Silverstein, E., and Sulebele, G. (1969b), *Biochemistry 8*, 2543.
Silverstein, E., and Sulebele, G. (1969c), *Biochim. Biophys. Acta 185*, 297.
Silverstein, E., and Sulebele, G. (1969d), 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL-261.
Silverstein, E., and Sulebele, G. (1970a), *Biophys. J. 10*, 123a.
Silverstein, E., and Sulebele, G. (1970b), *Biochemistry 9*, 274, 283.
Smith, E. L., Landon, M., Piszkiewica, D., Brattin, W. J., Langley, T. J. and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Struck, J., and Sizer, I. W. (1960), *Arch. Biochem. Biophys. 86*, 260.
Tomkins, G. M., Yielding, K. L., Curran, J. F., Summers, M. R., and Bitensky, M. W. (1965), *J. Biol. Chem. 240*, 3793.

Affinity Labeling of the Active Sites of Δ^5-Ketosteroid Isomerase Using Photoexcited Natural Ligands†

Richard J. Martyr‡ and William F. Benisek*

ABSTRACT: In attempts to use natural ligands which bind to macromolecules as their own affinity reagents, we have explored the use of photoexcited ketones. The activity of Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* is only slightly reduced when the enzyme is irradiated with ultraviolet light of wavelengths above 300 nm unless steroid ketones which are competitive inhibitors of the enzyme are present. The order of effectiveness of steroid ketones in promoting photoinactivation is 3-oxo-4-estren-17β-yl acetate > 17β-hydroxy-4-estren-3-one > 17β-hydroxy-4-androsten-3-one $\gg$ 1-cyclohexen-2-one, which parallels the order of affinity of these substances for the enzyme's active site. The competitive inhibitors 3β-hydroxy-5-pregnen-20-one and 3-methoxy-1,3,5(10)-estratrien-17β-ol do not support photoinactivation at a significant rate. The inactivation promoted by 3-oxo-4-estren-17β-yl acetate is slowed in the presence of the competitive inhibitor 3β-hydroxy-5-androstene-17β-carboxylic acid. Sephadex chromatography of isomerase photoinactivated in the presence of 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate showed that radioactivity became associated with the protein during the photoinactivation, whereas enzyme which was incubated with the steroid in the dark did not become associated with appreciable radioactive material. These results suggest that photoinactivation proceeds by excitation of the keto group of an active-site bound 3-keto steroid followed by chemical reactions between enzyme functional groups and the electronically excited ketone. One or more of these reactions may involve covalent attachment of the steroid to the enzyme.

Affinity labeling of the binding sites for small molecules (ligand binding sites) on protein molecules has in numerous instances helped to identify functional group components of such sites or to identify which protein in a complex mixture of proteins contained the site of interest (Shaw, 1970). The accuracy of detailed structural interpretations based on affinity labeling is contingent upon two conditions. The first is that the site which is labeled by the affinity reagent is the same as that which binds the natural ligand. The second is that the affinity reagent noncovalently binds to the site congruently with the mode of binding of the natural ligand.

Frequently, grafting of a chemically reactive functional group onto the molecular structure of the natural ligand in order to produce a potential affinity reagent results in a sufficiently substantial structural alteration such that either one or both of the conditions mentioned may not be met.

One could avoid the possibility of not satisfying these conditions if one employed the natural ligand as its own affinity reagent. Usually this is not feasible since the natural ligand does not contain a suitably chemically reactive group. However, many natural ligands including substrates, allosteric effectors, and hormones contain functional groups which, when promoted to electronically excited states by absorption of light of appropriate wavelength, are converted from chemically inert to chemically reactive species. Ligand molecules which contain such functional groups could then function as their own affinity reagents when illuminated with radiation of the correct wavelengths. Affinity labeling based on electronic excitation of the natural ligand would, then, be a three-step process (Scheme I): (1) noncovalent binding of the natural

SCHEME I

$$\text{protein} + \text{ligand} \rightleftharpoons \text{protein}\cdot\text{ligand}$$

$$\text{protein}\cdot\text{ligand} \overset{h\nu}{\rightleftharpoons} \text{protein}\cdot\text{ligand}^*$$

$$\text{protein}\cdot\text{ligand}^* \longrightarrow \text{chemically modified protein}$$

ligand to its appropriate site on a macromolecule; (2) absorption of light by the bound ligand resulting in its electronic excitation; (3) reaction of the excited ligand with a functional group(s) in its binding site.

Ligand molecules which contain ketone groups possess the properties which could allow them to be used as affinity reagents. When irradiated with light in the wavelength range 280–320 nm, ketones are excited to a diradical-like state which

† From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616. *Received February 12, 1973.* Supported by a grant from the National Institutes of Health (AM-14729).

‡ Supported by National Institutes of Health Predoctoral Traineeship GM-119-14.

can undergo a variety of reactions with nearby functional groups (Turro *et al.*, 1972). Two of the reactions typical of excited ketones are described in the Appendix. One of these reactions, the intermolecular type II process, leads to addition of a hydrocarbon C–H group across the carbon–oxygen double bond of the ketone. This reaction and some others which electronically excited ketones can undergo would lead to covalent coupling of the ketone to neighboring functional groups of the binding site.

This paper reports initial studies on the application of these ideas to the affinity labeling of the steroid binding sites of Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* (EC 5.3.3.1). This system was chosen for this initial investigation because the enzyme is small, well characterized, readily purified, and binds steroid ketones. The primary structure has been determined by Benson *et al.* (1971). Recently, Büki *et al.* (1971) have reported successful affinity labeling of the active site of Δ^5-ketosteroid isomerase using the steroidal alkylating reagent, 3-oxo-6β-bromo-4-androsten-17β-yl acetate.

The Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* catalyzes the allylic isomerization of Δ^5-3-keto steroids to their Δ^4 isomers. Recent studies have shown that the pure enzyme at concentrations below 1 mg/ml exists as a dimer of identical mol wt 13,394 polypeptide chains (Talalay, P., personal communication; Beckman, E., and Benisek, W. F., unpublished experiments). A wide variety of steroids are competitive inhibitors of the enzyme and are presumed to bind at the substrate binding sites. Among these competitive inhibitors are the Δ^4-3-keto steroids which are the products of isomerase-catalyzed reactions. The research on the structure and mechanism of action of this enzyme has recently been thoroughly reviewed (Talalay and Benson, 1972).

The experiments described in this paper were designed to determine if photoexcited Δ^4-3-keto steroids inactivated isomerase, whether the inactivation was dependent upon the binding of the steroid at the active site, and whether there was covalent attachment of the steroid to the enzyme molecule.

Materials and Methods

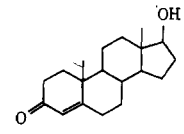
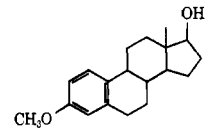
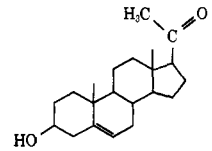
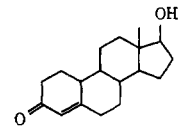
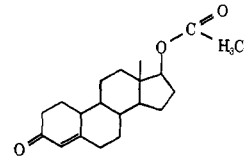
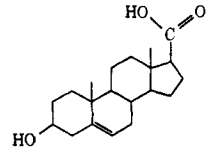
Δ^5-Ketosteroid isomerase was purified from *P. testosteroni* by the method of Jarabak *et al.* (1969). The protein appeared to be homogeneous when examined by electrophoresis in polyacrylamide gel either in the presence or absence of sodium dodecyl sulfate. Sedimentation velocity measurements in the analytical ultracentrifuge showed a single symmetrical sedimenting boundary using either schlieren optics or the ultraviolet scanning attachment. The specific activity of enzyme preparations was approximately 60,000 units per milligram of protein when assayed as described below.

Unlabeled steroids were obtained from Steraloids, Inc., and were used without further purification. 4-[^{14}C]-3-Oxo-4-estren-17β-yl acetate was obtained from New England Nuclear Corp. The specific activity was 50 mCi/mmol. Before use it was diluted with unlabeled 3-oxo-4-estren-17β-yl acetate to a specific activity of 16,000 cpm per microgram. All other chemicals were reagent grade commercial products.

Protein concentration was determined by measurement of the absorbance of solutions at 280 nm. The extinction coefficient of isomerase was taken to be 0.413 ($\epsilon_{1\,cm}^{0.1\%}$) (Kawahara *et al.*, 1962). All molar concentrations of enzyme are expressed in terms of polypeptide chains of mol wt 13,394.

Enzyme activity was determined by a modification of the method of Kawahara *et al.* (1962). Our modifications included assaying at 30° rather than 25° and supplementing the standard assay solution with 2.5 mM disodium ethylenediaminetetraacetate. In our laboratory, addition of EDTA decreases the blank rate obtained in the absence of enzyme and increases slightly the enzymatic rate. All enzymatic rates were corrected for the rate obtained in the absence of enzyme.

TABLE I: K_I Values and Structural Formulas of Steroids.

Systematic Name	Structural Formula	K_I (μM)
1-Cyclohexen-2-one		>5000[a]
17β-Hydroxy-4-androsten-3-one		48
3-Methoxy-1,3,5(10)-estratrien-17β-ol		22
3β-Hydroxy-5-pregnen-20-one		17
17β-Hydroxy-4-estren-3-one		13
3-Oxo-4-estren-17β-yl acetate		7
3β-Hydroxy-5-androstene-17β-carboxylic acid		7

[a] No inhibition of the enzyme reaction was detected when the concentration of 1-cyclohexen-2-one was 250 μM and the substrate concentration was 18 μM. A rate decrease of 5% would have been detectable in the enzyme assays.

K_I values for each steroid ketone were determined by the method of Dixon (1953). Initial rates were measured as a function of inhibitor concentration at each of three different substrate concentrations. The K_I values obtained are summarized in Table I along with the systematic names and structural formulas for each steroid and 1-cyclohexen-2-one.

Irradiation of enzyme and steroid mixtures or enzyme alone was carried out using an apparatus in the laboratory of Professor Kenneth Hancock, Department of Chemistry, University of California, Davis, Calif. The light source consisted of a 450-W medium-pressure mercury–xenon discharge lamp (Hanovia Model 679A) cooled by circulation of water through

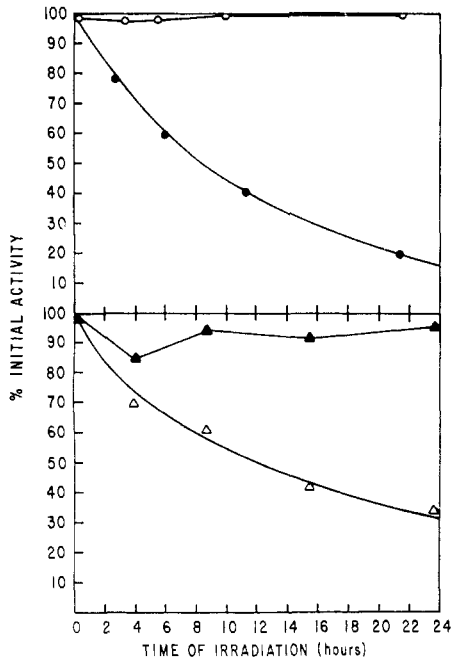


FIGURE 1: Ultraviolet irradiation of isomerase in the presence and absence of 17β-hydroxy-4-estren-3-one and oxygen. Final concentrations in the reaction mixture of 300 μl were: isomerase, 29 μM; 17β-hydroxy-4-estren-3-one, 0 or 122 μM; potassium phosphate, 0.045 M; EDTA, 0.0022 M; pH 7.0; ethanol, 3.3% (v/v). Symbols used are: enzyme + air (○); enzyme + steroid + air (●); enzyme + nitrogen (▲); enzyme + steroid + nitrogen (△). The temperature of the reaction mixture during irradiation was −1° (sample not frozen) to 4°.

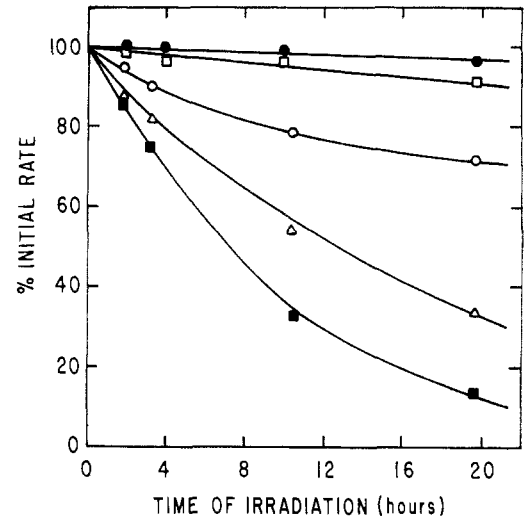


FIGURE 2: Ultraviolet irradiation of isomerase in the presence of various Δ^4-3-keto steroids. The final concentrations and reaction conditions were: pH 7.0; sodium phosphate, 0.04 M: ethanol, 3.3% (v/v); isomerase, 5 μM; temperature, 18–26°. The concentrations of α,β-unsaturated ketones were: none (●); cyclohex-2-en-1-one, 330 μM (□); 17β-hydroxy-4-androsten-3-one, 23 μM (○); 17β-hydroxy-4-estren-3-one, 24 μM (△); 3-oxo-4-estren-17β-yl acetate, 21 μM (■); total volume, 150 μl. The per cent of isomerase active sites complexed with α,β-unsaturated ketone under reaction conditions were: (●) 0% (□) <1%; (○) 32%; (△) 62%; (■) 71%.

a quartz jacket. The enzyme solution was contained in a Pyrex nuclear magnetic resonance (nmr) tube obtained from Kontes Glass Co. The sample tubes were positioned in a silvered Pyrex dewar flask which had an unsilvered "window" on the side facing the light source. There was a total thickness of 4.4 mm of Pyrex between the sample and the light source, which allowed less than 1% of light of wavelength less than 300 nm to reach the sample (Calvert and Pitts, 1966). The temperature of the interior of the dewar was regulated by blowing cooled dry nitrogen gas through the dewar flask. The temperature of the interior of the dewar was measured by means of a thermocouple. During irradiations the temperature was found to vary ±5°. For all experiments reported in this paper, the distance between the light source and the sample tubes was 8.5–9.0 cm. In some experiments, irradiation was carried out in the absence of oxygen. This was accomplished by flushing the sample in its nmr tube with nitrogen for a minimum of 7 hr at 4° before irradiation. During irradiation of anaerobic solutions, samples were withdrawn for enzyme assay be means of a hypodermic syringe fitted with a long piece of polyethylene capillary tubing while a nitrogen barrier was maintained over the solution. Samples thus obtained were diluted immediately with neutral 1% bovine serum albumin prior to assay for enzyme activity.

When it was desired to compare rates of inactivation promoted by different steroids, irradiation of the reaction mixtures to be compared was performed simultaneously. Thus, any fluctuations in temperature and light intensity were identical for such samples. In the apparatus employed up to three samples could be simultaneously irradiated. When it was necessary to compare more than three reaction mixtures a "monitor" reaction was included with each set of three reactions irradiated at a given time. Thus, any rate variations due to temperature differences or light intensity differences between separate irradiations could be corrected for by comparison of the rates of inactivation observed for the "monitor" reactions. Specific reaction conditions are described in the legends to figures summarizing the results of experiments.

Samples for radioactivity determination were placed in polyethylene vials. Aquasol (New England Nuclear Corp.) (10 ml) was added and after at least a 0.5-hr cooling period the samples were counted using a Packard Model 3375 scintillation counter.

Results

Photoinactivation of Isomerase. 17β-Hydroxy-4-estren-3-one, the product of the ketosteroid isomerase reaction when 17β-hydroxy-5-estren-3-one is the substrate, is a competitive inhibitor of isomerase.

Figure 1 shows the effect of irradiation of isomerase on the activity of the enzyme when carried out in the presence and absence of 122 μM 17β-hydroxy-4-estren-3-one. Each irradiation was performed using reaction mixtures equilibrated with either air or nitrogen as described under Materials and Methods. Two facts are apparent from consideration of these results. First, isomerase activity is not significantly altered by irradiation under the set of conditions employed unless the steroid is present. In the presence of this steroid a progressive inactivation process occurred. Incubation of isomerase and 17β-hydroxy-4-estren-3-one in the dark for 24 hr produced no inactivation of the enzyme. The rate of the inactivation process was somewhat greater in the presence of oxygen but the reaction still occurred under anaerobic conditions.

If excitation of active-site bound steroid was a necessary prerequisite to enzyme inactivation as required by the reactions in Scheme I, and if a series of molecules containing the 3-keto-4-ene chromophore but differing in affinity for isom-

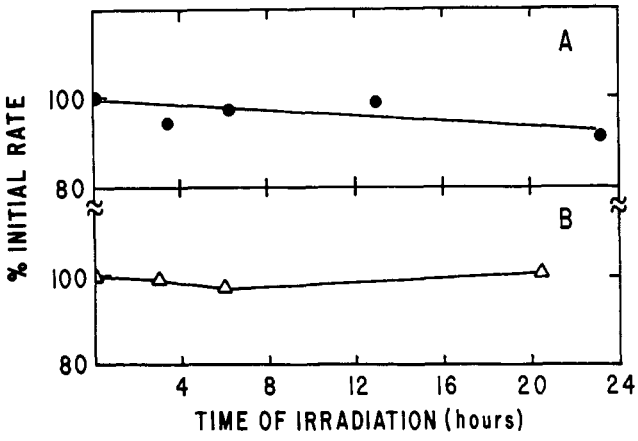


FIGURE 3: Ultraviolet irradiation of isomerase in the presence of steroids not containing the 3-keto group. Panel A, final concentrations and reaction conditions: pH 7.0; sodium phosphate, 0.04 M; ethanol, 3.3% (v/v); isomerase, 5 μM; 3-methoxy-1,3,5(10)-estratrien-17β-ol, 23 μM (●). The temperature range was 15–25°. Irradiation was carried out in the presence of atmospheric oxygen. Panel B, final concentration and reaction conditions: pH 7.0; potassium phosphate, 0.045 M; EDTA, 0.0022 M; ethanol, 3.3% (v/v); isomerase, 29 μM; 3β-hydroxy-5-pregnen-20-one, 21 μM (△). The temperature range was 1–6°. Nitrogen was blown over this sample for 11 hr prior to irradiation. The irradiation was carried out under a nitrogen barrier. The per cent of active sites complexed with steroid was: (●) 50%; (△) 39%.

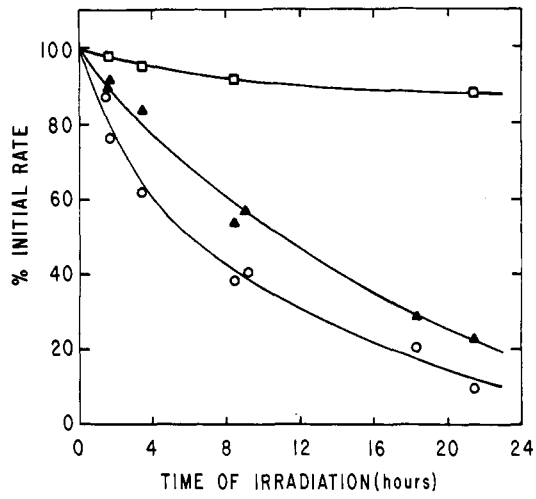


FIGURE 4: Effect of 3β-hydroxy-5-androstene-17β-carboxylic acid on photoinactivation of isomerase promoted by 3-oxo-4-estren-17β-yl acetate. The final concentrations were: pH 7.0; sodium phosphate, 0.04 M; ethanol, 3.3% (v/v); isomerase. 5 μM; 3-oxo-4-estren-17β-yl acetate, 21 μM (○); 3-oxo-4-estren-17β-yl acetate, 21 μM + 3β-hydroxy-5-androstene-17β-carboxylic acid, 42 μM (▲); 3β-hydroxy-5-androstene-17β-carboxylic acid, 42 μM (□). The temperature was 17–28°; final volume, 150 μl. Each keto steroid promoted inactivation curve represents a fit to the data from two separate irradiations. The per cent of isomerase active sites complexed to 3-oxo-4-estren-17β-yl acetate was: (○) 71%; (▲) 34%; (□) 0%.

erase was tested as photoinactivators, one would find that at a fixed concentration of steroid ketone, the higher the affinity of the steroid for the enzyme, the more rapid should be the photoinactivation. However, if a nonspecific process was responsible for inactivation then all cyclic α,β-unsaturated cyclohexenones should inactivate isomerase at comparable if not precisely equal rates. Figure 2 shows the results of an experiment in which isomerase was irradiated in the presence of several keto steroids and 1-cyclohexen-2-one. The concentration of each steroid was near 25 μM and that of cyclohexenone was 330 μM. Comparison of the inactivation curves obtained in the presence of each of these substances (Figure 2) shows that the effectiveness in promoting photoinactivation of isomerase correlates with the affinity of the unsaturated ketone for the enzyme. It is noteworthy that 1-cyclohexen-2-one, which does not detectably bind to the active site of isomerase, is only slightly active as a promoter of the photoinactivation even though it was tested at more than ten times the concentration of the steroidal ketones. Thus, nonspecific reactions between photoexcited keto groups or their photochemically altered products and enzyme cannot be responsible for the inactivation of the enzyme. Rather, it is necessary to postulate that the binding of the ketone-containing molecule to the active site is a necessary condition for inactivation by light. Such would be the case if excitation of bound steroid was an obligatory step in inactivation.

Alternately, one might suggest that *any* steroid which bound to the enzyme would, by means of some induced conformation change in the protein, convert the protein from an ultraviolet (uv)-resistant state to a uv-sensitive state. This possibility was tested by comparing the inactivation rate observed in the presence of 17β-hydroxy-4-estren-3-one with those found in the presence of 3β-hydroxy-5-pregnen-20-one and 3-methoxy-1,3,5(10)-estratrien-17β-ol, steroids which do not contain the 3-keto-4-ene chromophore. When isomerase was irradiated in the presence of either of these competitive inhibitors (Figure 3) little or no inactivation of the enzyme was found. The concentrations of 3β-hydroxy-5-pregnen-20-one and 3-methoxy-1,3,5(10)-estratrien-17β-ol were chosen so that approximately 50% of the enzyme active sites were complexed with steroid in each case. Our failure to observe inactivation in the presence of these steroids would suggest that steroid binding alone is not sufficient for effectively promoting photoinactivation. It should be noted that 3-methoxy-1,3,5-(10)-estratrien-17β-ol possesses an A-ring chromophore which has an extinction coefficient in the vicinity of 300 nm similar to that of 17β-hydroxy-4-estren-3-one.

Effect of a Competitive Inhibitor on Rate of Photoinactivation. If photoinactivation occurs *via* excitation of bound steroid ketone then the presence of a steroid which binds to the active site but does not promote photoinactivation should reduce the rate of photoinactivation promoted by the steroid ketone alone. To test this prediction the rate of inactivation of isomerase by 3-oxo-4-estren-17β-yl acetate was measured in the presence and absence of the competitive inhibitor 3β-hydroxy-5-androstene-17β-carboxylic acid. Figure 4 summarizes the results obtained. Clearly, the presence of 3β-hydroxy-5-androstene-17β-carboxylic acid reduces the inactivation rate substantially. Assuming the initial inactivation rate is proportional to the concentration of the enzyme–3-oxo-4-estren-17β-yl acetate complex (as required for an affinity labeling process) the presence of 3β-hydroxy-5-androstene-17β-carboxylic acid should have reduced the initial rate of inactivation to 48% of that obtained in its absence at the concentrations of steroids and enzyme employed. The initial rate of inactivation was observed to drop to about half that measured in the absence of 3β-hydroxy-5-androstene-17-β-carboxylic acid, in satisfactory agreement with the theoretical prediction.

Covalent Coupling of Steroid and Enzyme. The mechanism of photoinactivation of isomerase in the presence of 4,5-un-

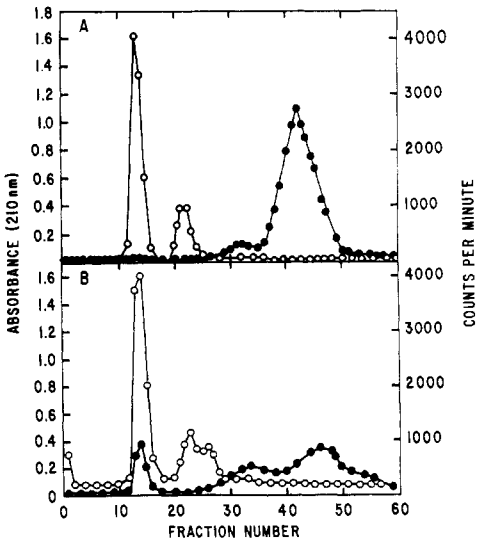


FIGURE 5: (A) Sephadex chromatography of isomerase incubated with 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate in the dark. The final concentrations of reactants were: isomerase, 29 μM; 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate, 122 μM; potassium phosphate, 0.045 M; EDTA, 0.0022 M; pH 7.0; ethanol, 3.3% (v/v); final volume, 0.30 ml. Under these conditions, 94% of the active sites were occupied by the steroid. Nitrogen was blown over the liquid surface in the nmr tube for 6 hr at 4°, the tube stoppered and stored in the dark at 4° for 42 hr. The reaction mixture was applied to a Sephadex G-25 column (diameter = 0.9 cm; bed volume = 15 ml) and eluted with 0.01 M KH_2PO_4, pH 7.0, at a flow rate of 5 ml/hr. Fractions (0.51 ml) were collected and their absorbances at 210 nm measured. The extinction coefficient of isomerase at 210 nm = 28.7 ($\epsilon^{0.1\%}_{1\ cm}$). Each fraction (200 μl) was added to 10 ml of Aquasol, cooled at least 0.5 hr, and counted in the scintillation counter. The measured counting efficiency in all vials was greater than 90%. Final enzyme activity was 100% of the initial activity: A_{210} (○); counts per min (●). (B) Sephadex chromatography of isomerase irradiated in the presence of 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate. The reaction conditions were identical with those described in A. Nitrogen was blown over the surface of the solution in the nmr tube for 19 hr at 4°. Irradiation was performed in a temperature range from −3° (sample not frozen) to 10°. The activity after irradiation was 51% of the initial activity. Chromatography, A_{210} determination, and sample counting were carried out as described as in Figure 5A: A_{210} (○); counts per minute (●). Sephadex chromatography of a sample containing only 2.5 mM EDTA in 34 mM potassium phosphate, pH 7.0, results in a peak absorbing at 210 nm eluting at fraction 20 similar in size to the small peaks seen near this position in A and B.

saturated 3-keto steroids which are competitive inhibitors of the enzyme is unknown to us at this time. However, since some of the possible photochemical processes would result in covalent attachment of the steroid to the protein (see Appendix) it was of interest to determine if such an attachment occurred during photoinactivation. For this purpose the photoinactivation of isomerase in the presence of 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate was investigated. This steroid was chosen for this study because it is a good competitive inhibitor of the enzyme (K_I = 7 μM) and is commercially available labeled with C-14. As shown in Figure 5B, when isomerase was irradiated under nitrogen in the presence of 4-[^{14}C]-3-oxo-4-estren-17β-yl acetate for 22 hr and then chromatographed on a column of Sephadex G-25, radioactive material eluted with the protein, whereas if irradiation was omitted but the steroid and enzyme were allowed to incubate at 4° in the dark for 42 hr and then chromatographed, very little radioactive material migrated with the protein (Figure 5A). During the irradiation period the enzyme's activity was reduced to 51% of the initial activity. In the dark control no loss of enzyme activity was detectable.

The stoichiometry of attachment of steroid to isomerase could be estimated from the total disintegrations per minute eluted with the protein peak after correction for the amount of radioactive material migrating with isomerase measured in the dark control. This calculation showed that the molar ratio of protein-linked steroid to inactivated protein active sites was 0.48. This raises the possibility that under the conditions under which the photoinactivation occurred approximately half of the photoinactivating events involved attachment of steroid to enzyme, presumably at the enzyme's active site.

Discussion

The study of binding and catalytic sites for small molecules on proteins and identification of functional groups adjacent to or within such sites have been approached by chemical modification of the protein using group specific reagents. One of the most successful routes to specific chemical modification of functional groups of active sites has been the use of active-site directed reagents or affinity reagents (Shaw, 1970), chemically reactive molecules which possess structures which resemble the structures of the natural substrates or ligand molecules. Following noncovalent binding to the relevant site on the protein, such reagents, ideally, react irreversibly with some functional group nearby. To the extent that the irreversible reaction is specific for the active-site group *vs.* similar groups not in or near the site, such a labeling process can serve to identify the modified group as a component of the site. The validity of the identification is a direct function of the structural resemblance between the reagent and the normal substrate. This resemblance also determines whether the reagent binds to the very same site as does the substrate or whether it binds to some other site on the protein surface. Thus, one needs to demonstrate the equivalence of the substrate binding site and the reagent binding site. Sometimes this can be established by showing that the reagent behaves as a competitive inhibitor of the enzyme if the binding site is also a catalytic site, but often the reagent is too chemically reactive for such experiments. In the labeling of binding sites which are not catalytic such an approach is not possible. Another approach is to demonstrate that the natural ligand inhibits the labeling process. This method is applicable both to catalytic and noncatalytic sites.

In addition to identifying functional group components of the binding site one would like to obtain information about the relative positions of atoms of the ligand and atoms of the functional group which is modified. This can sometimes be inferred from the known (or presumed) steric requirements of the particular labeling reaction involved. For example, it is often assumed that a labeling reaction which consists of a nucleophilic displacement on a saturated carbon atom involves "backside" attack by the protein nucleophile which is labeled (even though other mechanisms exist for nucleophilic substitution which have quite different steric requirements, such as the SN1 process or, in the case of allylic halides, the SN2′ process). One can employ a series of structurally related affinity reagents which have the reactive functionality located in different parts of the parent structure in the hopes of labeling different functional groups of the protein which are coresidents of the site. Then one might infer from the different structures of the reagents employed how the various protein functional groups were geometrically related to each other as well as how each was disposed relative to the natural ligand.

Such detailed analyses of labeling experiments depend on certain assumptions for their relevance to the structure of the natural ligand–protein complex. One, mentioned above, is that the reagent and the natural ligand bind at the same site. Another is that the reagent and natural ligand bind with the same orientation with respect to the protein functional groups. In the case of flexible ligands and affinity reagents one must also assume that the reagent and the natural ligand adopt the same conformation upon binding. It is customary to append a good leaving group such as bromo or iodo to a ligand's carbon skeleton in order to generate an affinity reagent which can alkylate a protein nucleophile. Although such a structural modification may seem innocuous when the structural formulas of the reagent and the corresponding natural ligand are compared, inspection of space filling molecular models shows that rather large changes in the surface contour of the molecule result from attachment of the larger halogen atoms. Such steric alterations could well influence the orientation and conformation of the bound species or even prevent binding altogether.

Presumably, the electronically excited keto steroids employed in this study are bound in the active site in a fashion completely congruent with that of their ground states, products of the isomerase reaction, thus minimizing the ambiguities inherent when structurally different affinity reagents are employed.

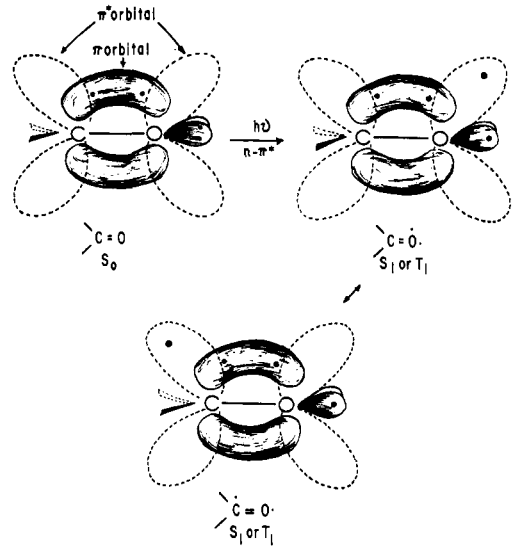
The existence of an inverse correlation between the rate of photoinactivation and the magnitude of the steroid–protein dissociation constant for a series of 3-keto-4-ene steroids and cyclohexenone (Figure 2) suggests that the inactivation process or processes are most probably dependent upon binding of the steroids to the active site.

The protective effect of 3β-hydroxy-5-androstene-17β-carboxylic acid (Figure 4) provides additional support for the conclusion that the photoinactivation occurs at the active site.

The experiments described in this paper have demonstrated that the active sites of the Δ^5-ketosteroid isomerase of *Pseudomonas testosteroni* can be inactivated using α,β-unsaturated 3-keto steroids, the products of the reaction catalyzed by this enzyme, as active-site directed reagents. However, binding alone is not sufficient for promotion of inactivation since 3β-hydroxy-5-pregnen-20-one and 3-methoxy-1,3,5(10)-estratrien-17β-ol, two steroids which do not contain the α,β-unsaturated 3-keto structure, but do bind to the enzyme's active sites (K_I = 17 and 22 μM, respectively), do not appreciably promote inactivation. The aromatic steroid even possesses an A-ring chromophore which absorbs light over a similar wavelength range as do α,β-unsaturated ketones.

In the case of 3-oxo-4-estren-17β-yl acetate the light-induced inactivation is accompanied by attachment of the steroid to the enzyme in approximately half of the activating events. This may indicate that inactivation consists both of processes which involve attachment of steroid and processes which do not involve such attachment. A candidate for an aerobic inactivation process not involving steroid attachment is steroid ketone sensitized photooxidation of the enzyme. If oxygen is sensitized by active-site bound steroid ketones, then any subsequent oxidation by the resulting singlet oxygen should be restricted to the immediate vicinity of the active site since the lifetime of singlet oxygen in solution is very short (Kearns, 1971). Such a bound ligand sensitized oxidation could constitute a useful method for labeling photooxidizable functional groups residing at the border of the ligand binding site and would provide structural information complementary to that derived from inactivation processes not involving oxygen.

SCHEME II



Reaction between the excited ketone and protein functional groups such as photoreduction (Yang and Yan, 1958), which does not involve molecular oxygen, can lead to chemical modification of the steroid binding site without covalent attachment of the steroid to the protein.

Our experiments have not identified the chemistry of the steroid attachment reaction or even whether more than one mode of attachment occurs. Such studies are now in progress. The reactions outlined in the Appendix only represent reasonable guesses which must be tested by further experimentation.

The fact that mammalian cells possess receptor proteins for a number of steroid hormones (Jensen and DeSombre, 1972) which bind specific keto steroids with high affinity and specificity encourages us to explore the range of applicability of this approach to site labeling. Use of radioactive steroids in photoaffinity labeling involving steroid attachment may be of some use in tagging keto steroid hormone receptor molecules in complex systems such as cells or cell contents from the appropriate target tissues. However, the investigation of complex systems must be preceded by studies of simpler systems. It may also be of some interest that a large number of nonsteroidal natural metabolites and effector substances contain ketone groups and might be used as their own affinity reagents by the photochemical approach. These would include the α-keto acids, keto sugars, and prostaglandins, to name a few examples.

Acknowledgment

The authors thank Dr. Kenneth Hancock for generously making available for this research a photochemical reactor and for several informative discussions.

Appendix

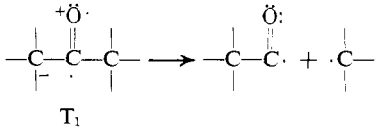
When irradiated by light of wavelength 300 ± 20 nm, ketones undergo electronic excitation *via* the processes outlined in Scheme II. Absorption of a quantum of light of wavelength 280–320 nm results in promotion of one of the nonbonding electrons of the oxygen to an antibonding π orbital. Initially, the promoted electron and the electron remaining in

the nonbonding orbital retain the antiparallel spins of the S_0 ground state. This first excited state is, like the ground state, a singlet state and is referred to as S_1. The lifetime of this state is short, 10^{-5}–10^{-9}. Particularly with ketones, a relatively efficient process called intersystem crossing occurs which involves a spin flip of one of the now unpaired electrons so that the two electrons have parallel spins. The result of intersystem crossing is an electronically (and vibrationally) excited triplet state referred to as T_1. The lifetime of T_1 is much greater than that of S_1, being about 10^{-3} sec, frequently long enough for chemical reactions involving T_1 to occur before T_1 decays back to S_0 *via* phosphorescence or *via* intersystem crossing to one of the higher vibrational levels of S_0.

For the purposes of protein chemistry two points are particularly important. First, the chemical properties of S_1 and T_1 are quite different from those of S_0, both being more reactive than S_0. Second, the lifetime of T_1 is long enough for it to react with appropriate nearby atoms at a significant rate, although reactions occurring *via* S_1 only have been detected (Turro *et al.*, 1972).

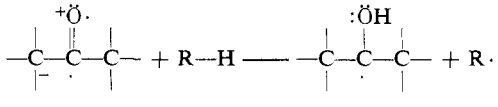
The chemistry of the T_1 states of ketones is quite diverse and has been treated in several reviews and monographs (Turro *et al.*, 1972; Kan, 1966). The first event in any photochemical reaction of a ketone is the production of S_1 and T_1. For $n \rightarrow \pi^*$ type excitation the process can be diagrammed in terms of the molecular orbitals involved as shown in Scheme II. Note that both the carbonyl carbon and the oxygen possess unpaired electrons in the S_1 and T_1 states, and thus can be regarded as diradicals.

The excess energy possessed by T_1 is comparable to or greater than the energy of single bonds. Consequently, one process which can occur is homolytic cleavage of the bond to an α carbon atom generating a radical pair. This is referred to as the type I reaction.

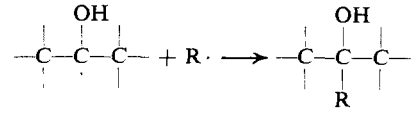


T_1

Another commonly observed reaction is the type II reaction which consists of hydrogen atom abstraction by the diradical-like T_1 state, either inter- or intramolecularly (Walling and Gibian, 1965).



The pair of radicals produced by the intermolecular type II process can then recombine in a subsequent step (Yang and Yan, 1958).



Since the hydrogen atom which was abstracted can be one bound to a carbon atom of R, the net result is addition of a hydrocarbon group across a ketone carbonyl group to yield a tertiary alcohol, a stable species. If the ketone occupies a ligand binding site on a protein and RH is a C–H bond-containing functional group of the protein, then attachment of the ketone to the site can occur by this mechanism.

An analogous process of hydrogen atom abstraction followed by radical recombination can be written for the radical pair generated by the type I α cleavage, again leading to covalent linkage of RH and a carbon atom of the ketone.

The reactivity of photoexcited ketones is not limited to hydrocarbon groups. Following type I cleavage, an intramolecular hydrogen atom migration can occur which generates a ketene. The well-known susceptibility of ketenes to nucleophilic addition by amines and hydroxyl groups permits formation of amide and ester linkages, respectively. Consequently, one might anticipate that appropriately juxtaposed hydroxyl and amino groups of proteins could be sites of affinity labeling as well.

References

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

Büki, K. G., Robinson, C. H., and Talalay, P. (1971), *Biochim. Biophys. Acta 242*, 268.

Calvert, J. G., and Pitts, J. N., Jr. (1966), Photochemistry, New York, N. Y., Wiley, p 748.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Jarabak, R., Colvin, M., Moolgavkar, S. H., and Talalay, P. (1969), *Methods Enzymol. 15*, 642.

Jensen, E. V., and DeSombre, E. R. (1972), *Annu. Rev. Biochem. 41*, 203.

Kan, R. O. (1966), Organic Photochemistry, New York, N. Y., McGraw-Hill.

Kawahara, F. S., Wang, S.-F., and Talalay, P. (1962), *J. Biol. Chem. 237*, 1500.

Kearns, D. R. (1971), *Chem. Rev. 71*, 395.

Shaw, E. (1970), *Enzymes, 3rd Ed., 1*, 91.

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

Turro, N. J., Dalton, J. C., Davies, K., Farrington, G., Hautala, R., Morton, D., Niemczyk, M., and Schore, N. (1972), *Accounts Chem. Res. 5*, 92.

Walling, C., and Gibian, M. J. (1965), *J. Amer. Chem. Soc. 87*, 3361.

Yang, N. C., and Yan, D-D. H. (1958), *J. Amer. Chem. Soc. 80*, 2913.