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AN ACTIVE-SITE-DIRECTED IRREVERSIBLE INHIBITOR OF Δ^5-3-KETOSTEROID ISOMERASE

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SUMMARY

In short-term experiments, 6β-bromotestosterone acetate is a competitive inhibitor of the crystalline Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni*. Upon prolonged incubation, 6β-bromotestosterone acetate causes a time- and concentration-dependent irreversible inactivation of the enzyme which is favored at elevated pH values. Protection against this inactivation is afforded by the simultaneous presence of 19-nortestosterone, a truly competitive inhibitor of the enzyme. Upon incubation with radioactive 6β-bromotestosterone 17-[^{3}H]acetate, the enzyme becomes labeled, and under suitable conditions more than one mole of steroid is bound to each mole of enzyme. These findings suggest that 6β-bromotestosterone 17-acetate is an active-site-directed irreversible inhibitor of Δ^5-3-ketosteroid isomerase. At least three species of inactive enzyme separable by polyacrylamide gel electrophoresis are formed during the inactivation process. It seems likely that these species reflect the binding of progressively increasing numbers of steroid molecules, and that eventually the labeled enzyme dissociates into its subunits.

INTRODUCTION

The crystalline Δ^5-3-ketosteroid isomerase (EC 5.3.3.1) of *Pseudomonas testosteroni* is a catalyst of special interest from a mechanistic viewpoint[1]. This enzyme promotes the conversion of a number of $\Delta^{5(6)}$- and $\Delta^{5(10)}$-3-ketosteroids to their corresponding Δ^4-3-ketosteroids by a direct intramolecular *cis* diaxial proton transfer from the C-4β to the C-6β position[1,2]. The protein, which has a molecular weight of approximately 40 800, consists of identical chains, probably three in number, each containing 125 amino acid residues[3,4]. Cyst(e)ine and tryptophan are absent, and there appears to be no prosthetic group. The catalytic activity is extraordinarily high (molecular turnover of $17.6 \cdot 10^6$ per min at 25° and pH 7.0). A number of phenolic steroids and 19-nortestosterone are very effective competitive inhibitors of the enzymatic isomerization of Δ^5-androstene-3,17-dione. Evidence based on spectral examination of the binding of inhibitors and on isotope exchange measurements sug-

gests that the reaction proceeds by way of an enolic intermediate[5]. More recent information supports the possibility of the participation of a histidine residue at the active site, and a model involving the alternate proton acceptor and proton donor functions of the tautomeric nitrogens of the imidazole ring has been proposed[1]. The primary sequence determination of the enzyme is nearing completion[3,4]. The finding of an active-site-directed irreversible inhibitor, 6β-bromotestosterone acetate, provides an approach to the delineation of the active site of the enzyme. This paper describes the inhibition and inactivation of Δ^5-3-ketosteroid isomerase by 6β-bromotestosterone acetate and supplies evidence that the steroid molecule becomes covalently bound to the enzyme. Preliminary information on the number of moles of steroid bound to the enzyme, and the consequences of this interaction on the tertiary structure of the protein, is described.

METHODS

Polyacrylamide gel electrophoresis

The electrophoresis was carried out in 9% gels (65 cm × 6 mm) in glass tubes according to the general procedure of DAVIS[6,7]. The gels were prepared by mixing the following: 2.4 ml of an aqueous solution of 30% acrylamide (Eastman 5521) and 0.8% *N,N'*-methylenebisacrylamide (Canalco 202); 1 ml of 0.30 M Tris–HCl gel buffer, pH 9.0; 5 μl of *N,N,N'N'*-tetramethylenediamine (Eastman 8178); 4.5 ml of water; and 0.1 ml of 10% $(NH_4)_2S_2O_8$ solution (Fisher). The sample (25–50 μl) was mixed in the space above the vertical gel with the following: 0.125 vol. (3–6 μl) of sample buffer (25.6 ml of 1 M phosphoric acid and 5.7 g of Tris base in 100 ml of H_2O, final pH 8.6); 50 μl of 30% sucrose in eight-fold diluted sample buffer; and 1 μl of 1.0% bromphenol blue, as tracking dye. The electrode buffer contained 0.6 g of Tris base and 2.88 g of glycine per l (pH 8.3). The samples were separated by electrophoresis at an applied current of 4 mA per gel for 2 h. The gels were stained with amido black 10 B (1.0 g in 100 ml of 7% acetic acid), and destained electrophoretically in 7% acetic acid.

Preparation of enzyme and assay of activity

The enzyme was prepared and crystallized as described previously[8]. The crystals were collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer, pH 7, and dialyzed against the same buffer. The clear enzyme solution was kept frozen until used. The enzyme concentration was determined from measurement of the absorbance at 280 nm, assuming that a solution containing 1.0 mg of protein per ml has an absorbance of 0.413. The molecular weight was assumed to be 40 800. Enzyme assays were carried out in systems of 3.0 ml volume containing: 100 μmoles of potassium phosphate buffer, pH 7.0; 0.175 μmole of Δ^5-androstene-3,17-dione in 0.05 ml methanol, and enzyme diluted in neutralized 1% bovine serum albumin. Absorbance measurements were made at 248 nm on a Gilford Model 2000 linear absorbance recorder and the activities determined from the initial linear portions of the curves, assuming ε_M of Δ^4-androstene-3,17-dione is 16 300 $M^{-1} \cdot cm^{-1}$ at 248 nm in water. The crystalline enzyme used for these studies converted 55 200 μmoles of substrate per mg of protein per min under the specified conditions.

Determination of enzyme-bound radioactivity

Sephadex G-25 columns were used for rapid separation of free 6β-bromotestosterone acetate from that bound by the enzyme. The columns (248 mm × 8 mm) were equilibrated with 0.01 M potassium phosphate buffer, pH 7, and eluted with the same buffer. Samples (25–50 μl) of the reaction mixture were applied directly to the columns, and fractions of 1 ml were collected. The enzyme was eluted in Fraction 5 or 6 (void volume of the column was 6 ml as determined with Dextran blue 2000). The radioactivity was determined on 1 ml of the effluent in 10 ml of BRAY's[9] solution on a Beckman Model LS-133 scintillation counter.

Localization of enzymatic activity and radioactivity on polyacrylamide gels

Immediately after electrophoresis the gels were removed from the glass tubes and sectioned lengthwise into two halves by cutting with a wire. For location of the protein band one half of the gel was stained with amido black 10B for 1 h and destained with 7% acetic acid overnight. The relative densities of the protein bands were determined with a Gilford 2000 linear absorbance recorder and a Gilford Model 2410 linear transport attachment for scanning gels. Once the protein bands had been located, the section of the duplicate half of the gel was cut into 1.27-mm slices. Each slice was agitated for 2 h with 1.0 ml of bovine serum albumin (10 mg/ml, neutralized with NaOH), and an aliquot removed for enzymatic assay. The radioactivity of slices could be reproducibly measured by shaking each slice with 1.0 ml of 0.01 M potassium phosphate (pH 7.0) for 3 h and then adding 10 ml of BRAY's solution.

Preparation of 6β-bromotestosterone acetate

Androsta-3,5-diene-3,17-diol diacetate[10]. 1 g of testosterone and 0.1 g of *p*-toluenesulfonic acid were dissolved in 10 ml of acetic anhydride. The reaction mixture was kept at 90° for 1 h, after which it was poured onto ice. When the ice melted and the acetic anhydride had decomposed, the mixture was extracted three times with equal volumes of ether. The combined ether extract was washed with several portions of saturated $KHCO_3$ solution (until it was acid-free) and twice with an equal volume of water. It was then dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was crystallized from ethanol after treatment with charcoal. After a second recrystallization the material showed a melting point of 136–144° (literature: 150–153°) (ref. 10) and $\lambda_{max}^{ethanol}$ 235 nm (ε_M = 20 000 $M^{-1} \cdot cm^{-1}$) and was homogeneous by thin-layer chromatography.

Bromination of androsta-3,5-diene-3,17-diol diacetate[11,12]. The pure enol acetate (430 mg) obtained above was dissolved in 17 ml of ether and added to a solution of 1.7 g of potassium acetate in 31 ml of 85% acetic acid, and the mixture was cooled to 0°. While stirring, 185 mg of bromine in 5.2 ml of glacial acetic acid were added dropwise over a 5-min period and after a further 5 min of stirring the reaction mixture was partially evaporated *in vacuo*, poured into ice water, and extracted three times with ether. The combined extract was washed with saturated $KHCO_3$ until neutral, then washed twice with half its volume of water, and dried over anhydrous Na_2SO_4 and evaporated. The dry residue was crystallized from ether-hexane. After two recrystallizations, the melting point was 120–127° (literature: 125–127°) (ref. 13) (Kofler block) and $\lambda_{max}^{ethanol}$ 248 nm (ε_M = 13 600 $M^{-1} \cdot cm^{-1}$).

The identity of the product was confirmed by NMR and mass spectrometry.

The NMR spectrum determined in [^{2}H]chloroform on a Varian HA 100 instrument gave the following signals (δ units, trimethylsilyl = 0): 0.92 (C-18 CH_3); 1.56 (C-19 CH_3); 2.05 (acetate CH_3) and a well-defined band attributable to vinylic proton at 5.92 with a band width of 1.8 cps. The last findings are consistent with the presence specifically of a 6β-bromo substituent and the absence of a 6β-proton[14,15].

Mass spectral analysis was carried out by direct probe inlet (70 V; 200°) into a Consolidated Electrodynamics Corporation Model 21-110 instrument. Molecular ions were detected at *m/e* = 408 and 410 in a 1:1 ratio, as required for a compound containing one atom of Br in its natural isotopic distribution. A prominent peak at *m/e* 329 is ascribed to loss of Br. A signal at *m/e* 287 reflects subsequent loss of 42 mass units, as would be expected from either the 17-acetate (elimination of C_2H_2O) or the A ring with its Δ^4-3-keto group (loss of C_3H_6). The last prominent peak in the high mass range occurs at *m/e* 269 and corresponds to a fragment ion formed by loss of bromine and acetic acid. The mass spectrum thus provides evidence for presence of one atom of bromine and one acetate group.

Preparation of 6β-bromotestosterone 17-[^{3}H]acetate. The preparation of this compound was carried out in a Y-shaped Pyrex tube of which only one arm was sealed initially. A chloroform solution of 25 mg of testosterone and 1 mg of *p*-toluenesulfonic acid was introduced into the sealed arm, and dried out as a thin film. An ampoule containing 25 mC of ^{3}H-labeled acetic anhydride (100 mC/mole; Amersham/Searle) was sealed into the second arm, and the system was evacuated and sealed. The seal of the ampoule of [^{3}H]acetic anhydride was broken under vacuum with the aid of a magnet and the acetic anhydride was driven into the steroid by cooling the arm containing the steroid and warming the rest of the system. The acetylation was allowed to proceed for 1 h at 90° in an oven. After the reaction was complete, the unreacted acetic anhydride and the acetic acid produced were driven back from the reaction arm, leaving a brown residue. The tube was opened and the reaction mixture was dissolved in carbon tetrachloride and applied to a dry column of Silica gel G (for dry column chromatography; Woelm). The column was eluted with hexane–ethyl acetate (33:17, v/v) and collected in fractions. Portions were dried out on Silica GF_{254} plates for monitoring the products by ultraviolet absorption. The first ultraviolet absorbing compound eluted from the column was the desired intermediate androsta-3,5-diene-3,17-diol 3,17-[^{3}H]diacetate. After the solvent had been evaporated, 17.6 mg of crystalline residue were obtained. After dilution with 40 mg of unlabeled androsta-3,5-diene-3,17-diol 3,17-diacetate, the mixture was dissolved in 3 ml of ether, to which were added 240 mg of potassium acetate and 4 ml of 85% acetic acid in water, preparatory to the bromination. A solution of bromine (17 mg) in 0.7 ml of glacial acetic acid was added dropwise with stirring to the mixture maintained at 0°. 10 min later the reaction mixture was diluted with 5 ml of water and extracted 4 times with 5-ml aliquots of ether. The ether extracts were combined, washed 4 times with 5-ml portions of saturated $KHCO_3$ and then twice with 5-ml aliquots of water, and evaporated. The dry residue (75 mg) was crystallized from a mixture of ether–hexane. After three further recrystallizations the 6β-bromotestosterone 17-[^{3}H]acetate was dissolved in dry benzene (1 mg/ml) and stored at −20°. The compound showed an absorption maximum of 248 nm in ethanol and had a specific activity of 30.5 μC/μmole. The radioactive material was homogeneous on thin layer chromatography but was not further characterized.

RESULTS AND DISCUSSION

A number of steroid derivatives potentially capable of alkylating nucleophilic groups of the isomerase were examined. Preliminary experiments revealed that the incubation of the enzyme with two bromosteroids susceptible to attack by nucleophilic groups caused significant and irreversible inactivation of the enzyme: 2ξ,6ξ-dibromoandrost-4-ene-3,17-dione and 6β-bromotestosterone 17-acetate. The latter compound, selected for intensive further study because it is a single well-characterized compound of known steric configuration, was a somewhat more powerful inhibitor than 2ξ,6ξ-dibromotestosterone (a mixture of isomers), and could be easily prepared at high specific activity with ^3H in the acetate moiety.

Influence of pH on inactivation of isomerase

The H^+ concentration of the medium has a marked effect on the rate of inactivation of the isomerase by 6β-bromotestosterone acetate. Fig. 1 shows that in the

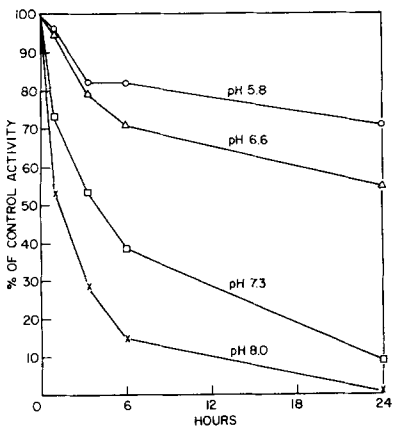


Fig. 1. Effect of pH on the inactivation of Δ^5-3-ketosteroid isomerase by 6β-bromotestosterone acetate. Reactions, which were carried out at 25° in the dark, contained the following components in final volumes of 0.2 ml: 100 mM potassium phosphate buffers at the pH values indicated; 1.2 μM isomerase; 50 μM 6β-bromotestosterone acetate in 2 μl of dioxane. Aliquots were withdrawn at stated intervals, diluted with 1% bovine serum albumin, and assayed under standard conditions (pH 7.0). The final concentration of 6β-bromotestosterone acetate in the assay cuvettes was negligible. The activity at each time point and pH was related to that of a control vessel which contained dioxane but no 6β-bromotestosterone acetate. The controls did not vary in activity by more than 10% over the time course of the experiment.

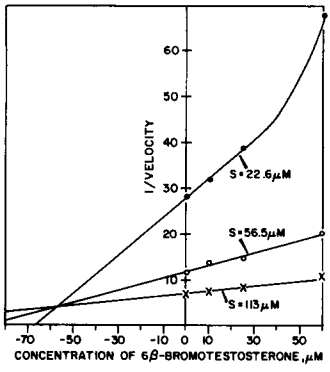
presence of a molar ratio of inhibitor to enzyme of 40, the loss of enzyme activity in 24 h at 25° was 29% at pH 5.8 and 99% at pH 8.

The results are compatible with the possibility that a nucleophilic group which is unprotonated is required for most efficient reaction, and a more detailed analysis of the reaction rates suggests that its pK_a is in the neighborhood of 7. At pH 8, which was selected for further experiments, and at room temperature in buffered aqueous solution, 6β-bromotestosterone acetate is relatively stable, and over a period of many hours it does not undergo any changes detectable on thin layer chromatography. The experiments recorded in Fig. 1 (together with similar experiments not shown)

also strongly suggest that the inhibition is irreversible, since in carrying out the assays, aliquots of the enzyme are diluted more than 100 000-fold so that the final concentration of 6β-bromotestosterone acetate in the assay cuvette is negligible.

Short-term inhibition experiments

Since under the conditions selected for study of inhibition both enzyme and steroid (by reason of solubility) are dilute, the rates of irreversible inhibition of the enzyme are relatively slow (Fig. 1) and 50% inhibition is attained only after several hours have elapsed. Thus it is possible to obtain some measure of the affinity of the 6β-bromotestosterone acetate for the enzyme in short-term experiments in which both substrate and inhibitor are present in the assay cuvette simultaneously, and the enzyme is used to initiate the reaction. Measurements of the initial reaction velocity (over the first 5 min period) were made at a range of concentrations of Δ^5-androstene-3,17-dione and of 6β-bromotestosterone acetate, but the system differed from the conventional assay system by being at pH 8.0 rather than at pH 7.0. The measurements were analyzed according to the graphical method of DIXON[16] (Fig. 2). The pattern was consistent with a competition between the substrate and the inhibitor, and an approximate K_i value for 6β-bromotestosterone of 55–60 μM. Calculation of



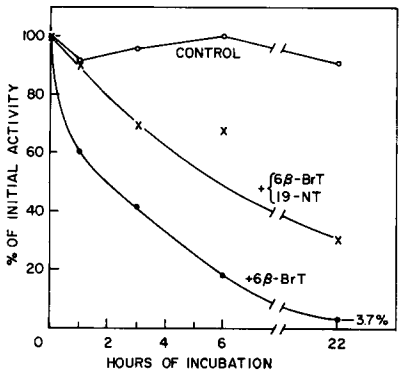


Fig. 2. Short-term measurements of the inhibition of Δ^5-3-ketosteroid isomerase by 6β-bromotestosterone acetate. The initial velocity of the isomerization reaction was measured in systems with a final volume of 3.0 ml containing: 33 mM potassium phosphate buffer, pH 8.0; 22.6, 56.5, or 113 μM Δ^5-androstene-3,17-dione in 0.1 ml of methanol; 10, 25, or 60 μM 6β-bromotestosterone acetate in 0.06 ml of dioxane; and $6 \cdot 10^{-5}$ μg of crystalline isomerase (not diluted with albumin) which was used to initiate the reaction. Initial rates of change of absorbance at 248 mμ were determined at 25° against blanks containing all ingredients except substrate. The reciprocal of the absorbance change in 5 min is plotted with respect to inhibitor concentration (μM) at various levels of substrate(s) in accordance with DIXON[16]. The K_i of 6β-bromotestosterone acetate determined from the graphic presentation is 57 μM.

Fig. 3. Inactivation of Δ^5-3-ketosteroid isomerase by 6β-bromotestosterone 17-acetate (6β-BrT) and protection by 19-nortestosterone (19-NT). Reaction vessels contained the following components in a final volume of 1 ml: 100 mM potassium phosphate buffer, pH 8.0; 4 μM isomerase; and 73 μM 6β-bromotestosterone acetate in 40 μl of dioxane with or without 100 μM 19-nortestosterone. The control vessel contained the dioxane but no steroids. The vessels were stored at 25° in the dark and aliquots were removed at indicated times, diluted into 1% neutralized bovine serum albumin solution, and assayed for activity. The activities are expressed as percentages of values at time 0. Independent controls showed that 19-nortestosterone did not affect the enzyme activity under these conditions. The steroids were so extensively diluted for the assay that essentially negligible concentrations were present in the assay cuvettes.

the K_m for Δ^5-androstene-3,17-dione from the limited measurements obtained gave a value similar to that previously reported at pH 7.0 (320 μM). It would be of interest to study the effect of pH on both K_m and K_i values which might provide information whether the greater rate of inactivation of the enzyme at higher pH (Fig. 1) is the result of a greater affinity for the inhibitory steroid rather than a favorable ionization state of the group on the enzyme that is being alkylated. Examination of Fig. 2 shows that there is significant deviation from linearity in the DIXON[16] plots at the highest concentrations of inhibitor and lowest concentration of substrate. Since these conditions would tend to favor irreversible inhibition, it seems likely that deviation from linearity may be due to irreversible inhibition, although unequivocal proof for this supposition has not been obtained.

Protection against inactivation by 6β-bromotestosterone acetate with 19-nortestosterone

Evidence for the interaction of 6β-bromotestosterone acetate with the steroid binding sites of the isomerase was also provided in an experiment in which a considerable degree of protection of the isomerase against inactivation was achieved by including 19-nortestosterone in the reaction system. It has been shown previously[5] that 19-nortestosterone (K_i = 5.2 μM) binds relatively tightly to the isomerase, in a manner competitive with the substrate, and that a spectral shift of the chromophore of the inhibitor occurs when it combines with stoichiometric quantities of the enzyme. In the presence of 73 μM 6β-bromotestosterone acetate about 80% of the enzyme activity was destroyed in 6 h at 25° and pH 8.0, whereas if 100 μM 19-nortestosterone was present simultaneously, the loss in activity was reduced to about 50% (Fig. 3).

Incorporation of 6β-bromotestosterone 17-[^{3}H]acetate into isomerase protein

Proof that the inhibitor molecules become firmly attached, presumably in a covalent manner, to the enzyme protein was obtained by demonstrating that the incorporation of radioactive 6β-bromotestosterone acetate into the protein was concentration-dependent and that the label could not be removed by gel filtration. Crystalline isomerase was incubated at pH 8.0 and 25° for 25 h with three concentrations of 6β-bromotestosterone acetate which were considerably lower than those used in the experiments described above. The rate of loss of isomerase activity increased with the concentration of inhibitor. At levels of 6β-bromotestosterone acetate equal to, twice, or 4 times the amount of enzyme, the residual enzyme activity was, respectively, 83.5, 76.1, and 43% of the initial activity. Aliquots of the reaction mixture were subjected to gel filtration on Sephadex G-25 columns and the quantity of free and enzyme-bound radioactivity was determined. It can be seen from Table I that the quantity of radioactive steroid present in the macromolecular fraction increased with the concentration of the inhibitor. If the ratio of the amount of radioactive steroid bound to the total amount of enzyme is computed, the averages of 0.41, 0.82, and 1.89 moles of steroid per mole of total amount of enzyme protein added are obtained at the 3 different concentrations of steroid. Since all the reaction systems still have sizable quantities of fully active enzyme (see below), the reasonable assumption is made that the radioactive steroid is largely bound to inactive enzyme, the proportion of the latter may be computed from the activity measurements. The number of moles of steroid bound per mole of inactive enzyme is computed to be 2.4, 3.4, and 3.3 moles, respectively, for the three concentrations of the inhibitor.

TABLE I

EFFECT OF VARYING CONCENTRATION OF 6β-BROMOTESTOSTERONE 17-[^{3}H]ACETATE ON INCORPORATION OF LABEL INTO ISOMERASE

Reactions were carried out in final volumes of 0.50 ml containing 12.5 nmoles of crystalline isomerase, 50 μmoles of potassium phosphate, pH 8.0, and 20 μl of dioxane containing the indicated amounts of 6β-bromotestosterone 17-[^{3}H]acetate (specific activity, 6800 counts/min per nmole). Incubations were conducted at 25° for 25 h in the dark. The separation of free from enzyme-bound radioactive steroid was accomplished on Sephadex G-25 columns as described under METHODS. 25 μl aliquots of reaction mixture were applied to each Sephadex G-25 column.

Reaction mixture	*Final enzyme activity (% of control)*	*Steroid added (nmoles)*	*Enzyme-bound steroids (nmoles)*	*Free steroid (nmoles)*	*Total steroid recovered* (nmoles)*	*Molar ratio of bound steroid per mole of total enzyme*
1	83.5	10.6	5.09	5.82	10.9	0.41
2	76.1	20.9	10.3	11.3	21.6	0.82
3	43	48.2	23.6	26.6	50.2	1.89

* The amount of steroid recovered is based on the sum of the radioactivities found in Sephadex fractions containing free and enzyme-bound steroid.

On the basis of either calculation, it may be concluded that more than 1 mole of inhibitor can become bound to each mole of enzyme. Since the gel electrophoresis findings to be presented below reveal the existence of at least 3 different species of protein containing radioactive steroid, it is not possible at this point to draw conclusions on the number of sites to which the radioactive inhibitor is bound, or on the relation of the number of steroid molecules bound to the enzymatic activity of the particular protein species.

Enzymatic activity and electrophoretic behavior of isomerase species obtained after treatment with 6β-bromotestosterone 17-acetate

Examination of the products of the interaction of isomerase with 6β-bromo testosterone acetate was undertaken with the aid of polyacrylamide gel electrophoresis under the conditions described under METHODS. The native enzyme migrated toward the anode and invariably gave a single and discrete band under these conditions. Preliminary studies in which the concentration of inhibitor and the time of interaction were varied revealed that as the enzyme was progressively inactivated the band having the migratory properties of the native enzyme (E) decreased in intensity, and that there was an orderly appearance of a band which migrated somewhat more slowly than the native enzyme (I_1), which in turn was gradually replaced by a second band (I_2) which migrated even more slowly than the I_1 band. At the same time, in most experiments, a third band (I_3) appeared that migrated more rapidly than any of the preceding species. Enzyme activity is found in bands E and I_1, but I_2 and I_3 are invariably devoid of activity.

Fig. 4 illustrates polyacrylamide gel electrophoresis of a sample of isomerase that had been treated for 24 h at 25° with 6β-bromotestosterone 17-acetate at a ratio of steroid to enzyme concentration of 3.86 (Sample 3, Table I). An aliquot of this reaction mixture which retained 43% of the initial enzyme activity was subjected to polyacrylamide gel electrophoresis. The gel was sliced longitudinally, one-half was

stained, and the protein peaks were identified by scanning in a densitometer (Fig. 4, upper). The other half was cut into thin slices and the radioactivity in each slice was determined (Fig. 4, lower). Radioactivity is associated with each of the positions in which protein is found in the stained sections. If the densitometer trace is integrated, it becomes clear that the highest specific radioactivity, and therefore presumably the most heavily labeled material, is to be found in the I_3 band. In view of the fact that these experiments were conducted under mild conditions which could not have caused

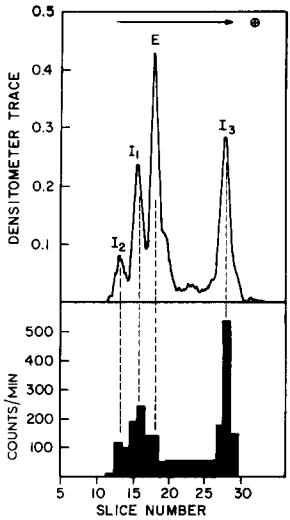


Fig. 4. Polyacrylamide gel electrophoresis of Δ^5-3-ketosteroid isomerase that had been treated with 6β-bromotestosterone 17-[^{3}H]acetate for 24 h at pH 8.0 and at a molar ratio of inhibitor to enzyme of 3.86. Reaction mixture 3 of Table I was dialyzed against 0.01 M potassium phosphate, pH 7.0. An aliquot (50 μl) containing 3630 counts/min and 0.125 nmole of protein was subjected to electrophoresis as described under METHODS. The gel was split longitudinally and one half was stained with amido black 10B and its densitometric tracing is shown in the upper part of the figure. The other half was sliced into 1.27 mm sections, and the radioactivity of each slice was determined (lower bar graph). Active enzyme was found associated only with band E (which corresponds to the migration of native enzyme) and band I_1. The designations of bands I_1, I_2 and I_3 are explained in the text. Migration of the bands was toward the anode (right).

a cleavage of the primary structure of the enzyme, it seems plausible that I_3 represents the enzyme dissociated into its subunits. In order to resolve this question, it will be necessary to isolate each fraction and to determine the precise number of steroid molecules bound to it. One explanation for these findings is that the different bands represent different degrees of alkylation of the enzyme by the inhibitory steroid. Since even the band corresponding to the native enzyme contains radioactivity, and it may be a mixture of native enzyme and that containing some covalently bound inhibitor, it seems possible that the materials migrating in regions I_1, I_2 and I_3 represent increasing degrees of substitution with inhibitor molecules. The material migrating more rapidly than the native enzyme (I_3) appears to have a higher specific radioactivity than the other materials, and probably represents dissociation of the enzyme into subunits.

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