the Kitz and Wilson model for time-dependent, irreversible inhibition by 8-BDB-TADP (K_{i} = 314 μM, first order maximal rate constant k_{obs} = 7.8 × 10^{-3} s^{-1}). NADH (50 μM) significantly protects isomerase from inactivation by 8-BDB-TADP (100 μM). The isomerase is inactivated more rapidly by 8-BDB-TADP as the concentration of the affinity alkylator increases from 67 μM (t_{1/2} = 8.4 min) to 500 μM (t_{1/2} = 2.4 min). In sharp contrast, the 3β-HSD activity is inactivated more slowly as the concentration of 8-BDB-TADP increases from 67 μM (t_{1/2} = 4.8 min) to 500 μM (t_{1/2} = 60.0 min). We hypothesized that the paradoxical kinetics of 3β-HSD inactivation is a consequence of the activation of isomerase by 8-BDB-TADP via a nucleotide-induced shift in enzyme conformation. Biophysical support for an NADH-induced conformational change was obtained using stopped-flow fluorescence spectroscopy. The binding of NADH (10 μM) quenches the intrinsic fluorescence of the enzyme protein in a time-dependent manner (rate constant k_{app} = 8.1 × 10^{-1} s^{-1}, t_{1/2} = 85 s). A time lag is also observed for the activation of isomerase by NADH. This combination of affinity labeling and biophysical data using nucleotide derivatives supports our model for the sequential reaction mechanism; the cofactor product of the 3β-HSD reaction, NADH, activates isomerase by inducing a conformational change in the single, bifunctional enzyme protein.

3β-Hydroxy-Δ5-steroid dehydrogenase (3β-HSD).

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1 The abbreviations used are: 3β-HSD, 3β-hydroxy-Δ5-steroid dehydrogenase; 8-BDB-TADP, 8-[4-(bromo-2,3-dioxobutyl)thio]adenosine 5'-diphosphate; FSBBA, 5'-[5-(2-fluorosulfanyl)benzyl]adenosine; 2u-BAP (2u-bromoacetoxypregesterone), 2u-bromoacetoxyc-3,20-dione; PIPES, 1,4-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid.
employed previously to study nucleotide binding by pyruvate kinase (10) and isocitrate dehydrogenase (11, 12). Demonstration that the binding of substrate triggered a time-dependent quenching of protein fluorescence (13) or stimulation of enzyme activity (14) provided evidence for a ligand-induced conformational change in Escherichia coli dihydrololate reductase. The unprecedented use of both kinetic data from affinity labeling and spectroscopic measurements of coenzyme binding furnishes a definitive test of our hypothesis that NADH induces a conformational change that is critical to the sequential reaction mechanism of 3β-HSD/isomerase.

EXPERIMENTAL PROCEDURES

Materials—Steroid hormones, pyridine nucleotides, ADP, and DEAE-Sephadex were purchased from Sigma; 5-pregnen-3,20-dione and 5-androsten-3,17-dione were from Steraloids Inc. (Wilton, NH); reagent grade salts and analytical grade solvents were from Fisher; 1,4-dibromo-2,3-butanedione was from Aldrich; AG50W-X4 (H+), ion exchange gel was from Bio-Rad. Glass-distilled, deionized water was used in all aqueous solutions.

Enzyme Purification—3β-HSD/isomerase was purified from human placental microsomes by our previously described method (1). The purified enzyme, which expresses both 3β-HSD and isomerase activities, is a homogeneous protein according to SDS-polyacrylamide gel electrophoresis, the NH2-terminal sequence of amino acids, and fractionation of each activity during gel filtration chromatography (1, 15). The "UV-invisible" nonionic detergent, Genapol C-100 (0.2% w/v), was substituted for UV-absorbing nonionic detergent, Emulgen 913 (0.2% w/v), as the eluting reagent during the DEAE chromatography to provide purified enzyme suitable for fluorescence spectroscopy. The enzyme purified using the Genapol C-100 was identical to enzyme purified using Emulgen 913 according to the 3β-HSD and isomerase specific activities as well as the mobility of the single protein band during SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Bradford (16) using bovine serum albumin as the standard.

Synthesis and Stability of 8-BDB-TADP—The affinity alkylating nucleotide was synthesized as previously described by DeCamp et al. (10). The identity of each product in the three-step synthesis was verified by thin layer chromatography on cellulose sheets developed with isobutyric acid:concentrated NH4OH:H2O (66:1:33) and by ultraviolet spectrastra. The identity and purity of the 8-BDB-TADP were verified by the thin layer chromatography system (single spot, Rf = 0.63), ultraviolet spectrophotometry (λmax = 278 nm), and determination (11) of bromide content (1.1:1.0 molar ratio of hydrolyzable bromide to 8-BDB-TADP). These values agree with those previously reported for 8-BDB-TADP (10).

The decomposition rate of 8-BDB-TADP in 0.03 M MES buffer, pH 7.0, was determined by the loss of bromide at 22°C as previously described (11). The half-time for bromide loss was 56 min, in agreement with the reported half-life of 50 min under similar conditions (10).

Inactivation and Assay of the Enzyme—I-nactivation of the pure enzyme (1.0 μM) was carried out in experimental incubations that contained 8-BDB-TADP (67.0–500.0 μM), added in 0.03 M MES buffer, pH 6.0, at a 4 x final concentration) dissolved in 0.05 x PIPES buffer, pH 7.0, 20% glycerol, at 22°C. I-identical control incubations contained ADP in place of the 8-BDB-TADP. The inactivation of each activity was plotted as time versus log % of the initial (zero time) enzyme activity in the experimental mixture.

In protection studies, the control and experimental mixtures contained the same protein 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 Km or Ki, measured for 3β-HSD or isomerase activity to facilitate competition with a subsaturating concentration of 8-BDB-TADP (100.0 μM).

In the nucleotide activator analysis (7), the stimulation of isomerase activity was measured at 241 nm using 5-pregnen-3,20-dione (12.0 μM) as substrate and kinetically equivalent conditions (0.5 x Kact) of NADH alone (1.2 μM), 8-BDB-TADP alone (170 μM), or a mixture of the two activators (each at 0.5 x Kact).

Nonspecific inactivation by 8-BDB-TADP was evaluated by preincubating the enzyme with ethyl bromoacetate (100.0 μM) for 30 min following the addition of 8-BDB-TADP (100.0 μM) using the conditions described above for enzyme inactivation. The rates of isomerase inactivation by 8-BDB-TADP were compared with or without preincu-
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| Nucleotide analog | $K_{act}$ | $V_{max}$ |
|-------------------|----------|----------|
| 8-BDB-TADP        | 337.9 μM | 2114.4 nmol/min/mg |
| NADH              | 2.4 μM   | 609.5 nmol/min/mg   |
| NAD*              | 14.4 μM  | 394.3 nmol/min/mg   |
| ADP               | 523.9 μM | 71.7 nmol/min/mg    |
| FSBA              | 152.6 μM | 58.7 nmol/min/mg    |

Isomerase activity (241 nm, pH 7.4, 15.0 μg 5-pregnen-3,20-dione, 5.0 μg of enzyme) was measured in duplicate incubations containing 8-BDB-TADP (0.03–0.31 mM) or ADP (0.10–0.40 mM). The strong UV absorbance of 8-BDB-TADP and ADP limited the upper nucleotide concentrations to the values indicated above. The kinetic constants were calculated using the method of Lineweaver and Burk. The $K_{act}$ values are reported as $K_{act}$ values to reflect the fact that the nucleotides are unconverted activators of isomerase rather than substrates that are converted to products.

The assay measured the activation of isomerase activity by NADH (0.01–0.10 mM), NADH (0.02–0.20 mM), or FSBA (0.025–0.125 mM) to obtain these previously published kinetic constants (7).

μM, 100/1 alkylator to enzyme molar ratio) was preincubated with the enzyme for 30 min (with less than 10% inactivation), and then 8-BDB-TADP (100.0 μM) was added to the same incubation mixture. Isomerase activity was rapidly inactivated (t_1/2 = 5.0 min) by 8-BDB-TADP at the same rate measured for 8-BDB-TADP (100.0 μM) without preincubation with ethyl bromoacetate (data not shown). These observations are evidenced that 8-BDB-TADP inactivates isomerase with good specificity.

The Kitz and Wilson analysis (9) determined an inhibition constant (Ki = 314 μM) and a first-order maximal rate constant (k_inh = 7.8 × 10^{-3} s^{-1}) for the inactivation of isomerase by 8-BDB-TADP. In contrast to the first-order kinetics of isomerase inactivation, the inactivation of 3β-HSD by 8-BDB-TADP exhibited an intriguing reversal of the expected kinetics that could not be analyzed by the Kitz and Wilson model.

Fluorescence Spectroscopy—The change in the intrinsic fluorescence of the enzyme protein induced by the binding of NADH was measured by stopped-flow fluorescence spectroscopy (Fig. 4). The initial binding event was visible as the very rapid voltage change during the first second (Fig. 4, inset). The subsequent gradual change in voltage over 200 s characterized the NADH-induced conformational change in the enzyme protein (k_obs = 8.1 × 10^{-3} s^{-1}, t_1/2 = 85 s) and is represented by the best-fitting curve in Fig. 4.

Protection Studies—NADH significantly slowed the rate of inactivation of both 3β-HSD (Fig. 2A) and isomerase (Fig. 2B) by 8-BDB-TADP. NAD* only marginally protected 3β-HSD from inactivation (Fig. 2A) and failed to protect isomerase (Fig. 2B). The appropriate substrate steroid moderately slowed the inactivation of 3β-HSD (Fig. 2A) or isomerase (Fig. 2B) by 8-BDB-TADP.

Time-dependent Activation of Isomerase—When the enzyme was preincubated with isomerase substrate steroid, the addition of NADH activated isomerase gradually over 1 min (t_1/2 = 20 s). A time-dependent increase in isomerase activity was also measured when NADH and isomerase substrate were added simultaneously to enzyme preincubated in buffer alone (t_1/2 = 18 s). In contrast, isomerase immediately exhibited maximal activity without the time-dependent lag when the substrate steroid was added to enzyme that had been preincubated with NADH (Fig. 3).
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**FIG. 2.** Protective effects of cofactors and substrate steroids against the inactivation of 3β-HSD and isomerase by 8-BDB-TADP. 3β-HSD (panel A) and isomerase (panel B) activity were measured in incubations of enzyme (1.0 μM) with 100.0 μM 8-BDB-TADP alone (●) and in identical mixtures with 8-BDB-TADP plus 50.0 μM NADH (□), 150.0 μM NAD⁺ (○), or substrate steroid (●). 10.0 μM pregnenolone for 3β-HSD or 150.0 μM 5-androstene-3,17-dione for isomerase. The unprotected control mixture (□) contained ADP in place of 8-BDB-TADP, and the protected control mixtures (○) also included the appropriate cofactor or steroid. The percent of initial (zero time) enzyme activity is plotted on a logarithmic scale along the ordinate, and time is represented by the linear scale on each abscissa.

**FIG. 3.** Time-dependent activation of isomerase by NADH. Purified 3β-HSD/isomerase was preincubated (2.0 min) with isomerase substrate (10.0 μM 5-pregnen-3,20-dione), and NADH (2.4 μM) was added to start the reaction (●). In another identical assay, the enzyme was preincubated in buffer, and the reaction was started by adding a mixture of the substrate steroid and NADH (□). In another identical assay, the enzyme was preincubated with NADH, and the isomerase substrate was added to start the reaction (○). Additional experimental conditions are described in the text. The isomerase activity (nmol of progesterone formed) was measured at 15 equal intervals during the first minute and at 4 equal intervals during the second minute to obtain the enzyme velocity versus time plots. The half-time (t½) of the time-dependent activation of isomerase was determined from the abscissa intercept of a straight line (not shown) extrapolated from the linear portion of the plot (1–2 min). The values are the means from duplicate experiments.

**FIG. 4.** Stopped-flow fluorescence spectroscopy of 3β-HSD/isomerase after the addition of NADH. Drive syringe 1 contained 1.2 μM pure enzyme in 0.02 M potassium phosphate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.2% Genapol C-100. Drive syringe 2 contained 20.0 μM NADH in 0.02 M potassium phosphate buffer, pH 7.4. After firing the syringes at zero time, the reaction cell contained a 1:1 mixture of the contents of each. Additional experimental details are described in the text. The inset shows the first 10 s of the NADH-induced change in fluorescence. The quenching of protein fluorescence was measured as volts with positive values representing decreasing intrinsic fluorescence. The figure shows a representative plot from three determinations.

**FIG. 5.** Determination of the stoichiometry and dissociation constant for the binding of NADH to 3β-HSD/isomerase using fluorescence spectroscopy. The enzyme (3.0 μM) was titrated with successive additions of NADH (0.5–17.2 μM). The decrease in intrinsic enzyme fluorescence produced by titration with NADH was measured by excitation at 290 nm and emission at 330 nm. Further experimental detail and the construction of the plot are described in the text. The stoichiometry was calculated from the abscissa intercept (3.0 μM enzyme, and the dissociation constant was calculated from 1/slope of the best-fitting straight line through the points (●).

It was not possible to use 8-BDB-TADP in similar studies of stopped-flow or intrinsic fluorescence spectroscopy because of the strong ultraviolet absorbance of 8-BDB-TADP (extinction coefficient = 19,000 M⁻¹ cm⁻¹ at λmax = 278 nm) and the high 8-BDB-TADP concentrations (Kact = 338 μM) needed to perform the experiments.

**DISCUSSION**

As our studies of purified human placental 3β-HSD/isomerase with affinity alkylators have progressed (5, 7, 8, 18–20), it has become increasingly clear that the two-step enzyme mechanism is more complex than a dehydrogenase reaction followed by an isomerase reaction along a single protein with separate substrate and coenzyme sites for each activity. In addition to the NADH/NAD⁺ protection (5, 8) and the secosteroid inactivation (8) studies discussed above, NADH (20) and pregnenolone (19) protected the same tryptic peptides (Arg-250, Lys-175) in the enzyme from affinity radioalkylation by 2-bromo[2-¹⁴C]BAP for access to the Arg-250, Lys-175 in the enzyme from affinity radioalkylation by 2β-brom[2-¹⁴C]acetoxypregesterone (2β-¹⁴C][BAP]. Moreover, NADH shifted the binding of 2β-¹⁴C][BAP to radiolabel a histidine in the Lys-135 peptide, while pregnenolone simply competed with 2β-¹⁴C][BAP for access to the Arg-250 and Lys-175 peptides (19, 20). This shift in affinity radiolabeling that was produced by NADH protection, but not by pregnenolone pro-
tection, provided further support for our hypothesis: NADH formed by the 3β-HSD reaction induces a conformational change in the enzyme protein that activates isomerase. The hypothesis has now been definitively tested by measuring the inactivation of 3β-HSD and isomerase with the NADH site-directed, affinity alkylating nucleotide, 8-BDB-TADP, as well as by obtaining direct spectroscopic measurements of the time-dependent activation of isomerase by NADH.

The mixed activator analysis and protection studies are consistent with 8-BDB-TADP binding at the NADH site on the enzyme. NADH completely protected against the inactivation of both 3β-HSD and isomerase by all other affinity alkylators we have studied (2α-BAP (5), FSBA (7), and secoeriodiol (8)). Complete protection against inactivation is an unusual observation and suggests that NADH protected in these cases by inducing a conformational change in the enzyme rather than by simple competition. The significant, but less than complete, protection seen with 8-BDB-TADP plus the mixed activator results suggest that NADH directly competes with this affinity alkylator.

NAD+ failed to protect either activator from 8-BDB-TADP. There has been no significant protection by NAD+ against any alkylator we have studied thus far, with the notable exception of the inactivation of 3β-HSD by FSBA (7). NAD+ slowed the FSBA inactivation of 3β-HSD by 3-fold but did not significantly protect isomerase from inactivation by FSBA. Although both FSBA and 8-BDB-TADP are alkylating analogs of adenosine, the respective alkylating groups are located at “opposite” ends of the adenosine molecule (8-position of adenine in 8-BDB-TADP versus the 5'-position of ribose in FSBA). FSBA is the weakest activator of isomerase in the group of nucleotide-analogs studied (Table I). Finally, FSBA inactivates 3β-HSD by the expected first-order kinetics (not “reverse” kinetics) because FSBA does not significantly activate isomerase. The current study with 8-BDB-TADP indicates that FSBA binds at the NAD+ site when the enzyme is in the 3β-HSD conformation, whereas 8-BDB-TADP binds at the NADH site after the 8-substituted nucleotide induces the enzyme to assume the isomerase conformation.

Because 8-BDB-TADP is a highly efficacious activator of isomerase, a definitive model has been developed to explain the “reverse” kinetics of 3β-HSD inactivation by the affinity alkylating nucleotide. According to this model, the concentration-dependent activation of isomerase by increasing levels of 8-BDB-TADP (I) converts progressively more molecules of enzyme from the 3β-HSD conformation (E) into the isomerase form (E-I). Enzyme alkylated in the isomerase form (E-I) retains significant dehydrogenase activity when an aliquot from the alkylated enzyme mixture is diluted 10-fold in the 3β-HSD assay cuvette, where the dehydrogenase reaction is favored at pH 9.7 (3β-HSD optimum) with pregnenolone as substrate. However, enzyme alkylated in the isomerase conformation has no activity during the isomerase assay because the conformation is not shifted to the dehydrogenase form under these incubation conditions (at the isomerase optimal pH 7.5 with 5-pregnen-3α,20α-dione as substrate). This model is illustrated by the following reaction scheme:

\[
\text{3β-HSD assay:} \quad E + I \rightarrow E^* + I \rightarrow E^{-} - I \rightarrow E^{-} - I (\text{active for 3β-HSD}) \quad \text{Isomerase assay:} \quad E^{-} - I (\text{inactive for isomerase})
\]

As the concentration of 8-BDB-TADP is increased, more alkylated enzyme exists in the active E^*-I form during the assay used to measure 3β-HSD inactivation. Thus, the induction of the isomerase conformation (E-I) by the reversible binding of 8-BDB-TADP to the enzyme is directly responsible for the decrease in the rate of 3β-HSD inactivation as the concentration of 8-BDB-TADP increases.

At each of the 8-BDB-TADP concentrations used (67–500 μM), a portion of the enzyme molecules remains in the dehydrogenase form (E) during the inactivation. Because formation of the reversible enzyme-alkylator complex induces the isomerase conformation (E-I), the enzyme in the dehydrogenase form is inactivated by 8-BDB-TADP via a bimolecular mechanism (E + I → E-I). Based on the measurements of 3β-HSD inactivation, enzyme alkylated by 8-BDB-TADP while in the dehydrogenase conformation (E-I) has no activity in the 3β-HSD assay and partial activity in the isomerase assay.

The inactivation of isomerase by 8-BDB-TADP fits the equation for biphasic enzyme inactivation (10),

\[
E/E_0 = (1 - F)e^{-k_{fast}(t)} + (F)e^{-k_{slow}(t)}
\]

where kfast and kslow represent the values of kobs that were measured for the inactivation of isomerase and 3β-HSD, respectively, at each concentration of 8-BDB-TADP (determined from Fig. 1, A and B). The variable F represents the fractional residual activity of isomerase. The calculated curves fit the data points measured for the inactivation of isomerase (Fig. 6B) until less than 20% of the initial activity remains. At this point, the observed data points are higher than the predicted curves because all available enzyme in the isomerase

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Figure 6: The inactivation of 3β-HSD and isomerase by 8-BDB-TADP fit curves predicted by equations for one-phase and two-phase enzyme inactivation, respectively. The 3β-HSD (panel A) or isomerase (panel B) activity of the enzyme (1.0 μM) was inactivated by 67.0 μM I, 100.0 μM I, 200.0 μM I, and 500 μM I of 8-BDB-TADP. The inactivation of 3β-HSD fits the equation for single-phase enzyme inactivation, E/E0 = (1 - P)e^{-k_{obs}(t)} + P, where kobs represents the value of kobs that was measured for 3β-HSD inactivation over time at each 8-BDB-TADP concentration (determined from Fig. 1A). The inactivation of isomerase fits the equation for biphasic enzyme inactivation, E/E0 = (1 - F)e^{-k_{fast}(t)} + (F)e^{-k_{slow}(t)}}, where kfast and kslow represent the values of kobs that were measured for the inactivation of isomerase and 3β-HSD, respectively, at each concentration of 8-BDB-TADP (determined from Fig. 1, A and B). The remaining parameters and the significance of the fits are discussed in the text. Fractional enzyme activity (E/E0, E0 = 1.0) is plotted on a logarithmic scale along each ordinate, and time is represented by the linear scale on each abscissa.
form (E⁻¹) has been inactivated as E⁻ → I, leaving a mixture of E and E⁻ → I. Because the biphase inactivation equation assumes that the alkylated enzyme (E⁻ → I or E⁻ → I) has no activity, the observed data points exceed the values of the calculated points due to the partial isomerase activity of enzyme alkylated in the 3β-HSD form (E⁻ → I).

The equation for biphase enzyme inactivation overestimates the observed rate of 3β-HSD inactivation. However, the data fits the equation for single-phase enzyme inactivation (Fig. 6A),

\[
E/E_0 = (1 - P)e^{-k_{obs}t} + P
\]

(Eq. 2)

where \(k_{obs}\) represents the value of \(k_{obs} = 0.693/ t_{1/2}\) that was measured for 3β-HSD inactivation at each 8-BDB-TADP concentration (determined from Fig. 1A). The variable P represents the fractional residual 3β-HSD activity when an inactivation plateau is reached. Because 8-BDB-TADP decomposes relatively slowly (\(t_{1/2} = 56\) min) compared to the rates of inactivation measured for 3β-HSD, reagent decomposition causes the observed data points to exceed the predicted values only at the lower 8-BDB-TADP concentrations (67 and 100 \(\mu\)M) after 20 min of inactivation.

The need to switch from a two-phase to single-phase equation to fit the data obtained for the inactivation of 3β-HSD supports the concept that enzyme alkylated in the isomerase conformation has full activity in the 3β-HSD assay. Because a greater proportion of enzyme molecules are in the isomerase conformation as the 8-BDB-TADP concentration increases (Table 1), higher concentrations of 8-BDB-TADP inactivate 3β-HSD more slowly than lower concentrations to yield the "reverse" kinetics of 3β-HSD inactivation.

Our hypothesis that NADH activates isomerase by inducing a conformational change in the enzyme protein is indirectly supported by this affinity labeling study: 8-BDB-TADP binds at the NADH site, activates isomerase, and produces the reverse kinetics of 3β-HSD inactivation. Moreover, direct evidence for the NADH-induced conformational change has been obtained by the time dependence of both the activation of isomerase (Fig. 3) and the quenching of intrinsic protein fluorescence by NADH (Fig. 4). The fact that the time frame for the fluorescence change (\(t_{1/2} = 85\) s) is longer than for the activation of isomerase by NADH (\(t_{1/2} = 20\) s) suggests that a point is reached during the conformational change where the isomerase substrate is brought into proper juxtaposition with the amino acid residues that catalyze the reaction. Once that threshold is reached, isomerization proceeds at the maximal rate.

The stoichiometry of 1 mol of NADH bound/mol of enzyme dimer can be interpreted in two ways: 1) NADH induces the isomerase conformation in just one of the two subunits or 2) both subunits form a single NADH site when the enzyme is in the isomerase form. Whether one or both subunits participate in the isomerase activity will require studies of tertiary and quaternary structure by nuclear magnetic resonance spectroscopy or x-ray crystallography.

The inactivation data obtained with the NADH site-directed affinity alkylation, 8-BDB-TADP, complement the direct measurements of the NADH-induced activation of isomerase to validate our proposed mechanism for the sequential 3β-HSD/isomerase activity. As the 3β-HSD activity oxidizes the 3β-hydroxy-5-ene steroid (pregnenolone or dehydroepiandrosterone) to the 3-oxo-5-ene intermediate, NAD⁺ is reduced to form NADH. This NADH induces a conformational change in the enzyme protein that activates isomerase to produce the 3-oxo-4-ene steroid (progesterone or androstenedione). After the product steroid and NADH dissociate, the enzyme converts back to the dehydrogenase form and can again catalyze the reaction sequence. Understanding how the enzyme shifts from the first to the second reaction in the sequence will help us evaluate the relationship between the individual reaction mechanisms for 3β-HSD and isomerase, which are currently being studied in our laboratory.

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