The kinetic mechanism catalysed by homogeneous rat liver 3 α -hydroxysteroid dehydrogenase

Evidence for binary and ternary dead-end complexes containing non-steroidal anti-inflammatory drugs

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Rat liver 3 α -hydroxysteroid dehydrogenase (3 α -HSD) (EC 1.1.1.50) is an NAD(P)⁺-dependent oxidoreductase that is potentially inhibited at its active site by non-steroidal anti-inflammatory drugs (NSAIDs). Initial-velocity and product-inhibition studies performed in either direction at pH 7.0 are consistent with a sequential ordered Bi Bi mechanism in which pyridine nucleotide binds first and leaves last. This mechanism is supported by fluorescence titrations of the E·NADH complex, and by the failure to detect the binding of either [³H]androsterone or [³H]androstenedione to free enzyme by equilibrium dialysis. Dead-end inhibition studies with NSAIDs also support this mechanism. Initial-velocity studies with indomethacin show that this drug is an uncompetitive inhibitor against NAD⁺, but a potent competitive inhibitor against androsterone, indicating the ordered formation of an E·NAD⁺·indomethacin complex. Calculation of the individual rate constants reveals that the binding and release of pyridine nucleotide is rate-limiting and that isomerization of the central complex is favoured in the forward direction. Equilibrium dialysis experiments with [¹⁴C]indomethacin reveal the presence of two abortive NSAID complexes, a high-affinity ternary complex corresponding to E·NAD⁺·indomethacin ($K_d = 1\text{--}2\ \mu\text{M}$ for indomethacin) and a low-affinity binary complex corresponding to E·indomethacin ($K_d = 22\ \mu\text{M}$ for indomethacin). Since indomethacin has a low affinity for free enzyme, the formation of this abortive binary complex does not complicate kinetic measurements which are made in the presence of NAD⁺, but may contribute to the inhibition of the enzyme by NSAIDs. Using either *pro-R*-[4-³H]NADH or *pro-S*-[4-³H]NADH as cofactor, radiolabelled androsterone was formed only when the *pro-R*-[4-³H]NADH was used, confirming that purified 3 α -HSD is a Class A dehydrogenase.

INTRODUCTION

Hydroxysteroid dehydrogenases are a family of NAD(P)⁺-linked oxidoreductases that interconvert alcohols and ketones on the steroid nucleus and side chain in a positional and stereoselective manner (Talalay, 1963). The mammalian enzymes are believed to play pivotal roles in the regulation of steroid hormone action (Taurog *et al.*, 1975; Jacobi *et al.*, 1977; Funder *et al.*, 1988), and 3 α -hydroxysteroid dehydrogenase (3 α -HSD) belongs to this group. 3 α -HSD has been shown to convert the potent androgen (5 α -dihydrotestosterone) into the weak androgen (3 α -androstenediol) (Scheme 1) (Dorfman & Dorfman, 1963; Liao *et al.*, 1973) and as a result may terminate androgen action *in vivo*.

3 α -HSD from rat liver cytosol represents one of the more thoroughly characterized mammalian hydroxysteroid de-

hydrogenases (Penning *et al.*, 1986). The enzyme can be purified in milligram quantities (Penning *et al.*, 1984), and its abundance has led to the assignment of additional functions to the rat liver enzyme. 3 α -HSD displays dual pyridine nucleotide specificity (NAD⁺ and NADP⁺) and accounts for the major dihydrodiol dehydrogenase and aromatic ketone reductase activities present in rat liver cytosol (Penning *et al.*, 1984). In addition, it displays 9-, 11- and 15-hydroxyprostaglandin dehydrogenase activity (Penning & Sharp, 1987). The enzyme also has the novel property of being potentially inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) in rank order of their pharmacological potencies (Penning & Talalay, 1983), and evidence has accumulated to suggest that 3 α -HSD may be an additional target for these drugs (Penning *et al.*, 1986). The enzyme has been crystallized for X-ray studies, and its cDNA has been cloned and sequenced (Isaacson *et al.*, 1988; Pawlowski *et al.*, 1991a,b). The deduced amino acid sequence shows 69 % sequence identity with bovine lung prostaglandin F synthase (Watanabe *et al.*, 1988; Pawlowski *et al.*, 1991b) and further points to a role for the enzyme in the transformation of inflammatory prostanoids.

In general, hydroxysteroid and hydroxyprostaglandin dehydrogenases are pyridine-nucleotide-linked enzymes which can display either ordered or random Bi Bi sequential kinetic mechanisms. Sequentially ordered Bi Bi mechanisms in which nucleotide binds first and leaves last have been described for the

Scheme 1. Reaction catalysed by 3 α -HSD

Abbreviations used: 3 α -HSD, 3 α -hydroxysteroid dehydrogenase [3 α -hydroxysteroid:NAD(P)⁺ oxidoreductase (EC 1.1.1.50)]; androsterone, 5 α -androstan-3 α -ol-17-one; androstenedione, 5 α -androstan-3,17-dione; testosterone, 17 β -hydroxyandrost-4-en-3-one; indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid; NSAIDs, non-steroidal anti-inflammatory drugs; vinyl ketone, 1-(4-nitrophenyl)-2-propen-1-one.

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Pseudomonas testosteroni dehydrogenases displaying 3 α -hydroxysteroid (Skålhegg, 1975) and 3(17) β -hydroxysteroid (Levy *et al.*, 1987) activities. In addition, rat ovarian 20 α -hydroxysteroid dehydrogenase (Pongsawasdi & Anderson, 1984) and mouse liver 3 α -HSD (Hara *et al.*, 1988) have been shown to display ordered Bi Bi mechanisms. By contrast, human placental 17 β -hydroxysteroid dehydrogenase has been shown to follow a random Bi Bi sequential mechanism (Betz, 1971). Kinetic studies on purified human placental NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase are consistent with an ordered Bi Bi mechanism with NAD⁺ binding first (see Hansen, 1976, for a review).

Studies from this laboratory have implied that rat liver 3 α -HSD may follow a random Bi Bi kinetic mechanism. It can be inactivated by a series of bromoacetyl-steroids (Penning *et al.*, 1987, 1991) and bromoacetamido-NSAID analogues (Askonas *et al.*, 1988, 1990) in the absence of NAD⁺. In addition, both NAD⁺ and indomethacin (a potent NSAID) protect the enzyme against inactivation by these affinity-labelling agents. However, analysis of these protection data gave estimates of the K_d for indomethacin which were inconsistent with that determined by Dixon-plot analysis for this drug. Furthermore, the K_d values deduced for bromoacetamido-NSAIDs from inactivation experiments were also inconsistent with those obtained under initial-velocity conditions (Askonas *et al.*, 1988). These discrepancies could be explained by the formation of two different complexes, i.e. low-affinity binary complexes between free enzyme and the NSAIDs or their bromoacetamido analogues, and high-affinity ternary complexes between E·NAD⁺ and NSAIDs or their bromoacetamido analogues. These findings could be rationalized by a random mechanism in which the alpha and beta terms are significant, or by an ordered mechanism in which NSAIDs bind with low affinity to form abortive binary complexes with free enzyme.

Since 3 α -HSD satisfies many of the criteria expected of a target enzyme for NSAIDs, it is important to document the kinetic mechanism for this enzyme and identify all the complexes through which NSAIDs may exert their inhibitory effects. Results presented here suggest that the kinetic mechanism of 3 α -HSD is predominantly ordered, with pyridine nucleotide binding first and leaving last. Dead-end ternary complexes, equivalent to E·nucleotide·NSAID, are observed which can suppress the reaction in the forward or reverse direction. The formation of low-affinity E·NSAID and E·bromoacetamido-NSAID complexes is also substantiated; however, these binary complexes are dead-end complexes for which no productive analogous complex forms during the natural course of the enzyme reaction. This information has been integrated into a model of inhibition of 3 α -HSD by NSAIDs and may provide additional insights into the mechanism of action of these drugs.

EXPERIMENTAL

Materials

[9,11-³H]Androsterone (60 Ci/mmol) and [2-¹⁴C]indomethacin (37.7 mCi/mmol) were purchased from du Pont/NEN (Boston, MA, U.S.A.). [4-¹⁴C]Testosterone (57.0 mCi/mmol) and [4-³H]NAD⁺ (3.0 Ci/mmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle) and yeast alcohol dehydrogenase were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Grade I pyridine nucleotides were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Unlabelled steroids were purchased from Steraloids (Wilton, NH, U.S.A.). 1-(4-Nitrophenyl)-2-propen-1-one (vinyl ketone) was synthesized as previously described (Ricigliano & Penning, 1989).

Preparation and assay of enzyme

3 α -HSD was purified to homogeneity as previously described (Penning *et al.*, 1984). The final specific activity of the enzyme used in these kinetic studies was 1.93 μ mol of androsterone oxidized/min per mg under standard assay conditions [75 μ M-androsterone, 2.3 mM-NAD⁺, 100 mM-potassium phosphate buffer, pH 7.0, and 4% acetonitrile (v/v) in a 1.0 ml system at 25 °C]. The reaction was started by the addition of enzyme, and the rate of formation of NADH (ϵ 6270 M⁻¹·cm⁻¹) monitored at 340 nm over 5 min using a Gilford 260 u.v.-visible recording spectrophotometer.

Analysis of kinetic data

Enzyme-kinetic data from initial-velocity, product-inhibition and dead-end-inhibition studies were fitted to the appropriate rate equation with the FORTRAN computer programs described by Cleland (1977, 1979). Initial-velocity data were analysed for sequential (eqn. 1 below) and rapid equilibrium ordered (eqn. 2 below) mechanisms by the SEQUEN and EQORD programs respectively. The product and dead-end inhibition data were fit to eqns. 3–5:

$$v = \frac{V_m AB}{K_A^i K_B + K_A B + K_B A + AB} \quad (1)$$

$$v = \frac{V_m AB}{K_A^i K_B + K_B A + AB} \quad (2)$$

$$v = \frac{V_m A}{K_A \left(1 + \frac{I}{K_i^s}\right) + A} \quad (3)$$

$$v = \frac{V_m A}{K_A + A \left(1 + \frac{I}{K_i^i}\right)} \quad (4)$$

$$v = \frac{V_m A}{K_A \left(1 + \frac{I}{K_i^s}\right) + A \left(1 + \frac{I}{K_i^i}\right)} \quad (5)$$

which correspond to competitive (COMP program), uncompetitive (UNCOMP program) and linear non-competitive (NONCOMP program) inhibition respectively. Patterns shown in the Results section are those judged to be the best fit according to the criteria of Cleland (1979). The kinetic constants are defined as follows: K_A and K_B are the Michaelis-Menten constants for NAD⁺ (A) and androsterone (B) respectively, K_A^i is the dissociation constant for NAD⁺, K_B^i is the dissociation constant for the inhibitor (I) derived from the slope of the double-reciprocal plot, K_i^i is the corresponding constant from the intercept, and V_m is the maximum velocity.

Synthesis of [³H]androstanedione

[³H]Androsterone was converted enzymically into [³H]-androstanedione as follows. The system contained 2.82 ml of 100 mM-sodium pyrophosphate buffer (pH 10.0), 2.3 mM-NAD⁺, 75 μ M-[³H]androsterone (100 μ Ci) and 4% acetonitrile (v/v). Reactions were initiated by the addition of 7.9 μ g of purified 3 α -HSD and were allowed to run to completion. At the end of each reaction, each system was extracted with 2 $\times$ 1.5 ml of ethyl acetate, and the extracts were pooled and dried *in vacuo*. The specific radioactivity of the [³H]androstanedione was confirmed by using it as a substrate in the reverse reaction. Final specific radioactivity was estimated to be 2.22 $\times$ 10⁵ c.p.m./nmol. The radiochemical purity of [³H]androstanedione was confirmed by t.l.c. in benzene/ethyl acetate (4:1, v/v) (R_f 0.625).

Equilibrium-dialysis studies

3 α -HSD, stored in a glycerol-containing buffer (Penning *et al.*, 1984), was dialysed against 20 mM-potassium phosphate buffer (pH 7.0)/1 mM-EDTA/1 mM-2-mercaptoethanol/5 % acetonitrile (v/v) at 4 °C. The activity of 3 α -HSD was re-determined after dialysis, and the enzyme was diluted appropriately with dialysis buffer. Solutions of the radioligands were prepared daily in the above buffer. Aliquots of 3 α -HSD (100 μ l) were subjected to equilibrium dialysis against 1.0 ml of dialysis buffer in the presence of increasing concentrations of radioligand at 25 °C in the presence or absence of saturating concentrations of nucleotides. At appropriate time intervals, 10 μ l samples were removed from the sample and diffusate and subjected to liquid-scintillation counting of radioactivity. At equilibrium, triplicate measurements were taken for liquid-scintillation counting, and the enzyme concentration was re-determined. Equilibrium-dialysis experiments with indomethacin were conducted in an EMD 101B 1/10 Equilibrium Microvolume Dialyzer apparatus (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

Synthesis of *pro-R*- and *pro-S*-[4-³H]NADH

Stereospecifically labelled *pro-R*-[4-³H]NADH and *pro-S*-[4-³H]NADH were synthesized enzymically from [4-³H]NAD⁺ by standard methods. *pro-R*-[4-³H]NADH was synthesized from [4-³H]NAD⁺ with glyceraldehyde-3-phosphate dehydrogenase (Type-B stereochemistry) (Loewus *et al.*, 1956), and *pro-S*-[4-³H]NADH was synthesized utilizing yeast alcohol dehydrogenase (Type-A stereochemistry) (Loewus *et al.*, 1953). The specific radioactivities were determined spectrophotometrically and found to be 1.01×10^4 c.p.m./nmol for the *pro-R*-[4-³H]NADH and 6.88×10^3 c.p.m./nmol for the *pro-S*-[4-³H]NADH.

RESULTS AND DISCUSSION

Initial-velocity studies

Initial-velocity measurements were determined for the forward and reverse directions by varying the respective concentrations of nucleotide and steroid and monitoring the reaction at pH 7.0. The data were then analysed by the SEQUEN and EQORD programs described by Cleland (1979). In all cases the data fitted best to the SEQUEN analysis. Plots of $1/v$ versus $1/[\text{androsterone}]$ (Fig. 1a) and replots of the same data as $1/v$ versus $1/[\text{NAD}^+]$ give a family of lines intersecting beneath the x-axis. By contrast, plots of $1/v$ versus $1/[\text{androstanedione}]$ (Fig. 1b) and replots of the same data as $1/v$ versus $1/[\text{NADH}]$ give a family of lines intersecting above the x-axis. These plots are consistent with a sequential mechanism for 3 α -HSD. The kinetic constants for the forward and reverse reactions calculated by the SEQUEN program from eqn. (1) are shown in Table 1. The reaction velocity is seen to be 3.3 times faster in the forward direction. However, the K_m values for substrates in the reverse direction are considerably lower than those observed for the corresponding substrates in the forward direction, i.e. the K_m^{NADH} is 33 μ M versus $K_m^{\text{NAD}^+}$ of 760 μ M, and the $K_m^{\text{androstanedione}}$ is 1.7 μ M versus the $K_m^{\text{androsterone}}$ of 47 μ M.

Product-inhibition studies

Product-inhibition studies with androstanedione and NADH (Table 2a) were conducted in order to establish the exact order of substrate binding and hence derive the kinetic mechanism. A complete product-inhibition study was attempted; however, under the experimental conditions employed, it was not possible to generate a complete data set, owing to the limited solubility of androsterone. Despite these limitations, the data sets for all product-inhibition studies were fitted to the COMP, NONCOMP and UNCOMP programs. In some cases, the σ value (a measure

Fig. 1. Initial-velocity patterns

(a) The initial velocity of androsterone oxidation was measured under conditions in which the androsterone concentration was kept constant and the NAD⁺ concentration was varied. The concentrations of NAD⁺ were: $\blacktriangle$, 2.47 mM; $\square$, 1.23 mM; $\bullet$, 0.697 mM; $\triangle$, 0.496 mM; $\blacksquare$, 0.386 mM; $\circ$, 0.316 mM. (b) The initial velocity of androstanedione reduction was measured under conditions in which the androstanedione concentration was kept constant and the NADH concentration was varied. The concentrations of NADH were: $\blacktriangle$, 169 μ M; $\square$, 84.0 μ M; $\bullet$, 56.0 μ M; $\triangle$, 43.7 μ M; $\blacksquare$, 34.8 μ M; $\circ$, 28.8 μ M. Each assay was initiated by the addition of 3 α -HSD (1.53 μ g) and each reaction was performed in 100 mM-potassium phosphate buffer, pH 7.0. The experimental points shown are the means of triplicate determinations and the lines shown are those fitted to eqn. (1) by the SEQUEN program as described in the text.

Table 1. Kinetic constants and standard errors for the reaction catalysed by 3 α -HSD

Values were determined from initial-velocity measurements as a fit to eqn. (1) by the SEQUEN program. Abbreviations used: K_m , Michaelis constants for the respective substrates; K_i , inhibition (dissociation) constants; $V_{\text{max}}^{\text{f}}$, [maximum velocity for the forward direction (oxidation of androsterone by NAD⁺)] was 3.012 ± 0.186 μ mol/min per mg. $V_{\text{max}}^{\text{r}}$, [maximum velocity for the reverse direction (reduction of androstanedione by NADH)] was 0.894 ± 0.056 μ mol/min per mg.

Substrate	K_m (μ M)	K_i (μ M)
Androsterone	47.4 ± 4.9	$17.8 \pm 3.7^*$
NAD ⁺	759.9 ± 97.2	285.1 ± 51.7
Androstanedione	1.74 ± 0.75	$10.4 \pm 2.9^*$
NADH	32.6 ± 6.3	$193.9 \pm 98.5^\dagger$

* K_i values for androsterone and androstanedione were calculated from the equation $K_i^{\text{A}} K_B = K_A K_i^{\text{B}}$, assuming a random mechanism. Results show that a random mechanism does not apply.

† The value has been determined to be 160 μ M by fluorescence titration (see the text).

Table 2. Product- and dead-end-inhibition patterns

The kinetic patterns were determined as described in the text. The patterns were designated as follows: MT, mixed type (non-competitive); C, competitive; UC, uncompetitive, —, no inhibition; ND, not determined against the variable substrate; RE, rapid equilibrium.

Inhibition	Kinetic pattern	Inhibitor†	Variable A (NAD ⁺)*		Variable B (androsterone)*	
			Unsaturated B	Saturated B	Unsaturated A	Saturated A
(a) Product	Observed‡	P	MT	ND	C	C
		Q	C	ND	C	—
	Ordered Bi Bi	P	MT	UC	MT	MT
		Q	C	C	MT	—
	Random Bi Bi	P	MT	MT	MT	MT
		Q	MT	MT	MT	MT
	Theorell–Chance	P	MT	—	C	C
		Q	C	C	MT	—
(b) Dead-end	Observed	P	MT	—	C	C
		Q	C	—	C	—
	Bi Bi Dead-end EAP	Q	C	—	C	—
		—	—	—	—	—
	Observed	Indo.	UC	—	—	C
		V.K.	C	—	—	MT
	Ordered Bi Bi	Indo.	UC	—	—	C
		V.K.	C	—	—	MT
	Random Bi Bi	Indo.	MT	—	—	C
		V.K.	C	—	—	MT

* The substrates were NAD⁺ (A) and androsterone (B).

† Inhibitors used were androstenedione (P), NADH (Q), indomethacin (Indo.) and 1-(4-nitrophenyl)-2-propen-1-one (vinyl ketone) (V.K.).

‡ Four concentrations of each unsaturating substrate were used.

of the fit) obtained with the NONCOMP program was lower than the σ value obtained with the COMP program. However, when the NONCOMP fit gave errors for the K_1^s term in excess of 50 % of the estimated value, it was concluded that this term was insignificant in the rate equation. In these cases it was decided that the competitive fit was the more appropriate (Cleland, 1979). The observed patterns of product inhibition are compared with the predicted patterns for several kinetic mechanisms (Segel, 1975) in Table 2(a). It should be noted that the closest fit of the product-inhibition patterns is to a rapid equilibrium random Bi Bi mechanism with a dead-end EAP complex.

Dead-end inhibition studies

Dead-end inhibition studies were also conducted to elucidate the kinetic mechanism. Two different classes of inhibitors were chosen, NSAIDs (which are known competitive inhibitors against androsterone) (Penning *et al.*, 1984) and the aromatic vinyl ketone [1-(4-nitrophenyl)-2-propen-1-one], which competes for, and alkylates, the NAD⁺-binding site of 3 α -HSD (Ricigliano & Penning, 1990). As tabulated in Table 2(b), indomethacin displays competitive inhibition against androsterone ($K_1^s = 0.74 \mu\text{M}$) (Fig. 2a) and uncompetitive inhibition against NAD⁺ ($K_1^s = 0.57 \mu\text{M}$) (Fig. 2b). In the reverse direction, indomethacin exhibits the same inhibition patterns. Thus, under initial-velocity conditions, the enzyme forms E·NAD(H)·indomethacin complexes. By contrast, the vinyl ketone displays apparent competitive kinetics against NAD⁺ and mixed-type kinetics versus androsterone (Ricigliano & Penning, 1990). In this case, an E·(vinyl ketone) complex is formed. The results of these dead-end inhibition studies are compared with the predicted patterns of inhibition for enzymes displaying either random or ordered kinetic mechanisms in Table 2(b). These results clearly indicate that 3 α -HSD follows an ordered Bi Bi mechanism, with NAD⁺ binding first.

Fig. 2. Dead-end-inhibition studies

(a) The kinetic patterns for the inhibition of 3 α -HSD by indomethacin at a saturating concentration of NAD⁺ (2.3 mM) when androsterone is varied at pH 7.0. The lines represent the data fitted to eqn. (3) by the COMP program as described in the text. The concentrations of indomethacin were: $\blacklozenge$, 0 μM ; $\times$, 1.1 μM ; $\blacksquare$, 2.2 μM ; $\circ$, 3.3 μM ; $\blacktriangle$, 4.4 μM . (b) The kinetic patterns for the inhibition of 3 α -HSD by indomethacin at a constant concentration of androsterone (75 μM) when NAD⁺ is varied. The lines represent the data fitted to eqn. (4) by the UNCOMP program as described in the text. The concentrations of indomethacin were: $\blacklozenge$, 0 μM ; $\times$, 1.62 μM ; $\blacksquare$, 3.24 μM ; $\circ$, 4.86 μM ; $\blacktriangle$, 6.18 μM .

Measurement of binary and ternary complexes on the normal kinetic path

The preceding sections clearly show that product-inhibition and dead-end-inhibition studies give inconsistent results with regard to the identity of the kinetic mechanism. The product-inhibition studies suggest a random Bi Bi mechanism with a dead-end EAP complex, whereas the dead-end-inhibition studies support an ordered Bi Bi mechanism. To distinguish between these alternatives, attempts were made to measure the binary complexes predicted by each mechanism. It is possible to measure the E·NADH binary complex by fluorescence titration. The K_d values obtained for the E·NADH and E·NADPH complexes by this means have been previously reported and are 160 μM and 195 nm respectively (Ricigliano & Penning, 1990). The value for the E·NADH complex confirms the value predicted by initial-velocity studies (Table 1). In addition, attempts were made to measure the formation of E·[^3H]androsterone and E·[^3H]androstanedione complexes under equilibrium-dialysis conditions. No formation of either binary complex (E·steroid) could be detected at the limit of steroid solubility (75 μM for androsterone and 50 μM for androstanedione). The inability to form an E·steroid complex at concentrations of steroid used for the initial-velocity studies effectively rules out a random Bi Bi mechanism.

Attempts were also made to measure dead-end ternary complexes that might contribute to the kinetic mechanism. However, no binding of [^3H]androsterone to E·NADH and no binding of [^3H]androstanedione to E·NAD $^+$ could be detected under conditions of equilibrium dialysis. Thus, EAP and EBQ dead-end ternary complexes do not participate in the kinetic mechanism of 3 α -HSD. These observations further negate interpreting the product-inhibition studies as supporting a rapid equilibrium random Bi Bi mechanism with the formation of a dead-end EAP complex.

Analysis of the individual rate constants for an ordered Bi Bi mechanism

The aforementioned studies support a sequential ordered Bi Bi kinetic mechanism for rat liver 3 α -HSD, with nicotinamide nucleotide binding first and leaving last. By using the values obtained from the SEQUEN program in Table 1, it was possible to calculate the individual rate constants for the mechanism (Table 3) (Segel, 1975). These rate constants predict that the enzymic reaction is freely reversible at pH 7.0, that the binding and release of nicotinamide nucleotide is rate-limiting and that isomerization of the central complex is favoured in the forward direction.

Table 3. Individual rate constants for the reaction catalysed by 3 α -HSD

Values for the individual rate constants were obtained from the kinetic constants (Table 1) (Segel, 1975). The corresponding location of each step is shown in Scheme 2.

Forward direction (androsterone oxidation)	Value	Reverse direction (androstanedione reduction)	Value
k_{+1}	$1.346 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$	k_{-4}	$9.313 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$
k_{+2}	$3.488 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$	k_{-3}	$4.575 \times 10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$
k_{+p}	102.3 min^{-1}	k_{-p}	30.36 min^{-1}
k_{+3}	235.8 min^{-1}	k_{-2}	145.4 min^{-1}
k_{+4}	180.6 min^{-1}	k_{-1}	38.37 min^{-1}

The product-inhibition patterns described in Table 2(a) can be justified by an ordered mechanism when the values of the individual rate constants are considered. At a constant concentration of androsterone, androstanedione is a mixed inhibitor against NAD $^+$ (no EAP), and NADH is a competitive inhibitor of NAD $^+$, as predicted by a simple ordered Bi Bi mechanism (Table 2a). By contrast, the product-inhibition patterns observed at constant NAD $^+$ concentrations are inconsistent with those predicted for a simple ordered mechanism. When androstanedione is varied against androsterone, competitive patterns are observed at both unsaturating and saturating concentrations of NAD $^+$. An ordered Bi Bi mechanism would predict mixed patterns, but a Theorell–Chance mechanism would predict competitive product-inhibition patterns. This would suggest that, as in a Theorell–Chance mechanism, the rate-limiting step in the kinetic mechanism of 3 α -HSD may be the release of NADH (Q), a suggestion substantiated by the individual rate constants (Table 3). They reveal that steroid is released very quickly from the central complex, while the release of pyridine nucleotide may well be a rate-limiting step. These findings support the product inhibition patterns observed. Advocacy of a Theorell–Chance mechanism, however, is ruled out because the criteria $K_m^A = K_i^A$, $K_m^Q = K_i^Q$, $k_{-2} \gg k_{-1}$, and $k_3 \gg k_4$ (Segel, 1975) do not hold (Tables 1 and 3). These interpretations of the product-inhibition patterns still fail to describe why competitive inhibition patterns are observed when NADH is varied against androsterone at unsaturating fixed concentrations of NAD $^+$. This can be explained by the individual rate constants which show that NADH ($k_{-4} = 9.31 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$) binds seven times faster than NAD $^+$ ($k_{+1} = 1.35 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$) to free enzyme at pH 7.0. Thus NADH will compete with NAD $^+$ until NAD $^+$ is saturating. The enzyme is then predominantly in the E·A form and no inhibition by NADH is expected, and this is what is observed.

Measurement of dead-end NSAID complexes

Dead-end inhibition patterns observed with indomethacin support the ordered Bi Bi mechanism and predict the formation of E·NAD(P)(H)·indomethacin complexes. By using [^{14}C]indomethacin as radioligand, the ternary complexes E·NAD $^+$ ·[^{14}C]indomethacin and E·NADH·[^{14}C]indomethacin were measured directly. Scatchard (1949) analysis of the binding of indomethacin to the E·NAD $^+$ complex gave a K_d of 2.02 μM and a B_{max} corresponding to 0.7 binding sites per enzyme monomer (results not shown).

During the course of the equilibrium-dialysis studies, a binary E·[^{14}C]indomethacin complex was also demonstrated. The K_d for indomethacin obtained for this binary complex was 21.4 μM , with a B_{max} corresponding to 0.9 sites per enzyme monomer (results not shown). This K_d value is 10–30-fold higher than the value obtained for the E·NAD $^+$ ·indomethacin complex. The formation of this E·indomethacin complex by a side shunt does not contribute significantly to the inhibition patterns observed, since inhibition studies are routinely performed in the presence of NAD $^+$ concentrations which are 8-fold above its K_d . Under these conditions, the enzyme is effectively trapped in its high-affinity state for indomethacin, namely E·NAD $^+$. However, if NAD $^+$ concentrations were to fall significantly below its K_d (288 μM), the formation of E·indomethacin may contribute to the inhibition observed with this drug and other NSAIDs.

The formation of dead-end binary complexes is also supported by affinity-labelling studies with bromoacetoxy-steroids (Penning *et al.*, 1987, 1991) and bromoacetamido-NSAID analogues (Askonas *et al.*, 1988, 1990). In each case, these active-site directed agents are capable of inactivating free enzyme, yielding K_i values for the resulting E·I complex significantly higher than

this implies that binary E-steroid complexes bind nucleotide (as predicted by a random mechanism), the E'-steroid complex formed by covalent modification represents a complex for which no analogous kinetically significant complex occurs.

Our model also predicts the kinetic patterns observed with the active-site-directed bromoacetoxy-steroids and bromoacetamido-NSAID analogues (Scheme 2). These compounds will bind to free enzyme with low affinity and can cause enzyme inactivation. Inactivation can be retarded by both NAD⁺ and ligands that compete for the steroid-binding site, e.g. indomethacin. These data imply that formation of the binary complexes E·NAD⁺ or E·indomethacin deplete the amount of free enzyme available for inactivation. The concentrations of NAD⁺ required to obtain protection against inactivation are at or near the K_d value for the nicotinamide nucleotide, consistent with an ordered mechanism. By contrast, the concentration of indomethacin required to obtain protection is much higher than the K_d observed for the E·NAD⁺·indomethacin complex, and indicates that indomethacin and the bromoacetylating agents compete for the same low-affinity form of free enzyme. In addition, initial-velocity studies with these affinity-labelling agents (in the presence of saturating NAD⁺) indicate that these agents are competitive inhibitors with androsterone, uncompetitive inhibitors with NAD⁺, and yield much lower K_i values for the E·NAD⁺ complex than for E alone (Penning *et al.*, 1987, 1991). These data can be summarized by simply stating that the E·NAD⁺ complex has a much higher affinity for ligands that occupy the steroid-binding site than free enzyme.

The kinetic mechanism predicted in this study for 3 α -HSD offers important insights into the nature of inhibition mediated by NSAIDs. Our findings suggest that NSAIDs, as well as acting as competitive inhibitors with androsterone, can effectively slow the forward and reverse reactions by forming dead-end ternary complexes equivalent to E·nucleotide·NSAID. In the absence of nucleotide, NSAIDs can inhibit the enzyme by forming non-productive binary complexes. This mode of inhibition is in contrast with that reported for the inhibition of 15-hydroxyprostaglandin dehydrogenase from bovine lung, in which indomethacin and aspirin were found to be non-competitive inhibitors with prostaglandin E₂. Since aspirin has been reported to compete for the NAD⁺-binding site for several dehydrogenases (Einarsson *et al.*, 1974), it was inferred that NSAIDs inhibit 15-hydroxyprostaglandin dehydrogenase by binding at this site. This is not the case for 3 α -HSD. Although 3 α -HSD has significant sequence identity with prostaglandin F synthase (Watanabe *et al.*, 1988; Pawlowski *et al.*, 1991b), which is a member of the aldehyde reductase family (Hayashi *et al.*, 1989), NSAIDs do not potently inhibit prostaglandin F synthase or the aldehyde reductases. These studies indicate that 3 α -HSD represents a novel target for NSAID inhibition and that this inhibition is mediated by the formation of binary and ternary dead-end drug complexes.

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