Teraoka, G., & Kitagawa, T. (1981) *J. Biol. Chem. 256*, 3969–3977.
Terner, J., & Reed, D. E. (1984) *Biochim. Biophys. Acta 789*, 80–86.
Terner, J., Sitter, A. J., & Reczek, C. M. (1985) *Biochim. Biophys. Acta 828*, 73–80.
Tien, M., & Kirk, T. K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2280–2284.
Tien, M., Kirk, T. K., Bull, C., & Fee, J. A. (1986) *J. Biol. Chem. 261*, 1687–1693.
Van Wart, H. E., & Zimmer, J. (1985) *J. Am. Chem. Soc. 107*, 3379–3381.

Affinity Alkylation of 3-Oxo-Δ^5-steroid Isomerase by Steroidal 3β-Oxiranes: Identification of the Modified Amino Acid by Reduction with Hydroxyborohydride†

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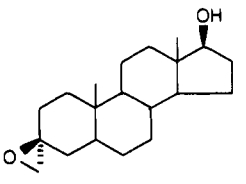
ABSTRACT: The steroidal 3β-oxirane (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**) is an active site directed irreversible inhibitor of the 3-oxo-Δ^5-steroid isomerase from *Pseudomonas testosteroni*. Two steroid-bound peptides (TPS_1 and TPS_2) were isolated by high-performance liquid chromatography (HPLC) from the trypsin digest of enzyme inactivated with **1β**. The modified tryptic peptides (residues 14–45 of the enzyme) were further digested with chymotrypsin, each giving rise to a single steroid-containing product (CPS_1 and CPS_2, respectively) derived from residues 31 to 45 of the enzyme. The modified chymotryptic peptides were isolated by HPLC, and the peptide–steroid ester linkage was reduced with sodium hydroxyborohydride. Amino acid analysis of the reduced peptides gave ca. 0.5 residue of homoserine and one less residue of aspartic acid than the corresponding unreduced peptides. Sequence analysis of both reduced chymotryptic peptides revealed that homoserine was located at position 8 in the peptide sequence, corresponding to residue 38 of the enzyme. The finding that the steroidal 3β-oxirane, like the 17β-oxiranes, inactivates the isomerase via esterification of aspartic acid-38 is strong evidence that this enzyme binds steroids in at least two orientations.

The 3-oxo-Δ^5-steroid isomerase (EC 5.3.3.1) of the soil bacterium *Pseudomonas testosteroni* catalyzes the conversion of a variety of 3-oxo-Δ^5-steroids to their corresponding Δ^4-isomers (Talalay & Benson, 1972; Batzold et al., 1976). This enzyme is of particular interest because it is a remarkably potent catalyst, having a turnover number (4.4×10^6 min^{-1}) that places it among those enzymes that may be classified as "diffusion controlled" (Fersht, 1985). Unlike its mammalian counterpart, which is an integral membrane protein (Murota et al., 1971; Ford & Engel, 1974), the bacterial isomerase is soluble in water, is readily obtainable in pure form, and has been extensively characterized. The isomerase exists in dilute solution as a dimer of identical subunits having a monomer M_r of 13 394 (Benson et al., 1971). The primary structure of the enzyme has been reported by Benson et al. (1971) and was partially confirmed by Ogez et al. (1977), although there is some disagreement on the assignment of residues near the N-terminus. Westbrook et al. (1984) have recently reported the crystal structure of the isomerase at 6-Å resolution and demonstrated that the steroid binding site of each monomer is located near the contact interface of the dimer.

The catalytic mechanism of the bacterial isomerase has been the focus of much research in recent years, and several workers have employed active site directed irreversible inhibitors to identify amino acid residues that may play a role in catalysis. Talalay and co-workers have located asparagine-57 at the active site on the basis of their work with the suicide inactivator 5,10-secoestr-5-yne-3,10,17-trione (Penning et al., 1981, 1982; Penning & Talalay, 1981). Benisek and co-workers have shown that photoinactivation of the isomerase by 3-oxo-4-estren-17β-yl acetate is accompanied by chemical modification of aspartic acid-38 (Martyr & Benisek, 1975; Ogez et al., 1977; Hearne & Benisek, 1985). In recent work from our laboratory on the active site directed inactivation of the isomerase by 3β- and 17β-steroidal oxiranes (Pollack et al., 1979; Bevins et al., 1980), we have shown that inactivation occurs by formation of an ester linkage between the steroid and a carboxylic acid residue of the enzyme (Kayser et al., 1983; Bevins et al., 1984). In addition, we have identified aspartate-38 as the nucleophile involved in the reaction with the 17β-oxiranes (Kayser et al., 1983).

We report here that aspartate-38 is also the amino acid residue esterified by the 3β-oxiranyl steroid (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**). Specifically esterified



1β

enzyme was digested with trypsin and chymotrypsin, and the

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ester linkage was reduced with sodium hydroxyborohydride. The modified active site amino acid was then identified as the reduction product homoserine.

Materials and Methods

General procedures concerning the purification of the isomerase, trypsin digestion, peptide mapping, and amino acid analysis by high-performance liquid chromatography (HPLC) have been described previously (Kayser et al., 1983; Bevins et al., 1984). The synthesis of (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol and the inactivation of the isomerase have also been previously described (Pollack et al., 1979). Chymotrypsin, dried cells of *Pseudomonas testosteroni*, and dansylated amino acids were purchased from Sigma. Trypsin, pretreated with L-1-(*p*-toluenesulfonamido)-2-phenylethyl chloromethyl ketone, was obtained from Worthington. HPLC-grade solvents were purchased from J. T. Baker. Water was purified by filtering glass distilled water through a Waters Associates Norganex resin cartridge. μBondapak reverse-phase C_{18} columns were purchased from Waters Associates. Sodium borohydride (99+%), acetanilide, methylbenzamide, cyclohexanecarboxamide, and dansyl chloride were obtained from Aldrich. All other solvents and reagents were of reagent grade. The specific activity of the isomerase used in this study was ca. 47 000 units/mg.

DL-*5-Hydroxynorvaline* was prepared by the method of Gaudry (1951), mp 214.5–215 °C (lit. mp 215 °C; Gaudry, 1951).

Preparation of the Hydroxyborohydride Solutions. Sodium borohydride was prepared by the method of Reed and Jolly (1977) with a simplified version of the quenched-flow reactor described by Reed et al. (1974) made entirely of glass. Glass tubing 5 mm in diameter and sealed at the top served as the reaction vessel. Two inlet tubes for the mixing of $NaBH_4$ and HCl were fused at an angle to the top of the vessel. A third inlet tube for the introduction of NaOH to quench the reaction was joined at an angle to the vessel 5 mm below the upper inlet tubes, and immediately above an outlet tube. Inlet and outlet tubes were 3 mm in diameter. The reactant solutions were contained in Erlenmeyer flasks, which were fitted with needle valves rigged for simultaneous pressurization from a single nitrogen cylinder. The needle valves were adjusted to deliver each reagent at ca. 11 mL/s. The reagent solutions were precooled to 0 °C, and the product was collected in a beaker on ice. The first several milliliters of effluent from the flow reactor were discarded, as a short time was needed to establish a steady flow from all three reagent reservoirs. Solutions of 1.5 M $NaBH_4$, 3.0 M HCl, and 3.0 M NaOH were used to prepare the hydroxyborohydride. The concentrations of BH_3OH^- and residual BH_4^- in the resulting solution were determined by hydrogen gas evolution as described by Reed and Jolly (1977). The concentration of hydroxyborohydride in solutions synthesized in the quenched-flow reactor was ca. 0.25 M, while the concentration of unreacted borohydride was generally less than 0.1 M.

Isomerase Inactivation. Isomerase (30 μM, in 0.01 M phosphate buffer, pH 7.0) was incubated with **1**β (32 μM) for 3 h at room temperature. Enzyme thus treated contained less than 1% residual activity.

Digestion of Modified and Unmodified Enzyme with Trypsin. Samples of both active (27 nmol) and inactivated enzyme (54 nmol) were denatured by heating for 10 min at 100 °C, cooled, and then incubated at room temperature with trypsin (6%, w/w of protein) in 5 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer (pH 7.8). The digestion was terminated after 18 h by the addition of sufficient 1 N HCl to reduce the pH to ca. 3.

The tryptic peptides were analyzed by HPLC on a C_{18} reverse-phase column (30 cm × 7.8 mm) with gradient elution for 30 min from 30 to 60% acetonitrile/water with 6 mM sodium phosphate (pH 3.0) in the water and 1% 2-propanol throughout, with a flow rate of 2 mL/min. Peptides were detected by monitoring the UV absorbance at 205 nm. The two peptides that were present in the tryptic digest of the modified enzyme and not in the digest of the unmodified enzyme were collected. These peptides were neutralized by addition of sufficient 0.1 N NaOH to raise the pH to ca. 6 and were concentrated, with removal of the acetonitrile, under a stream of nitrogen.

Digestion of Modified and Unmodified Tryptic Peptides with Chymotrypsin. Samples of the tryptic peptides derived from enzyme alkylated by **1**β (TPS_1 and TPS_2) and the corresponding unmodified tryptic peptide (TP), each containing ca. 20 nmol, were treated with 40 μg of chymotrypsin at pH 8.0 (15 mM phosphate buffer) and room temperature. The digestion was stopped after 18 h by the addition of sufficient 1 N HCl to reduce the pH to ca. 3.

The products of the chymotrypsin digestion were analyzed, and the steroid-containing peptides were isolated as described for the tryptic digests, except that a 15-min gradient from 35 to 40% acetonitrile was used for elution of the steroid-bound chymotryptic peptides, and a 10-min gradient from 10 to 20% acetonitrile was used for elution of the corresponding unmodified chymotryptic peptide. The collected peptides were neutralized and concentrated, and the acetonitrile was removed under a stream of nitrogen.

Treatment of Chymotryptic Peptides with Sodium Hydroxyborohydride. Aliquots of ca. 300 μL of each of the modified and unmodified chymotryptic peptides (CPS_1, CPS_2, and CP), containing 12–20 nmol, and 25 μL of acetonitrile were added to 750 μL of a freshly prepared solution of sodium hydroxyborohydride (adjusted to pH 11.9 with 1 M phosphoric acid). The solutions were stirred at 0 °C, and 40-μL aliquots were quenched by addition of 20 μL of 1 N HCl after reaction for 30 min, 1 h, and 18 h.

The products of treatment with hydroxyborohydride were chromatographed as described above by gradient elution for 20 min from 10 to 20% acetonitrile. The collected peptides were neutralized, and the acetonitrile was removed in a stream of nitrogen or argon.

Acid Hydrolysis and Dansylation of Peptides. Aliquots of the collected peptides (1–4 nmol) were lyophilized and hydrolyzed in 100 μL of constant-boiling HCl at 105–110 °C for 18 h. The peptide hydrolysates were lyophilized to remove the HCl, redissolved in 200 μL of H_2O, and lyophilized again. The residues were dansylated by a modification of the method of Tapuhi et al. (1981). Samples were dissolved in 200 μL of lithium carbonate buffer (40 mM, pH 9.5) and treated with 100 μL of dansyl chloride solution (1.5 mg/mL in acetonitrile). After 1 h the reaction was stopped by the addition of either 10 μL of leucine or 10 μL of methylamine (0.1 M aqueous solution). Sufficient 1 N HCl was added to reduce the pH to ca. 3 prior to HPLC analysis.

Dansylation of Homoserine and 5-Hydroxynorvaline. Dansylation of homoserine and 5-hydroxynorvaline was carried out according to the procedure of Tapuhi et al. (1981).

HPLC Analysis of Dansyl Derivatives. Dansylated amino acid derivatives were initially separated on a μBondapak C_{18} column (7.8 mm × 30 cm) under isocratic elution for 15 min with 30% aqueous acetonitrile containing 15 mM sodium phosphate buffer (pH 3.5) in the water followed by a 5-min

gradient to 40% aqueous acetonitrile containing 15 mM phosphoric acid (pH 2.5) in the water, with 1% 2-propanol throughout (flow rate 2 mL/min). The dansyl derivatives of serine and homoserine coeluted under these conditions and nearly coeluted with the derivatives of aspartic and glutamic acids. The dansyl derivatives of serine, homoserine, aspartic acid, and glutamic acid were collected and reseparated on two μBondapak C_{18} columns (3.9 mm × 30 cm) connected in tandem, under isocratic elution in 20% aqueous acetonitrile with 1% 2-propanol and 15 mM phosphoric acid (pH 2.5) in the water (flow rate 1 mL/min). Dansyl derivatives were detected by monitoring the UV absorption at 220 nm.

Treatment of Model Amides with Sodium Hydroxyborohydride. Acetanilide (0.5 mmol dissolved in 5 mL of methanol) was stirred at 0 °C with 5 mL of a 0.26 M solution of hydroxyborohydride (pH 11.9). After 17 h an aliquot was removed and extracted with ether. The ether layer was concentrated and analyzed by gas chromatography. Methylbenzamide (0.5 mmol) was dissolved in 2 mL of methanol and treated for 23 h at 0 °C with 13.5 mL of a 0.20 M solution of the reducing reagent. The resulting solution was extracted with ether; the ether layer was concentrated and analyzed by gas chromatography. Cyclohexanecarboxamide (0.5 mmol) was dissolved in 2.5 mL of methanol and stirred with 21 mL of a 0.25 M solution of hydroxyborohydride. After 48 h at 0 °C, an aliquot was extracted with chloroform; the organic layer was concentrated and analyzed by gas chromatography. Analyses were carried out on a Varian Model 1400 gas chromatograph on a 2 m × $^1/_8$ in. 10% OV 101 packed column with temperature programming and a thermal conductivity detector. The carrier gas was helium maintained at a flow rate of ca. 15 mL/min.

Sequence Analysis of Hydroxyborohydride-Treated Chymotryptic Peptides. Aliquots containing ca. 1 nmol of the major peptide product from reduction of each of the steroid-bound chymotryptic peptides and from the corresponding unmodified peptide were lyophilized and analyzed by automated sequential Edman degradation on an Applied Biosystems Model 470A sequencer. Phenylthiohydantoin derivatives were detected on an Applied Biosystems Model 120A PTH analyzer.

Results

Proteolytic Digestion of Inactivated Isomerase and Isolation of the Chymotryptic Peptides. Isomerase was inactivated by treatment with **1**β for 3 h, denatured, and treated with trypsin. HPLC analysis of the tryptic digest showed two peaks (TPS_1 and TPS_2), which were not present in the tryptic map of the unmodified isomerase. The tryptic map of the unmodified enzyme contained a single major peptide (TP), which did not appear in the chromatogram of digested inactivated enzyme. We have previously demonstrated that each of the peptides TPS_1 and TPS_2 contains steroid and is derived from the peptide TP, which corresponds to residues 14–45 of the amino acid sequence (Bevins et al., 1984). Digestion of the tryptic peptides TP, TPS_1, and TPS_2 with chymotrypsin in each case gave rise to two detectable peptide products. One of these products eluted at 39% acetonitrile in all three cases. The elution pattern of the other product varied in the three samples: that from TP (CP) eluted at 15% acetonitrile, that from TPS_1 (CPS_1) at 35% acetonitrile, and that from TPS_2 (CPS_2) at 37% acetonitrile. Incubation of CPS_1 and CPS_2 for 1 h at room temperature and pH 13 caused complete loss of the peaks at 35 and 37% acetonitrile, with the appearance of a peak at 15% acetonitrile. This result indicates that CPS_1 and CPS_2 contain steroid and are derived from the same peptide as CP. The peak eluting at 39% acetonitrile was unchanged by treatment with base.

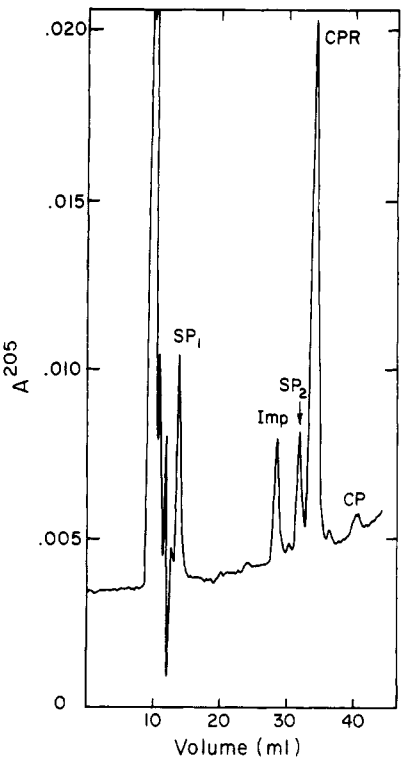


FIGURE 1: HPLC analysis of CPS_2 after treatment with sodium hydroxyborohydride (pH 11.9) for 1 h. A 40-μL aliquot of the reaction mixture was removed, the reaction was quenched by adding 20 μL of 1.0 N HCl, and 20 μL of this solution was injected. The peptides were eluted under a 20-min gradient from 10 to 20% aqueous acetonitrile with 6 mM phosphate buffer (pH 3) in the water and 1% 2-propanol throughout.

Hydroxyborohydride Treatment of Chymotryptic Peptides. Samples of the chymotryptic peptides CP, CPS_1, and CPS_2 were incubated at 0 °C with 0.18 M sodium hydroxyborohydride. Aliquots were removed and acidified to ca. pH 3 for HPLC analysis after 30 min, 1 h, and 18 h. Treatment of the steroid-bound peptides with hydroxyborohydride gave rise in each case to one major peptide peak, which eluted in the chromatogram at 13.5% acetonitrile. This peptide (CPR) was present after only 30 min and reached a maximum level after ca. 1 h. Figure 1 shows the chromatogram of a sample of CPS_2 after treatment with hydroxyborohydride for 1 h. Treatment of the unmodified chymotryptic peptide CP with hydroxyborohydride for up to 18 h produced no substantive change in the elution pattern. Amino acid analysis of the CPR samples from the reduction of both CPS_1 and CPS_2 showed a peak corresponding to homoserine, whereas the sample of CP reisolated after treatment with the reducing reagent for 1.5 h did not. None of these samples contained 5-hydroxynorvaline.

The amino acid compositions of the products of the reaction of hydroxyborohydride with CP, CPS_1, and CPS_2 are summarized in Table I. CPS_1 not treated with the hydroxyborohydride is included for comparison, as well as an analysis of CP. These latter peptide samples both gave an amino acid analysis consistent with residues 31–45 of the sequence (Benson et al., 1971; Ogez et al., 1977), as did the sample of CP isolated after treatment with hydroxyborohydride. This composition is also consistent with the predicted site of chymotrypsin cleavage of the tryptic peptide from which it was derived. The amino acid analyses obtained for the reduced steroid-bound peptides from CPS_1 and CPS_2 differed from the analyses for

CP	Ala-Asp-Asp-Ala-Thr-Val-Glu-Asp-Pro-Val-Gly-Ser-Glu-Pro-Arg
CPR from CPS_1	Ala-Asp-Asp-Ala-Thr-Val-Glu-Hse-Pro-Val-Gly-Ser-Glu-Pro
CPR from CPS_2	Ala-Asp-Asp-Ala-Thr-Val-Glu-Hse-Pro-Val
Residues 31-45	Ala-Asp-Asp-Ala-Thr-Val-Glu-Asp-Pro-Val-Gly-Ser-Glu-Pro-Arg

FIGURE 2: Sequence of sodium hydroxyborohydride reduced chymotryptic peptides from unmodified enzyme (CP) and of enzyme alkylated with **1**β (CPR). No signal was observed in cycle 15 for the sequence of CPR from CPS_1. The analysis of the CPR sample from CPS_2 was terminated after 10 cycles. Also shown is the sequence of residues 31–45, as reported by Ogez et al. (1977).

Table I: Amino Acid Analyses of Modified and Unmodified Chymotryptic Peptides

amino acid	untreated		BH_3OH^- treated[c,d]			
	CP[a]	CPS_1	CP	CPR from CPS_1	CPR from CPS_2	theoretical[e]
Arg	0.91	1.25	1.07	1.11	1.10	1
Ser	1.89	1.15	1.14	0.93	1.01	1
Hse				0.46	0.47	
Asp	2.74	2.94	3.05	1.97	1.80	3
Glu	2.22	1.86	1.94	2.23	2.14	2
Thr	0.99	0.68	1.04	1.28	1.14	1
Gly	2.56	0.93	0.93	1.10[d]	1.10	1
Ala	1.76	2.03	2.02	2.07	2.04	2
Pro	1.96	*b*	1.91	2.06	2.29	2
Val	2.42	2.14	2.26	1.92	1.93	2

[a] Analysis by Sequemat, Inc. This sample was analyzed in the initial phase of our work. Samples obtained at that time frequently were contaminated with trace impurities, leading to high values for Ser and Gly. [b] The relative amount of proline could not be calculated because the peak was obscured by dansylmethylamine present in analysis. [c] The relative amounts of amino acids of BH_3OH^--treated samples reflect the average of relative amounts calculated from two samples. [d] The glycine peak of one sample was obscured by dansylamide. [e] Residues 31–45 (Benson et al., 1971; Ogez et al., 1977).

Table II: Amino Acid Analyses of Other Products from Treatment of CPS_1 and CPS_2 with Sodium Hydroxyborohydride

amino acid	SP_1 from		SP_2 from	
	CPS_1	CPS_2	CPS_1	CPS_2
Arg	0.37	0.36		
Ser	1.17	1.06	1.25	1.33
Hse			0.44	0.77
Asp			1.76	2.04
Glu	1.02	1.06	1.60	1.59
Thr	0.37	0.34	0.73	1.00
Gly	0.95	0.89	0.96	1.06
Ala		0.39	2.59	2.66
Pro	0.86	0.73	1.45	1.78
Val	0.56	0.49	2.22	1.64

CP and unreduced CPS_1 in that the samples of CPR isolated from both CPS_1 and CPS_2 showed one residue less of aspartic acid and ca. 0.5 residue of homoserine.

Figure 2 shows the results of the sequence analysis of samples of CPR isolated after treatment of CPS_1 and CPS_2 with sodium hydroxyborohydride. Also shown is the sequence of a sample of CP reisolated after treatment with the reducing agent. The sequence of the CPR samples differs from that of CP only at position 8, corresponding to residue 38, where an aspartic acid of CP is replaced by a homoserine in the CPR samples.

At long reaction times of CPS_1 and CPS_2 with hydroxyborohydride, the peak at 13.5% acetonitrile decreased in size while other peaks in the chromatogram increased, most notably a peak that eluted near the solvent front (SP_1) and another that eluted at 13% acetonitrile (SP_2) (Figure 1). These products were isolated after 18-h treatment with hydroxyborohydride and were analyzed for their amino acid composition (Table II). The analyses for SP_1 and SP_2 are consistent with peptides derived from the chymotryptic peptide containing portions of residues 31–45. The peptide SP_1 has the composition Ser-Glu-Gly-Pro-Val, which corresponds to residues 39–43 or 40–44. The composition of SP_2 is consistent with residues 31–44 of the amino acid sequence, with one Asp being converted to Hse.

A peak (labeled Imp) eluting between 12 and 12.5% acetonitrile in the HPLC analyses of all hydroxyborohydride-treated samples was also present in blank runs. A sample of this peak was isolated for amino acid analysis and showed no amino acids above background.

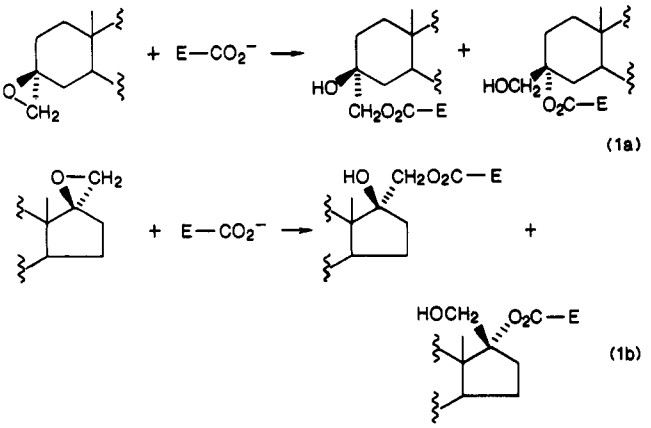
Lack of Reactivity of Sodium Hydroxyborohydride toward Model Amides. Several model amides were tested with hydroxyborohydride under conditions similar to those used with the peptide samples. No amine products were detected in gas chromatographic analyses of the simple amides acetanilide, methylbenzamide, and cyclohexanecarboxamide after treatment with hydroxyborohydride for up to 48 h, while the amount of amide was undiminished.

Discussion

Steroid–Peptide Ester Linkage of Chymotryptic Peptides. Previous reports from this laboratory have described the active site directed irreversible inactivation of 3-oxo-Δ^5-steroid isomerase by both 3β- and 17β-oxiranyl steroids (Pollack et al., 1979; Bevins et al., 1980, 1984, 1986; Kayser et al., 1983; Bantia et al., 1985). The reactions of the 3β- and 17β-oxiranes with the isomerase were shown to be remarkably similar. In each case the stoichiometry of the inactivation was determined to be 1 mol of inhibitor bound/mol of enzyme subunit (Kayser et al., 1983; Bevins et al., 1984), and the covalent bond formed during the inactivation of the enzyme by both series of inhibitors was shown to be an ester linkage (Kayser et al., 1983; Bevins et al., 1984; Bantia et al., 1985). Trypsin digestion of the enzyme inhibited with either of the oxiranes gave rise to two distinct steroid-bound tryptic peptides, both of which were shown to be derived from residues 14–45 of the reported sequence (Kayser et al., 1983; Bevins et al., 1984).

For the reaction of the isomerase with both the 3β- and the 17β-oxiranes, two different products were formed. Analysis of the products of base-catalyzed hydrolysis of enzyme inactivated with ^{18}O-labeled oxiranes has shown that these products result from attack of an enzymic carboxylate on the 2′-carbon and either the 3- or the 17-carbon (eq 1; Kayser et al., 1983; Bevins et al., 1984; Bantia et al., 1985). On the basis of the similarity of these two reactions, we proposed that the 17β-oxiranes modify the same amino acid as the 3β-oxiranes.

We have previously identified the amino acid modified by inactivation of the isomerase with 17β-oxiranes as aspartate-38 (Kayser et al., 1983). In that study, the chromophoric properties of those steroids provided a tag for identification of the steroid-bound peptides during the course of exhaustive proteolytic digestion. As **1**β does not contain a UV chromophore, it was necessary to find another way to trap the car-

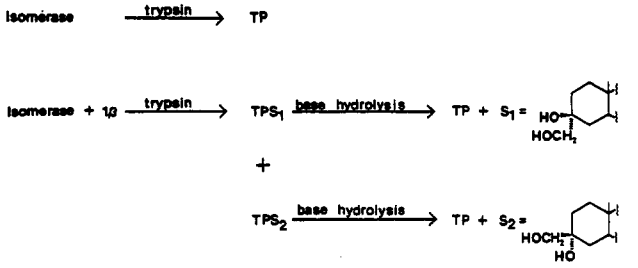


boxylate side chain involved in the reaction and render it identifiable. Although radioactive labeling of the steroid would allow monitoring of the digestion, protease-catalyzed hydrolysis of the ester linkage had been a complication during the study of the 17β-oxiranes and was a potential obstacle to the identification of the enzyme residue esterified by the 3β-oxiranes as well. Thus, we preferred a more general method that might also be applicable to other systems involving ester linkages and in which the lability of the ester bond would not be a problem.

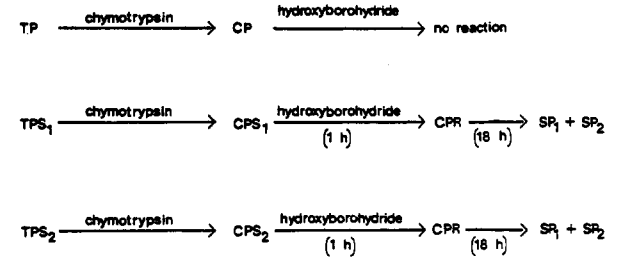
For more than 2 decades the classical approach to the identification of ester linkages in proteins has been to cleave the ester with hydroxylamine and to convert the resulting hydroxamic acid to a primary amine with the Lossen rearrangement, as described by Gallop et al. (1960). Blumenfeld and Gallop (1962) proposed the existence of both α- and β-linked aspartyl esters in collagen, using evidence obtained from hydroxylamine cleavage and the Lossen rearrangement. Gross and Morell (1966) used this method to show that the inactivation of pepsin by *p*-bromophenacyl bromide involves esterification of an aspartic acid residue. Feinstein et al. (1969) used the Lossen rearrangement to convert carboxylate residues of trypsin into amines. Meloche et al. (1978) identified the active site glutamic acid of 2-keto-3-deoxygluconate-6-phosphate aldolase specifically esterified by bromopyruvate using hydroxylamine cleavage and the Lossen rearrangement, and more recently, Harris and Wilson (1983) used this procedure to identify a glutamic acid in the active site of angiotensin I converting enzyme. Hydroxylamine, however, has been shown to catalyze the cleavage of certain peptide bonds, especially Asn–Gly bonds (Butler, 1969; Bornstein & Balien, 1970; Blodgett et al., 1985), and the conclusions of Blumenfeld and Gallop (1962) on collagen have been questioned, since collagen contains numerous Asn–Gly linkages. Additionally, poor yields of diaminobutyric and diaminopropionic acids and significant amounts of background conversion (Feinstein et al., 1969) limit the generality of this method.

An alternative approach for discriminating between aspartate and glutamate esters is reduction of the esterified protein, with conversion of the carboxylate side chain to a hydroxymethylene function. The involvement of an aspartate residue in the catalytic mechanism of pepsin has been determined by reduction of an esterified carboxylate with lithium borohydride in tetrahydrofuran (Gross & Morell, 1966), and Eshdat et al. (1974) have used sodium borohydride in aqueous solution to convert the specifically esterified aspartic acid-52 of lysozyme to homoserine. Sodium borohydride, however, is not a general reducing agent for esters, and in most examples in the literature describing reduction of esters with sodium borohydride the reactions were carried out in organic solvents at reflux temperatures (Brown & Rapoport, 1963; Brown et al., 1982; Soai et al., 1982). In addition, the insolubility of

Scheme I



Scheme II



many proteins and peptides in organic solvents limits the usefulness of these reagents. Thus, failure to reduce an ester linkage with lithium or sodium borohydride should probably not be interpreted as evidence against the presence of such a linkage. A more attractive reducing agent is one that is soluble in aqueous solution and will readily reduce esters in this medium.

In 1977, Reed and Jolly reported the facile reduction of esters in aqueous solution at 0 °C using the hydroxyborohydride ion, an intermediate in the stepwise hydrolysis of sodium borohydride (Gardiner & Collat, 1965). Utilizing this reagent, we have been able to convert the enzymic carboxylate side chain esterified by the 3β-oxiranyl steroid (3*S*)-spiro-[5α-androstane-3,2′-oxiran]-17β-ol to a hydroxymethylene function and to subsequently identify the chemically modified residue and its position in the amino acid sequence of the isomerase. Although we could not achieve satisfactory results using either hydroxylamine and the Lossen rearrangement or reduction with either sodium or lithium borohydride, reduction with sodium hydroxyborohydride was quite satisfactory.

We have previously reported that trypsin digestion of isomerase modified with **1**β produces two steroid-bound peptides (TPS_1 and TPS_2), each of which is derived from the peptide containing residues 14–45 of the enzyme (Bevins et al., 1984). Base-catalyzed hydrolysis of each of these peptides produced a different steroid, showing that there is isomerization at the steroid end of the linkage (Scheme I). We have now further digested the tryptic fragments with chymotrypsin and found that two peptides are produced in each case. One of these peptides, which eluted in HPLC at 39% acetonitrile, was also produced upon chymotrypsin digestion of the unmodified tryptic peptide TP and thus can be ruled out as containing steroid. The elution pattern of the other product varied in the three digests (CP at 15%, CPS_1 at 35%, and CPS_2 at 37% acetonitrile), indicating that both CPS_1 and CPS_2 contain steroid (Scheme II). This conclusion was verified by treating CPS_1 and CPS_2 with base; in each case base hydrolysis of the ester linkage released the steroids, giving rise to a product that coeluted with CP. Amino acid analysis of a sample of CP revealed that it is derived from residues 31–45 of the sequence, consistent with the chymotryptic cleavage sites reported for the isomerase by Benson et al. (1971). Sequence analysis of a sample of CP confirmed its identity. This portion of the isomerase sequence includes two disputed identifications.

Benson et al. (1971) reported asparagine at positions 32 and 38 while Ogez et al. (1977) found aspartic acid at these positions. Our analysis agrees with that of Ogez et al. (1977).

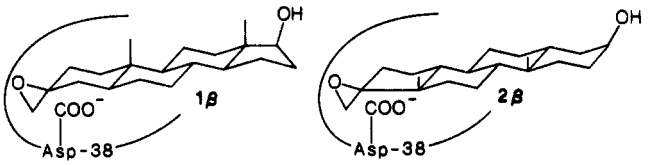
In order to trap the carboxylate side chain esterified by **1β**, samples of CPS_1 and CPS_2 were treated with sodium hydroxyborohydride. For **1β** bound to an aspartic acid residue, reduction of the ester linkage would yield homoserine. Similarly, a glutamic acid esterified by **1β** would be reduced to 5-hydroxynorvaline. The major product isolated from both of these reactions, CPR, differed in its amino acid composition from CP in the conversion of one residue of aspartic acid to homoserine, implicating an aspartic acid residue in the ester linkage. Sequence analyses of samples of CPR isolated from the reduction of CPS_1 and CPS_2 revealed that in both cases the aspartic acid residue at position 8 of the peptide, corresponding to aspartic acid-38 of the isomerase, had been converted to homoserine. Thus, the nucleophilic side chain of the isomerase that reacts with the spiro-oxiranyl group of **1β** to produce both products is aspartic acid-38.

Prolonged (18-h) treatment of both CPS_1 and CPS_2 with sodium hydroxyborohydride gave rise to two additional peptide products (SP_1 and SP_2) (Scheme II). Amino acid analysis revealed these products to be peptides composed of portions of residues 31–45 (SP_2 also contained homoserine). Two possible side reactions that could produce this result are (1) hydrolysis of the peptide backbone catalyzed by the alkaline reaction conditions and (2) reduction of some of the peptide bonds to amine functions, which would be impervious to subsequent acid hydrolysis. To investigate the possibility that sodium hydroxyborohydride reduces amide linkages, some simple organic amides were treated with the reducing reagent. In no case was any amine product detected after treatment of model amides with hydroxyborohydride for 18–48 h, suggesting that hydrolysis of the peptide bonds was responsible for the appearance of the additional products. By limiting the reaction time of the chymotryptic peptides to 1 h, we were able to optimize the yield of the desired reduction products.

The alkaline conditions (pH 11.9) employed in the reduction of the esterified peptides might lead to the expectation that base-catalyzed hydrolysis of the ester linkage would be a major complication in the procedure. While in preliminary experiments as much as 50% hydrolysis was observed, experience in preparation of the reductant and scrupulous care in maintaining the reaction temperature at 0 °C eliminated most of the problem, with less than 5% of the reactions reported here being attributable to ester hydrolysis.

Significance of Aspartic Acid-38 as the Site of Reaction with the 3β-Oxiranyl Steroid. We have previously described the mechanism of reaction of the steroid isomerase with 3β- and 17β-oxiranyl steroids, showing that a carboxylate of the enzyme reacts at the α-face of the steroid nucleus for both types of inhibitors (Bevins et al., 1984; Bantia et al., 1985). Two products result in each case, one from attack of the carboxylate at the oxiranyl 2′-carbon and the other from attack at the spiro 3- or 17-carbon. For enzyme inactivated with the 17β-oxirane (17*S*)-spiro[4-androstene-17,2′-oxiran]-3-one, the site of attachment at both the 2′- and 17-carbons was found to be aspartic acid-38 (Kayser et al., 1983). These results show that the residue alkylated by the 3β-oxirane (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol, at both the 2′- and 3-carbons, is also aspartic acid-38.

On the basis of the similarity in the mechanism of inactivation by the 3β- and 17β-oxiranes, such as **1β** and **2β**, we have postulated (Bevins et al., 1980, 1984; Bantia et al., 1985) that the bacterial isomerase can bind steroids in two modes: (1) with the A ring proximal to the catalytic machinery of the isomerase and (2) backward, with the D ring in the normal A-ring binding site. Other lines of evidence support the



backward binding hypothesis. An analogue of the competitive inhibitor equilenin, containing the 17β-oxiranyl functional group, exhibits changes in its fluorescence (Bevins et al., 1980) and UV spectra (Bevins et al., 1986) consistent with two binding modes. These changes can be explained by initial reversible binding of the oxirane in the A-ring mode, causing spectral changes that decay as the steroid ultimately binds in the D-ring mode, irreversibly inactivating the enzyme. The finding that the 3β-oxirane **1β** inactivates the isomerase via esterification of the same residue modified in the inactivation by the 17β-oxiranes is definitive evidence that two modes of steroid binding operate in the interaction of the isomerase with its steroid ligands.

Backward binding has also been proposed for other steroid transforming enzymes. Sweet and Samant (1980) and Strickler et al. (1980) have reported dual activity at a single steroid binding site for the enzyme 3α,20β-hydroxysteroid dehydrogenase and have proposed two modes of binding that differ by a 180° longitudinal rotation about an axis parallel to the steroid nucleus. Our results for the isomerase, on the other hand, require that the two modes of binding differ by rotation about an axis perpendicular to the steroid nucleus as the isomerase reacts with both the 3β- and 17β-oxiranes at the α-face of the steroid.

Studies of the X-ray crystal structures of isomeric 3β- and 17β-oxiranyl steroids (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17β-ol (**1β**) and (17*S*)-spiro[5α-androstane-17,2′-oxiran]-3β-ol (**2β**) show that the oxiranyl oxygens of both inhibitors occupy positions relative to the plane of the steroid nucleus similar to that of the 3-oxo group of the substrate 5-androstene-3,17-dione.[1] This similarity has important implications for the catalytic mechanism of the isomerase and the mechanism of its inactivation by the oxiranyl steroids. Malhotra and Ringold (1965) and Viger et al. (1981) have proposed that there is an acidic residue at the active site that protonates the 3-oxo group of substrate. Such a residue should also be capable of protonating the epoxide oxygen of the 3β- and 17β-oxiranes, which are otherwise quite inert toward nucleophilic attack (Cook et al., 1968), activating them toward ring opening. The similarity in position of the oxiranyl oxygens of the 3β- and 17β-oxiranes as well as the similar overall shape of **1β** and "backward" **2β** suggests how the isomerase might be fooled into recognizing the A and D rings of the steroidal oxiranes interchangeably.

Recently we reported the pH dependence of the isomerase for substrate catalysis and for inhibition by the 3β-oxirane (3*S*)-spiro[5α-androstane-3,2′-oxiran]-17-one (**7β**) (Pollack et al., 1986). The pH–rate profiles for both the isomerization and the inactivation reactions generated similar values for the p*K*'s of the free enzyme and of the enzyme–substrate/inhibitor complex, suggesting that both reactions may be governed by the same catalytically important carboxylate side chain (Pollack et al., 1986). The finding that aspartate-38 of the

[1] S. Kashino, H. Katz, J. P. Glusker, R. M. Pollack, and P. L. Bounds, unpublished results.

isomerase is the residue esterified by **1β**, which differs from **7β** only in the oxidation state of the substituent at the 17-position, clearly identifies this catalytically important residue. We have suggested that this carboxylate is located at the α-face of the bound steroid and that it promotes catalysis by electrostatic stabilization of protonated ketone intermediates (Bevins et al., 1984). The rationale for this mechanism is based partly upon the fact that the enzyme is not inactivated by the corresponding 3α-oxiranes (Pollack et al., 1979), indicating that the isomerase can distinguish between the α- and β-faces of bound steroids. Recent X-ray work of Westbrook,[2] however, suggests that this assumption may be incorrect and that aspartate-38 may, in fact, be able to function as the proton-transfer agent in the isomerization.

Linden and Benisek (1986) have reported the amino acid sequence of the 3-oxo-Δ^5-steroid isomerase from *Pseudomonas putida*, demonstrating only 34% overall homology between the related *putida* and *testosteroni* enzymes. However, 100% homology was found in a region containing aspartate-38, residues 33–41. These authors suggest that this single highly conserved portion within these enzymes must be important for catalysis.

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Registry No. **1β**, 2066-43-5; EC 5.3.3.1, 9031-36-1; L-Asp, 56-84-8; $NaBH_3OH$, 16903-32-5.

REFERENCES

Bantia, S., Bevins, C. L., & Pollack, R. M. (1985) *Biochemistry 24*, 2606–2609.

Batzold, F. H., Benson, A. M., Covey, D. F., Robinson, C. H., & Talalay, P. (1976) *Adv. Enzyme Regul. 14*, 243–267.

Benson, A. M., Jarabak, R., & Talalay, P. (1971) *J. Biol. Chem. 246*, 7514–7525.

Bevins, C. L., Kayser, R. H., Pollack, R. M., Ekiko, D. B., & Sadoff, S. (1980) *Biochem. Biophys. Res. Commun. 95*, 1131–1137.

Bevins, C. L., Bantia, S., Pollack, R. M., Bounds, P. L., & Kayser, R. H. (1984) *J. Am. Chem. Soc. 106*, 4957–4962.

Bevins, C. L., Pollack, R. M., Kayser, R. H., & Bounds, P. L. (1986) *Biochemistry 25*, 5159–5164.

Blodgett, J. K., Loudon, G. M., & Collins, K. D. (1985) *J. Am. Chem. Soc. 107*, 4305–4313.

Blumenfeld, O. O., & Gallop, P. M. (1962) *Biochemistry 1*, 947–959.

Bornstein, P., & Balien, G. (1970) *J. Biol. Chem. 245*, 4854–4856.

Brown, H. C., Narasimhan, S., & Choi, Y. M. (1982) *J. Org. Chem. 47*, 4702–4708.

Brown, M. S., & Rapoport, H. (1963) *J. Org. Chem. 28*, 3261–3263.

Butler, W. T. (1969) *J. Biol. Chem. 244*, 3415–3417.

Cook, C. E., Corley, R. C., & Wall, M. E. (1968) *J. Org. Chem. 33*, 2789–2793.

Eshdat, Y., Dunn, A., & Sharon, N. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1658–1662.

[2] E. M. Westbrook, personal communication.

Feinstein, G., Bodlaender, P., & Shaw, E. (1969) *Biochemistry 8*, 4949–4955.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, New York.

Ford, H. C., & Engel, L. L. (1974) *J. Biol. Chem. 249*, 1363–1368.

Gallop, P. M., Siefter, S., Lukin, M., & Meilman, E. (1960) *J. Biol. Chem. 235*, 2619–2627.

Gardiner, J. A., & Collat, J. W. (1965) *Inorg. Chem. 4*, 1208–1212.

Gaudry, R. (1951) *Can. J. Chem. 29*, 544–551.

Gross, E., & Morell, J. L. (1966) *J. Biol. Chem. 241*, 3638–3642.

Harris, R. B., & Wilson, I. B. (1983) *J. Biol. Chem. 258*, 1357–1362.

Hearne, M., & Benisek, W. F. (1985) *Biochemistry 24*, 7511–7516.

Hoare, D. G., Olson, A., & Koshland, D. E., Jr. (1968) *J. Am. Chem. Soc. 90*, 1638–1643.

Kayser, R. H., Bounds, P. L., Bevins, C. L., & Pollack, R. M. (1983) *J. Biol. Chem. 258*, 909–915.

Linden, K. G., & Benisek, W. F. (1986) *J. Biol. Chem. 261*, 6454–6460.

Malhotra, S. K., & Ringold, H. J. (1965) *J. Am. Chem. Soc. 87*, 3228–3236.

Martyr, R. J., & Benisek, W. F. (1975) *J. Biol. Chem. 250*, 1218–1222.

Meloche, H. P., Monti, C. T., & Hogue-Angeletti, R. A. (1978) *Biochem. Biophys. Res. Commun. 84*, 589–594.

Murota, S., Fenselau, C. C., & Talalay, P. (1971) *Steroids 17*, 25–37.

Ogez, J. R., Tivol, W. F., & Benisek, W. F. (1977) *J. Biol. Chem. 252*, 6151–6155.

Penning, T. M., & Talalay, P. (1981) *J. Biol. Chem. 256*, 6851–6858.

Penning, T. M., Covey, D. F., & Talalay, P. (1981) *J. Biol. Chem. 256*, 6842–6850.

Penning, T. M., Heller, D. N., Balasubramanian, T. M., Fenselau, C. C., & Talalay, P. (1982) *J. Biol. Chem. 257*, 12589–12593.

Pollack, R. M., Kayser, R. H., & Bevins, C. L. (1979) *Biochem. Biophys. Res. Commun. 91*, 783–790.

Pollack, R. M., Bantia, S., Bounds, P. L., & Kaufman, B. M. (1986) *Biochemistry 25*, 1905–1911.

Reed, J. W., & Jolly, W. L. (1977) *J. Org. Chem. 42*, 3963–3965.

Reed, J. W., Ho, H. H., & Jolly, W. L. (1974) *J. Am. Chem. Soc. 96*, 1248–1249.

Soai, K., Oyamada, H., & Ookawa, A. (1982) *Synth. Commun. 12*, 463–467.

Strickler, R. C., Covey, D. F., & Tobias, B. (1980) *Biochemistry 19*, 4950–4954.

Sweet, F., & Samant, B. R. (1980) *Biochemistry 19*, 978–986.

Talalay, P., & Benson, A. M. (1972) *Enzymes (3rd Ed.) 6*, 591–618.

Tapuhi, Y., Schmidt, D. E., Lindner, W., & Karger, B. L. (1981) *Anal. Biochem. 115*, 123–129.

Viger, A., Coustal, S., & Marquet, A. (1981) *J. Am. Chem. Soc. 103*, 451–458.

Westbrook, E. M., & Sigler, P. B. (1984) *J. Biol. Chem. 259*, 9090–9095.

Westbrook, E. M., Piro, O. E., & Sigler, P. B. (1984) *J. Biol. Chem. 259*, 9096–9103.