Photoaffinity Modification of Δ^5-3-Ketosteroid Isomerase by Light-Activatable Steroid Ketones Covalently Coupled to Agarose Beads†

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ABSTRACT: In order to identify the minor site(s) of photoattachment of unsaturated steroid ketones to Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni*, we have developed a solid-state photoaffinity labeling technique. Two solid-state reagents, *O*-carboxymethylagarose–ethylenediamine–succinyl-17β-*O*-19-nortestosterone and *O*-carboxymethylagarose–ethylenediamine–succinyl-17β-*O*-4,6-androstadien-3-one, have been synthesized. Under anaerobic conditions, isomerase bound to these resins is photoinactivated by UV light ($\lambda > 290$ nm) whereas isomerase bound to *O*-carboxymethylagarose–ethylenediamine–deoxycholate or isomerase in the presence of *O*-carboxymethylagarose–ethylenediamine–acetate is almost completely stable to irradiation under the same conditions. Photoinactivation under anaerobic condition promoted by the resin-bound steroid ketones results from a reaction at the active site since the competitive inhibitor, sodium cholate, which does not absorb light above 290 nm, provides protection toward photoinactivation. Preliminary analysis of isomerase that has been photolyzed in the presence of *O*-carboxymethylagarose–ethylenediamine–succinyl-17β-*O*-4,6-androstadiene-3-one has established that the enzyme is converted to at least two different forms. One form binds more tightly to the resin than does the native enzyme. This form can be eluted by a sodium dodecyl sulfate containing buffer. The second form is not eluted by this buffer but can be released from the resin by cleavage of the ester bond linking the steroid to the derivatized agarose. We presume that the latter form is covalently coupled to the resin-linked steroid. In the presence of oxygen, additional nonspecific inactivation reactions occur, but these can be suppressed by the singlet oxygen trap, L-histidine. The application of solid-state photoaffinity reagents to some areas of receptor isolation and characterization is discussed.

The enzyme Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* has been chemically modified by a number of affinity and photoaffinity reagents in studies designed to identify functional groups comprising its active site (Ogez et al., 1977; Covey & Robinson, 1976; Penning et al., 1981; Penning & Talalay, 1981; Pollack et al., 1979; Bevins et al., 1980). These studies have indicated that aspartic acid-38 and asparagine-57 are components of the steroid binding site.

In our studies of the photoinactivation of this enzyme by light-activated steroid ketones (Ogez et al., 1977; Martyr, 1974; Benisek et al., 1980), we observed a minor reaction, which, unlike the major process that involves the photodecarboxylation of aspartate-38, is distinguished by the formation of a stable covalent linkage of steroid and polypeptide. The minor reaction appeared to be at the active site since a nonchromophoric competitive inhibitor provided a significant reduction of the extent of covalent coupling (Martyr, 1974). Thus, it was of interest to us to identify the site or sites of photoattachment. This has proved to be a difficult task because only 5–15% of the enzyme molecules undergo photoattachment to the steroid reagent during the course of photoinactivations stimulated by 19-nortestosterone acetate or testosterone (Martyr, 1974).

In order to circumvent the problems that attend the low efficiency of covalent attachment in these reactions, we have combined elements of the techniques of covalent chromatography (Brocklehurst et al., 1974; Blumberg & Strominger, 1974; Voss et al., 1974) and photoaffinity labeling (Chowdry & Westheimer, 1979) into a solid-phase photoaffinity labeling procedure designed to facilitate the selective retrieval of polypeptides and peptide fragments that contain the site(s) of covalent photoattachment.

Our method uses light-activatable photoaffinity reagents (e.g., 19-nortestosterone and Δ^6-testosterone) that are covalently linked via their 17β-hydroxyl groups to agarose via an ester bond to a succinyl–ethylenediamine–*O*-carboxymethyl leash. The isomerase is applied to a small column of the photoreagent, and the resulting enzyme–reagent complex is irradiated with light of $\lambda \geq 290$ nm. Forms of the enzyme that are not covalently attached are eluted from the column by washes of the competitive inhibitor sodium cholate and the denaturants sodium dodecyl sulfate ($NaDodSO_4$) and/or guanidinium chloride. Then, polypeptides that are still retained by the resin due to a steroid–polypeptide covalent bond are released by mild alkaline hydrolysis of the succinyl–steroid ester bond.

In the present study, we wish to report our initial applications of this strategy to the characterization of the 3-ketosteroid-dependent photoinactivation of *P. testosteroni* Δ^5-3-ketosteroid isomerase. We find that this enzyme is inactivated by ultraviolet light specifically when bound to agarose resins bearing 19-nortestosterone and Δ^6-testosterone ligands. Protection of the enzyme from photoinactivation by the competitive inhibitor sodium cholate provides evidence that the resin-dependent inactivations are active site specific.

In addition to this specific application, we wish to point out generally applicable potentially advantageous features of solid-state affinity reagents in the identification and characterization of binding sites on proteinaceous receptors.

Experimental Procedures

Materials. Δ^5-3-Ketosteroid isomerase of pI 4.75 was purified from progesterone-induced *P. testosteroni* following an adaptation (Ogez et al., 1977) of the method of Jarabak et al. (1969) as modified by Benson et al. (1974). When analyzed by $NaDodSO_4$–urea gel electrophoresis or by pH 4–6 gel isoelectric focusing, a single Coomassie Blue staining band was observed.

19-Nortestosterone 17β-hemisuccinate was synthesized by succinylation of 19-nortestosterone (Steraloids, Inc.) by a

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modification of the procedure of Giannini & Fedi (1960). A 1-g sample of 19-nortestosterone that had been dried in vacuo was mixed with 3 g of succinic anhydride (freshly recrystallized from acetic anhydride and dried in vacuo) in 10 mL of dry pyridine. An atmosphere of dry N_2 was maintained in the reaction vessel. The temperature was maintained at 100 °C for 6 h at which time the reaction was judged to be completed by thin-layer chromatography on Merck silica gel 60 F_{254} with chloroform–methanol, 9:1, as solvent. After the reaction mixture was permitted to cool to ambient temperature and to stand overnight, its contents were poured into 75 mL of H_2O. The pH of the resulting brownish suspension was adjusted to 8–9 by addition of 50% NaOH under vigorous stirring. The resulting clear yellow-brown solution was filtered through Whatman No. 1 paper, and the filtrate was acidified to pH 3.0 by addition of concentrated HCl, resulting in the precipitation of a tan material, crude 19-nortestosterone 17β-hemisuccinate. The precipitate was collected by centrifugation, and it was washed extensively with several 25-mL portions of 1 mM HCl. The washed solid was dried overnight by lyophilization. The weight of the dry solid was approximately 1.2 g. Further purification was achieved by adsorption chromatography on a 1.5 × 55 cm column of silica gel (Baker, 60–200 mesh) equilibrated and eluted with 5% methanol in chloroform. The steroid-containing fractions were located by absorbance measurements at 248 nm of small aliquots diluted with water. These fractions were combined and evaporated to dryness, and the resulting, nearly colorless residue was crystallized from dichloroethane–cyclohexane (mp 168–171 °C [lit. (Giannini & Fedi, 1960) mp 170–171 °C]). The resulting crystalline material migrated as a single species when subjected to thin-layer chromatography as described earlier. The ultraviolet and proton magnetic resonance spectra of the material were consistent with the structure of 19-nortestosterone 17β-hemisuccinate. The λ_{max} was 248 nm (ϵ = 1.69 × 10^4 M^{-1} cm^{-1})(10% ethanol in water). The 500-MHz proton magnetic resonance spectrum of a deuteriochloroform solution contained the following characteristic resonances (δ = parts per million relative to tetramethylsilane at δ = 0): δ 0.85 (s, 3.12, C-18 methyl), 2.63 (complex m, 2.2, succinyl methylene), 2.67 (complex m, 2.2, succinyl methylene), 4.64 (t, 1, J = 8 Hz, 17α proton), 5.83 (s, 1.01, C-4 vinyl proton).

Δ^6-Testosterone hemisuccinate (4,6-androstadien-3-one 17β-hemisuccinate) was a product of Steraloids, Inc. The melting point was 250–253 °C. The λ_{max} was 293 nm (ϵ = 2.35 × 10^4 M^{-1} cm^{-1})(10% ethanol in water). The 500-MHz proton magnetic resonance spectrum, obtained in deuteriochloroform solution exhibited the following characteristic resonances (δ = parts per million relative to tetramethylsilane at δ = 0): δ 0.87 (s, 2.8, C-18 methyl), 1.12 (s, 3.0, C-19 methyl), 2.64 (complex m, 1.9, succinyl methylene), 2.68 (complex m, 1.9, succinyl methylene), 4.67 (t, 1.01, J = 8 Hz, 17α proton), 5.68 (s, 0.94, C-4 vinyl proton), 6.08 (d, 1.04, J = 10 Hz, C-6 vinyl proton), 6.12 (d, 1.04, J = 10 Hz, C-7 vinyl proton). A more complete and detailed analysis of the spectra of these and some related steroids will be reported elsewhere.

Sodium deoxycholate and sodium cholate were products of Sigma Chemical Co. Sodium acetate was the AR grade of Mallinckrodt. Dioxane was 99+% Gold Label from Aldrich. CM-Bio-Gel A (*O*-carboxymethylagarose), 100–200 mesh, Na^+ form, having a carboxyl content of 20 mM, was lot no. 18925 from Bio-Rad Laboratories. Ethylenediamine (98%) was purchased from Mallinckrodt and was used without further purification.

Synthesis of N-(2-Aminoethyl)-O-carboxamidomethylagarose. *O*-Carboxymethylagarose was coupled to ethylenediamine by a carbodiimide-mediated amidation reaction. The resin was first washed with water on a sintered glass funnel. Then, 50 mL of settled resin was suspended in 90 mL of H_2O containing 6 g of ethylenediamine. The pH of the stirred suspension was adjusted to 4.7 with concentrated HCl. Three 0.58-g portions of 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride were added to the continuously stirred suspension at 8–12-h intervals. The reaction was terminated by extensive washing with H_2O. The amino resin was stored as a suspension in water at 2–4 °C. The amino- and carboxyl-group contents of the amino resin were estimated by the pH titration method of Inman (1974) and were found to be 20.8 and <0.1 μmol/mL of gravity-settled resin, respectively. Thus, essentially all of the initial carboxyl groups had been converted to *N*-(2-aminoethyl) amides.

Synthesis of Steroidal and Acetyl Resins. The steroidal and acetyl resins were synthesized by coupling 19-nortestosterone hemisuccinate, Δ^6-testosterone hemisuccinate, deoxycholate, and acetate to *N*-(2-aminoethyl)-*O*-carboxamidomethylagarose with a carbodiimide to drive formation of amide bonds between the carboxylic acids and the resin amino groups. Generally, 2 mL of gravity-settled resin was mixed with 2 mL of dioxane. The appropriate carboxylic acid or its sodium salt was added (2.08 μmol), and the pH meter reading was adjusted to 7.0 with 0.1 M HCl or NaOH as required. The reaction was initiated by the addition of 41.6 μmol of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. Usually 1–2 equal additional portions of carbodiimide were added to the gently stirred suspension at 16-h intervals. This procedure achieves acylation of only a small portion (≤15%) of the total amino groups. In order to suppress nonspecific ionic binding of protein to the resins, the remaining free amino groups were blocked by exhaustive acetylation. Thus, 1.0 mL of 3 M sodium acetate buffer, pH 4.8, was added to the reaction mixture, followed by 0.192 g of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride. The suspension was gently stirred for 3 h. The resin was transferred to a sintered funnel and washed with copious quantities of 50% dioxane–water. The washings were checked by measurement of the absorbance at 248 nm in order to ensure that all unbound steroid was removed. Then, the resins were washed with distilled water and stored at 4 °C until needed. The peracetylated resins gave negative tests for primary amines when treated with 2,4,6-trinitrobenzenesulfonate (Inman, 1974).

The 19-nortestosterone and Δ^6-testosterone contents of the NTS-[1] and Δ^6TS-resins were estimated by alkaline hydrolysis, solvent extraction of the liberated steroid, and determination of the steroid concentration by ultraviolet spectrophotometry. The technique was standardized against accurately known amounts of the steroid hemisuccinates that were subjected to the same procedure. Thus, 0.1–0.3 mL of gravity-settled NTS- or Δ^6TS-resin was suspended in 1.5 mL of 0.1 M NaOH. With continuous magnetic flea stirring, the suspension was incubated in a boiling water bath for 15 min. After cooling to room temperature, the base was neutralized with 0.15 mL of 1.0 M HCl. The resulting resin suspension was extracted 4 × 1.5 mL with ethyl acetate, and the combined extracts were taken to dryness under a gentle jet of N_2 gas. The residue was redissolved in an appropriate sized volume of ethanol, and an aliquot of this solution was diluted with a known volume of

[1] Abbreviations: NTS, 19-nortestosterone succinyl; Δ^6TS, Δ^6-testosterone succinyl; DOC, deoxycholyl; Ac, acetyl; NMR, nuclear magnetic resonance.

water. The ultraviolet absorption spectrum of the aqueous dilution was recorded, and the absorbances at 248 and 293 nm were used to calculate the total amount of 19-nortestosterone or Δ^6-testosterone, respectively, released from the resin. The steroid contents are expressed as micromolar, i.e., nanomoles of steroid per milliliter of gravity-settled resin. Thin-layer chromatography on silica gel ($CHCl_3$–MeOH, 9:1) showed that the only steroids present in the ethyl acetate extracts were 19-nortestosterone or Δ^6-testosterone.

Other Materials. NMR tubes, 5 mm (4.24-mm i.d.), used as the source of borosilicate glass tubing from which the minicolumns (see below) were constructed were obtained from Kontes Glass Co., catalog no. 897193-000. Teflon antivortex plugs and NMR tube caps were purchased from Wilmad Glass Co. Teflon cloth was from Glenco Scientific. Intramedic polyethylene tubing (i.d. 0.86 mm) and 20-gauge Luer stub adapters were purchased from Clay Adams. The photochemical reactor used for all experiments was the same as that described previously (Benisek, 1977).

Assembly of Minicolumns. Minicolumns were constructed from 15-cm lengths of glass tubing obtained by cutting the bottoms off of NMR tubes. The bottom support for the resin was constructed from an antivortex plug that had been shortened by removal of 2.5 mm from the end having the larger i.d. hole. This end was oriented uppermost in the assembled column, and the plug was positioned at one end of the piece of glass tubing. The upper end of the plug was overlaid by a disk of Teflon cloth. Into the lower end of the plug, containing the small i.d. hole, was pressed a 1-cm length of stainless steel tubing cut from a 20-gauge Luer stub adapter. A 10–15-cm piece of polyethylene tubing was pressed onto the free end of the stainless steel tube, and it served as the outlet line from the column. Finally, an ordinary thumbtack served as a leak-proof plug at the end of the outlet line. When a loaded column was to be irradiated, the outlet line and its associated thumbtack were removed, and the free end of the stainless-steel tube was stoppered by a ca. 5-mm piece of polyethylene tubing that had been heat sealed at one end.

Resin was loaded into the minicolumns as a dilute slurry in water. Bed heights of 2–4 cm were employed. Prior to the application of isomerase samples, the columns were equilibrated with 0.3 M potassium phosphate buffer, pH 7.00.

Procedures for Irradiation and Elution. Most irradiations were performed under anaerobic conditions since it was found that the presence of oxygen decreased the active site specificity of the photoinactivations, presumably by permitting nonspecific photooxidation to occur (see results). Anaerobic conditions were established in one of two ways. Under conditions in which the enzyme would bind tightly to resin, the enzyme was applied in air-equilibrated 0.3 M potassium phosphate, pH 7.00. Then, the column was washed extensively with several column volumes of nitrogen-purged phosphate buffer while maintaining a nitrogen atmosphere above the surface of the liquid and resin bed. Assay of the effluent washes from NTS, Δ^6TS, and DOC columns showed that in the absence of sodium cholate, negligible ($\ll$1%) quantities of active isomerase were eluted by this procedure. Under conditions in which the isomerase did not bind firmly to the column (e.g., enzyme in the presence of sodium cholate or enzyme applied to acetyl resin), a different deoxygenation procedure was employed. Prior to application of the enzyme, the resin was eluted with several column volumes of N_2-purged 0.3 M potassium phosphate, pH 7.0. The stock enzyme solution in 0.3 M KP_i, pH 7.0, usually containing sodium cholate was depleted of dissolved oxygen by passive diffusion for at least 48 h at 4 °C in a tightly closed container containing a physically separate solution of 10% pyrogallol and 10% NaOH in water (Willis, 1969) as an oxygen trap.[2] Then, with a gas-tight syringe inserted through the Teflon-lined rubber-septum closure, an aliquot of the enzyme solution was transferred to the top of the resin bed, which was maintained under a blanket of nitrogen. The aliquot was permitted to drain into the resin bed by gravity. Then the column was tightly capped and prepared for irradiation.

Columns were irradiated for 2–18 h with a 450-W Hanovia lamp as described previously (Benisek, 1977). Dark controls were kept in a closed cupboard during the irradiation procedure. Following irradiation or dark incubation, the columns were eluted with two or more 1.0-mL portions of 0.5 mM sodium cholate in 0.3 M potassium phosphate, pH 7.0. Generally, two 1.0-mL portions eluted all of the elutable enzyme. The enzyme activity in the eluates was determined by the method of Kawahara et al. (1962) as modified slightly by Martyr & Benisek (1973).

Sequential Elution of Enzyme from Resin by Sodium Cholate, Sodium Dodecyl Sulfate, and Alkaline Hydrolysis. First, the irradiated columns were eluted with five to eight 1-mL aliquots of 0.5 mM sodium cholate in 0.3 M potassium phosphate, pH 7. The columns were then washed with five to eight 1-mL aliquots of distilled water to completely remove the sodium cholate. Next, the resin was removed from the columns and transferred to conical glass centrifuge tubes. One milliliter of 1 sodium dodecyl sulfate in 0.01 M sodium acetate, pH 4.9, was added, and the resin suspensions were incubated at 37 °C for 2 h with stirring. Subsequently, the tubes were centrifuged for 5–10 min at top speed in a clinical swinging-bucket centrifuge to sediment the resin, and the supernatant–sodium dodecyl sulfate solution was removed with a Pasteur pipet. The resin was washed with two additional 1-mL portions of the sodium dodecyl sulfate solution by resuspension followed by recentrifugation, and the three 1-mL aliquots were pooled. Then, the resin was washed with three 2-mL aliquots of distilled water, again by resuspension and centrifugation, to remove the sodium dodecyl sulfate. The water washes were pooled. Finally, the resin was suspended in 2 mL of 1% triethylamine hydrochloride, pH 10.5. The suspensions were incubated for 3 h at 30 °C with stirring. Then, after a centrifugation of the suspension to pellet the resin, the triethylamine hydrochloride was removed. The resin was subsequently washed with two 1-mL aliquots of triethylamine hydrochloride, and the three fractions were pooled.

The fractions from each of the three treatments were assayed for protein content with fluorescamine (Nakai et al., 1974). The standard curve was established with isomerase as the standard protein. In some cases, the samples were acid hydrolyzed prior to assay in order to increase the sensitivity

[2] The time required for the diffusion of oxygen molecules from the enzyme stock solution can be estimated from the equation, $\overline{\delta^2} = 2D\Delta t$, derived by Gosting (1956) following the theory of Brownian motion developed by Einstein (1908). δ = the distance diffused by a solute; $\overline{\delta^2}$ = the mean square diffusion displacement; D = the diffusion coefficient in centimeters squared per second; and Δt = the time interval to achieve a particular $\overline{\delta^2}$. The depth of the enzyme stock solution was no more than 2.0 cm. The diffusion coefficient of O_2 in 1% NaCl at 18 °C is close to 2×10^{-5} cm^2/s. If these values and the above equation are used, the time for O_2 to diffuse from the bottom to the top of the solution would be Δt = 28 h. Since the diffusion coefficient of O_2 in air is 10^4 times larger than it is in aqueous solution, the gas phase above the enzyme solution is essentially instantly depleted of O_2 by the oxygen-trapping solution. Thus, an incubation time of 48 h is sufficient for extensive deoxygenation of the enzyme solution.

Table I: Photoinactivation of Steroid Isomerase in the Presence of Various Agarose Derivatives: Recovery of Applied Isomerase Activity (%)

treatment	NTS-resin[a]	Δ^6TS-resin[d,e]	DOC-resin	Ac-resin
irradiated 18 h	50.2, 53.6, 49.4,[b] 63.2[c]	10.8, 11.6, 11.7, 11.6, 11.3	92.7	98.4, 96.2
not irradiated	99.5, 117.3, 92.7,[b] 94.8[c]	92.9, 82.6, 104.3, 106.1, 99.2	99.9	110.0, 116.1, 99.8

[a] [NTS] = 355 μM unless indicated otherwise. [b] [NTS] = 341 μM. [c] [NTS] = 269 μM. [d] [Δ^6TS] = 327 μM. [e] The irradiation time was 2 h.

of the protein determinations. Furthermore, it was necessary to lyophilize the triethylamine hydrochloride fractions to remove a volatile fluorescamine-positive amine that interfered with the assays.

Results

Binding of Isomerase to Agarose Derivatives. In preliminary experiments the ability of the four resins to bind isomerase was assessed qualitatively. Minicolumns of resin-bed dimensions 4.2 × 25 mm were prepared using NTS-resin, Δ^6TS-resin, DOC-resin, and Ac-resin. Approximately 20 μg of isomerase in 0.3 M potassium phosphate, pH 7.0, was applied to each column equilibrated with the same buffer. Subsequent elution of these columns with several column volumes of 0.3 M potassium phosphate, pH 7.0, resulted in negligible (≪1%) amounts of isomerase activity in the recovered fractions from the three steroidal agarose derivatives, whereas all of the applied isomerase activity was recovered in the eluate from the Ac-resin. These results demonstrate that isomerase is essentially quantitatively bound to the steroidal resins but only weakly, if at all, bound to Ac-resin under these solvent conditions.

In addition, a quantitative measurement of the maximum binding capacities of the three steroidal resins was made by adding successive aliquots of enzyme in 0.3 M potassium phosphate, pH 7.0, to the appropriate steroidal column until substantial quantities of isomerase activity were detected by enzyme assay of the effluent. All resins bound 10–20 mg/mL of isomerase, which is equivalent to a bound monomer concentration of 0.75–1.5 mM. All of the following irradiation experiments were conducted with steroid concentrations that were 2 orders of magnitude less than these maximum capacities.

Inhibition of Isomerase by Steroids. In order to determine if the resin-bound steroids were capable of binding at the active site of isomerase, a study was made of the effects of 19-nortestosterone succinate and Δ^6-testosterone succinate on the kinetics of the isomerization of 5-androsten-3,17-dione (Kawahara et al., 1962). Isomerization rates were measured at 5-androsten-3,17-dione concentrations ranging from 41 to 203 μM. Inhibitor concentrations were varied over the following ranges: 19-nortestosterone succinate, 0–134 μM; Δ^6-testosterone succinate, 0–108 μM. The solvent was 0.033 M potassium phosphate–3% v/v methanol. The data were analyzed by Dixon plots (Dixon, 1953) fitted by a linear-regression program. The data for all steroid inhibitors were satisfactorily fitted ($r \geq 0.98$) by linear fits to the $1/v$ vs. [I] plots, and for each steroid, the plots intersected in a closely clustered array of points, indicating that these steroids were competitive inhibitors. The K_I values obtained were as follows: 19-nortestosterone hemisuccinate, 33 ± 2 μM; Δ^6-testosterone hemisuccinate, 85 ± 10 μM.

Effect of Resin Ligand on Recovery of Activity after Photolysis. The abilities of the four agarose derivatives to stimulate a light-dependent loss of enzyme activity recoverable in sodium cholate eluates, hereafter referred to as "inactivation", were compared. A substantial number of irradiations and dark controls were performed for a fixed time of 18 h or for 2 h in the case of Δ^6TS-resin. The results of these experiments are given in Table I. The NTS-resin and the Δ^6TS-resin stimulated loss of enzyme activity whereas the DOC- and Ac-resins did not significantly affect the recovery of active isomerase. Thus, loss of activity required a ligand to which isomerase would bind and which bore a near-ultraviolet light absorbing group. Recoveries from the dark controls were in all but one case greater than 90%, indicating that the loss process was light dependent. We conclude that the loss of enzyme activity in the sodium cholate eluates is the result of one or more photochemical reactions between the enzyme and the enone or dienone chromophores of NTS-resin and Δ^6TS-resin, respectively.

Kinetics of Inactivation Stimulated by NTS-Resin and Δ^6TS-Resin. The data of Table I indicated that Δ^6TS-resin was more effective in stimulating enzyme inactivation than was NTS-resin. In order to more precisely delineate the kinetics of these inactivation reactions, the time-course of enzyme inactivations promoted by NTS-resin and Δ^6TS-resin was investigated. In view of the desirability of rigorously excluding oxygen from the reaction medium (see below) and of the inability to obtain a representative and quantitative sampling of the heterogeneous reaction mixtures involved, we prepared separate minicolumns for each time point in the kinetics study. All columns received the same amount of enzyme, and the irradiations of all columns of a single resin type were conducted simultaneously. At appropriate times, individual columns were removed from the reactor and placed in darkness. Then, all of the columns were eluted with 0.3 M potassium phosphate, pH 7.0, containing 0.5 mM sodium cholate. The results obtained are given in Figure 1. As expected, the loss of activity achieved with the Δ^6TS-resin (inset) was much more rapid than that obtained with the NTS-resin. The half-time for Δ^6TS-resin-promoted inactivation was approximately 30 min while the half-time for the NTS-resin-dependent inactivation was approximately 15 h. It is presumed that this large rate difference is due to the much higher extinction coefficient of the conjugated dienone chromophore of Δ^6-testosterone compared to that of the enone chromophore of 19-nortestosterone at wavelengths longer than 290 nm.

Effect of Ligand Concentration on Extent of Photoinactivation Sensitized by NTS-Resin. Resin samples having different contents of nortestosterone moieties were prepared by varying the amount of carbodiimide and the pH used in the standard synthesis of NTS-resin. Identical amounts of isomerase were applied to these samples, and the minicolumns were irradiated under anaerobic conditions for 18 h. As the concentration of steroid is increased (0, 46, 193, and 355 μM), the extent of inactivation increases (7, 14, 23, and 48%). This trend provides further evidence that it is the steroid moiety that is responsible for most or all of the photoinactivation. The 7% loss of activity obtained with the NTS-free resin in this experiment is qualitatively consistent with the results obtained with the Ac-resin and DOC-resin shown in Table I. This suggests that the enzyme is weakly photolabile in the absence of light-absorbing steroids.

Table II: Inhibitory Effects of Sodium Cholate on NTS- and Δ^6TS-Dependent Photoinactivations of Steroid Isomerase

resin	treatment	% of appld act. at a [sodium cholate] (mM) of 0	0.5	2.0	3.4
NTS	irradiated 18 h	63.2,[b] 56.5,[c] 63.9,[d] 59.2,[d] 51.7,[d] 25.7[a]	77.6[b]	85.7,[c] 85.0,[d] 86.1,[d] 81.4[d]	57.4[a,e]
NTS	not irradiated	94.8,[b] 100.9,[c] 95.0,[b] 110.0[a,e]	95.0[b]	100.8,[c] 101.1[d]	101.5[a,e]
Δ^6TS	irradiated 2 h	23.7,[f] 13.5[g]			94.5,[f] 95.1[g]
Δ^6TS	not irradiated	108,[f] 118[g]			97.6,[f] 104[g]

[a] A different lamp and Pyrex filter were used for these photolyses. [b] [NTS] = 269 μM. [c] [NTS] = 341 μM. [d] [NTS] = 394 μM. [e] [NTS] = 355 μM. [f] [Δ^6TS] = 319 μM. [g] [Δ^6TS] = 327 μM.

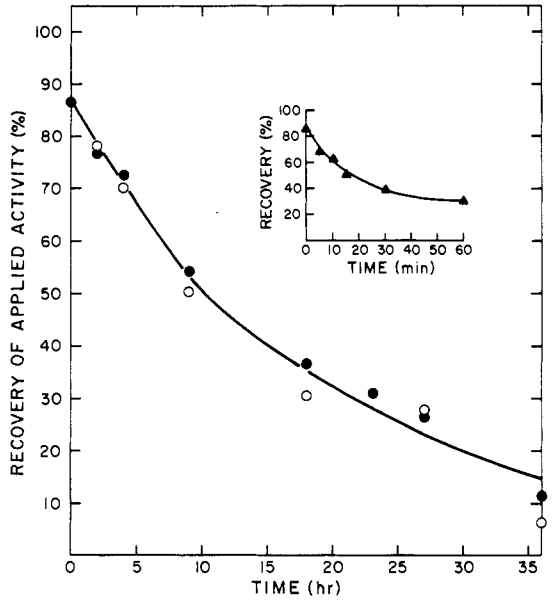

FIGURE 1: Kinetics of photoinactivation of steroid isomerase in the presence of NTS-resin and Δ^6TS-resin. Approximately 800 units of isomerase were applied to 4.2 × 35 mm columns of NTS-resin ([NTS] = 350 μM (●) or 394 μM (○)) and 600 units to 4.2 × 35 mm columns of Δ^6TS-resin ([Δ^6TS] = 319 μM (▲)). The columns were rendered anaerobic by perfusion with N_2-purged 0.3 M potassium phosphate, pH 7.0, and irradiated with a 450-W Hanovia mercury arc lamp through 4.4 mm of Pyrex glass as a filter for wavelengths below 290–300 nm. At suitable times, columns were removed from the reactor and eluted with three 1.0-mL portions of 0.495 mM sodium cholate in 0.3 M potassium phosphate, pH 7.0. The eluates were assayed separately and the activities in each summed to give the total cholate-elutable isomerase activity for each time point. The data for Δ^6TS-resin are shown in the inset.

Effect of Sodium Cholate on Extents of Photoinactivation Sensitized by NTS and Δ^6TS. In order to determine whether the photoinactivation processes stimulated by NTS-resin and Δ^6TS-resin were at the active site, the effects of sodium cholate, a competitive inhibitor of the enzyme (K_I = 8.5 μM), on the extent of photoinactivation were assessed. Table II shows the effects of three concentrations of sodium cholate on the degree of inactivation obtained with various NTS-resin preparations and of one concentration of sodium cholate on the inactivation in the presence of two Δ^6TS-resin preparations. In both cases, significant protection of enzyme activity is afforded by sodium cholate, with nearly complete protection by 3.4 mM cholate. These data would suggest, therefore, that under anaerobic conditions the inactivation processes are probably occurring at the active site.

Effects of Oxygen and L-Histidine on Photoinactivation Promoted by Δ^6TS-Resin. Photolysis of isomerase bound to Δ^6TS-resin equilibrated with air or 100% oxygen resulted in a much greater extent of inactivation after only 1 h than that obtained under oxygen-free conditions after 2 h. Moreover,

Table III: Effect of Oxygen on Extent of Δ^6TS-Resin-Dependent Photoinactivation of Steroid Isomerase

treatment	atm	% of appld act. at a [sodium cholate] (mM) of 0	3.4
irradiated 1 h	air	3.8	63.9
irradiated 1 h	O_2	0.7	47.2
not irradiated	air	91.6	104.0
not irradiated	O_2	94.0	103.0

Table IV: Effect of L-Histidine on Aerobic Δ^6TS-Resin-Dependent Photoinactivation of Steroid Isomerase

[L-His] (mM)	atm	% of appld act. at a [sodium cholate] (mM) of[a] 0	3.4
0	air	2.0	48.6
2.5	air	21.3	96.1
5.0	air	26.4	
10.0	air	25.6	
0	N_2	21.8	91.4

[a] The time of irradiation was 45 min. The [Δ^6TS] was approximately 320 μM.

the ability of 3.4 mM cholate to protect the enzyme activity was markedly reduced relative to the protection under anaerobic conditions. The results are collected in Table III. We note that the higher the partial pressure of oxygen, the more extensive is the inactivation and the less effective is 3.4 mM cholate in providing protection of activity. This pattern of results strongly indicates that oxygen promotes new inactivation processes in addition to those that occur under anaerobic conditions and that these additional processes are not inhibited by cholate. This would suggest that the additional processes are not active site located.

One possibility for an oxygen-dependent photochemical reaction is chromophore- (dye) sensitized photooxidation (Means & Feeney, 1971). Mäkinen & Mäkinen (1982b) have recently made a survey of inhibitors of chromophore-sensitized photooxidation, and these workers have found that various histidine-type compounds are very efficient scavengers of the singlet oxygen that is believed to be the active oxidant in these reactions. In order to assess the importance of singlet oxygen in the aerobic inactivations, a study of the effect of L-histidine of the extent of inactivation and the protective effects of sodium cholate was made. A set of minicolumns containing Δ^6TS-resin, enzyme, and various concentrations of L-histidine were prepared and irradiated simultaneously for 45 min in the photochemical reactor. As a control, an oxygen-free minicolumn loaded with the same amount of isomerase was prepared and irradiated with the aerobic group. The recoveries of enzyme in the cholate eluates are presented in Table IV. These results show that 2.5 mM histidine supresses the oxy-

Table V: Sequential Elution of Isomerase Protein from Irradiated and Nonirradiated Columns of Δ^6TS-Resin

expt no.	enzyme applied (units)	treatment	recovery of activity or protein[a] (% of applied) act. in sodium cholate	protein in sodium cholate	protein in $NaDodSO_4$	protein in alkaline hydrolysate	total protein
1	9 600	irradiated 2 h	12.6	19.2	27.5	16.7[b]	63.4
2	9 600	not irradiated	87.1	98.4	1.8	0.0[b]	100.2
3	30 200	irradiated 2 h	16.0	22.3	29.3	21.6	73.2
4	30 200	not irradiated	99.5	79.2	0.7	0.0	80.9

[a] Fluorescamine assay was used to estimate isomerase protein. The standard curve was based on native isomerase as the protein standard. [b] Fluorescamine assay was performed after complete hydrolysis of protein by acid (5.7 M HCl, 110 °C, in vacuo, 20 h). The standard curve was determined with isomerase acid hydrolysate.

gen-dependent inactivation processes and restores the ability of sodium cholate to afford full protection of the enzyme as it does under anaerobic conditions.

Hydrolysis of Steroid Ester Bond under Relatively Mild Conditions. In the determination of the steroid content of NTS- and Δ^6TS-resins, very harsh conditions (ca. 0.094 M NaOH, 100 °C, 15 min) were used to release steroid from the resin by hydrolysis of the succinylsteroid ester group. Such conditions would be much too vigorous for the isolation of undegraded proteins or peptide fragments that had undergone photocoupling to the resin steroid moieties. Consequently, milder conditions of hydrolysis were investigated.

Small aliquots of NTS-resin or Δ^6TS-resin were incubated in various buffers at 30 °C, and after suitable periods of time, the resin was pelleted by centrifugation. An aliquot of the supernatant solution was diluted into 3.0 mL of 0.1 M sodium acetate buffer, pH 4.7, in order to terminate the reaction and to achieve a concentration of steroid suitable for UV spectrophotometric assay at 248 mm in the case of NTS-resin and 293 nm in the case of Δ^6TS-resin. It was found that treatment with 0.1 M sodium carbonate/bicarbonate or triethylamine hydrochloride, pH 10.5, at 30 °C for 3 h resulted in quantitative release of the steroids from the resins. A similar incubation in 0.1 M phosphate buffer, pH 7.3–7.5, resulted in no detectable release of steroid from NTS-resins.

Fates of Isomerase due to Irradiation on Δ^6TS-Resin. As a first step toward characterization of the chemical events associated with enzyme photoinactivation, we have attempted to estimate what fraction of isomerase that is irradiated in the presence of Δ^6TS-resin becomes covalently coupled to the resin and what fraction suffers modifications that do not lead to covalent attachment. Samples of isomerase were applied to minicolumns of Δ^6TS-resin and either irradiated or incubated in the dark. Then, the irradiated columns and their dark controls were eluted sequentially with sodium cholate, sodium dodecyl sulfate, and triethylamine hydrochloride, pH 10.5. The sodium cholate eluate should contain unreacted enzyme plus photomodified products that are still cholate elutable. The sodium dodecyl sulfate eluate should contain photomodified forms that are not cholate elutable but can be released from the resin by denaturation of the protein under nonhydrolytic conditions. The triethylamine hydrochloride eluate should contain photomodified species that are resistant to the previous elutions but that are detached from the solid phase by conditions already shown (see above) to hydrolyze the succinyl ester bound of the Δ^6TS-resin. These latter forms would represent species of isomerase that have become covalently linked to the steroid moiety during photolysis. The results of our initial experiments are given in Table V. It is found that irradiation leads to the appearance of protein in all three elution fractions, suggesting that photolysis in the presence of Δ^6TS-resin not only results in covalent attachment of enzyme to the resin but also leads to a form of the enzyme that is noncovalently but more tightly bound to the resin than is native isomerase.

Discussion

The purposes of this investigation were to prepare light-activatable steroid resins incorporating a cleavable arm, to determine whether such resins support photoinactivation of steroid isomerase as do similar steroids in free solution, and to obtain evidence bearing on the active site specificity of the inactivating processes. In the present study, we have established that NTS-resin and Δ^6TS-resin promote a photochemical inactivation of the steroid isomerase. The reaction or reactions involved in the inactivation processes promoted by both resins under anaerobic conditions appear to be focused at the active site since the isomerase competitive inhibitor, sodium cholate, affords to the enzyme substantial protection in the case of NTS-resin and virtually complete protection in the case of Δ^6TS-resin. On the other hand, in the case of the Δ^6TS-resin-promoted reaction under aerobic conditions, the rate of enzyme inactivation is substantially greater than it is in the absence of oxygen, and protection by sodium cholate is partial. These results suggest that oxygen promotes inactivation processes at sites other than the enzyme's active site, perhaps sensitized photooxidation. Chromophore-sensitized photooxidation involves the intermediacy of singlet oxygen, and ketones have been shown to be effective sensitizers of photooxidation reactions of proteins (Mäkinen & Mäkinen, 1982a). Histidine and several other imidazole-containing compounds have been shown to be efficient quenchers of ketone-sensitized photooxidation (Mäkinen & Mäkinen, 1982b) of lactoperoxidase. As shown in Table IV, 2.5 mM and larger concentrations of L-histidine were found to suppress the oxygen-stimulated nonspecific inactivation processes and to result in full protection by sodium cholate under aerobic conditions.

The anaerobic inactivation process(es) appear(s) to require a UV light absorbing chromophore on the resin since DOC-resin and Ac-resin were essentially ineffective in promoting isomerase photoinactivation. Similar conclusions for the steroid ketone sensitized photoinactivation of isomerase in free solution were reached by Martyr & Benisek (1973).

The nature of the anaerobic inactivation remains to be elucidated. Several types of reaction will result in inactivation as defined[3] in the present study. These reaction types are (1)

[3] Although we have used the term "inactivation" to refer to the loss of enzyme activity recoverable in the cholate eluates, it should be recognized that any process that results in a cholate-resistant attachment of the enzyme to the photoaffinity resin will be scored as inactivation, even if the resin-attached enzyme is still catalytically competent. This is in contrast to the situation in homogeneous solution in which similar coupling reactions that do not reduce the catalytic activity are not scored as inactivation.

covalent linkage of the enzyme to the steroid via a functional group of the active site, (2) covalent linkage of the enzyme to the steroid via a functional group not at the active site, (3) modification of an active site residue with concomitant site inactivation without covalent attachment of the enzyme to the resin, and (4) modification of an enzyme functional group by a reaction that does not involve covalent bonding to the resin but that increases the affinity of the enzyme for the resin steroid to such an extent that the modified enzyme is not elutable by sodium cholate. The fact that cholate protects the enzyme would seem to suggest that (2) is unlikely for the system studied, but it is conceivable that allosteric effects associated with the binding of cholate to the active site could via a conformational change attenuate the photoreaction at an extrasite residue. Clearly, additional experiments involving accurate determination of the extent of covalent attachment to the resin are needed in order to distinguish among the four possibilities of reaction type. These experiments are in progress.

The bulk of the results reported in this paper refer only to the sodium cholate elutable enzyme. This includes the unmodified enzyme as well as any photomodified enzyme that remains loosely bound to the resin. However, as shown in Table V, we have found that there are at least two additional forms of photomodified enzyme: one form that becomes tightly but (presumably) noncovalently bound to the resin and that may be removed by sodium dodecyl sulfate treatment and the other form that becomes covalently attached to the resin and that can be removed from the resin along with the steroidal affinity ligand after mild hydrolysis with triethylamine. Thus far, little progress has been made in this area of investigation. The initial task is to quantitate the portion of the total enzyme that becomes modified to each of these forms. The results obtained so far have not been quantitatively reproducible; however, a qualitative picture emerges. One-fourth to one-third of the enzyme is released by each of the three treatments of the photolyzed sample, while significant amounts of protein are found only in the sodium cholate fractions of the dark controls. In addition, it may be that the specific activity of the enzyme in the sodium cholate fractions of the irradiated samples is lower than that of the enzyme that was originally applied. This implies that these fractions contain modified enzyme; it is likely that they contain unmodified enzyme as well.

A sample set of data may be found in Table V. It illustrates part of the problem faced in quantitating the protein: the total recoveries of protein are incomplete. It is possible that the sodium dodecyl sulfate and triethylamine treatments are not completely removing the enzyme, so studies examining the rate of release of the enzyme from the resin during these treatments must be performed. Finally, after all of the enzyme is accounted for and is adequately separated, studies aimed at investigating the nature of the modifications can be initiated.

The use of solid-phase reagents in protein and peptide chemistry has been previously reported by several groups of workers. For example, Rubenstein et al. (1976) have prepared polyacrylamide derivatives bearing *S*-sulfenyl chloride groups that react specifically with tryptophan residues in peptides and have used these polymers for the purification of tryptophan peptides from complex mixtures. In a similar vein, Shechter et al. (1977) used [(chloroacetamido)ethyl]polyacrylamide under acidic conditions to isolate methionine peptides by S-alkylation and to immobilize several proteins via their methionine residues. Brocklehurst et al. (1974) have prepared agarose derivatives containing reactive mixed disulfides and have used such a resin to purify various SH-containing enzymes. Blumberg & Strominger (1974) have used a penicillin–agarose conjugate to isolate penicillin-reactive proteins from membranes of *Bacillus subtilis*. Voss et al. (1974) have synthesized an agarose derivative bearing a *p*-nitrophenyl phosphate moiety and have used it to purify acetylcholinesterase. These examples have generally been referred to as "covalent chromatography" since they have been developed with the task of purification in mind. Alternatively, one could regard the reagents of Blumberg & Strominger (1974) and Voss et al. (1974) as "solid-phase affinity reagents" since the reactive groups are active site specific covalent bond forming reagents. It is likely that these reagents could be used to label and identify specific amino acid residues in the catalytic sites of their targets, D-alanine carboxypeptidase and acetylcholinesterase.

Following completion of the studies reported in this paper, we discovered that the concept of solid-state affinity labeling had been previously enunciated by Singh et al. (1979). These workers reported the synthesis of a succinimidyl ester agarose derivative and the use of this amino group reactive resin to immobilize proteins. Release of the proteins was accomplished by chemical cleavage of phenyl ester bonds in the arm. Singh et al. (1979) pointed out the possibility of preparing a similar resin bearing an active site directed reagent moiety on a cleavable arm and using it to localize and identify active site residues of target proteins.

Seen in this light, the present studies are significant in their recognition of the potential utility of solid-phase reagents as affinity-labeling media and in the introduction of the approach of solid-phase *photo*affinity labeling as a tool for identification of binding-site residues. In addition, solid-phase reagents should be an aid in "fishing" for macromolecules bearing biologically interesting ligand binding sites. As an example of a system from the steroid receptor field to which solid-state photoaffinity labeling may be applicable as an aid to the isolation of the receptor, one may cite the recent report of Sadler & Maller (1982). These workers found that 17,21-dimethyl-19-norpregn-4,9-diene-3,20-dione (R5020), a synthetic progestin having a dienone chromophore, was a photoaffinity-labeling agent for a steroid receptor on the surface of *Xenopus* oocytes that is thought to mediate a steroid-stimulated cell division. Godeau et al. (1978) observed that a steroid covalently linked to an insoluble polymer will elicit the same biological response of cell division by *Xenopus* oocytes as do soluble steroids. By photochemical techniques like those we have developed, an R5020-like steroid linked to an insoluble polymer could be used to isolate the macromolecule from the cell surface to which steroids bind.

Registry No. Δ^5-3-Ketosteroid isomerase, 9031-36-1; 19-nortestosterone, 434-22-0.

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Kinetics of RNA Replication†

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ABSTRACT: The reaction kinetics of single-stranded RNA replication were investigated by means of analytical and computer simulation methods. A model reaction mechanism is proposed that is in accord with the extensive experimental data available for the replication of various templates by the enzyme Qβ replicase. Despite the complexity of this mechanism, conventional concepts of steady-state and dynamic enyzme catalysis and plausible values of the rate and stability constants for the elementary reactions suffice to provide detailed understanding of RNA replication kinetics. The main features can be described with simple formulas that are analogous to traditional descriptions of enzyme kinetics.

Self-reproduction is the basis of genetic information transfer and thus also of natural selection and evolution. Indeed, the earliest self-reproductive systems able to organize themselves in a selective fashion were most likely composed of short single-stranded RNA molecules (Eigen, 1971). It is therefore especially interesting to study the mechanism of single-stranded RNA replication. The detailed mechanism of the autocatalytic growth process that occurs for self-replicating RNA molecules, i.e., for RNA species whose plus and minus strands can both the replicated by the same enzyme, is important for their selection and evolution.

However, while the kinetics of enzyme catalysis have been studied in great detail and for a broad variety of reactions since the subject was given its theoretical foundation by Michaelis and Menten in 1913, enzyme-catalyzed, template-instructed polymerization reactions have had little attention from kineticists. This is due in part to the intrinsic mechanistic complexity of polymerization reactions and in part to the difficulty of finding a suitable prototype reaction for experimental studies. The latter problem has now been solved by the establishment of the reaction mechanism of the in vitro replication of suitable RNA strands by the enzyme Qβ replicase. A theoretical analysis of the reaction mechanism of RNA replication based upon the available experimental results is presented in this paper.

Experimental Background

The first replication system to be studied in vitro was the replication of RNA-containing bacteriophages. In contrast to the long-known RNA-containing plant viruses like TMV, whose replication mechanism is still unclear (Hirth & Richards, 1981), several groups succeeded shortly after the discovery of RNA-containing bacteriophages (Loeb & Zinder, 1961) in detecting RNA synthesis in cellular extracts of infected cells (Haruna et al., 1963; Weissmann et al., 1963a,b; August et al., 1963). An enzyme that specifically amplified viral RNA while ignoring host RNA was partially purified from infected cells (Haruna et al., 1963; Spiegelman & Doi, 1963) and called RNA replicase (Spiegelman & Hayashi, 1963). The replicase of phage Qβ proved to be a relatively stable enzyme and was therefore adopted for most in vitro experiments (Haruna & Spiegelman, 1965a,b). Spiegelman et al. (1965) showed that the product of in vitro replication

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