Evidence that enzyme-generated aromatic Michael acceptors covalently modify the nucleotide-binding site of 3 α -hydroxysteroid dehydrogenase

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The non-steroidal allylic and acetylenic alcohols 1-(4'-nitrophenyl)prop-2-en-1-ol (I) and 1-(4'-nitrophenyl)prop-2-yn-1-ol (II) are oxidized by homogeneous 3 α -hydroxysteroid dehydrogenase to the corresponding $\alpha\beta$ -unsaturated ketones 1-(4'-nitrophenyl)prop-2-en-1-one (III) and 1-(4'-nitrophenyl)prop-2-yn-1-one (IV), which then inactivate the enzyme selectively with high affinity; low effective partition ratios are observed for the parent alcohols [Ricigliano & Penning (1989) *Biochem. J.* **262**, 139–149]. Inactivation of 3 α -hydroxysteroid dehydrogenase by compound (I) displays an NAD⁺ concentration optimum. Scavenging experiments indicate that the enzyme-generated inactivators (III) and (IV) alkylate the enzyme via a release-and-return mechanism. Several lines of evidence suggest that compounds (III) and (IV) covalently modify the NAD(P)⁺-binding site. First, micromolar concentrations of NAD(P)H offer substantial protection against enzyme inactivation mediated by Michael acceptors (III) and (IV). In these protection studies K_a measurements for NAD(P)H approached those measured by fluorescence titration of free enzyme. Secondly, under initial-velocity conditions compounds (III) and (IV) act essentially as competitive inhibitors of NAD⁺ binding, and as mixed competitive or non-competitive inhibitors against androsterone binding. Thirdly, enzyme inactivated with either compound (III) or compound (IV) fails to bind to NAD⁺ affinity columns (e.g. Affi-gel Blue). Under the same conditions of chromatography native enzyme and enzyme affinity-labelled at the steroid-binding site with 17 β -bromoacetoxy-5 α -dihydrotestosterone is retained on the affinity column. A kinetic scheme that represents the inactivation of the homogeneous dehydrogenase by the enzyme-generated alkylators (III) and (IV) is presented.

INTRODUCTION

In a previous paper (Ricigliano & Penning, 1989) the concept that non-steroidal substrates of hydroxysteroid dehydrogenases (HSDs) could effectively be used to develop mechanism-based inactivators of this group of enzymes was presented. Some of the inherent advantages of non-steroids include the ready availability of starting materials, the diversity of electronic and molecular properties and, frequently, the ease with which reactive groups can be introduced into the molecule. In the earlier study latent Michael acceptors based on a single aromatic ring, 1-(4'-nitrophenyl)prop-2-en-1-ol (I) and 1-(4'-nitrophenyl)prop-2-yn-1-ol (II), were oxidized by homogeneous 3 α -HSD of male rat liver cytosol to yield the corresponding $\alpha\beta$ -unsaturated ketones, 1-(4'-nitrophenyl)prop-2-en-1-one (III) and 1-(4'-nitrophenyl)prop-2-yn-1-one (IV). The oxidation products acted as enzyme-generated inactivators, producing irreversible inactivation, and the parent alcohols gave low effective partition coefficients. In addition, the chemically prepared Michael acceptors (III) and (IV) were shown to inactivate 3 α -HSD rapidly in a stoichiometric fashion.

The present paper provides evidence that the enzyme-generated inactivators (III) and (IV) are released from the site of oxidation, and ultimately bind and alkylate the nicotinamide nucleotide-binding domain of 3 α -HSD.

MATERIALS AND METHODS

Nucleotides were purchased from Pharmacia-LKB Bio-

technology Products (Piscataway, NJ, U.S.A.). Unlabelled steroids were purchased from Steraloids (Wilton, NH, U.S.A.). [4-¹⁴C]Testosterone (57.0 mCi/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Affi-gel Blue was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

The inactivators 1-(4'-nitrophenyl)prop-2-en-1-one and 1-(4'-nitrophenyl)prop-2-yn-1-one were synthesized as described previously (Ricigliano & Penning, 1989). Fresh solutions of inactivators and nucleotides were prepared daily and their concentrations determined spectrophotometrically. 17 β -Bromo[¹⁴C]acetoxy-5 α -dihydrotestosterone (specific radioactivity 6.6 $\times$ 10³ c.p.m./nmol) was synthesized by the method of Penning *et al.* (1987) from bromo[1-¹⁴C]acetic acid.

Homogeneous 3 α -HSD from male Sprague-Dawley rat liver was prepared and stored by the method of Penning *et al.* (1984). Portions (0.4 ml) of the enzyme (4.86 mg/ml) were kept frozen at -80 °C and thawed as needed. Before use enzyme was dialysed overnight against three changes of 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, to remove glycerol and 2-mercaptoethanol.

The standard spectrophotometric assay for determining 3 α -HSD activity was conducted at 25 °C in a 1 ml system containing 75 μ M-androsterone and 2.3 mM-NAD⁺ in 100 mM-potassium phosphate buffer, pH 7.0, with 4 % (v/v) acetonitrile as a co-solvent. The formation of reduced nicotinamide nucleotide was monitored at 340 nm, by using a molar absorption coefficient of 6270 M⁻¹·cm⁻¹.

Abbreviations used: E, 3 α -HSD, 3 α -hydroxysteroid:NAD(P)⁺ oxidoreductase (EC 1.1.1.50) or 3 α -hydroxysteroid dehydrogenase; HSD, hydroxysteroid dehydrogenase; allylic alcohol, 1-(4'-nitrophenyl)prop-2-en-1-ol (I); acetylenic alcohol, 1-(4'-nitrophenyl)prop-2-yn-1-ol (II); enone or vinyl ketone, 1-(4'-nitrophenyl)prop-2-en-1-one (III); acetylenic ketone, 1-(4'-nitrophenyl)prop-2-yn-1-one (IV); Br-DHT, 17 β -bromoacetoxy-5 α -dihydrotestosterone; androsterone, 3 α -hydroxy-5 α -androstane-17-one.

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RESULTS AND DISCUSSION

Inactivation of 3 α -HSD by the allylic alcohol (I) in the presence of various concentrations of NAD⁺

Inactivation of 3 α -HSD by the allylic and acetylenic alcohols has an obligatory requirement for NAD⁺. Previous studies published by us indicate that in the presence of 1.2 mM-NAD⁺ the allylic alcohol can promote 50 % loss of enzyme activity in 10 s at saturation and that the acetylenic alcohol can promote 50 % loss of enzyme activity in 9.9 min at saturation (Ricigliano & Penning, 1989). [In the earlier study Fig. 2(a) should have a time scale in minutes and Fig. 2(b) in hours, and not both in hours as originally published.] By studying the rate of enzyme inactivation in the presence of the allylic alcohol and various concentrations of NAD⁺, it was found that 0.5–1.0 mM-NAD⁺ was optimal for inactivation and that higher nicotinamide nucleotide concentrations significantly retarded the inactivation event (Fig. 1). This result was unexpected, because raising the NAD⁺ concentration should increase the rate of inactivator formation, and hence the rate of inactivation. Similar results are observed with the acetylenic alcohol.

Inactivation of 3 α -HSD by allylic alcohol (I) in the presence of a nucleophilic scavenger

Inactivation of 3 α -HSD by 470 μ M-allylic alcohol (I) plus NAD⁺ was conducted in the presence of increasing amounts of the scavenging nucleophile 2-mercaptoethanol (Fig. 2). 2-Mercaptoethanol provides a concentration-dependent protection, presumably owing to the ability of the nucleophile to scavenge the enzyme-generated Michael acceptor (III). Similar results were obtained with the acetylenic alcohol (II) (results not

Fig. 1. Pseudo-first-order rate constant for the inactivation of 3 α -HSD by the allylic alcohol (I) is dependent on the NAD⁺ concentration

The pseudo-first-order rate constants (k_{app}) for the inactivation of 3 α -HSD by the allylic alcohol in the presence of variable concentrations of NAD⁺ were determined as follows. A series of time-dependent inactivations of 3 α -HSD were conducted in 60 μ l portions of 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, with 5% (v/v) acetonitrile as a co-solvent, containing 4.8 μ M (0.16 mg of protein/ml) dialysed enzyme, various concentrations of NAD⁺ and 400 μ M-allylic alcohol. Samples were incubated at 25 °C, and portions were removed and diluted into the standard 1 ml androsterone assay. Dilution was never less than 100-fold. Estimates of the rate of enzyme inactivation were made by plotting log[activity (% of initial enzyme activity)] versus time for each concentration of NAD⁺, from which the pseudo-first-order rate constant was calculated. The Figure shows a plot of the pseudo-first-order rate constant for enzyme inactivation k_{app} , (min⁻¹) versus [NAD⁺].

Fig. 2. Time-dependent inactivation of 3 α -HSD by the allylic alcohol (I) in the presence of various concentrations of 2-mercaptoethanol

Time-dependent inactivations of 3 α -HSD were conducted in 30 μ l portions of 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, with 5% (v/v) acetonitrile as a co-solvent, containing 4.8 μ M (0.16 mg of protein/ml) dialysed enzyme, the indicated concentration of 2-mercaptoethanol and 470 μ M-allylic alcohol. Samples were incubated at 25 °C, and at the indicated times portions were removed and diluted into the standard 3 α -HSD assay to determine the amount of enzyme activity remaining. In all cases dilution was never less than 100-fold.

shown). These data suggest that the enzyme-generated Michael acceptors are forced to dissociate from the enzyme before inactivation, and that these compounds inactivate the enzyme by a release-and-return mechanism. With this hypothesis in mind it became crucial to determine the ultimate site of alkylation.

Inactivation of 3 α -HSD by the enone (III) in the presence of nicotinamide nucleotide

The inactivation of 3 α -HSD by the enone (III) can be retarded markedly in a concentration-dependent manner by both NADH and NADPH. A representative protection experiment conducted with enone (III) is shown in Fig. 3. Attempts to extract the nicotinamide nucleotide-binding constants from protection studies have met with limited success for two reasons. First, the curvilinear nature of the inactivation data makes it difficult to estimate k_{app} as the concentration of NAD(P)H is varied. Secondly, the rapid rate of enzyme inactivation by enone (III) in the absence of nicotinamide nucleotide makes accurate measurements of the limiting rate constant for enzyme inactivation (k_{inact}) difficult (Ricigliano & Penning, 1989). Despite these difficulties, transformation of the data gave K_d values for NADH which ranged from 11 to 46 μ M and K_d values that ranged from 90 to 132 nM for NADPH. The upper limits given for these measurements approach the K_d values determined fluorimetrically (Fig. 4 and Table 1). This data should be contrasted with the inability to observe protection against inactivation by the enone with ligands that are mutually exclusive with androsterone (prednisolone, indomethacin etc.). These findings imply

Fig. 3. Time-dependent inactivation of 3 α -HSD by the enone (III) in the presence of NADH

Inactivation of 3 α -HSD (4.76 μ M) by the enone (III) was conducted in 30 μ l portions of 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, with 4% (v/v) acetonitrile as a co-solvent, containing the indicated concentration of NADH. After the initial activity was determined, inactivator was added, and the sample was incubated at 25 $^{\circ}$ C. At the indicated times, portions were withdrawn and diluted at least 100-fold into cuvettes containing all of the components of the standard 3 α -HSD assay except androsterone. To determine the amount of enzyme activity remaining, assays were initiated by the addition of androsterone. In all cases dilution was never less than 100-fold. No time points are shown for the inactivation of enzyme by the enone in the absence of NADH. The first point on this line is below the abscissa.

that the vinyl ketone and acetylenic ketone prefer to alkylate free 3 α -HSD (E) rather than the E-nucleotide complex. The ability of nucleotide to protect indicates either that the Michael acceptors bind 3 α -HSD in a competitive fashion with respect to nucleotide, or that an enzyme-nucleotide-Michael acceptor complex, if formed, does not yield inactivated enzyme at an appreciable rate.

Fig. 4. Fluorescence titration of 3 α -HSD by NADH

Samples of rat liver 3 α -HSD (500 μ l; 12.4 μ M) were titrated in a Perkin-Elmer 535 spectrofluorimeter by the incremental addition of NADH. The excitation spectra were recorded at the emission maximum (333 nm) and emission spectra were recorded at the excitation maximum (283 nm). An asymmetric cuvette having an excitation path of 2 mm and an emission path of 1 cm was employed for all measurements. The total volume of added nucleotide did not exceed 5% of the sample volume. The Δ fluorescence at 333 nm was used to calculate the amount of NADH bound, assuming a 1:1 stoichiometry of enzyme to nucleotide. The resulting data were subsequently plotted by the method of Scatchard (1949).

Table 1. Dissociation constants determined for the binding of reduced nicotinamide nucleotide to 3 α -HSD

The dissociation constants for the binding of NAD(P)H to free 3 α -HSD were determined either fluorimetrically as described in the legend to Fig. 4 or from protection studies in which NAD(P)H was used to retard the rate of enzyme inactivation mediated by the enone (III).

Nucleotide	Dissociation constant (K_d)	
	Fluorescence titration	Protection studies
NADH	158 μ M	11–46 μ M
NADPH	195 nM	90–132 nM

Initial-rate studies in the presence of various concentrations of the enone (III) and the acetylenic ketone (IV)

Initial-rate measurements of NADH production were conducted in the presence of enone or acetylenic ketone at several NAD $^{+}$ concentrations and at a fixed androsterone concentration (75 μ M). The data, shown as primary and secondary Dixon plots (Fig. 5), indicate both the acetylenic ketone and the enone are competitive inhibitors of NAD $^{+}$ binding, yielding K_d values of 7 μ M for the enone and 4 μ M for the acetylenic ketone. Alternatively, initial-rate measurements of NADH production were conducted in the presence of enone or acetylenic ketone at several androsterone concentrations and at a fixed concentration of NAD $^{+}$ (2.3 mM). These data indicate that both of the Michael acceptors are mixed competitive/non-competitive inhibitors of androsterone binding (Fig. 6). The curvature in these plots is presumably due to the difficulty of obtaining good initial-rate data in the presence of enzyme inactivation. Because of the curvature, it was necessary to take tangents to the curves as they approach the ordinate for the secondary-plot construction. These data imply that the enone and acetylenic ketone compete with NAD $^{+}$ for the nicotinamide nucleotide-binding domain and not for the steroid-binding site.

The ability to observe competitive behaviour between NAD $^{+}$ and the Michael acceptors suggests that E-NAD $^{+}$ does not form a ternary complex with the Michael acceptors. This rules out protection mechanisms in which the ternary complex forms but the enzyme nucleophile and Michael acceptor fail to react. It also implies that the nucleotide protection data in Fig. 3 do not result from formation of a ternary complex, and thus result from the competition of nucleotide and alkylator for free enzyme.

Accessibility of the nicotinamide nucleotide-binding site after alkylation of the enzyme with either Michael acceptor (III) or (IV) or the steroid affinity-labelling agent 17 β -bromoacetoxy-5 α -dihydrotestosterone (Br-DHT)

The preceding protection data and initial-rate studies strongly suggest that the Michael acceptors alkylate the nicotinamide nucleotide-binding site. To assess the availability of this site, titrations of the enzyme's tryptophan fluorescence by NAD(P)H were performed on native enzyme, enzyme inactivated with the enone (III) and enzyme inactivated with the steroid affinity alkylator Br-DHT (Penning *et al.*, 1987). Fluorescence titrations of native enzyme have already been described (Fig. 4 and Table 1). Fluorescence titrations of 3 α -HSD inactivated by either Michael acceptor (III) or (IV) were unfortunately futile, owing to the quenching effect of the nitrophenyl ring on the protein fluorescence signal. Thus fluorescence titrations could not be used to determine the accessibility of the nicotinamide nucleotide-

Fig. 5. Initial-rate studies with the vinyl ketone (III) or acetylenic ketone (IV) in the presence of various NAD^+ concentrations

Assays were initiated by the addition of dialysed enzyme to cuvettes containing 1 ml of 100 mM-potassium phosphate buffer, pH 7.0, containing $75 \mu\text{M}$ -androsterone, with 4% (v/v) acetonitrile as a co-solvent, the indicated concentrations of NAD^+ and either the indicated concentration of vinyl ketone (a) or the indicated concentration of acetylenic ketone (b). The rate of NADH formation was monitored at 340 nm. The initial-rate data shown were plotted by the method of Dixon (1953). Where it was difficult to determine the slope of the primary plots because of enzyme inactivation, linear regression of the points nearest the ordinate was used to estimate the slope. These slopes were then replotted in the secondary plots (c) and (d) as suggested by Segal (1975).

binding site. Fluorescence titrations of Br-DHT-inactivated 3α -HSD were possible, and yielded a K_d for NADH binding to the covalent enzyme-steroid complex that was experimentally identical with the K_d for NADH binding to free enzyme, K_d $137 \mu\text{M}$. Titration of the Br-DHT-inactivated enzyme confirmed that modification of the enzyme at the steroid-binding site does not preclude binding of nucleotide to its binding site, and it confirmed that Br-DHT could be employed as a control for agents that modify the steroid-binding site.

Affinity chromatography of 3α -HSD on Affi-gel Blue: evidence that the site of covalent modification is the nucleotide-binding domain

Affinity chromatography of 3α -HSD on the immobilized nucleotide analogue Cibacron Blue (Affi-gel Blue) was used instead of fluorescence titration as a test for occupancy of the nucleotide-binding site. Native enzyme adheres with high affinity to the immobilized dye (Fig. 7a), requiring a high salt concentration, 1.5 M-KCl, for removal. Like native enzyme, enzyme inactivated with the radiolabelled steroid affinity alkylator Br-[^{14}C]DHT also binds the dye matrix tightly, requiring 1.5 M-KCl for removal (Fig. 7b). By contrast, enzyme inactivated by the enone or the acetylenic ketone is eluted in the void volume, indicating it has no affinity for the dye matrix (Figs. 7c and 7d). The negative deflections observed in the chromatograms are artifacts of the high-salt buffers employed.

From the data obtained with the Br-[^{14}C]DHT-inactivated

enzyme it is clear that ligands that bind the steroid-binding site do not prevent binding to an immobilized nucleotide analogue. In direct contrast, 3α -HSD inactivated by either the enone or the acetylenic ketone no longer binds to the affinity medium. This study suggests that the Michael acceptors (III) and (IV) and nucleotide bind to the same site of the enzyme rather than to distinct sites.

Kinetic model of the inactivation of 3α -HSD by Michael acceptors (III) and (IV)

In order to construct a concise model of the interactions between 3α -HSD and the aromatic Michael acceptors (III) and (IV), fundamental information describing the kinetic mechanism had to be obtained. The kinetic mechanism has been determined in this laboratory. Initial-velocity data for the NAD^+ -dependent oxidation of androsterone and product-inhibition studies are consistent with an ordered mechanism in which nicotinamide nucleotide binds first and leaves last (L. J. Askonas, J. W. Ricigliano & T. M. Penning, unpublished work). Data from equilibrium binding studies (Fig. 8) also support an ordered mechanism, since 3α -HSD binds [^{14}C]testosterone, a known competitive inhibitor of androsterone binding, only in the presence of nicotinamide nucleotide. The mechanism may further be described as sequential rather than ping-pong, on the basis of the nature of NAD^+ -linked dehydrogenase mechanisms, excluding those with a prosthetic group such as a flavin (Dalziel, 1975). Scheme 1 describes the interaction of 3α -HSD with the

Scheme 1. Kinetic mechanism to explain the inactivation of 3 α -HSD by the enzyme-generated aromatic Michael acceptors

In this Scheme E represents 3 α -HSD, Ei represents inactivated enzyme, St-OH represents a 3 α -hydroxysteroid; St=O represents a 3-oxosteroid, Ar-OH represents a latent Michael acceptor, Ar=O represents a Michael acceptor, and NAD⁺ and NADH represent oxidized and reduced nicotinamide nucleotide respectively.

aromatic Michael acceptors (III) and (IV) in the presence or in the absence of the substrates, NAD⁺ and androsterone, on the basis of an ordered sequential mechanism.

The upper cycle of Scheme 1 depicts the oxidation of a 3 α -hydroxysteroid by 3 α -HSD in the presence of NAD⁺ in an ordered sequential sequence. The alkylator (Ar=O) binds preferentially to free enzyme, rather than to E-NAD⁺ or the ternary E-nucleotide-steroid complex (on the basis of protection and

competitive inhibition studies). The scheme predicts that in the initial-rate studies Ar=O is competitive with NAD⁺ and non-competitive/mixed competitive with steroid, as is observed experimentally.

Initially, more complicated kinetic patterns were expected because the Michael acceptors should bind to the steroid-binding site where they are produced during the enzymic oxidation of their latent precursors, as well as to the nucleotide-binding site.

Fig. 6. Initial-rate studies with the vinyl ketone (III) or acetylenic ketone (IV) in the presence of various androsterone concentrations

Assays were initiated by the addition of dialysed enzyme to cuvettes containing 1 ml of 100 mM-potassium phosphate buffer, pH 7.0, containing 2.3 mM-NAD⁺, with 4% (v/v) acetonitrile as a co-solvent, the indicated concentrations of androsterone and either the indicated concentration of vinyl ketone (a) or the indicated concentration of acetylenic ketone (b). The rate of NADH formation was monitored at 340 nm. The initial-rate data shown were plotted by the method of Dixon (1953). The slopes of the lines in the primary plots were replotted in the secondary plots (c) and (d).

Fig. 7. Affinity chromatography of native and inactivated 3α -HSD on the immobilized nucleotide analogue Cibacron Blue

Four 120 μ l samples of 3α -HSD (1.0 mg/ml) in 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, with 4% (v/v) acetonitrile as a co-solvent, were treated with no additions (a), 80 μ M-Br-[14 C] DHT (b), 80 μ M-vinyl ketone (c) or 80 μ M-acetylenic ketone (d). After 20 min of incubation at 25 $^{\circ}$ C the samples containing enzyme inactivator had lost more than 90% of their activity. The samples were then applied to Affigel Blue columns equilibrated with 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-2-mercaptoethanol. Following sample application, the column was washed with elution buffer containing 1.5 M-KCl to elute 3α -HSD, and then subsequently washed with elution buffer containing 3.0 M-KCl. Fractions (0.63 ml) were assayed for activity in the standard 3α -HSD assay. The A_{280} (—) is shown for the chromatograms (a–d). The percentage of initial enzyme activity recovered (● · ●) is shown for native enzyme in chromatogram (a); no other samples had detectable enzyme activity. The elution of 14 C radioactivity is shown for chromatogram (b) (Δ – Δ).

Fig. 8. Equilibrium binding of testosterone to 3α -HSD in the presence of NADH

Portions (100 μ l) of 3α -HSD (13 μ M) were subjected to equilibrium dialysis against 0.5 ml of 1 mM-EDTA/10 mM-potassium phosphate buffer, pH 7.0, with 6% (v/v) acetonitrile as a co-solvent, containing 1.4 mM-NADH and increasing concentrations of [14 C]testosterone (71 500 c.p.m./nmol) (0–75 μ M). At 0, 5, 6, 8 and 24 h, 10 μ l samples were withdrawn from both the sample and diffusate and were analysed by scintillation counting. As shown in the inset, the samples had reached equilibrium with the diffusate by 8 h. The resulting data were plotted by the method of Scatchard (1949).

Apparently, the affinity of the active Michael acceptors for the nucleotide-binding site is substantially higher than their affinity for the steroid-binding site in the presence of NAD $^{+}$, effectively simplifying the kinetics.

If the latent Michael acceptors (I) and (II) (Ar–OH) are substituted for steroidal substrate in the upper cycle of Scheme 1, then the released product will be an active Michael acceptor (Ar=O). Under these conditions Scheme 1 correctly predicts a nucleotide concentration optimum for 3α -HSD inactivation by the latent Michael acceptors. Two opposing processes are involved in the NAD $^{+}$ concentration optimum. One process, the oxidation of the latent Michael acceptors, requires NAD $^{+}$; the second, alkylation of free 3α -HSD by the active Michael acceptors, is inhibited by nucleotides. Because both of these processes occur simultaneously during the inactivation of 3α -HSD by the latent Michael acceptors, and have opposing NAD $^{+}$ concentration maxima, an optimum concentration for NAD $^{+}$ results.

Many of our findings concerning the inactivation of 3α -HSD by the aromatic Michael acceptors are accounted for in the essential features of Scheme 1 as follows. (a) The mechanism of 3α -HSD is described as ordered and sequential. (b) Experimental initial-rate measurements indicate that the Michael acceptors are non-competitive/mixed competitive with steroid, and competitive with nucleotide. (c) Nucleotide protects 3α -HSD from time-dependent inactivation by the alkylators. (d). Inactivation of 3α -HSD by the allylic and acetylenic alcohols should display

an optimum for NAD^+ concentration. (e) Because the active alkylators are released into solution before they can recombine and inactivate enzyme, 2-mercaptoethanol provides protection of 3α -HSD from inactivation by the latent Michael acceptors in the presence of NAD^+ .

The conclusion that inactivation of 3α -HSD by the active Michael acceptor occurs by alkylation of the nucleotide-binding site is supported by nucleotide protection data, the competitive binding kinetics for the Michael acceptors versus NAD^+ and affinity-chromatography studies of native and inactivated 3α -HSD. The observation of an optimum NAD^+ concentration for inactivation of 3α -HSD by the latent Michael acceptors is also consistent with the alkylation site being in the nucleotide-binding domain.

It is of importance to note that the inactivation of 3α -HSD by the active Michael acceptors (III) and (IV) occurs by alkylation of free enzyme. This pathway appears to be off the principal ordered kinetic path of the enzymic reaction, unless the active Michael acceptors are considered to be nucleotide analogues. Like the aromatic Michael acceptors, Br-DHT also appears to inactivate 3α -HSD through the formation of an enzyme-steroid complex that comprises a side shunt off the principal kinetic path. The same phenomena may also be observed for other HSDs interacting with mechanism-based and/or affinity alkylators. For example, $3\alpha,20\beta$ -HSD from *Streptomyces hydrogenans* (Betz & Warren, 1968), and rat ovarian 20α -HSD (Pongsawasdi & Anderson, 1984) are believed to catalyse steroid transformation by ordered kinetic mechanisms with nucleotide binding first, yet both enzymes are inactivated by steroid affinity alkylators in the absence of added nucleotide (Ganguly & Warren, 1971; Sweet *et al.*, 1972; Arias *et al.*, 1973; Strickler *et al.*, 1975; Covey *et al.*, 1986; Ricigliano & Penning, 1986).

Two possibilities are suggested by these observations. First, complexes of enzyme and inactivator, other than those for which analogous substrate complexes have been described, may be of importance in the inactivation of HSDs with ordered mechanisms. These reversible complexes may arise either from alterations in the order of inactivator binding and release relative to the order of substrate binding and release, or through dead-end shunts. Such reversible complexes may be transient in nature, and in some instances evidence of their existence comes only from the detection of the resultant inactivated enzyme. This implies that the rate of covalent bond formation is of the same order of magnitude or exceeds the dissociation rate of the reversible enzyme-inactivator complex. Secondly, each of these enzymes may actually have a minor random component to their

mechanism, although the kinetically significant route for the conversion of steroids occurs by an ordered mechanism with nucleotide binding first. Although $3\alpha,20\beta$ -HSD has a principally ordered mechanism (Betz & Warren, 1968), it does form a small amount of E-steroid binary complex that goes on to produce product (Betz & Taylor, 1970).

Auchus & Covey (1986) have asserted that only those dehydrogenases that form kinetically significant concentrations of the binary E-inactivator complex are susceptible to true suicide inactivation. Since dehydrogenases with ordered mechanisms bind nucleotide first and release it last, their assertion carries with it the implication that enzymes that act by an ordered mechanism are refractory to steroid-based suicide inactivators. By contrast, dehydrogenases that catalyse substrate turnover by a random mechanism may be better candidates for steroid-based suicide inactivation. Our studies and those of others suggest that knowledge *a priori* of a target enzyme's kinetic mechanism may not reliably predict the success of a suicide substrate or mechanism-based inactivator.

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