Affinity-labelling of the anti-inflammatory drug and prostaglandin-binding site of 3 α -hydroxysteroid dehydrogenase of rat liver cytosol with 17 β - and 21-bromoacetoxysteroids

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The homogeneous 3 α -hydroxysteroid dehydrogenase of rat liver cytosol binds prostaglandins with low micromolar affinity at its active site and is competitively inhibited by the non-steroidal and steroidal anti-inflammatory drugs [Penning, Mukharji, Barrows & Talalay (1984) *Biochem. J.* **222**, 601–611]. To examine the portion of this binding site that accommodates the glucocorticoid side chain, we have synthesized 17 β -bromoacetoxy-5 α -dihydrotestosterone (BrDHT) and 21-bromoacetoxydesoxycorticosterone (BrDOC) as affinity-labelling agents. Both these agents promote rapid inactivation of the purified enzyme in a time- and concentration-dependent manner. Analyses of the inactivation progress curves gave estimates of K_i for the inactivators and half-life ($t_{1/2}$) for the enzyme at saturation (τ) as follows: $K_i = 33 \mu\text{M}$ and $\tau = 18 \text{ s}$ for BrDHT, and $K_i = 10 \mu\text{M}$ and $\tau = 203 \text{ s}$ for BrDOC. Under initial-velocity conditions BrDHT and BrDOC act as competitive inhibitors, yielding K_i values identical with those measured in the inactivation experiments. Both indomethacin and prostaglandin E_2 can protect the enzyme from inactivation, yielding K_i values for these ligands consistent with those measured independently by competitive-inhibition studies. These data confirm that the bromoacetoxysteroids label the active site, which is coincident with the prostaglandin- and anti-inflammatory-drug-binding site. Neither gel filtration nor extensive dialysis restores activity to the enzyme inactivated with either affinity-labelling agent. Use of radioactive BrDHT or BrDOC, in which either the steroid portion is labelled with ^3H or the bromoacetate portion is labelled with ^{14}C , indicates that inactivation is accompanied by a stoichiometric incorporation of 0.7–1.0 molecules of inhibitor per enzyme monomer. The linkage that forms between the dehydrogenase with either [^{14}C]BrDHT or [^{14}C]BrDOC is stable to acid and base treatment. Complete acid hydrolysis of the enzyme inactivated with [^{14}C]BrDHT, followed by amino acid analyses, indicates that 87% of the radioactivity is eluted with carboxymethylcysteine. An almost identical result is obtained with [^{14}C]BrDOC, where at least 75% of the radioactivity is eluted with this amino acid. Thus BrDHT and BrDOC alkylate at least one reactive cysteine residue at the active site that may be of functional importance in binding the glucocorticoid side chain.

INTRODUCTION

The 3 α -hydroxysteroid dehydrogenase (EC 1.1.1.50) of rat liver cytosol is an NAD(P) $^{+}$ -dependent oxidoreductase that catalyses the interconversion between 3 α -hydroxy- and 3-oxo-steroids. Rat liver represents an abundant source of this protein, and milligram quantities can be readily purified (Penning & Talalay, 1983; Penning *et al.*, 1984). A number of diverse functions have been assigned to the purified enzyme, and these include: reduction of 5 α -dihydrotestosterone to 3 α -androstane-3 α -diol (Hoff & Schreffers, 1973), reduction of 5 β -dihydrocortisol to tetrahydrocortisol (Tomkins, 1956*a,b*) and, most recently, the oxidation of the isomeric pair of *trans*-7,8-dihydrodiols derived from the metabolism of benzo[*a*]pyrene *in vivo* (Smithgall *et al.*, 1986). Thus the purified dehydrogenase catalyses the first step in androgen metabolism, the second step in glucocorticoid metabolism and oxidizes potent proximate carcinogens to as-yet-unidentified products.

A rather surprising feature of the purified 3 α -hydroxysteroid dehydrogenase is its potent inhibition by both the non-steroidal and steroidal anti-inflammatory drugs, which appear to bind at the active site (Penning & Talalay, 1983; Penning *et al.*, 1984). Both these classes of drugs inhibit the enzyme in rank order of their therapeutic potency, and the IC_{50} (concentration causing half-maximal inhibition) values observed with many of these agents are lower than their peak plasma concentrations. These earlier findings suggested that the enzyme could be used to predict the potency of anti-inflammatory drugs. Indeed, the ability to use the NADPH-linked reduction of 5 β -dihydrocortisol as a specific reaction to assay the enzyme in rat liver cytosol has led to the development of a rapid spectrophotometric screen for non-steroidal-anti-inflammatory-drug potency (Penning, 1985).

The ability of the purified 3 α -hydroxysteroid dehydrogenase to be potentially inhibited by anti-inflammatory drugs has also led to the suggestion that the enzyme may

Abbreviations used: 3 α -hydroxysteroid dehydrogenase, 3 α -hydroxysteroid:NAD(P) $^{+}$ oxidoreductase (EC 1.1.1.50); androsterone, 5 α -androstane-3 α -ol-17-one; 5 α -dihydrotestosterone, 5 α -androstane-17 β -ol-3-one; BrDHT, 17 β -bromoacetoxy-5 α -dihydrotestosterone (17 β -bromoacetoxy-5 α -androstane-3-one); desoxycorticosterone, 21-hydroxypregn-4-ene-3-one; BrDOC, 21-bromoacetoxydesoxycorticosterone (21-bromoacetoxy-pregn-4-ene-3-one); prostaglandin E_2 , (5Z,11 α ,13E,15S)-11,15-dihydroxy-9-oxoprostano-5,13-dien-1-oic acid; indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindol-3-ylacetic acid.

be a target for these drugs *in vivo* (Penning & Talalay, 1983). The observation that the indomethacin-sensitive dehydrogenase is widely distributed in rat tissues supports this hypothesis (Smithgall & Penning, 1985). The dehydrogenase is also competitively inhibited by low-micromolar concentrations of the prostaglandins. K_i values reported for the prostanoids range from 0.8 to 12 μM and are similar to K_m values displayed by a variety of prostaglandin dehydrogenases for their substrates (Nakano *et al.*, 1969; Hansen, 1976; Yuan *et al.*, 1980). We have some information that the dehydrogenase displays hydroxyprostaglandin dehydrogenase activity (T. M. Penning & R. B. Sharp, unpublished work). These findings may provide a link between the inhibition of this enzyme by the anti-inflammatory drugs and regulation of prostanoid levels in inflammation.

The abundance of the purified 3α -hydroxysteroid dehydrogenase, its ability to predict the potency of anti-inflammatory drugs and its ability to interconvert prostaglandins has prompted the commencement of X-ray-crystallographic studies. In concert we have been prompted to elucidate the topography of the enzymes' active site by using a classical affinity-labelling approach. The information generated from these studies may provide a better understanding of the architecture of an anti-inflammatory-drug- and prostaglandin-binding site and may provide clues for the development of new anti-inflammatory agents.

It is recognized that the presence of the 17β side chain of glucocorticoids (17β -21-dihydroxy-C-20-ketone) is essential for their anti-inflammatory activity (Wolff, 1982). To examine the topography of the dehydrogenase active site that surrounds this side chain we have synthesized two affinity-labelling agents that contain alkylating groups in this portion of the steroid molecule, 17β -bromoacetoxy- 5α -dihydrotestosterone (BrDHT) and 21-bromoacetoxydesoxycorticosterone (BrDOC). The selection of bromoacetoxysteroids was based on the technology developed by Warren and his co-workers (Sweet *et al.*, 1972; Arias *et al.*, 1973; Strickler *et al.*, 1975), who have successfully affinity-labelled the active site of the $3\alpha,20\beta$ -hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* with similar reagents.

In the present paper we evaluate BrDHT and BrDOC as affinity labels for the purified 3α -hydroxysteroid dehydrogenase. The amino acid(s) alkylated by these agents at the prostaglandin- and anti-inflammatory-drug-binding site have been identified. This represents the first report of the affinity-labelling of a mammalian 3α -hydroxysteroid dehydrogenase.

MATERIALS AND METHODS

All steroids were purchased from Steraloids (Wilton, NH, U.S.A.). [$1,2,4,5,6,7(n)^3\text{H}$]Dihydrotestosterone (135.0 Ci/mmol), [$1\alpha,2\alpha(n)^3\text{H}$]desoxycorticosterone (48.0 Ci/mmol) and bromo[1^{14}C]acetic acid (56.0 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, U.S.A.). [1^{14}C]Glycollic acid (calcium salt; 40.0 mCi/mmol) was a product of ICN Radiochemicals (Irvine, CA, U.S.A.). Prostaglandin E_2 was a product of Bio-Mol, Philadelphia, PA, U.S.A. Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and Sephadex G-25F was a product of Pharmacia (Piscataway, NJ, U.S.A.). Pyridine ('Gold-label'), [^3H]chloroform, tetramethylsilane and dicyclo-

hexylcarbodi-imide were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All solvents were of u.v. grade and were obtained from Burdick-Jackson (Media, PA, U.S.A.). Budget-Solve scintillation fluid was purchased from Research Products International (Mount Pleasant, IL, U.S.A.).

All n.m.r. spectra were recorded on a Bruker WM-360 MHz spectrometer, in [^3H]chloroform with a deuterium field lock relative to tetramethylsilane. I.r. spectra were recorded in chloroform on a Perkin-Elmer 521 spectrometer with an extended range interchange. Absorption bands are listed as strong (s), medium (m), weak (w) and broad (b). Reactions were monitored by t.l.c. conducted on silica-gel GF 250 μm plates (10 cm $\times$ 2.5 cm) (Analtech, Newark, DE, U.S.A.).

Steroid synthesis

Synthesis of 17β -bromoacetoxy- 5α -dihydrotestosterone.

5α -Dihydrotestosterone (0.2 mmol) was dissolved in 3.0 ml of methylene chloride (CaH-distilled). The mixture was stirred on ice and 0.2 mmol of bromoacetic acid dissolved in 1.0 ml of methylene chloride was then added. Upon further stirring a slight molar excess of dicyclohexylcarbodi-imide in 1.0 ml of methylene chloride was added to initiate the reaction. After 5 min on ice the mixture turned cloudy, indicating the precipitation of some dicyclohexylurea; at this time a catalytic quantity of pyridine (10 μl) was added and the mixture was stirred on ice for a further 120 min. The reaction was monitored by t.l.c. in benzene/ethyl acetate (4:1, v/v), and the plates were sprayed with methanolic H_2SO_4 . Under these conditions the starting material had an R_F of 0.27, whereas the product had an R_F of 0.62. Once the reaction was complete, the solvent was removed under a stream of N_2 and the residue was dissolved in 4.0 ml of cold acetone. The insoluble dicyclohexylurea was removed by filtration and the filtrate which contained the product was evaporated to dryness *in vacuo*. The product was recrystallized from the residue in methanol. I.r.: (cm^{-1}), absence of O-H stretch at 3600 cm^{-1} (b); C=O stretch at 1690 (s) with shoulder at 1650 ; C-C(=O)-O stretch at 1275 (s) and O-C-C stretch at 1100 (s) . N.m.r.: δ (p.p.m.)([^3H]chloroform) 0.80 (3 s, C-18 $-\text{CH}_3$), 1.05 (3 s, C-19 $-\text{CH}_3$), 3.58 (2 s, Br- CH_2 -C=O) and 4.70 (1t, C-17 $-\text{CH}$).

Synthesis of 21-bromoacetoxydesoxycorticosterone.

This compound was synthesized in a similar manner to that described for the synthesis of 17β -bromoacetoxy- 5α -dihydrotestosterone. The reaction was monitored by t.l.c. as described above; the starting material had an R_F of 0.16, and the product had an R_F of 0.43. Recrystallization from methanol afforded yellow flocculent crystals. I.r.: (cm^{-1}) absence of O-H stretch at 3500 cm^{-1} b, and O-C-C stretch at 1100 (s) . The additional C=O stretch of the ester was hidden within the existing carbonyl stretches. N.m.r.: δ (p.p.m.) ([^3H]chloroform), 0.70 (3 s, C-18 $-\text{CH}_3$), 1.06 (3 s, C-19 $-\text{CH}_3$), 4.0 (2 s, Br- CH_2 -C=O), 4.6 (1d, C-21 $-\text{CH}_2$), 4.8 (1d, C-21 $-\text{CH}_2$) and 5.75 (1 s, C-4 $-\text{CH}$). U.v. λ_{max} 240 nm ($\epsilon = 17000\text{ M}\cdot\text{cm}^{-1}$).

Synthesis of [^3H]17 β -bromoacetoxy- 5α -dihydrotestosterone. This synthesis was conducted in the manner described above by using 25 mg of [^3H]5 α -dihydrotestosterone diluted to a specific radioactivity of 0.63 mCi/

mmol. The specific radioactivity of the [^3H]17 β -bromoacetoxy-5 α -dihydrotestosterone after three recrystallizations was 495 c.p.m./nmol.

Synthesis of [^{14}C]17 β -bromoacetoxy-5 α -dihydrotestosterone. This synthesis was conducted in the manner described above by using 14.5 mg of unlabelled steroid and 6.95 mg of bromo[1- ^{14}C]acetate diluted to a specific radioactivity of 5.0 mCi/mmol. The specific radioactivity of the [^{14}C]17 β -bromoacetoxy-5 α -dihydrotestosterone after three recrystallizations was 6560 c.p.m./nmol.

Synthesis of [^3H]21-bromoacetoxydesoxycorticosterone. This synthesis was conducted in the manner described above by using 28.4 mg of labelled steroid, [^3H]desoxycorticosterone diluted to a specific radioactivity of 2.3 mCi/mmol. The specific radioactivity of [^3H]21-bromoacetoxydesoxycorticosterone after three recrystallizations was 2313 c.p.m./nmol.

Synthesis of [^{14}C]21-bromoacetoxydesoxycorticosterone. This synthesis was conducted in the manner described above by using 16.5 mg of unlabelled steroid and 6.95 mg of bromo[1- ^{14}C]acetate diluted to a final specific radioactivity of 5.0 mCi/mmol. The specific radioactivity of 21-bromo[1- ^{14}C]acetoxydesoxycorticosterone after three recrystallizations was 6766 c.p.m./nmol.

Enzyme studies

Purification of 3 α -hydroxysteroid dehydrogenase. 3 α -Hydroxysteroid dehydrogenase was purified to homogeneity from male Sprague-Dawley-rat liver cytosol as described by Penning *et al.* (1983). The final specific activity was 1.5 μmol of androsterone oxidized/min per mg under standard assay conditions (see below).

Standard enzyme assay. The standard spectrophotometric assay for the 3 α -hydroxysteroid dehydrogenase was conducted in 1.0 ml systems containing: 75 μM -androsterone, 2.3 mM-NAD $^+$, 4% (v/v) acetonitrile and 100 mM-potassium phosphate buffer, pH 7.0. The reaction was initiated by the addition of the enzyme and the absorbance of the reduced nicotinamide (NADH) nucleotide was measured at 340 nm on a Gilford 260 UV/Vis recording spectrophotometer at 25 $^\circ\text{C}$. The absorbance change was monitored over 5 min and converted into nmol of product formed/min by using an ϵ value of 6270 $\text{M}^{-1}\cdot\text{cm}^{-1}$ for NADH. Under these conditions non-enzymic rates were negligible.

Inactivation experiments. Purified 3 α -hydroxysteroid dehydrogenase (0.169 mg) was diluted to 1.0 ml and dialysed overnight against three changes of 10 mM-potassium phosphate, pH 7.0, containing 1 mM-EDTA. The enzyme was divided into 100 μl portions and the inactivation event was initiated by the addition of bromoacetoxy steroid to give final concentrations of inhibitor that ranged from 2.5 to 50 μM in 6% acetonitrile. The enzyme solution was then incubated at 25 $^\circ\text{C}$, and at various times 2–10 μl samples were removed and diluted into the standard enzyme assay of 1.0 ml. Because of these substantial dilutions (100–500-fold), any further alkylation events are effectively quenched. The determination of enzyme activity in these assays was thus a measure of enzyme inactivation. Plots

of percentage activity remaining versus time gave estimates of the half-life of the enzyme ($t_{1/2}$). These data were further analysed to give estimates of the $t_{1/2}$ at saturation (τ) and K_i (dissociation constant for the reversible enzyme-inhibitor complex). For further details of the analyses, see the Results section.

Protection studies. In these experiments a fixed concentration of BrDHT or BrDOC was used to inactivate the homogeneous 3 α -hydroxysteroid dehydrogenase. Incubations were then repeated in the presence of increasing concentrations of protecting ligand (e.g. indomethacin or prostaglandin E $_2$). From the various progress curves, estimates of the initial velocity of inactivation were made and plotted against the concentration of protecting ligand in a manner formally analogous to the Dixon plots used for the analyses of competitive-inhibition data. From the slope of these plots the K_i values for the protecting ligand was computed (Penning *et al.*, 1981).

Competitive-inhibition studies. K_i values for competitive inhibitors were derived from initial-velocity measurements in which the concentration of inhibitor was varied over three fixed substrate (androsterone) concentrations. Primary Dixon plots were constructed and the slopes of these lines were then plotted against the reciprocal of the substrate concentration to yield a secondary plot. The K_i for the competitive inhibitor was calculated from the slope of the secondary plot as described by Siegel (1975).

Amino acid analyses. Large quantities of enzyme (10.0–15.0 nmol, M_r 34000) were inactivated with bromo[^{14}C]acetoxy steroid. Excess radiolabelled steroid was removed by gel-exclusion chromatography, and the peak protein fractions were pooled, checked for stoichiometry by liquid-scintillation counting and dialysed against three changes of distilled water overnight. The aqueous samples were then freeze-dried and the dried residues were hydrolysed in 6 M-HCl for 24 h. The acid was removed by drying *in vacuo* and the residue was taken up in sodium citrate buffer, pH 2.2, for amino acid analysis on a Beckman 6300 high-performance amino acid analyser. After amino acid detection with ninhydrin, the effluent was collected at 12 s intervals. Fractions were then dissolved in Budget-Solve and radioactivity quantified by liquid-scintillation counting. Calibration of the dead volume between the detector and fraction collector was achieved by using a cysteic acid standard. It was estimated that the time interval that exists between the detector and fraction collector was approx. 12 s, or one fraction. This knowledge permitted accurate superimposition of the radioactive scan over the ninhydrin trace.

Radioactivity measurements. ^3H and ^{14}C radioactivity were counted on a Tracor 43 analytic liquid-scintillation counter, which has a machine efficiency of 53% for ^3H and 95% for ^{14}C . All samples were measured as corrected c.p.m. Aqueous samples were counted for radioactivity in Budget-Solve, and organic samples were counted for radioactivity in a toluene-based scintillation

fluid [4 g of 2,5-diphenyloxazole (PPO) and 200 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP/litre of toluene].

RESULTS

Inactivation of homogeneous 3α -hydroxysteroid dehydrogenase by bromoacetoxysteroids

Previous studies indicated that the homogeneous 3α -hydroxysteroid dehydrogenase is sensitive to competitive inhibition by steroidal and non-steroidal anti-inflammatory drugs (Penning & Talalay, 1983; Penning *et al.*, 1984). Knowing that a 17β side chain (17β -21-dihydroxy-C-20-ketone) is required for glucocorticoid activity (Wolff, 1982), steroids containing a bromoacetoxy moiety either at C-17 or C-21 were synthesized (e.g. BrDHT and BrDOC) as affinity-labelling agents. These compounds have the potential to label that part of the dehydrogenase active site concerned with the accommodation of the glucocorticoid side chain. Both BrDHT

and BrDOC inactivate the purified 3α -hydroxysteroid dehydrogenase (Figs. 1a and 1b) in a rapid time-dependent manner. Semilogarithmic plots of enzyme activity remaining versus time show that $t_{1/2}$ values can be as short as a few minutes. At lower concentrations of inactivator, the progress curves for inactivation show marked curvature, attributable to the almost stoichiometric amounts of enzyme and inhibitor present in the incubation. At higher concentrations of inactivator the progress curves are almost linear and appear to indicate pseudo-first-order kinetics. In analysing this data it is assumed that, in each progress curve, the initial velocity of enzyme inactivation is a function of $k[EI]$, where k is a pseudo-first-order rate constant and $[EI]$ is the concentration of the enzyme-inactivator complex. To obtain estimates of these pseudo-first-order rate constants (k_{app} values), tangents were drawn to the progress curves. This method, if anything, yields underestimates of the true velocity of inactivation. Analyses of a double-reciprocal plot of $1/k_{app}$ versus $1/[I]$ gave a measure of $1/k_{+2}$ (limiting rate constant for inactivation)

Fig. 1. Inactivation of homogeneous 3α -hydroxysteroid dehydrogenase with BrDHT and BrDOC

Homogeneous 3α -hydroxysteroid dehydrogenase (0.49 nmol) in 100 μl of 10 mM-potassium phosphate, pH 7.0, containing 1 mM-EDTA was incubated at 25 °C in the presence of increasing concentrations of either BrDHT (2.5–25 μM) (a) or BrDOC (2.5–30 μM) (b). Bromoacetoxysteroids were dissolved in 6% acetonitrile. Over the time course indicated, 2–10 μl samples were removed from the incubation medium and diluted directly into the standard enzyme assay, and the amount of enzyme activity remaining was determined (see the Materials and methods section). Rate constants for inactivation were calculated by drawing tangents to the inactivation progress curves. These estimates of the pseudo-first-order rate constants were plotted against the concentration of the inactivator to yield the double-reciprocal plot for the inactivation of 3α -hydroxysteroid dehydrogenase by either BrDHT (c) or BrDOC (d).

and $1/K_i$ (dissociation constant for the EI complex). Generation of the straight lines shown in Figs. 1(c) and 1(d) indicates that, with both BrDHT and BrDOC, the process of inactivation is saturable and therefore dependent on the total concentration of enzyme. Data transformed in this manner yields the following kinetic constants: $K_i = 33.0 \mu\text{M}$ and $k_{+2} = 40.0 \times 10^{-3} \text{ s}^{-1}$ for BrDHT, and $K_i = 10.0 \mu\text{M}$ and $k_{+2} = 3.0 \times 10^{-3} \text{ s}^{-1}$ for BrDOC. These limiting rate constants predict that, at saturation, exceedingly short half-lives exist for the enzyme. Thus the $t_{1/2}$ for the enzyme when saturated with BrDHT is 18 s and that with BrDOC is 203 s.

It should be emphasized that it is not possible to change the conditions of the inactivation experiment such as to reduce the curvature observed in the progress curves. This would require either decreasing the concentration of the enzyme or increasing the concentration of the steroid inactivator in the incubation; neither is possible. At lower concentrations of enzyme it would not be possible to measure enzyme activity accurately at the end of the experiment. At higher concentrations of steroid the inactivators are insoluble.

Evidence that BrDHT and BrDOC label the prostaglandin- and anti-inflammatory-drug-binding site of 3 α -hydroxysteroid dehydrogenase

Under initial-velocity conditions in which the concentration of androsterone ($75 \mu\text{M}$) and nicotinamide nucleotide (2.3 mM) are saturating, the bromoacetoxy-steroids can act as competitive inhibitors of catalytic quantities of enzyme (Figs. 2a and 2b). Under such conditions the bromoacetoxy-steroids presumably bind to the enzyme in a rapid reversible fashion, but are prevented from initiating an alkylating event by substrate. In these competitive-inhibition studies the K_i values determined for the bromoacetoxy-steroids are similar to those determined in the inactivation experiments. These kinetic analyses suggest that the bromoacetoxy-steroids label the active site.

Micromolar concentrations of both indomethacin and prostaglandin E_2 also protect the enzyme from inactivation mediated by high concentrations of the bromoacetoxy-steroids [Figs. 3(a) and 3(b) (only the data obtained with BrDHT as inactivator are shown)]. Although complete protection with either indomethacin or PGE_2 is only observed at concentrations greater than $100 \mu\text{M}$, it should be emphasized that, in these experiments, this represents only a 10–20 fold excess over the concentration of enzyme and a meagre 2–3-fold excess over the concentration of inactivator. These experiments suggest that powerful protection with these ligands is being achieved. K_i values obtained for indomethacin and PGE_2 from these protection studies are comparable with those measured directly for these ligands in competitive-inhibition studies (Table 1). These kinetic data indicate that the bromoacetoxy-steroids affinity-label the anti-inflammatory-drug- and prostaglandin-binding site of the enzyme, which appears to be coincident with the active site.

Evidence that inactivation of the 3 α -hydroxysteroid dehydrogenase results in covalent attachment of the steroid

Several lines of evidence suggest that BrDHT and BrDOC covalently modify the dehydrogenase active site. In experiments in which the dehydrogenase is inactivated

with either BrDHT or BrDOC to within less than 5% of its initial activity, enzyme activity cannot be restored after substantial (100-fold) dilution into the assay system. Further, enzyme activity is not restored after extensive dialysis against 10 mM -potassium phosphate, pH 7.0, containing 1 mM -EDTA or by gel filtration. During these manipulations control enzyme retains full activity.

Stoichiometric incorporation of radiolabelled bromoacetoxy-steroids into the active site of 3 α -hydroxysteroid dehydrogenase

Radioactive probes were synthesized in which either the steroid nucleus of BrDHT and BrDOC was labelled with ^3H or the bromoacetoxy portion of BrDHT and BrDOC was labelled with ^{14}C . When these agents were

Fig. 2. Competitive inhibition of 3 α -hydroxysteroid dehydrogenase by BrDHT and BrDOC

Initial velocities of androsterone oxidation were measured at three fixed substrate concentrations (15 – $75.0 \mu\text{M}$) while the concentrations of either BrDHT (0 – $40 \mu\text{M}$) (a) or BrDOC (0 – $20 \mu\text{M}$) (b) were varied. In each instance the assays were performed under the standard conditions described in the Materials and methods section. The final concentration of organic solvent was 6% acetonitrile.

Fig. 3. Indomethacin and prostaglandin E₂ protect 3 α -hydroxysteroid dehydrogenase from inactivation mediated by BrDHT

Homogeneous 3 α -hydroxysteroid dehydrogenase (0.32 nmol) in 100 μ l of 10 mM-potassium phosphate, pH 7.0, containing 1 mM-EDTA, was incubated with 10.0 μ M-BrDHT in 6% acetonitrile in the presence of increasing amounts of indomethacin (0–100 μ M). At various times, 2–10 μ l samples were removed and diluted into the standard enzyme assay mixture. The amount of enzyme activity remaining was determined (a) after correcting the initial velocities for the inhibition due to the presence of indomethacin in the enzyme assay. These corrections were made from the appropriate dose–response curve. In a similar set of experiments, homogeneous enzyme (1.0 nmol) in 100 μ l was incubated in the presence of 50 μ M-BrDHT in the presence of increasing amounts of prostaglandin E₂ (0–144 μ M). At various times, 2–10 μ l samples were removed and diluted into the standard enzyme assay mixture, and the amount of enzyme activity remaining was determined (b). The final concentration of prostaglandin E₂ present in these assays had no effect on the initial velocities observed. Rate constants for inactivation were calculated by drawing tangents to the inactivation progress curves. These estimates of the pseudo-first-order rate constants were plotted against the concentration of indomethacin (c) or prostaglandin E₂ (d); slopes from these secondary plots were used to calculate the K_i values for indomethacin and prostaglandin E₂.

used to inactivate the dehydrogenase, subsequent gel-exclusion chromatography to remove excess steroid indicated that the inactivation event was accompanied by the incorporation of 0.7–1.0 molecules of inactivator per enzyme monomer (results not shown). This suggests that a discrete amino acid is being labelled at the active site by both the steroid and bromoacetoxy moieties of BrDHT and BrDOC. Since the same stoichiometry is observed with all four probes, this suggests that transfer of the carboxymethyl group with subsequent release of the steroid is not facilitated by nucleophilic amino acids at the active site.

Stability of the covalent linkage that forms between the bromoacetoxysteroids (BrDHT and BrDOC) and 3 α -hydroxysteroid dehydrogenase

In these experiments 6.5–12.0 nmol of homogeneous 3 α -hydroxysteroid dehydrogenase were inactivated with

either [¹⁴C]BrDHT or [¹⁴C]BrDOC; excess steroid was removed by gel filtration, and the [¹⁴C]carboxymethylated enzyme was subjected to a variety of treatments to establish the stability of the covalent linkage. With either preparation, the inactivated enzyme retained its label when exposed to extremes of acidity. The covalent bond was somewhat less stable in alkali. This demonstrates that the carboxy group of aspartate or glutamate was not carboxymethylated, since the resulting ester should have been cleaved under the acid conditions employed with subsequent release of radiolabel.

Evidence that covalent modification of the 3 α -hydroxysteroid dehydrogenase results in a large conformational change

The u.v. spectrum of the homogeneous dehydrogenase is not unusual and shows a λ_{max} at 280 nm attributable

Table 1. Comparison of dissociation constants (K_d values) determined for indomethacin and prostaglandin E_2 by various methods

Dissociation constants for the competitive inhibitors indomethacin and prostaglandin E_2 were determined from Dixon plots using androsterone as substrate (as described in the Materials and methods section). These constants were also derived from inactivation experiments in which various concentrations of either indomethacin (0–100 μ M) or prostaglandin E_2 (0–150 μ M) were used to protect homogeneous 3 α -hydroxysteroid dehydrogenase from inactivation by either 50 μ M-BrDOC or 10 μ M-BrDHT.

Method of derivation	Competitive inhibitor	K_d (μ M)
Dixon plot	Indomethacin	1–3
	Prostaglandin E_2	10.0
Protection studies with BrDHT	Indomethacin	6.0
	Prostaglandin E_2	5.0
Protection studies with BrDOC	Indomethacin	6.0
	Prostaglandin E_2	7.0

to the absorbance of the aromatic amino acids as well as a large end absorbance due to the presence of the peptide bonds. After inactivation with BrDHT the absorbance of the aromatic amino acids appears to be unaffected; however, the end absorbance undergoes a substantial red shift accompanied by a 4–5-fold increase in intensity. Similar changes are observed in the u.v. after inactivation of the purified dehydrogenase with BrDOC. Since the absorbance of the aromatic amino acids is unperturbed by inactivation with either BrDHT or BrDOC, these amino acids would appear to be distant from the steroidal ligands. The large change in absorbance observed in the far u.v. may suggest that a substantial conformational change occurs as a result of the inactivation event.

Identification of the amino acid alkylated by BrDHT and BrDOC

Inactivation of a large quantity of homogeneous dehydrogenase with [^{14} C]BrDHT followed by subsequent removal of excess steroid by gel-exclusion chromatography afforded a sample that was freeze-dried and subjected to complete acid hydrolysis in 6 M-HCl for 24 h. The sample was then subjected to amino acid analyses and the effluent from the analyser was collected for liquid-scintillation counting. It was found that most of the radioactivity (85–87% of the total) was associated with a single amino acid with a retention time of 6.0 min (Fig. 4). This amino acid is co-eluted with carboxymethylcysteine. An almost identical result was observed from four separate analyses of [^{14} C]BrDHT-inactivated dehydrogenase.

When enzyme inactivated with [^{14} C]BrDOC was subjected to a similar procedure, most of the radioactivity (75–77%) was also found to be co-eluted with carboxymethylcysteine (results not shown). This result suggests that both affinity ligands predominantly label cysteine residue(s) at the active site.

With both labels the remainder of the radioactivity (13–25%) appears to be associated with a ninhydrin-negative peak that is eluted at 2.4 min. At the present time this peak remains to be identified; it is not co-eluted

with cysteic acid or glycollic acid, and it is also unlikely to be thioglycollic acid, since the unidentified peak has a shorter retention time than that compound.

Fig. 4. Identification of the amino acid alkylated by [^{14} C]BrDHT

Homogeneous 3 α -hydroxysteroid dehydrogenase (21.0 nmol/1.5 ml) was inactivated by 98% by 100 nmol of [^{14} C]BrDHT as described in the text. Excess steroid was removed by gel-exclusion chromatography, the radio-labelled protein fractions, which contained one molecule of steroid/enzyme monomer, were pooled and dialysed against water. An aqueous sample was prepared and subjected to amino acid analysis as described in the Materials and methods section. (a) Ninhydrin trace (—) and ^{14} C radioactivity (---) of a portion of the hydrolysed sample; (b) ^{14} C radioactivity detected after amino acid analysis of a second portion of the hydrolysed sample; (c) ninhydrin trace of 10.0 nmol of cysteic acid and carboxymethylcysteine standards (—) plus radioactive detection of a [^{14} C]glycollic acid standard (---).

DISCUSSION

The present paper provides the first successful account of the affinity labelling of the active site of a mammalian 3α -hydroxysteroid dehydrogenase. This enzyme is of interest, since it is involved in both androgen (Hoff & Schreiffers, 1973) and polycyclic-aromatic-hydrocarbon (Smithgall *et al.*, 1986) metabolism. One novel property of the enzyme is its potent inhibition by prostaglandins and anti-inflammatory drugs (Penning & Talalay, 1983). The abundant supply of this enzyme in rat liver cytosol indicates that it represents a useful model for mapping the topography of an anti-inflammatory-drug- and prostaglandin-binding site.

We have employed BrDHT and BrDOC to label amino acid residues that may be of functional importance in accommodating the glucocorticoid side chain at the active site. The kinetic data presented indicate that both agents label the appropriate site. The rates of inactivation observed are exceptionally high; other studies in which bromoacetoxysteroids were used to affinity-label hydroxysteroid dehydrogenases indicate that half-lives generally range from 20 min to 10 h (Sweet *et al.*, 1972; Arias *et al.*, 1973; Strickler *et al.*, 1975). Radiolabelled probes indicate that a stoichiometric incorporation of label accompanies the inactivation of 3α -hydroxysteroid dehydrogenase. This is observed irrespective of whether the steroid nucleus or the bromoacetate group is radio-labelled. With each probe carboxymethylcysteine is the amino acid predominantly labelled. Whether both BrDHT and BrDOC label an identical cysteine residue remains to be determined. This will require proteolytic digestion and peptide mapping of enzyme inactivated with either BrDHT or BrDOC.

The use of bromoacetoxysteroids to label the active site of hydroxysteroid dehydrogenases was originally described by Warren and his colleagues (Sweet *et al.*, 1972; Arias *et al.*, 1973; Strickler *et al.*, 1975). In those earlier studies the $3\alpha/20\beta$ -hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* was affinity-labelled with 6β , 11α - or 16α -bromoacetoxypregesterone, and the carboxymethylated amino acids identified were shown to be cysteine, methionine and histidine respectively. With the use of a 16α -bromoacetoxypregesterone, the active site of the $17\beta/20\alpha$ -hydroxysteroid dehydrogenase of human placenta has also been affinity-labelled (Strickler *et al.*, 1981). In this particular instance a dicarboxymethylated histidine was the result of the alkylation event. On the basis of studies with 16 -bromoacetoxypregesterone, a histidine residue would appear to be important in binding the steroid D-ring at the active site of 20α - and 20β -hydroxysteroid dehydrogenase. This present study demonstrates that a cysteine residue may be important in binding the steroid side chain at the 3α -hydroxysteroid dehydrogenase active site.

Although we have used a classical approach in our studies, this is the first time such agents have been used to probe the structure of a prostaglandin- and/or anti-inflammatory-drug-binding site. The linkage that forms between either BrDHT or BrDOC appears to be exceptionally stable to acid and somewhat stable to alkali. Such stability suggests that both probes could be used with success to isolate and sequence an active-site peptide of the dehydrogenase. This information will provide further clues to the architecture of a binding site capable of binding proximate carcinogens (*trans*-dihydrodiols of polycyclic aromatic hydrocarbons), prostaglandins and anti-inflammatory drugs.

This work was supported by National Institutes of Health Grant GM 33464. K.E.C. was supported by Training Grant GM 07229. Amino acid analyses were conducted at the University of Pennsylvania Protein Chemistry Laboratory, which is supported in part by a University BRSG grant, RR-05415, a Medical School BRSG grant, R-07083, and a Research Resources Equipment Grant, RR-01412. We thank Ms. Caryn Glusman for expert technical assistance in running the amino acid analyser.

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