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Inhibition of Rat Liver Steroid 5α-Reductase by 3-Androstene-3-carboxylic Acids: Mechanism of Enzyme–Inhibitor Interaction

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ABSTRACT: The interactions of a series of newly discovered inhibitors of Δ^4-3-oxo-steroid 5α-reductase (SR; EC 1.3.1.30), the 3-androstene-3-carboxylic acids (steroidal acrylates), have been studied by using a solubilized rat liver enzyme preparation. As exemplified by one member of this series, 17β-[*N*,*N*-diisopropylcarbamoyl)androst-3,5-diene-3-carboxylic acid (**1a**), the dead-end inhibition patterns of selected compounds in this class are best evaluated by a linear uncompetitive kinetic model versus either substrate, testosterone (T) or NADPH. These results were interpreted within the context of the preferentially ordered kinetic mechanism for rat liver SR to arise from the association of inhibitor to the binary complex of enzyme and $NADP^+$. This proposed inhibition mechanism was supported by data from double-inhibition experiments implicating the synergistic binding of steroidal acrylate and $NADP^+$ to SR. Further evidence for the preferential formation of this ternary complex was obtained from filtration binding assays with [^{3}H]-**1a**, where radioligand association to protein was greatly enhanced in the presence of $NADP^+$. The amount of [^{3}H]-**1a** binding to protein was proportional to the specific activity of SR in the enzyme preparations, and the estimated dissociation constant from binding data by Scatchard analysis (K_d = 25 nM) was comparable to the inhibition constants estimated for SR activity (K_i = 12–26 nM). From the pH profile for inhibition of the solubilized liver SR with **1a**, it is proposed that the anion of the steroidal acrylate (pK_1 = 4.7 ± 0.2) is the active inhibitory species, coordinating to a protonated active site functionality (pK_2 = 7.5 ± 0.1). On the basis of data from similar experiments with structural analogues of **1a**, the determinants for binding recognition and inhibitory potency are compared to structural features of the putative enzyme-bound intermediate states. These compounds represent a potential therapeutic alternative in the treatment of 5α-dihydrotestosterone specific androgen dependent disease states.

Since the publication of papers describing the phenotypic characteristics of humans deficient in 5α-dihydrotestosterone (DHT)[1] (Imperato-McGinley et al., 1974; Walsh et al., 1974) ample evidence has accumulated supporting the proposal that this product of testosterone (T) metabolism is the principal androgen for the trophic growth and support of the prostate (Imperato-McGinley et al., 1979) and function of the sebacious gland (Sansone & Reisner, 1971). In addition, unusually high levels of DHT have been correlated with diseases such as benign prostatic hypertrophy (BPH) (Geller et al., 1976; Wilkin et al., 1980), acne (Sansone & Reisner, 1971), male pattern baldness (Bingham & Shaw, 1973), and female hirsutism (Kuttenn et al., 1977). Furthermore, recent results with pharmacological models have provided support for the hypothesis of DHT as a potent, tissue-specific androgen (Brooks et al., 1986; Blohm et al., 1986; Rittmaster et al., 1987). From this proposal of androgen action has evolved the concept that administration of specific antagonists of DHT could pose as effective therapy for these metabolic disorders. One approach toward this goal would be through the blockade of DHT biosynthesis. Here, the most attractive target is Δ^4-3-oxo-steroid 5α-reductase [steroid 5α-reductase (SR); EC 1.3.1.30], the NADPH-dependent enzyme that converts T into DHT.

Several classes of SR inhibitors with varying potency have been described. Those compounds that have been best characterized both in vitro and in DHT responsive model systems include the 6-methylene derivatives of progesterone and testosterone (Kadohama et al., 1983; Petrow et al., 1983) and the 3-keto-4-diazo-5α-dihydro steroids (Blohm et al.,

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[1] Abbreviations: T, testosterone; DHT, 5α-dihydrotestosterone; SR, Δ^4-3-oxo-steroid 5α-reductase (EC 1.3.1.30); MK-906, 17β-[*N*-(2-methyl-2-propyl)carbamoyl]-4-aza-5α-androst-1-en-3-one; **1a**, 17β-(*N*,*N*-diisopropylcarbamoyl)androst-3,5-diene-3-carboxylic acid; **2a**, 17β-[*N*-(2-methyl-2-propyl)carbamoyl]androst-3,5-diene-3-carboxylic acid; dpm, disintegrations per minute.

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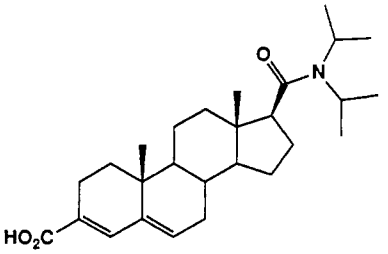
1986), both putative irreversible enzyme inhibitors, and the 3-oxo-4-aza steroids (Liang et al., 1985), a class of reversible SR inhibitors.

Our own foray to discover novel modulators of DHT biosynthesis which could be of therapeutic utility has resulted in the identification of 17β-substituted 3-androstene-3-carboxylic acids, such as 17β-(*N*,*N*-diisopropylcarbamoyl)androst-3,5-diene-3-carboxylic acid (**1a**), as potent inhibitors of human



1a

(Metcalf et al., 1989) and rat (Levy et al., 1989) prostatic SR. Preliminary steady-state inhibition experiments with the prostatic enzyme activities have suggested that inhibition of SR activity by these compounds results from the formation of a ternary complex consisting of enzyme, $NADP^+$, and inhibitor. Unfortunately, further mechanistic studies with enzyme from these sources were precluded by the low levels of SR activity in the prostatic microsomal preparations. Consequently, rat liver SR was chosen as a model system with which to better elucidate the interactions between steroidal acrylate and enzyme. Toward this end, a study of the kinetic mechanism and pH characteristics of solubilized rat liver SR has been detailed (Levy et al., 1990); the conclusions described therein are used in the interpretation of results presented in this paper.

Expanding on this previous work, we now describe the results of experiments designed to elucidate the characteristics of interaction between the solubilized rat liver SR and the steroidal acrylates. As previously proposed, the preferential association of enzyme and inhibitor within a ternary complex containing $NADP^+$ is supported with data derived from steady-state inhibition and filtration binding analyses. In addition, by use of pH profiles of SR inhibition by **1a**, the ionization state of an enzyme-bound steroidal acrylate has been elucidated. Discussion of these results is presented in the context of both the kinetic and chemical mechanisms of SR catalysis.

Experimental Procedures

Materials

[4-^{14}C]Testosterone (55–57 mCi/mmol) and sodium boro[^{3}H]hydride (75 Ci/mmol) were purchased from Amersham Corp. and New England Nuclear (NEN). Econosolve II and Aquasol 2 were obtained from NEN. Sephadex G-25 (medium) was purchased from Pharmacia. Other chemicals and enzymes were purchased from Sigma Chemical Co. or Aldrich Chemicals. Protein concentrations were estimated by the method of Bradford (1976) using the Bio-Rad protein dye assay with bovine serum albumin as the protein standard. Radioactivity was determined with either a Beckman LS-5801 scintillation counter calibrated to disintegrations per minute (dpm) with Beckman standards or with a System 2000 Bioscan imaging scanner (Bioscan, Washington, DC) Single-wavelength UV/vis spectral changes were monitored on a Gilford 260 spectrophotometer. Multiple-wavelength spectra were recorded with a Hewlett-Packard 8450A spectrophotometer with a diode array detector. Analyses of enzyme reactions were performed on prechanneled silica TLC plates containing a preabsorbing region (Si250F-PA, Baker).

Methods

Filtration Binding Assays with [^{3}*H*]-**1a**. All filtration binding assays were conducted with solubilized rat liver SR preparations at 0–4 °C. For these experiments, all steroids and [^{3}H]-**1a** in ethanol were deposited in test tubes, and the solvent was removed to dryness in a Savant rotary evaporator. To each tube was added cold incubation buffer (20 mM sodium phosphate, pH 7.5) containing 200 μM NADPH or $NADP^+$ and an aliquot of solubilized SR (12.5 μg of protein). The mixtures were incubated on ice to allow the establishment of equilibrium between protein and ligand. An aliquot of the incubation was transferred to a prewashed (20 mM sodium phosphate, pH 7.5) Whatman glass fiber filter (GF/F; pore size 0.7 μm) within a Hoefer FH240 filtration unit. The solution passed through the filter within 2 s under a vacuum of 200–300 mmHg provided by a vacuum pump. The filter was rinsed with three times 6 mL of cold incubation buffer and transferred to a vial containing 15 mL of Aquasol 2. The vials were shaken for 1 h to make the filters transparent, and the radiochemical contents were determined in a Beckman LS-5801. With this procedure, virtually all protein was retained by the filter; no protein could be detected in filtrates using the Bradford protein assay. Concentrations of [^{3}H]-**1a** and solubilized protein were chosen such that in the presence of $NADP^+$ 2–10% of the radioligand was retained on the filter. In most of the experiments, identical assays omitting $NADP^+$ were performed for comparison. In the absence of protein, the amount of radiolabeled ligand captured was less than 1% of that in the final incubation.

Gel Filtration Analysis of the Binding of Steroidal Acrylate **1a** *to Solubilized Rat Liver SR.* Chromatography on a column (1.35 × 49 cm) of Sephadex G-25 (medium gel size) was used to evaluate the reversibility of SR inhibition by steroidal acrylates. The column was equilibrated with 20 mM sodium phosphate buffer containing 20% glycerol (v/v) at pH 7.5; all column elutions used this equilibration buffer. As indicated in the individual experiments, 200 μM $NADP^+$ was included in the equilibration/elution buffer. As a general procedure, an aliquot of the solubilized rat liver preparation (125 μg of protein) was incubated at 0–4 °C for 10 min in the presence of 25 nM [^{3}H]-**1a** in a total volume of 1 mL of buffer corresponding to the elution conditions. An aliquot (0.9 mL) of the incubation mixture was removed and applied to the Sephadex G-25 column. Column fractions (1.4 mL) were evaluated for protein concentration, SR activity, and radioactive content (0.2 mL of eluate in 15 mL of Aquasol 2). Identical procedures omitting the inhibitor were run as controls for relative protein and activity recovery. In a second set of experiments, unlabeled **1a** or **2a** (5 nM) was preincubated with enzyme for 10 min followed by the addition of [^{3}H]-**1a** at final concentration of 25 nM. After a second 10-min incubation, the mixture was evaluated as described above.

Data Analysis. The assignment of inhibition patterns was made by best fit of experimental data to linear inhibition models (Levy et al., 1990) using the FORTRAN programs described by Cleland (1979). Data for pH profiles that decreased with a slope of 1 and −1 at high and low pH were fitted by the BELL program. Double-inhibition experiments at constant substrate concentrations were analyzed by eq 1 (Yonetoni

$$v_i = v_0/[1 + I/K_I + J/K_J + IJ/(\beta K_I K_J)] \quad (1)$$

& Theorell, 1964; Northrop & Cleland, 1974). For this equation, I and J are the concentrations of the two variable

Table I: Comparison of Inhibition Potency of Steroidal Acrylates between Prostatic and Hepatic Steroid 5α-Reductase Activities

compound	double bonds	R	other functionality	$K_{i,app}$[a] (nM) rat prostatic[b] (microsomal)	rat liver[c,d] (microsomal)	rat liver[c] (solubilized)	human prostatic[b] (microsomal)
1a	$\Delta^{3(4),5(6)}$	CO_2H		30	21	24	9
1b	$\Delta^{1(2),3(4)}$	CO_2H	5α-H	65	37	30	62
1c	$\Delta^{3(4)}$	CO_2H	5α-H	70	45	30	30
1d	$\Delta^{2(3)}$	CO_2H	5α-H	110	ND	30	85
1e	$\Delta^{2(3),4(5)}$	CO_2H		120	50	35	52
1f	$\Delta^{3(4),5(6)}$	CO_2H	10β-H	140	ND	105	50
1g	$\Delta^{3(4),5(10)}$	CO_2H	19-nor methyl	290	ND	140	110
1h		CO_2H (3β)	3α-H, 5α-H	340	80	76	220
1i	$\Delta^{4(5)}$	CO_2H (3β)	3α-H	2000	850	690	200
1j	$\Delta^{3(4),5(6)}$	$CONH_2$		4100	5500	7700	>5000
2a	$\Delta^{3(4),5(6)}$	CO_2H		23	11	5	32
2b	$\Delta^{3(4),5(6)}$	CHO		4200	4500	2000	4100
2c	$\Delta^{3(4),5(6)}$	CH_2OH		5300	4700	4500	4200

[a] The apparent inhibition constants ($K_{i,app}$) were determined as described under Methods by Dixon analyses (Dixon, 1953) at constant concentrations of substrates. Inhibitor concentrations were varied from 5 to 10 000 nM. [b] Substrate concentrations were 1.2 μM T and 400 μM NADPH with rat and human prostatic SR. Assays with human microsomes were conducted in 50 mM sodium citrate, pH 5.5; with rat prostatic SR, the incubation buffer was 20 mM sodium phosphate, pH 6.6. All incubations with the prostatic enzymes were at 37 °C. [c] With the hepatic enzymes, substrate concentrations were 1 μM T and 200 μM NADPH. Incubations were conducted at 30 °C in 20 mM sodium phosphate buffer, pH 7.5. [d] Inhibition not determined (ND).

inhibitors, v_0 and v_i are the velocities in the absence and presence of compounds I and J whose apparent dissociation constants are K_I and K_J, respectively, and β is an experimentally derived term that represents the degree of binding cooperativity between the two inhibitors. The direct binding of radiolabeled inhibitor was evaluated by Scatchard analysis (Segel, 1975) according to

$$L_b/L_f = (1/K_d)L_b + N/K_d \quad (2)$$

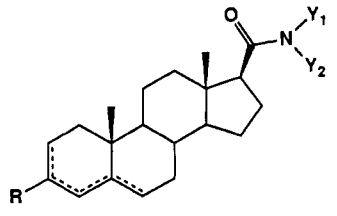
where L_b and L_f represent the concentration of bound and free radioligand respectively, N is the total concentration of ligand binding sites, and K_d is the dissociation constant of the ligand from the complex. The dissociation constant for a competing nonradioactive ligand, K_c, in direct filtration-binding experiments was calculated from

$$K_c = CD_{50}/(1 + L/K_d) \quad (3)$$

where L is the concentration of the radiolabeled reference compound with dissociation constant K_d as defined for eq 2 and CD_{50} represents the concentration of competing ligand required to displace 50% of the radiolabeled reference. Curve fitting of experimental data to eqs 1 and 2 was accomplished with a nonlinear regression procedure using the Marquardt algorithm (Marquardt, 1963) in the SAS statistical analysis software package (SAS Institute, Inc., Cary, NC).

Results

Inhibition of Rat Liver SR by Steroidal Acrylates. All of the 3-carboxy steroids (**1a–h, 2a**) proved to be potent inhibitors



1: $Y_1 = Y_2 \equiv -CH(CH_3)_2$

2: $Y_1 = -C(CH_3)_3$, $Y_2 \equiv -H$

of the rat liver SR activities, characterized by inhibition constants below 1 μM. Despite differences in the absolute potency of these compounds with SR associated with rat prostatic microsomes, the rank orders of inhibition vis-à-vis hepatic enzyme, as presented in Table I, are virtually identical. With each enzyme source, the more potent inhibition was shown to be associated with analogues that are unsaturated at C-3, C-4, and/or C-5. For example, the two most potent inhibitors, **1a** and **2a**, each contain sp^2 hybridization from C-3 through C-6; compound **1c**, possessing sp^2 hybridization at C-3 and C-4 with a 5α-H ring junction, is less potent, while the fully saturated 3β-carboxy steroid, **1h**, inhibits SR activity only at relatively higher concentrations. In addition, the 3-formamide derivative (**1j**) of **1a** and the 3-formyl and 3-(hydroxymethyl) variants of **2a** (**2b** and **2c**, respectively) demonstrated reduced interactions with SR (Table I), indicating that the presence of an acidic functionality at C-3 is imperative for potent enzyme inhibition within this series of compounds.

The kinetic patterns obtained from dead-end inhibition studies with several of the 3-carboxy steroids are summarized in Table IIA. Compounds **1a**, **2a**, **1c**, and **1h** were shown to be best evaluated by the linear uncompetitive kinetic model upon variation of either substrate, T or NADPH, with the microsomal associated enzyme activity. The dead-end inhibition patterns of compounds **1a**, **1c**, and **2a** versus both T and NADPH with solubilized liver SR also were shown to be uncompetitive. The inhibition constants from these dead-end inhibition evaluations (Table II) were quite comparable to those first estimated by Dixon analysis (Dixon, 1953) (Table I). In contrast to the results from the steroidal 3-carboxylic acids, dead-end analysis versus T with the 3-(hydroxymethyl) steroid **2c** clearly demonstrated a slope effect; fit of the data to a linear competitive pattern (K_m = 0.9 ± 0.1 μM; K_{is} = 2.9 ± 0.2 μM) was better than that to the noncompetitive model (K_m = 1.1 ± 0.1 μM; K_{is} = 3.6 ± 0.5 μM; K_{ii} = 19 ± 9 μM) on the basis of the relatively large error associated with the K_{ii} value in the latter analysis. Slope effects similarly were observed with the amide analogue, **1j**, of acrylate **1a**; here, too, the data best conformed to a competitive pattern versus T. The pattern of **2c** versus NADPH was uncompetitive (K_{ii} = 2.6 ± 0.3 μM; K_m = 11 ± 1 μM). As a control, it was confirmed that the 4-aza steroid, 17β-*N*-(2-methyl-2-propyl)carbamoyl-4-aza-5α-androst-1-en-3-one (MK-906), is a competitive inhibitor versus T and uncompetitive against NADPH with both types of hepatic enzyme preparation (Levy et al., 1990).

Since dead-end kinetic patterns with the steroidal acrylates have been interpreted to originate from the preferential binding

Table II: Steady-State Kinetic Evaluations of Steroid 5α-Reductase Inhibitors with Rat Liver Enzyme

			(A) dead-end inhibition analyses: inhibition constants (nM) and patterns[c,e] for variable substrate[b]		(B) double-inhibition analyses[d,e]			
inhibitor (I)	enzyme[a]	pH	testosterone	NADPH	inhibitor (J)	K_I (nM)	K_J (μM)	β
1a	M	6.6	K_{ii} = 12 ± 1 (UC)	K_{ii} = 26 ± 2 (UC)	$NADP^+$	33 ± 2	100 ± 6	0.25 ± 0.04
1a	S	6.0	K_{ii} = 18 ± 2 (UC)	K_{ii} = 10 ± 1 (UC)	$NADP^+$	35 ± 3	97 ± 5	0.66 ± 0.14
1a	S	7.5	K_{ii} = 18 ± 1 (UC)	K_{ii} = 10 ± 2 (UC)	$NADP^+$	41 ± 2	89 ± 2	0.33 ± 0.07
1c	M	6.6	K_{ii} = 17 ± 1 (UC)	K_{ii} = 36 ± 2 (UC)	$NADP^+$	31 ± 5	86 ± 8	0.60 ± 0.20
1c	S	7.5	K_{ii} = 20 ± 2 (UC)	K_{ii} = 20 ± 3 (UC)	$NADP^+$	34 ± 3	40 ± 2	0.40 ± 0.10
1h	M	6.6	K_{ii} = 40 ± 5 (UC)	K_{ii} = 74 ± 5 (UC)	$NADP^+$	117 ± 7	145 ± 9	0.25 ± 0.04
1j	S	7.5	K_{is} = 1900 ± 320 (C)	ND	ND			
2a	M	6.6	K_{ii} = 6 ± 1 (UC)	K_{ii} = 15 ± 2 (UC)	$NADP^+$	12 ± 2	52 ± 4	0.45 ± 0.07
2a	S	6.5	K_{ii} = 4 ± 1 (UC)	K_{ii} = 8 ± 2 (UC)	$NADP^+$	6 ± 1	49 ± 2	0.44 ± 0.10
2a	S	7.5	K_{ii} = 10 ± 2 (UC)	K_{ii} = 17 ± 2 (UC)	$NADP^+$	23 ± 4	20 ± 3	0.37 ± 0.10
2c	S	7.5	K_{is} = 4600 ± 200 (C)	K_{ii} = 2600 ± 300 (UC)	ND			
MK-906	M	6.6	K_{is} = 5 ± 1 (C)	K_{ii} = 10 ± 2 (UC)	$NADP^+$	16 ± 2	129 ± 14	$>1 \times 10^4$
MK-906	S	7.5	K_{is} = 6 ± 1 (C)	K_{ii} = 13 ± 2 (UC)	**1a**	13 ± 1	0.035 ± 0.003	$>1 \times 10^4$

[a] Experiments were conducted with microsomal (M) or solubilized (S) rat liver SR. [b] As nonvariable substrate, the concentrations of NADPH and T were 200 and 1.0 μM, respectively; all assays were incubated at 30 °C in 20 mM sodium phosphate buffer. [c] The kinetic patterns were determined by best fit of data to the linear kinetic models as described by Cleland (1979); the indicated patterns are competitive (C), noncompetitive (NC), and uncompetitive (UC). In every experiment, the apparent Michaelis constants (K_a, K_b, or K_m) for the variable substrate were in excellent agreement with those from the other experiments and with their values as previously reported (Levy et al., 1990). [d] Double-inhibition experiments were conducted as described in the text and the data analyzed by eq 1. Substrate concentrations were held constant at 1 μM T and 25 μM NADPH; all assays were conducted at 30 °C. [e] Kinetic pattern was not determined (ND).

of steroidal acrylate to an enzyme–$NADP^+$ complex (see Discussion), double-inhibition experiments were undertaken to further investigate inhibitor–enzyme interaction. In several sets of experiments with both microsomal and solubilized hepatic SR, concentrations of $NADP^+$ and steroidal inhibitor were varied at constant levels of the substrates. Data from these experiments were evaluated by eq 1, an expression that is algebraically equivalent to that derived by Yonetani and Theorell (1964) describing two inhibitors that are competitive versus the same substrate. The β term in eq 1 represents the degree of cooperativity between the two inhibitors I and J: when $\beta < 1$, the binding of I and J is synergistic; if $1 < \beta < \infty$, binding of the two inhibitors demonstrates negative cooperativity; and as β approaches infinity (∞) the binding of the inhibitors I and J is mutually exclusive. For the case in which $\beta < \infty$, a plot of $1/v_i$ versus I (or J) at differing concentrations of the second inhibitor should yield a series of intersecting lines; as β approaches infinity, the curves of such a plot will become parallel. Data from double-inhibition experiments between $NADP^+$ and **1a**, **1c**, **1h**, and **2a** using the solubilized enzyme preparation gave intersecting linear curves; the computer-evaluated value for β between **2a** and $NADP^+$ at pH 7.5 was 0.37 ± 0.10, demonstrating a positive binding cooperativity between steroidal acrylate and $NADP^+$. Results with the other compounds, summarized in Table IIB, show that binding cooperativity was observed in each case; values for β were determined to be less than unity in every experiment. Thus, each of the steroidal 3-carboxylic acids binds to SR in a complex with $NADP^+$; the empirical values of $\beta < 1.0$ indicate that upon binding of one ligand binding of the second is enhanced. In contrast to the results with the carboxy steroid inhibitors, no binding cooperativity ($\beta > 10^4$) between MK-906 and either $NADP^+$ or **1a** was observed (Table IIB).

*Filtration Binding Assays with [^{3}H]-**1a** and Solubilized Hepatic SR.* Binding of the radiolabeled inhibitor **1a** to preparations of SR was used to further characterize the interactions of the steroidal acrylates between different forms of enzyme. Results from initial exploratory filtration binding experiments using rat prostatic and hepatic microsomes were inconsistent: while the binding of [^{3}H]-**1a** to liver microsomes was enhanced in the presence of $NADP^+$ and demonstrated saturation kinetics upon increasing levels of steroidal acrylate, there was poor correlation between bound radiolabel and protein concentration when the microsomal content was varied. These difficulties most likely arose from variable nonspecific lipid or protein absorption of [^{3}H]-**1a** which could not be dislodged by repeated washings of the filter. Lower levels of binding in the non $NADP^+$ dependent assays and the background were obtained if small quantities (<5%) of ethanol were included in the incubations and the washes; however, inclusion of the organic solvent was not compatible with the SR activity assays and was discontinued. Minimal binding was observed with the rat prostatic microsomal preparation; this lower association from that of the liver preparation presumably results from the 1000–3000-fold lower SR content found in prostatic tissue (Liang et al., 1983). Consequently, a solubilized preparation of rat liver SR activity was chosen for further binding experiments.

As shown in Figure 1A, binding of [^{3}H]-**1a** at pH 7.5 to a solubilized preparation of liver SR is significantly enhanced in the presence of 200 μM $NADP^+$. The radiolabel retained by protein upon filtration increases to a constant level when the concentrations of both [^{3}H]-**1a** and $NADP^+$ are raised (insert, Figure 1A); with both compounds, saturation of binding was observed. Since conditions have not been identified for the efficient solubilization of SR activity in the absence of NADPH or $NADP^+$, an unknown percentage of the radioligand seen to be associated to protein in the absence of the added cofactor also could be $NADP^+$ dependent. Binding is not promoted by comparable concentrations of NADPH, NAD^+, or NADH—further suggesting that $NADP^+$ is a positive effector for the association of **1a** to the protein. With the enzyme solubilized in the presence of NADPH, the extent of binding is dependent on the incubation time at 0–4 °C, where maximal binding is observed by 20 min. No dependence of binding with incubation time was seen when enzyme that had been solubilized in the presence of $NADP^+$ was used. Presumably, this time dependence of binding results from the requisite displacement of NADPH by $NADP^+$ prior to association with **1a**, in agreement with the observation that SR activity is better stabilized by the reduced cofactor (Levy et al., 1990). In another binding experiment demonstrating the cofactor preference, an NADPH generating system of glucose 6-phosphate and glucose-6-phosphate dehydrogenase

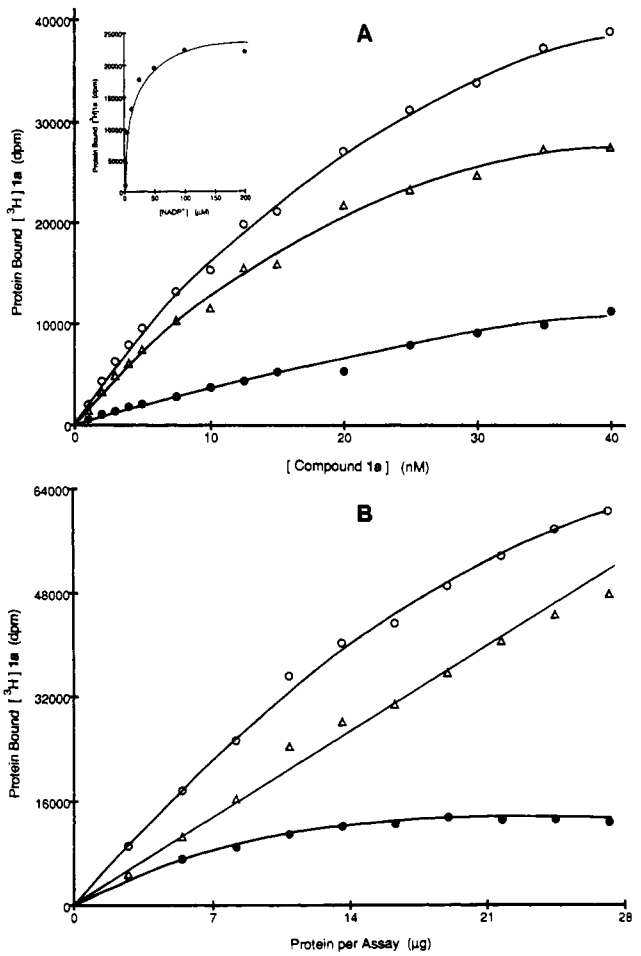


FIGURE 1: Filtration binding characteristics of [^{3}H]-**1a** to solubilized SR. The amount of [^{3}H]-**1a** associated with solubilized rat liver preparations of SR was determined by using the filtration binding assay as described in the text. The curves presented in (A) represent dependence of radioligand binding upon the concentration of **1a** in the absence (●) and the presence (○) of added 200 μM $NADP^+$. The third curve (△) represents the difference in binding between the assays plus and minus $NADP^+$. Depicted in the insert is the $NADP^+$-dependent binding saturation of **1a**. The curves in (B) represent association of inhibitor to protein in the presence (○) and absence (●) of $NADP^+$ along with the difference curve (△) representing the $NADP^+$-dependent binding component. For the experiments in (A), 12.5 μg of solubilized rat liver protein was employed. Incubations were conducted on ice for 20 min prior to filtration and washing.

was added to incubations orginally containing radioligand and $NADP^+$. Filtration binding analyses of these assays showed a decrease of protein-bound radioligand with time to control levels, corresponding to the glucose-6-phosphate dehydrogenase catalyzed conversion of $NADP^+$ to NADPH. Assessment of [^{3}H]-**1a** binding as a function of pH was not feasible since the background association of radioligand to the filter significantly increased below pH 6.0. Through extraction of the protein-bound radioactivity with ethyl acetate and analysis of the residue by TLC, >98% of the radiolabel was found to coelute with authentic **1a**. Thus, unmetabolized **1a** binds reversibly to the solubilized hepatic protein preparation.

Coincident addition of nonradioactive **1a** in the presence of $NADP^+$ reduced the amount of ^{3}H-labeled inhibitor associated with protein by filtration assay. In a competition experiment with 25 nM [^{3}H]-**1a**, only 25% of the radiolabel was bound to the protein in the presence of 92 nM unlabeled **1a** and less than 5% radioactivity was detected at concentrations in excess of 450 nM **1a**. Other steroidal acrylates similarly could compete for the inhibitor binding domain. With the addition of 10 and 100 nM **2a**, the binding of [^{3}H]-**1a** to the solubilized

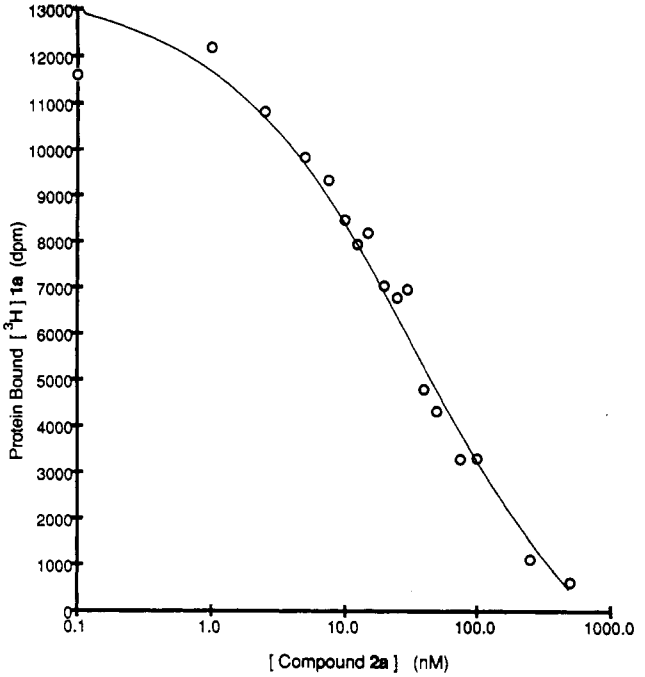


FIGURE 2: Displacement of protein-bound [^{3}H]-**1a** by **2c**. Enzyme (12.5 μg) and [^{3}H]-**1a** (25 nM) were preincubated with the indicated concentrations of inhibitor **2c** in 0.5 mL of 20 mM sodium phosphate, pH 7.5, on ice for 10 min prior to filtration. Control assays (minus $NADP^+$) were run simultaneously with those containing 200 μM $NADP^+$; the displayed curve represents the $NADP^+$-dependent binding as determined from the difference of the two sets of assays.

enzyme preparation was 77% and 22%, respectively. In contrast, no displacement of radioligand was observed at concentrations as high as 125 nM MK-906, 1 μM T, or 25 μM DHT in the presence of $NADP^+$.

The binding of radioligand also has been shown to be proportional to the protein content, and hence total SR activity, in the assays (Figure 1B). Scatchard analyses were performed with saturation binding data of **1a**, as that in Figure 1A, obtained under the previously established equilibrium conditions. By use of eq 2, dissociation constants (K_d) for **1a** and a relative concentration of binding sites for the steroidal acrylate could be estimated. With an enzyme preparation that had been solubilized in the presence of 200 μM NADPH (specific activity for SR of 3.2 nmol·min^{-1}·mg^{-1}), a single type of binding (−1/slope) with K_d of 25 nM was derived. The relative concentration of $NADP^+$-dependent steroidal acrylate binding sites, presumably equivalent to the number of SR catalytic sites, was determined to be 160 pmol/mg of protein in the solubilized liver preparation.[2] With the same enzyme preparation, a value of 8 μM for the K_d of $NADP^+$ at 25 nM [^{3}H]-**1a** was estimated by Scatchard analysis. Similarly, a single type of binding site was found with enzyme prepared with $NADP^+$ (specific activity of 0.8 nmol·min^{-1}·mg^{-1}) having a $K_d \sim 32$ nM for **1a** with a concentration of 48 pmol/mg of protein of ligand sites. Under comparable incubation conditions and protein content, the Scatchard analysis could not be undertaken with hepatic preparations solubilized in the absence of nicotinamide dinucleotide (specific activity of ~0.3 nmol·min^{-1}·mg^{-1}) since only minimal association of [^{3}H]-**1a**

[2] By use of the value of 160 pmol of binding sites/mg of protein (eq 2), the concentration of enzyme active sites in the standard filtration binding assay can be estimated at 2–3 nM. From this estimate, the concentrations of SR catalytic domains in activity assays were approximately 0.2–0.5 nM; thus, the enzyme concentration in the kinetic experiments is estimated to have been significantly lower than the concentrations of **1** or **2** required for enzyme inhibition (Tables I and II).

could be observed. Thus, the number of $NADP^+$-dependent binding sites for the steroidal acrylate **1a** is directly related to the specific activity of SR in the solubilized enzyme preparation.

The concentration-dependent displacement of [^{3}H]-**1a** (25 nM) by nonradioactive **2a** is shown in Figure 2. From these data, the concentration of **2a** required to displace half the reference ligand (CD_{50}) was estimated to be 25 nM. By use of eq 3, a dissociation constant (K_c) for the protein complex with **2a** in the presence of $NADP^+$ can be estimated to be 12 nM. The similarity between the dissociation constants for **1a** and **2a** determined by the filtration binding assay and their respective inhibition constants for SR activity with the solubilized enzyme (Tables I and II) suggests the same steroid binding site is responsible for both phenomena. The small differences between these dissociation and inhibition constants can be attributable to the differential protein content (10–15-fold higher for filtration binding than in the kinetic assay) in the respective experiments.

Recovery of SR Inhibition Activity by Gel Filtration. Gel filtration (Sephadex G-25) was employed to evaluate the reversibility of SR inhibition by the steroidal acrylates. In all the experiments, SR activity was found to coelute with the protein peak within the void volume of the column. With control incubations not including inhibitor, 72% of the protein and 55% of SR activity were recovered. These results were comparable for the incubations that included **1a**: without $NADP^+$, the recoveries of protein and SR activity were 68% and 58%, respectively; including $NADP^+$, 69% of the protein was recovered with a slightly higher yield (82%) of the enzyme activity. Thus, the activity of SR after gel filtration is the same both in the presence and in the absence of steroidal acrylate, indicating that inhibition of SR activity by **1a** is reversible. An additional observation is that the presence of both $NADP^+$ and **1a** affords protection from inherent SR inactivation during the course of the experiment, presumably by stabilizing the enzyme within a ternary complex.

Greater than 99.7% of the radiolabel dissociated from the protein upon gel filtration of enzyme preincubated with 25 nM [^{3}H]-**1a** and 200 μM $NADP^+$. A small amount (<0.2%) of radiolabel consistently coeluted in the void volume with solubilized protein, an observation that is independent of the presence of $NADP^+$. In comparison, this amount of radiolabel was ~10% of that for the $NADP^+$-independent and <5% of the $NADP^+$-dependent association to solubilized rat liver enzyme as determined with the filtration binding assay, where up to 10% of the ligand remained associated with protein. Upon preexposure of the solubilized protein to a low concentration (5 nM) of nonradioactive **1a** or **2a** followed by introduction of 25 nM [^{3}H]-**1a**, no tritium was observed in the void volume. Thus, there would appear to be a high-affinity association of the inhibitors within the solubilized liver prreparation that is independent of SR inhibition. No association of protein was observed with an early eluting shoulder of the main tritium peak. While the radiolabel that constitutes this shoulder could represent tightly bound [^{3}H]-**1a** which dissociates from protein during the course of gel filtration and subsequently elutes slightly before the originally unbound ligand, this interpretation seems unlikely since a similar profile was observed with [^{3}H]-**1a** in the absence of solubilized rat liver SR.

pH Dependence of Inhibition Constant for SR Activity with **1a**. The pH dependence of the inhibition constant of **1a** with solubilized rat liver SR is presented in Figure 3. Minimally, protonation states of two functionalities are observed in the

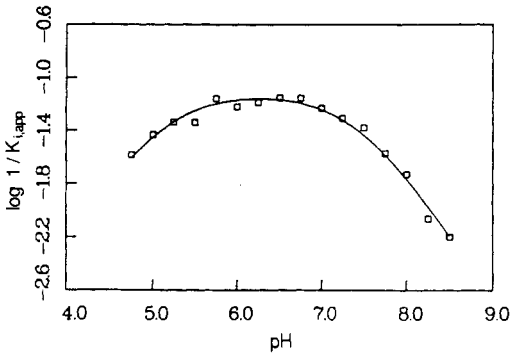


FIGURE 3: Dependence of the inhibition of solubilized SR by **1a** on pH. Solubilized SR was assayed in a constant ionic strength buffer in the presence of 1.0 μM T, 0.2 mM NADPH, a cofactor regnerating system, and varying concentrations of **1a**. Apparent values for the inhibition constant of **1a** ($K_{i,app}$ in units of nanomolar concentration) were determined by Dixon analysis throughout the pH range. The curve presented was evaluated with the BELL program (Cleland, 1979), yielding inflection points at pH 4.7 ± 0.2 and 7.5 ± 0.1. All activities were normalized to the protein content (2.2 μg) in the assays conducted at pH 7.5.

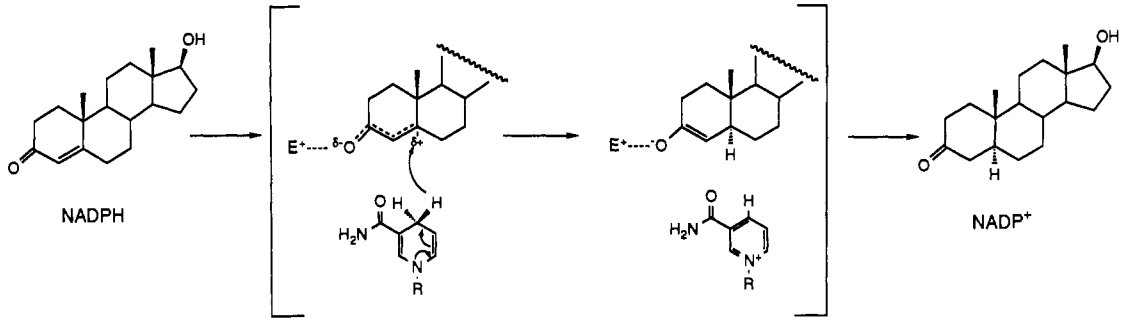
$1/K_i$ profile. A single deprotonation event at low pH (pK_1 = 4.7 ± 0.2) and a single protonation event at high pH (pK_2 = 7.5 ± 0.1) are required for SR inhibition. No loss of enzyme activity was observed upon preincubation within the extreme pH ranges followed by dilution and activity assay at neutral pH. Thus, the lower values for V_m within the highest and lowest pH ranges do not result from irreversible denaturation of the enzyme.

It would be anticipated that the carboxylic acid functionality of compounds such as **1a** and **2a** would have an acidity with a pK of less than 5.0 (Albert & Serjeant, 1962). By an independent spectrometric titration, the pK for **2a** has been estimated to be 4.8 ± 0.2. This ionization is in excellent agreement with the low pH deprotonation event (pK_1) shown to be required for SR inhibition by **1a**. Consequently, these data indicate that the inhibition of SR by the 3-carboxy steroids depends on the carboxylate anion of the steroidal inhibitor. Since no other group of the inhibitor molecule can account for the high pH protonation event (pK_2 = 7.5 ± 0.1) required for enzyme inhibition by **1a**, this inflection must be affiliated with the enzyme–$NADP^+$ binary complex. The protonated, electrophilic form of this functionality might coordinate to the carboxylate anion of the steroidal inhibitor through an ionic interaction, thereby stabilizing the enzyme-bound complex.

DISCUSSION

As a working model, the chemical mechanism of SR is proposed to involve the direct hydride transfer from NADPH to C-5 of T whereby the resulting 3,4-enolate could be stabilized by an enzyme-associated electrophile (Scheme I). Formation of a steroidal species with cationic character at C-5 by initial activation of the enone substrate (T) through coordination to this electrophilic center would facilitate the hydride transfer. Upon subsequent C-4 protonation of the enolate intermediate, DHT would be formed. According to Wolfenden (1972), chemically stable structural and electronic mimics of enzyme-bound reaction intermediates should demonstrate high enzyme affinity via exploitation of the specific interactions involved in the stabilization of the active site associated transition or intermediate states. It was through this approach that the concept of steroidal acrylate inhibitors evolved. Thus, the steroidal acrylates were designed to be analogues of the enzyme-bound enol or enolate state wherein the unsaturated 3-carboxyl functionality could represent a

Scheme I: Proposed Chemical Mechanism of Steroid 5α-Reductase



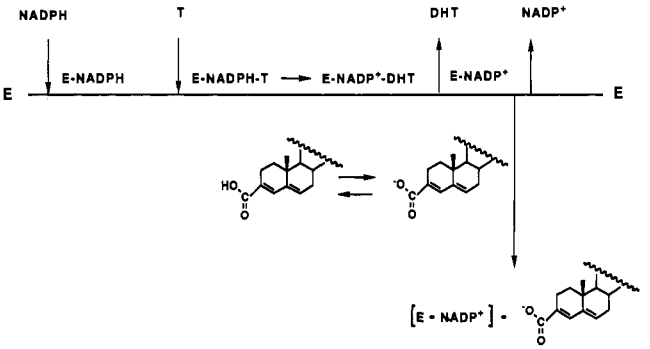
bound high energy state intermediate.

Anticipating the therapeutic utility of novel inhibitors for human disease states such as benign prostatic hypertrophy and acknowledging the potential for differences in binding affinities of steroids for enzyme derived from alternative sources (Liang et al., 1985), we focused our primary efforts on the inhibition of the SR activity from human hyperplastic prostatic tissue (Metcalf et al., 1989). As a potential model for inhibitor efficacy, SR from rat prostates has been used as a secondary enzyme evaluation (Levy et al., 1989). While useful for the characterization of compound potency, the prostatic enzyme preparations posed limitations on potential mechanistic studies of inhibitor–enzyme interaction; specifically, a tissue with higher concentration of enzyme activity was desired. Rat liver was chosen as the enzyme source for these studies since the level of its SR activity is several thousand fold greater than that in prostate (Liang et al., 1985). As summarized in Table I, a similar rank order of inhibitory potency for **1** and **2** was observed for rat prostatic, human prostatic, and rat hepatic SR activities. In addition, the dead-end inhibition patterns with **1a**, **2a**, and MK-906 have proven to be identical with each enzyme souce. Thus, although the absolute potency of the inhibitors may differ from one source to another, rat liver SR was thought to be useful as a mechanistic paradigm. The recent report that rat hepatic and prostatic steroid 5α-reductases are the same 29-kDa hydrophobic protein encoded by identical cDNA sequences (Andersson et al., 1989) supports this choice.

The linear uncompetitive inhibition patterns of the 3-carboxy steroids, **1a**, **1c**, **1h**, and **2a**, versus the two substrates require that the binding of inhibitor be downstream from the variable substrate or be separated by an irreversible step; consequently, binding of inhibitor must be kinetically isolated from that of both T and NADPH. In the context of the preferentially ordered kinetic mechanism proposed for rat liver SR (Levy et al., 1990), these uncompetitive dead-end inhibition patterns would be most consistent with the binding of inhibitor to the enzyme form generated upon first product (DHT) release or to the central complex. As depicted in Scheme II, the inhibition of rat liver SR by the 3-carboxy steroids could involve binding in a ternary complex to enzyme with $NADP^+$.

Unfortunately, since no conditions have been identified whereby SR can catalyze the reverse chemical reactions of DHT + $NADP^+$ → T + NADPH, simple confirmation of this mechanistic proposal by determination of the dead-end inhibition pattern versus DHT (theoretically expected to be competitive) was not possible. Instead, potential complementarity between the binding of $NADP^+$ and the 3-carboxy inhibitors to enzyme was investigated kinetically with multiple-inhibition analysis. As demonstrated by Northrup and Cleland (1974), data from double-inhibition experiments using two compounds, neither of which is competitive against a common substrate, can be evaluated by eq 1. The resulting

Scheme II: Mechanism of SR Inhibition by Steroidal Acrylates



intersecting curves indicate that the binding of $NADP^+$ and the 3-carboxy steroids is cooperative. With β values of less than 1 for **1a**, **1c**, **1h**, and **2a** (Table IIB) in the presence of the oxidized nicotinamide dinucleotide, the association of the two ligands to SR must be synergistic. While it is not our intention to suggest that other inhibitor complexes cannot or do not exist, it can be inferred that alternative complexes would involve less tightly associated components than has been demonstrated for the enzyme, $NADP^+$, and 3-carboxy steroid ternate.

Inhibition of SR by 3-oxo-4-aza steroids, such as 4-MA (Liang et al., 1983) and MK-906 (Liang et al., 1985), similarly results from formation of a dead-end ternary complex, although here the cofactor requirement is for NADPH, the reduced nicotinamide dinucleotide. Hence, cooperative combinations of enzyme with a 4-aza steroid and either $NADP^+$ or steroidal acrylate should be prohibited. From double-inhibition experiments with either compound pair, the experimental values for β ($>10^4$) are indistinguishable from infinity (Table IIB), thereby confirming the mutual exclusion of both $NADP^+$ and **1a** with MK-906 in their association to SR; neither $NAPD^+$ nor **1a** can bind to SR at the same time as does MK-906.

Enhanced binding of [^{3}H]-**1a** directly to the solubilized rat liver proteins demonstrates the same cofactor requirement as shown for inhibition of SR activity, with the level of inhibitor binding sites being proportional to the specific activity of the enzyme preparation. These findings, including estimates for the concentration of inhibitor binding sites, are in accord with previously published data using rat liver microsomes and [^{3}H]-4-MA (Liang et al., 1983). The binding affinities of [^{3}H]-**1a** (K_d = 25 nM) and **2a** (K_c = 12 nM) with the solubilized enzyme derived from filtration binding data are similar to the inhibition constants for both compounds (12–26 and 6–15 nM, respectively). Complete recovery of SR activity following gel filtration, relative to control values, confirms the reversible nature of the steroidal acrylate inhibition. A more definitive proof that inhibitor actually associates to SR in the binding assay would, of course, require further enzyme pu-

rification; the evidence in toto would suggest this to be likely. Hence, the model of SR inhibition as presented in Scheme II is supported mutually by results from the kinetic and filtration binding experiments.

Factors independent from those related to enzyme-bound intermediates, such as the C-17 substituent (Liang et al., 1985), can greatly influence ligand affinity; yet, within a series those compounds that can best mimic the enolate intermediate (Scheme I) in the T to DHT transformation are the better SR inhibitors. In this vein, the presence, placement, and degree of unsaturation have been shown to be critical toward inhibition potency of SR by the 3-carboxy steroids. A key structural feature to this binding energy is the sp^2 hybridization through C-3, C-4, and C-5. However, the functionalization that influences binding affinity to SR, such as the C-17 substituent, the C-5α ring junction, and the degree of unsaturation of the A/B rings, has minimal influence on the enzyme form to which inhibitor associates. It is the C-3 carboxylic acid which serves as the primary binding determinant to the enzyme–$NADP^+$ complex as demonstrated by comparisons between the dead-end and multiple-inhibition results (Table II) of **1a** ($\Delta^{3,5}$) with **1c** (Δ^3), **1h** (fully saturated), and **1j** (3-carboxamide) and **2a** (3-carboxylic acid) with **2c** [3-(hydroxymethyl)]. The change in the kinetic pattern between the carboxylic acids (**1a** and **2a**) and both amide **1j** and alcohol **2c** from uncompetitive to competitive (versus T) indicates differences in the mechanism of binding between the compound pairs (**1a** and **1j**; **2a** and **2c**) and suggests that a potential hydrogen-bond donor role for the C-3 carboxylic acid functionality is not the determinant in establishing preference for association to the enzyme–$NADP^+$ complex. It was important, consequently, to consider the protonation state of the carboxylic acid in SR inhibition.

The pH profile of SR inhibition indicates that two single ionizations, one protonation ($pK_2 = 7.5 \pm 0.1$) and one deprotonation ($pK_1 = 4.7 \pm 0.2$), are necessary for the binding of **1a**. A comparison of these inflections to those from the steady-state kinetic parameters of V_m and V_m/K_m, for which the values of both pK_1 (5.0–5.6) and pK_2 (7.9–8.9) are significantly higher (Levy et al., 1990), suggests that different molecular events are involved in inhibition of SR by **1a** than in substrate binding and catalysis or the inhibition by MK-906. Considering that the acidity ($pK = 4.8 \pm 0.2$) for the carboxylic acid of **2a** is indistinguishable from the pK_1 found in $1/K_i$ profile, the inhibition-dependent deprotonation must correspond to formation of the C-3 carboxylate anion of the inhibitor.[3] The second event at high pH in the $1/K_i$ profile would represent a protein-associated group. The anionic carboxylate of the steroid could coordinate to the protonated form of this functionality ($pK_2 = 7.5 \pm 0.1$), providing additional stabilization energy for the enzyme–inhibitor complex. This deprotonation appears to be kinetically distinguishable from that observed in the pH profiles with substrate and MK-906, yet the electrophile proposed to coordinate to the 3-oxo steroid during substrate binding and catalysis (E^+ in Scheme I) could be the same as that involved in stabilization of the SR–$NADP^+$–steroidal acrylate complex.

Our previous studies have suggested that observation of catalytic events by the steady-state parameters of V_m/K_m and/or V_m is limited by the kinetic events preceding release of $NADP^+$, the second product (Levy et al., 1990). As such, changes in protonation states that may influence the binding of an inhibitor to the enzyme–$NADP^+$ complex would not be observed in the pH profiles of these paraneters with the current assay. Implicit in this analysis is that binding of molecules to two different forms of enzyme–nicotinamide dinucleotide phosphate, each on opposite sides of this kinetic barrier as for MK-906 and **1a**, is influenced by the protonation states exposed within the individual complexes. It appears experimentally that association of steroid (T and MK-906) to the "substrate" side of SR—showing preference for E–NADPH—is more greatly influenced by the protonation states that effect cofactor binding; such events were not observed in the pH profile of SR inhibition by **1a**. However, these results may be somewhat misleading. The binding of **1a** under initial velocity conditions must occur prior to dissociation of the cofactor, making $NADP^+$ a "sticky" product; furthermore, association of the 3-carboxy steroid inhibitor to enzyme should decrease solvent accessibility to the nicotinamide dinucleotide phosphate binding site as observed with the inhibitor MK-906 (Levy et al., 1990). The combination of both these phenomena could shift the observed pK values involved in $NADP^+$ binding toward pH extremes that are not experimentally observable, thereby explaining the absence of such ionization in the inhibition profile.

It is intriguing that complementarity of charge between the cofactor and the inhibitor may account for selection of the binary complex to which an inhibitor would associate. While a direct ionic interaction with $NADP^+$ might strengthen binding of the anionic steroidal acrylate, it also is conceivable that the active-site presence of $NADP^+$ could confer an alteration within the steroid binding domain that would favor the presence of a C-3 unsaturated anion such as **1a** or **2a**. The implicit spatial constraints imposed on the cofactor within its binding site, as required by the transfer of hydride from nicotinamide to C-5 of T, would seem to make a strong direct charge interaction between the distal C-3 carboxylate of inhibitor and $NADP^+$ unlikely. Either through conformational modification of protein, charge-transfer complementation, or a combination of both, an electrophilic center that coordinates to the steroidal anion within the protein appears to be more accessible by having $NADP^+$ in the active site. From both the lack of product inhibition by DHT and the proposal that the androstene-3-carboxylic acids bind to the form of steroid 5α-reductase from which DHT is released, it can be inferred that the first product dissociating from the enzyme might be the 3,4-enolate of DHT which is subsequently protonated nonenzymatically at C-4 in solution; such a sequence of molecular events involved in product release effectively would make the reaction thermodynamically irreversible. Consequently, to designate the steroidal acrylates as transition-state inhibitors, reaction intermediate mimics or ground-state product analogues would be moot without further details of the molecular motions involved in the enzyme-catalyzed chemical conversion.

Pertinent here is a recent review dealing with protein–ligand charge interactions in the binding recognition of substrate and inhibitor to lactate dehydrogenase. Using genetically introduced modifications to specific active site associated amino acids, Clarke et al. (1989) have demonstrated that protein–ligand charge complementarity greatly affects binding and recognition. In this system, an active-site histidine (His 195) coordinates to the oxygen atom of substrate involved in the oxidation–reduction chemistry. Changes in single amino acids

[3] From such an interpretation, one can predict that the pK_1 values derived from pH profiles with analogues of inhibitors **1** and **2** would correlate with the acidity of the C-3 substituent. Independently, we have shown that with an equally potent steroidal inhibitor whose C-3 substituent would be anionic throughout the workable pH range ($pK < 2.0$) no inhibition-dependent deprotonation at low pH is observed. This result confirms that the low pH inflection (pK_1) seen in the inhibition of SR by **1a** corresponds to deprotonation of the steroidal carboxylic acid.

that interact with this histidine, thereby altering its effective acidity, have been shown to modify the catalytic efficiency and binding properties of both substrate (pyruvate and lactate) and inhibitor (oxamate). By extrapolation to the inhibition of SR, a similar set of intertwined molecular and electronic interactions between protein and active site bound ligands may underlie the empirical observation for maintenance of net neutrality of charge between steroidal inhibitor and nicotinamide dinucleotide cofactor in the determination of binding preference.

Such considerations of recognition and binding elements have led to concerns of target specificity. From this vantage point, several alternative enzyme systems have been considered, including two metabolic processes whose proposed catalytic mechanisms involve formation of enzyme-bound steroidal 3,4-enol(ate)s: removal of the C-4 methyl group from lanosterol in the pathway to cholesterol (Gibbons et al., 1982) and the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids catalyzed by the 3β-hydroxy-Δ^5-steroid dehydrogenase/3-keto-Δ^5-steroid isomerase complex (Talalay & Benson, 1972; Smith & Brooks, 1977). Of additional interest were systems that could utilize the common ligands of T, DHT, and/or nicotinamide dinucleotide phosphate, specifically steroid 5β-reductase [which also forms a steroidal-3(4)-enol(ate) intermediate] (Okuda & Okuda, 1984), 3α-hydroxysteroid dehydrogenase (Diserio et al., 1985), and aromatase (Fishman & Raju, 1981). In each of these systems, the steroidal acrylates (e.g., **1a**, **1h**, and **2a**) demonstrated minimal, if any, enzyme interaction.[4]

Inhibition of SR by the 3-androstene-3-carboxylic acids could prove to represent an effective therapeutic alternative in the treatment of pharmacological disorders for which DHT is considered to be a causal agent. While it is too early to assess clinical efficacy with the steroidal acrylates, the positive response in man of MK-906 (Vermeulen et al., 1989) would indicate that 3-androstene-3-carboxylic acids have a high probability of success in demonstrating in vivo utility. Particularly intriguing is the potential for differences in pharmacological efficacy with classes of SR inhibitors that function by alternative mechanisms. To address these issues, the in vivo pharmacological assessment of the steroidal acrylates is in progress.

Acknowledgments

We greatly appreciate the efforts of J. I. Heaslip for obtaining initial estimates of inhibition potencies, Dr. A. T. Greway for determining the inhibition pattern of compound **1j**, and J. M. Erb and H.-J. Oh for preparing several of the compounds discussed in this paper. We thank Dr. M. Soloway, Department of Urology, University of Tennessee, for providing us with samples of human prostatic tissue.

Supplementary Material Available

Methods describing the syntheses of the steroidal inhibitors, protocols for enzyme preparation, the SR assay and inhibition studies, and the determination of the acidity of compound **2a**; a scheme outlining the synthesis of [^{3}H]-**1a** (Scheme S-1) and figures with solubilized SR showing the dead-end inhibition pattern of compound **1a** versus T (Figure S-1), double-inhibition synergism between NADP$^+$ and compound **2a** (Figure S-2), and a gel filtration elution profile of SR and [^{3}H]-**1a** (Figure S-3) (9 pages). Ordering information is given on any current masthead page.

[4] For each system, potency of the inhibitors (0–10 μM) was determined by Dixon analyses (Dixon, 1953) at substrate concentrations equivalent to their K_m values. In none of the systems was the apparent inhibition constant for the steroidal acrylate found to be lower than the maximal inhibitor concentration; thus, for each of the alternative systems, $K_{i,app} > 10$ μM. in addition, no time-dependent loss of enzyme activities was observed upon preincubation with the steroidal acrylates. (M. A. Levy, M. Brandt, A. T. Greway, J. I. Heaslip, D. A. Holt, and B. W. Metcalf, unpublished results).

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Oxalyl Hydroxamates as Reaction-Intermediate Analogues for Ketol-Acid Reductoisomerase

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ABSTRACT: *N*-Hydroxy-*N*-isopropyloxamate (IpOHA) is an exceptionally potent inhibitor of the *Escherichia coli* ketol-acid reductoisomerase. In the presence of Mg^{2+} or Mn^{2+}, IpOHA inhibits the enzyme in a time-dependent manner, forming a nearly irreversible complex. Nucleotide, which is essential for catalysis, greatly enhances the binding of IpOHA by the reductoisomerase, with NADPH (normally present during the enzyme's rearrangement step, i.e., conversion of a β-keto acid into an α-keto acid, in either the forward or reverse physiological reactions) being more effective than NADP. In the presence of Mg^{2+} and NADPH, IpOHA appears to bind to the enzyme in a two-step mechanism, with an initial inhibition constant of 160 nM and a maximum rate of formation of the tight, slowly reversible complex of 0.57 min^{-1} (values that give an association rate of IpOHA, at low concentration, of 5.9×10^4 M^{-1} s^{-1}). The rate of exchange of [^{14}C]IpOHA from an enzyme-[^{14}C]IpOHA-Mg^{2+}-NADPH complex with exogenous, unlabeled IpOHA has a half-time of 6 days (150 h). This dissociation rate (1.3×10^{-6} s^{-1}) and the association rate determined by inactivation kinetics define an overall dissociation constant of 22 pM. By contrast, in the presence of Mn^{2+} and NADPH, the corresponding association and dissociation rates for IpOHA are 8.2×10^4 M^{-1} s^{-1} and 3.2×10^{-6} s^{-1} (half-time = 2.5 days), respectively, which define an overall dissociation constant of 38 pM. In the presence of NADP or in the absence of nucleotide (both in the presence of Mg^{2+}), the enzyme-IpOHA complex is far more labile, with dissociation half-times of 28 and 2 h, respectively. In the absence of Mg^{2+} or Mn^{2+}, IpOHA does not exhibit time-dependent inhibition of the reductoisomerase. These results parallel the effects that divalent metals and nucleotide have on the rearrangement step of this enzyme, which is greater than 3-fold more rapid in the presence of NADPH than in the presence of NADP and absolutely dependent on Mg^{2+}, and strongly suggest that IpOHA is a potent inhibitor of ketol-acid reductoisomerase by virtue of its structural similarity to the rearrangement transition state.

Ketol-acid reductoisomerase (EC 1.1.1.86), the second common enzyme in the biosynthetic pathway for branched-chain amino acids, catalyzes the reversible conversion of a β-keto-α-hydroxy-α-alkyl acid to an α,β-dihydroxy-β-alkyl acid with concomitant oxidation of NADPH[1] to NADP. The physiological substrates and products are α-acetolactate and 2,3-dihydroxy-3-methylbutyrate (DHMB) for valine and leucine biosynthesis and α-aceto-α-hydroxybutyrate and 2,3-dihydroxy-3-methylpentanoate (DHMP) for isoleucine biosynthesis, respectively. The reaction likely proceeds in two steps with rearrangement of a β-keto-α-hydroxy-α-alkyl acid to an α-keto-β-hydroxy-β-alkyl acid reaction intermediate [3-hydroxy-3-methyl-2-oxobutyrate (HMOB) or 3-hydroxy-3-methyl-2-oxopentanoate (HMOP) for leucine and valine biosynthesis or isoleucine biosynthesis, respectively] followed by hydride transfer. Although attempts to isolate the α-keto acid reaction intermediate have been unsuccessful (Arfin & Umbarger, 1969; Chunduru et al., 1989), the two steps of the enzymic reaction (rearrangement and hydride transfer) can be distinguished on the basis of their metal ion requirements. The reductoisomerase will reduce the putative α-keto acid intermediate (HMOB or HMOP) with NADPH, and this reduction requires either Mg^{2+} or Mn^{2+}, in contrast to the oxidation of NADPH by a β-keto acid (acetolactate or acetohydroxybutyrate) which has an absolute specificity for Mg^{2+} (Chunduru et al., 1989). This is a rather unusual enzymic

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[1] Abbreviations: IpOHA, *N*-hydroxy-*N*-isopropyloxamate; NADP, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; DHMB, 2,3-dihydroxy-3-methylbutyrate; DHMP, 2,3-dihydroxy-3-methylpentanoate; HMOB, 3-hydroxy-3-methyl-2-oxobutyrate; HMOP, 3-hydroxy-3-methyl-2-oxopentanoate; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid.

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