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# Chemical Modification of Rhodopsin and Its Effect on Regeneration and G Protein Activation†

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ABSTRACT: The studies reported are concerned with the functional consequences of the chemical modifications of the lysines and carboxyl-containing amino acids of bovine rhodopsin. The 10 non-active-site lysine residues of rhodopsin can be completely dimethylated and partially acetimidated (8–9 residues) with no loss in the ability of the proteins to activate the G protein when photolyzed or to regenerate with 11-*cis*-retinal. These modifications do not alter the net charge on the protein. Surprisingly, heavy acetylation of these lysines (eight to nine residues) with acetic anhydride, which neutralizes the positive charges of the lysine residues, yields a modified rhodopsin fully capable of activating the G protein and being regenerated. It is concluded that the non-active-site lysine residues of rhodopsin are not importantly and directly involved in interactions with the G protein during photolysis. However, this is not to say that they are unimportant in maintaining the tertiary structure of the protein because heavy modification of these residues by succinylation and trinitrophenylation produces proteins incapable of G protein activation, although the succinylated protein still regenerated. The active-site lysine of rhodopsin was readily modified and prevented from regenerating with 11-*cis*-retinal and with *o*-salicylaldehyde and *o*-phthalaldehyde/mercaptoethanol, two sterically similar aromatic aldehyde containing reagents which react by entirely different mechanisms. It is suggested that rhodopsin contains an aromatic binding site within its active-site region. Monoethylation, but not monomethylation, of the active-site lysine also prevented regeneration. Finally, the functional consequences of the chemical modification of the aspartate and glutamate residues were studied. These residues were modified by their carbodiimide-mediated coupling to (1) taurine, (2) glycine methyl ester, and (3) ethylenediamine, reagents which (1) do not alter the negative carboxyl group charge, (2) neutralize it, and (3) convert it into a positive charge, respectively. These modifications did not alter the abilities of the modified rhodopsin derivatives to activate the G protein after photolysis, but they did prevent it from regenerating with 11-*cis*-retinal after bleaching.

Rhodopsin is an integral membrane protein found in the disks of rod outer segments. This protein, of molecular weight 39K, contains a chromophore, 11-*cis*-retinal, covalently bound to lysine-296 by means of a protonated Schiff base (Hargrave et al., 1983). The absorption of a photon of light by rhodopsin results in the cis to trans isomerization of the chromophore (Hubbard & Kropf, 1958). This configurational change about the double bond is translated into a series of conformational changes in the protein (Wald, 1968). One of the spectroscopically defined conformers, metarhodopsin II (Parkes et al., 1979; Calhoon et al., 1981), interacts with and catalyzes a GTP for GDP exchange in a G protein, resulting in the activation of the latter (Fung & Stryer, 1980). This interaction with the G protein is the only known biochemical process directly affected by rhodopsin and serves as a probe for the activated form of the pigment (R*).[1] Activated G protein, in turn, releases a cGMP-specific phosphodiesterase from inhibition which results in the lowering of the free cGMP

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[1] Abbreviations: OPA, *o*-phthalaldehyde/mercaptoethanol; PIPES, 1,4-piperazinediethanesulfonic acid; PM-Rh, non-active-site lysine permethylated rhodopsin; R*, photochemically activated rhodopsin; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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concentration in the rod outer segment (Miller, 1981; Stryer, 1984). Thus, the absorption of a photon by rhodopsin leads to the hydrolysis of cGMP which apparently regulates the plasma membrane sodium channels (Fesenko et al., 1985). Activated G protein is assumed to be self-regulated by virtue of its GTPase activity (Fung & Stryer, 1980). Of substantial interest is the nature of the structure of rhodopsin and of the alteration of its three-dimensional structure triggered by the absorption of light as it relates to its function, measured by rhodopsin's ability to activate the GTPase activity of the G protein. One probe of structure–function relationships is through chemical modification of specific amino acids and classes of amino acids and determining the effect of these modifications on the function of rhodopsin as revealed by its ability to activate the G protein, the only current functional assay for R*. For example, it has recently been shown that exhaustive modification of the sulfhydryl groups of rhodopsin has no effect on its binding to the G protein but the modification of a single sulfhydryl residue of the $G_\alpha$ subunit abolishes this interaction (Hofmann & Reichart, 1985). This paper is concerned with studies on the functional consequences of modifications of the lysines and carboxyl-containing amino acids of rhodopsin.

There are at least 2 classes of lysine residues in rhodopsin. Of the total 11 lysine residues, lysine-296 is especially critical in that it is this lysine which forms a Schiff base with 11-*cis*-retinal (Hargrave et al., 1983). Most chemical modifications of this lysine would be expected to yield an opsin molecule incapable of forming a pigment, and it is shown here that certain aromatic aldehydes can be used to derivatize this lysine. The roles of the other lysine residues in the maintenance of the structure and function of rhodopsin are currently not understood. There are 5 aspartate and 17 glutamate residues, and the carboxyl-terminal end of the protein is unblocked (Hargrave et al., 1983). Special functions have been ascribed to two of these as yet identified residues. One carboxyl-containing residue must serve as the counterion to the positively charged Schiff base head group, and the second residue is thought to reside adjacent to $C_{11}$ of the retinoid and is presumed to be of great importance in controlling the $\lambda_{max}$ of light absorption for rhodopsin (Honig et al., 1979). It is noteworthy that of the 22 acidic amino acids, only 3 are thought to reside in the predominantly hydrophobic helices of the protein (Hargrave et al., 1983). Helix 3 contains two glutamate residues and helix 2 a single aspartate residue (Hargrave et al., 1983). It might be assumed that one or more of these residues interacts directly with the chromophore. The role(s) of the remaining aspartate and glutamate residues in the structural maintenance and function of rhodopsin is (are) also not currently understood. However, it is clear that lysine and glutamate and/or aspartate residues have a special role to play in the functioning of rhodopsin, and alterations of these residues either by site-specific mutagenesis or by chemical modification studies can be used to help define these roles.

## Materials and Methods

### *Materials*

Bovine retinas were obtained from Hormel, Inc. Formaldehyde, pyridine/borane, 1,4-piperazinediethanesulfonic acid (PIPES), sodium cyanoborohydride, glycine methyl ester, taurine, ethylenediamine, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide, and *o*-salicylaldehyde were all products of Aldrich Chemical Co. Concanavalin A–Sepharose, G-25 Sephadex, *o*-phthalaldehyde, mercaptoethanol, trinitrobenzenesulfonic acid, $N^\alpha$-acetyllysine, alanine, chloral hydrate, and GTP were all obtained from Sigma Chemical Co. [$^3$H]Taurine, [$^{14}$C]formaldehyde, and [$^{14}$C]acetaldehyde were obtained from New England Nuclear. Acetaldehyde and acetonylacetone were products of Fluka. Dodecyl β-D-maltoside and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO) were obtained from Behring Diagnostics. Methyl acetimidate was a product of Pierce Chemical Co. Acetic anhydride was obtained from Fisher. Succinic anhydride was a product of Eastman Kodak Chemical Co. Hydrofluor was obtained from National Diagnostics. Ammonyx LO was a gift from Onyx Chemical Co. [$^{32}$P]GTP was obtained from ICN or Amersham.

### *Methods*

Rod outer segment suspensions and solubilized rhodopsin were prepared by standard methods, as previously described (Calhoon & Rando, 1985). Unless otherwise stated, protein modifications were performed on solubilized rhodopsin in 10 mM 1,4-piperazinediethanesulfonic acid, pH 6.5, containing 100 mM NaCl and 6 mM dodecyl maltoside (PIPES/dodecyl maltoside buffer). All procedures involving unbleached rhodopsin were carried out under dim red light. Protein concentrations were measured spectrophotometrically, by using $\epsilon = 40000\ M^{-1}\ cm^{-1}$ at 500 nm or $\epsilon = 64000\ M^{-1}\ cm^{-1}$ at 278 nm (Applebury et al., 1974). UV/vis spectra were recorded with a Perkin-Elmer Model 552A spectrophotometer. Fluorescence spectra were recorded with a Perkin-Elmer Model 512 double-beam fluorescence spectrophotometer.

For bleaching and regeneration assays of modified proteins, aliquots (1 mL) of solubilized 2–10 μM rhodopsin were bleached for 10–20 min at 4 °C in the presence of 10 mM hydroxylamine using intense white light, or without hydroxylamine using orange light (same source filtered through a Corning 3-68 filter, cutoff at 540 nm). A 2–4-fold excess of 11-*cis*-retinal (0.6 mg/mL in 2-propanol) was added back to the bleached protein, and regeneration was allowed to proceed for 30 min at 20–25 °C or for 24 h at 4 °C. Figures for percentage regeneration reported here are the maximum observed for samples bleached using both methods.

*Non-Active-Site Lysine Modifications.* (*A*) *Methylation.* Rhodopsin (at concentrations of 10–30 μM) was methylated with the incorporation of 19–20 methyl groups following two rounds of reaction using 20 mM pyridine/borane and 2 mM formaldehyde each for 24 h at 20–25 °C (Longstaff & Rando, 1985). Modified protein was isolated by using a desalting column of G-25 Sephadex. Methyl group incorporation was measured by using [$^{14}$C]formaldehyde (1 Ci/mol). Protein samples were dialyzed against 10 mM HCl/0.5% (v/v) Ammonyx LO before scintillation counting (Hydrofluor).

(*B*) *Ethylation.* Ethylation was carried out by mixing 15–20 μM rhodopsin with 10 mM acetaldehyde and 20 mM pyridine/borane for 24 h at 20–25 °C. The extent of modification under these conditions was measured by using [$^{14}$C]acetaldehyde (1 Ci/mol), and samples were dialyzed and counted as above.

(*C*) *Acetimidation.* Acetimidation was based on the method of Wallace and Harris (1984) and was carried out with unsolubilized rod outer segment membranes. A standard rod outer segment preparation devised by others was used here as quoted in Calhoon and Rando (1985). Approximately 25% of the membranes, corresponding to 80–100 nmol of rhodopsin and an estimated 52 mol of amine/mol of rhodopsin (deGrip et al., 1973), were pelleted (30 min at 20000 rpm using a Beckman JA-20 rotor). The pellet was resuspended in 0.1 M sodium borate buffer, pH 10.0 (3.5 mL), and to this was added 100 mg of methyl acetimidate dissolved in 5 M NaOH (0.3

mL). the pH of the reaction mixture was readjusted to pH 10 with the addition of aliquots of 3 M HCl. The reaction was allowed to proceed for 90 min at 20–25 °C with gentle mixing, whereupon the suspension was centrifuged once more and the pellet washed twice with 10 mM Tris, pH 8.0, containing 2 mM dithiothreitol. Rhodopsin was solubilized by using 10 mM PIPES, pH 6.5, containing 0.1 M NaCl, 28 mM CHAPSO, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (2 mL) and applied to a column of concanavalin A (1 mL) run by a standard method as described previously (Calhoon & Rando, 1985).

The extent of lysine acetimidation was estimated from a subsequent methylation of available lysines in the solubilized protein using 2 mM [$^{14}$C]formaldehyde and 10 mM sodium cyanoborohydride. Labeled protein was dialyzed and counted as described previously for the methylation of rhodopsin.

(*D*) *Acetylation.* The acetylation procedure was based on the method described by Means and Feeney (1971a), using freshly distilled acetic anhydride. To a solution of 17.5 μM rhodopsin (1 mL) was added saturated sodium acetate (1 mL), and the mixture was cooled on ice. Over a period of 1 h, five aliquots (10 μL each) of acetic anhydride were added followed by a final addition of 20 μL. After this time, modified protein was isolated by gel filtration chromatography (at 4 °C) using a column of G-25 Sephadex. The extent of acetylation was estimated by methylating available, unreacted lysines using [$^{14}$C]formaldehyde and sodium cyanoborohydride as outlined previously for acetimidation.

(*E*) *Succinylation.* Succinylation was based on the method in Means and Feeney (1971a). To a solution of 14.5 μM rhodopsin (0.5 mL) was added 0.1 M sodium borate buffer, pH 9.3 (0.5 mL). Over a period of 70 min, six aliquots (10 μL each) of succinic anhydride (20 mg/mL) in dioxane were added with mixing, at 20–25 °C. The reaction was allowed to proceed for a total time of 90 min. The pH was monitored during the course of the reaction and was found to drop to a final value of 6.5. Modified protein was isolated by using a column of G-25 Sephadex. The extent of succinylation was measured by methylating unreacted lysines as described above for acetimidation.

(*F*) *Trinitrophenylation.* Trinitrophenylation was carried out by using the method of Snyder and Sobocinski (1975). To a solution of 20 μM rhodopsin (1 mL) was added 0.1 M sodium borate buffer, pH 9.3 (1 mL), followed by 0.03 M trinitrobenzenesulfonic acid solution (50 μL freshly made up in distilled water). The mixture was allowed to react for 3 h at 20–25 °C before modified protein was isolated by using a G-25 Sephadex column. The extent of trinitrophenylation was measured by carrying out a subsequent methylation using [$^{14}$C]formaldehyde as described above for acetimidation.

(*G*) *Reaction with OPA.* Rhodopsin (1 and 9 μM), alanine (2 μM), and $N^{\alpha}$-acetyllysine (2 μM) were reacted under the same conditions with OPA. *o*-Phthalaldehyde was dissolved in methanol (80 mg/mL) and added to the reaction mixture to give a final concentration of 6 mM (0.8 mg/mL). Mercaptoethanol was present at 15 mM (1 μL/mL). All reactions were performed in 10 mM PIPES/dodecyl maltoside buffer, pH 6.5, at 4 °C.

*Carboxyl group modifications using water-soluble carbodiimide* were based on the method of Hoare and Koshland (1967).

(*H*) *Taurine.* A solution of 31.4 μM rhodopsin (1.5 mL) was mixed with a solution of 1 M taurine (1.5 mL), made up by dissolving 1.5 mmol of taurine in 1.35 mL of buffer and adding to this 150 μCi of [$^{3}$H]taurine (150 μL). To this mixture was added 230 mg of carbodiimide to initiate the reaction. Final concentrations of reagents were 15.7 μM rhodopsin, 0.5 M [$^{3}$H]taurine (0.1 Ci/mol), and 0.4 M carbodiimide in an initial reaction volume of 3 mL. The pH of the solution was adjusted to 5.0–5.5 with 0.5 M HCl and maintained within this range throughout the reaction by adding small aliquots of 0.5 M HCl. The reaction was allowed to proceed for 2 h at 20–25 °C, whereupon protein was isolated from the reaction mixture by using a G-25 Sephadex column. [$^{3}$H]Taurine incorporation was measured following further dialysis of aliquots of modified bleached protein against 50 mM borate buffer, pH 9.5, containing 0.5% (v/v) Ammonyx LO. Protein solutions were solubilized in Hydrofluor for scintillation counting.

(*I*) *Glycine Methyl Ester.* An aliquot (2 mL) of rhodopsin solution was mixed with a solution of 4.55 M glycine methyl ester (0.67 mL) made by dissolving 3.14 g of glycine methyl ester in 1 M NaOH (5 mL) and adjusting the pH to 5.5 with 6 M HCl (final concentration of glycine methyl ester 4.55 M). Carbodiimide (205 mg) was added to begin the reaction. Final reagent concentrations were 18.5 μM rhodopsin, 1.14 M glycine methyl ester, and 0.4 M carbodiimide in an initial reaction volume of 2.67 mL. The pH of the solution was adjusted to 5.0–5.5 with 0.5 M HCl and maintained within this range during the reaction, which was for 2 h at 20–25 °C. Modified protein was isolated by using a desalting column of G-25 Sephadex and dialyzed against the PIPES/dodecyl maltoside buffer. Aliquots of this solution were lyophilized and hydrolyzed in 6 M HCl for amino acid analysis. The extent of carboxyl group modification was ascertained from the increase in glycine residues in the protein.

(*J*) *Ethylenediamine.* To an aliquot (1 mL) of 24.9 μM rhodopsin was added 2.5 M ethylenediamine solution (0.5 mL), made by diluting ethylenediamine (1 mL) in water (1 mL), neutralizing this solution using 6 M HCl, and diluting to a final volume of 6 mL. To this mixture was added 115 mg of carbodiimide, giving final concentrations of 16.6 μM rhodopsin, 0.83 M ethylenediamine, and 0.4 M carbodiimide in an initial reaction volume of 1.5 mL. The pH was readjusted to 5.0–5.5 and maintained within these limits throughout 2 h of reaction at 20–25 °C. Modified protein was isolated by using a desalting column of G-25 Sephadex. The extent of carboxyl group modification was estimated by methylating protein samples before and after modification using 20 mM pyridine/borane and 2 mM [$^{14}$C]formaldehyde (1 Ci/mol) for 24 h at 20–25 °C. Methylated proteins were dialyzed for 3–4 days against 10 mM HCl/0.5% (v/v) Ammonyx LO before aliquots were used for scintillation counting (Hydrofluor). The increase in $^{14}$C methylation for ethylenediamine-modified rhodopsin, as compared to unmodified protein, was used to assess the extent of ethylenediamine incorporation.

Spectra of carboxyl group modified proteins isolated by gel filtration showed varying degrees of contamination by UV absorbing reaction products (<350 nm) which were removed by further dialysis. Bleaching/regeneration experiments and GTPase activation assays were performed on protein samples which had been dialyzed for 2 days against PIPES/dodecyl maltoside buffer. However, in the case of taurine-modified rhodopsin, complete removal of UV-absorbing contaminants was only achieved after bleaching in the presence of 10 mM hydroxylamine and dialysis for a further 2 days against 10 mM HCl/0.5% (v/v) Ammonyx LO or 50 mM sodium borate, pH 9.5, containing 0.5% (v/v) Ammonyx LO.

(*K*) *Active-Site Lysine Modifications.* Active-site lysine modifications were performed by using permethylated rho-

dopsin following two rounds of methylation, 20–24 h at 20–25 °C, using 2 mM formaldehyde and 20 mM pyridine/borane (Longstaff & Rando, 1985). Protein was bleached in the presence of 10 mM hydroxylamine under intense white light at 4 °C for 10–20 min and isolated by using a desalting column of G-25 Sephadex. All reactions involving bleached protein (10–20 $\mu$M) were carried out at 4 °C. Chloral hydrate, acetaldehyde, acetonylacetone, and formaldehyde were used at a concentration of 10 mM. Where present (see Figure 3), pyridine/borane was used at a concentration of 20 mM. *o*-Salicylaldehyde was used at 10 mM or present as a saturating solution. All reactions were carried out in PIPES/dodecyl maltoside buffer, pH 6.5. Trinitrobenzenesulfonic acid was used as described above for reaction with non-active-site lysines, except protein was in PIPES/dodecyl maltose buffer, pH 6.5, not sodium borate, pH 9.3. OPA was used in reactions at three pH values, at pH 6.5 as described above for reaction with non-active-site lysines and at pH 7.5 and 8.9 where the pH was adjusted by adding 0.5 M sodium phosphate, dibasic, or 0.5 M sodium borate buffer, pH 9.5 (both 100 $\mu$L/mL of reaction mixture), respectively. Controls, without OPA and mercaptoethanol were treated in an identical manner.

For regeneration, aliquots of protein solution were diluted to 2–5 $\mu$M with PIPES/dodecyl maltoside buffer, and to this was added a 2–4-fold excess of 11-*cis*-retinal in 2-propanol (0.6 mg/mL). Regeneration was complete after 30 min at 20–25 °C or after 24 h at 4 °C. Regeneration was expressed as the percentage of initial regeneration observed before any reaction (routinely 95–100% of theoretical maximum).

*(L) G Protein Activation Studies.* Modified rhodopsin samples were added to egg yolk phosphatidylcholine to form vesicles using standard techniques as described in Calhoon and Rando (1985). An extract containing partially purified G protein was prepared from rod outer segments as the single light-activated GTPase (Hurley et al., 1981). GTPase activity of the G protein was assayed by standard techniques as previously described (Calhoon & Rando, 1985). All GTPase assays contained constant amounts of (modified) rhodopsin (0.75 $\mu$M) and G protein activity. The assays were performed in a total volume of 0.5 mL of 80 mM Tris (pH 8.0) containing 0.5 $\mu$M [$\gamma$-$^{32}$P]GTP ($10^5$ cpm/min) and 4 mM $MgCl_2$ at 30 °C. The reactions were quenched by adding 0.5 mL of 5% iced trichloroacetic acid, and the liberated release of [$^{32}$P]-phosphate was determined by the method of Thacher (1978). The release of $^{32}$P was linear with time as previously shown (Calhoon & Rando, 1985). Typically, the GTPase activities in the dark and light were, respectively, 1.5 and 9.0 pmol of GTP hydrolyzed/min. GTPase activity was maintained constant in all the assays, rather than protein concentration. However, approximately 6 $\mu$g of G protein preparation was added to each assay tube. Protein was determined by using the method of Bradford (1976). The assay conditions were empirically determined (1) to maximize the difference between activity in the light and dark, while (2) maintaining the linearity of the assay and (3) using the minimum amount of rhodopsin which yielded maximal activation of GTPase in the light (0.75 $\mu$M). Thus, rhodopsin activity was limiting in these assays, unlike the case in intact photoreceptors, allowing partial GTPase activation at a much higher partial bleach. A curve relating the response of GTPase activation to partial rhodopsin bleach was generated as in Calhoon and Rando (1985). Partial bleach was simulated by mixing various ratios of fully bleached and unbleached rhodopsin, keeping the total rhodopsin concentration constant. The curve for the G protein preparation used in this work was virtually identical with the dose–response curve reported in Calhoon and Rando (1985) and showed a linear range between approximately 2% and 50% photolyzed rhodopsin where partial activation of G protein could be observed. The linear range observed is to be expected at the relatively low GTP concentration used as predicted by Fung et al. (1980). A further factor here would be the use of vesicles where a significant fraction of the added rhodopsin would not be exposed for possible interaction with the G protein. This curve allowed calculation of the amount of unmodified rhodopsin that would be necessary to give rise to the GTPase activation found with the various modified rhodopsins under fully bleaching conditions.

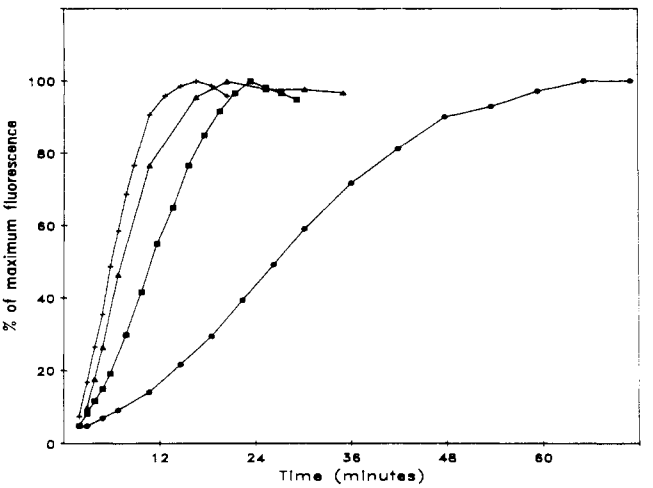



FIGURE 1: Reactivity of various primary amine groups with OPA and mercaptoethanol as measured by formation of the fluorescent product. Amines were 2 $\mu$M alanine (●), 2 $\mu$M $N^{\alpha}$-acetyllysine (■), and 1 $\mu$M (▲) and 9 $\mu$M (+) rhodopsin (non-active-site lysine $\epsilon$-amino groups). Other reagents were as described under Materials and Methods. The reaction was carried out at 4 °C in PIPES/dodecyl maltoside buffer, pH 6.5.

## RESULTS

*Non-Active-Site Amino Acid Modifications and Activation of the G Protein Lysine Modification.* The non-active-site lysine residues of rhodopsin can be reductively methylated with formaldehyde and pyridine/borane with the incorporation of approximately 20 methyl groups per rhodopsin molecule (Longstaff & Rando, 1985). As there are only 10 non-active-site lysine residues in rhodopsin, the incorporation of 20 methyl groups is the maximum possible. It is of interest to gauge the reactivity of these residues as compared to model amino acids and analogues. To these ends, the reaction of these non-active-site lysine residues was followed with OPA as a probe (Longstaff & Rando, 1985). This compound adducts to primary amino groups to form an easily quantifiable fluorescent derivative and, in fact, is of crucial importance in the separation and purification of active-site lysine monomethylated rhodopsin from its unmethylated analogue (Longstaff & Rando, 1985). As can be seen in Figure 1, the non-active-site lysine residues of rhodopsin are quite reactive toward this reagent. The rates of OPA reaction with the non-active-site lysines of rhodopsin occur at rates similar to that of $N^{\alpha}$-acetyllysine and faster than that with alanine (Figure 1).

Given that the non-active-site lysine residues of rhodopsin can be readily chemically modified, it was of interest to determine what effect these modifications would have on the function of the protein as measured by its ability to activate the G protein after photolysis. The modifications to lysine are shown in Scheme I. Permethylation of these lysine residues

Table I: Modification of Non-Active-Site Lysines and Effect on G Protein Activation[a]

| modification to lysine | lysines reacted (out of 10) | $A_{278}/A_{500}$ before reaction | $A_{278}/A_{500}$ after reaction | % regeneration of modified rhodopsin | % G protein activation relative to native rhodopsin |
|---|---|---|---|---|---|
| dimethyl | >9[b] | 1.8–1.9 | 1.8–1.9 | 90–100 | 105 |
| ethyl | 4.3 | 1.86 | 1.86 | 93 | 86 |
| acetimidyl | 6.7 | *c* | 1.85 | 85 | 69 |
| acetyl | 8.8 | 1.89 | 1.89 | 53 | 95 |
| succinyl | 8.2 | 1.88 | 1.98 | 62 | 3[e] |
| picryl | 5.2 | 1.81 | *d* | 0 | 2[e] |

[a] Chemical modifications of non-active-site lysines of rhodopsin and influence on ability to activate GTPase. Rhodopsin was reacted with a number of reagents (see Materials and Methods) to varying extents. All protein samples were able to withstand the reaction procedures with minimal denaturation or bleaching as shown by the ratio of the absorbance at 278 nm to the absorbance at 500 nm before and after reaction. Regeneration experiments and G protein activation assays were carried out independently. Regeneration after bleaching with hydroxylamine (see Materials and Methods) is expressed as the percentage of initial regeneration observed before reaction. The GTPase activity of the G protein was assayed in the presence of 0.75 $\mu$M (modified) rhodopsin in the dark or under room lights. Concentrations of the modified rhodopsins were calculated from the absorption at 500 nM. The relative G protein activation was calculated as follows: [(GTPase activity in the presence of the modified rhodopsin in light) − (activity with rhodopsin in dark)]/[(activity with native rhodopsin in light) − (activity with rhodopsin in dark)] × 100. [b] 8–9 with one round of methylation, >9 with two rounds. [c] The reaction was carried out with the rhodopsin in disk membranes. [d] Trinitrobenzene absorption interferes with this measurement. [e] Indistinguishable from zero by the assay used.

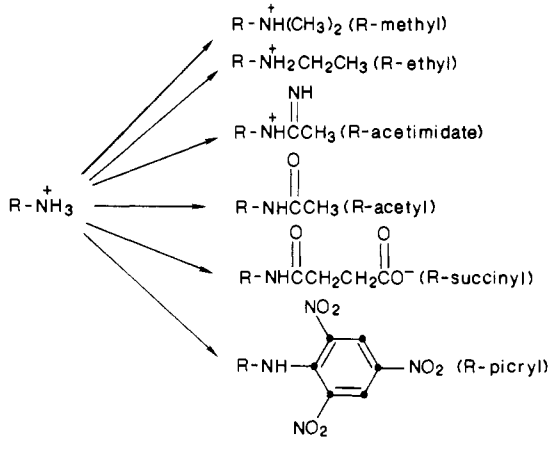
Table II: Modification of Carboxyl Groups and Effect on G Protein Activation[a]

| modification to carboxyl | carboxyls reacted (out of 22) | $A_{278}/A_{500}$ before reaction | $A_{278}/A_{500}$ after reaction | % regeneration of modified rhodopsin | % G protein activation relative to native rhodopsin |
|---|---|---|---|---|---|
| taurine | 12.1 | 1.88 | *b* | <5 | 105 |
| glycine methyl ester | 4.9 | 2.00 | 2.21 | 0 | 90 |
| ethylenediamine | 8.6 | 2.00 | 2.20 | <5 | 71 |

[a] Chemical modification of exposed carboxyl groups and influence on ability to activate the G protein (GTPase). Carboxyl groups were derivatized by using a water-soluble carbodiimide and an amine. Protein denaturation or bleaching was minimal under the reaction conditions employed, as judged by the loss of absorbance at 500 nm (see also footnote *b*). Regeneration experiments and G protein activation assays were carried out independently. Regeneration after bleaching with hydroxylamine (see Materials and Methods) is expressed as the percentage of initial regeneration observed before reaction. The GTPase activity of the G protein was assayed in the presence of 0.75 $\mu$M (modified) rhodopsin in the dark or under room lights. Concentrations of the modified rhodopsins were calculated from the absorption at 500 nM. The relative G protein activation was calculated as follows: [(GTPase activity in the presence of the modified rhodopsin in light) − (activity with rhodopsin in dark)]/[(activity with native rhodopsin in light) − (activity with rhodopsin in dark)] × 100. [b] UV-absorbing products were removed by dialysis after bleaching. The absorbance at 500 nm was little changed after reaction.

with formaldehyde and pyridine/borane, resulting in the dimethylation of virtually all of the 10 non-active-site lysines, does not have any measurable effect on the protein's ability to activate the G protein after photolysis (Table I). In addition, the bleached protein was readily regenerable with 11-*cis*-retinal (Table I). Similar results were obtained after polyethylation of rhodopsin, accomplished by treating rhodopsin with acetaldehyde and pyridine/borane (Table I). However, here only four ethyl groups were incorporated, although it must be remembered that diethylation reactions would not be expected (Means & Feeney, 1968). Acetimidation of rhodopsin using methyl acetimidate, under the conditions previously published, led to the incorporation of six to seven acetimidate moieties per rhodopsin molecule (deGrip et al., 1973). Like the reductive alkylation procedure, this modification also does not alter the net charge on the protein. This modification also had little effect on the ability of the modified protein to activate the G protein after photolysis and to regenerate (Table I). Most surprisingly, however, acetylation of rhodopsin with acetic anhydride, which led to the acetylation of nine lysine residues, also resulted in an active protein (Table I). Acetylation, of course, eliminates the positive charge on the lysine residues. However, not all lysine modifications resulted in active proteins. Succinylation of approximately eight lysines residues with succinic anhydride led to a modified rhodopsin incapable of activating the G protein although it is still capable of being regenerated with 11-*cis*-retinal (Table I). Here, a dramatic alteration in charge borne by the protein occurs because each modification event results in the conversion of a positive charge to a negative charge. Finally, modification

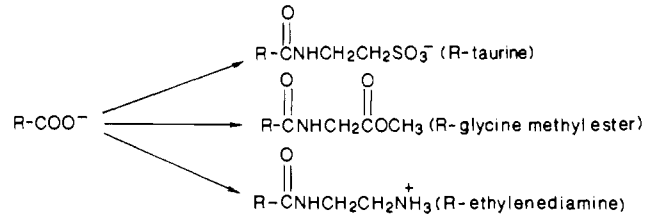
Scheme I




of the lysine residues with trinitrobenzenesulfonate led to the incorporation of approximately five hydrophobic trinitrobenzene moieties per rhodopsin molecule and resulted in a protein incapable of G protein activation and regeneration. Here again, this modification should lead to a rather pronounced alteration in protein structure due to the bulky, hydrophobic moiety introduced.

*Non-Active-Site Carboxyl Modifications and Activation of the G Protein.* Experiments similar to those described above were performed on the carboxyl-containing amino acid residues of rhodopsin which account for a total of 22 carboxyl residues in the protein (Table II) (Hargrave et al., 1983). Three reagents were chemically linked with a water-soluble carbo-

Scheme II




diimide to the free carboxyl groups, ethylenediamine, glycine methyl ester, and taurine as shown in Scheme II. Since a water-soluble carbodiimide was used here, it is presumed that only the nonburied acidic amino acids would be affected. The incorporation of taurine leaves the charge on the protein unperturbed, whereas glycine methyl ester increases the positive charge by one per molecule incorporated and ethylenediamine by two. Interestingly, the incorporation of these various molecules into rhodopsin resulted in bleachable pigments capable of activating the G protein nearly as well as rhodopsin itself. However, none of the modified rhodopsin molecules were capable of pigment regeneration with 11-*cis*-retinal after bleaching, suggesting that salt bridges may be involved in the refolding of the protein. It should be noted that the extents of the modification reactions were variable and less than quantitative (Table II). Further reaction was rendered problematic because of the limited stability of opsin.

*Active-Site Modification Studies.* To study the reaction of the active-site lysine of rhodopsin with chemical reagents, the non-active-site lysines must first be blocked. As already mentioned, multiple reductive methylations of rhodopsin with formaldehyde and pyridine/borane led to the incorporation of approximately 20 methyl groups and hence the complete dimethylation of these residues to form permethylated rhodopsin (PM-Rh) (Longstaff & Rando, 1985). Bleaching of this pigment leads to the liberation of the active-site lysine which is then susceptible to chemical modification. At 4 °C, the permethylated opsin was treated with several reagents capable of reacting with the active-site lysine residue. Simple monofunctional aldehydes such as acetaldehyde, chloral hydrate, and formaldehyde had little effect on subsequent regeneration with 11-*cis*-retinal (Figure 2). Thus, the Schiff bases formed between these aldehydes and the active-site lysine are reversed too readily to have an impact on the regeneration process. Aliphatic dialdehydes, such as acetonylacetone, which can potentially react irreversibly with the active-site lysine, are also without effect (Figure 2). When formaldehyde and pyridine/borane were added together, mono- and dimethylation of the active-site lysine occurred (Longstaff & Rando, 1985). The monomethylated rhodopsin was regenerable with 11-*cis*-retinal to form a new pigment absorbing at 520 nm. Reductive ethylation with [$^{14}$C]acetaldehyde and pyridine/borane led to a reduction in regeneration of the pigment to the extent of approximately 55% (Figure 2). Quantitation of $^{14}$C incorporation showed that 0.46 molecule of acetaldehyde was incorporated per opsin molecule (Table III). Since diethylation of amino groups does not occur (Means & Feeney, 1968), we conclude that active-site monoethylated opsin cannot form a Schiff base with 11-*cis*-retinal.

Given that aliphatic aldehydes such as formaldehyde can readily form reversible Schiff bases with the active-site lysine residue of rhodopsin as revealed by the reductive alkylation studies, it should be possible to find aldehydes which do form stable Schiff bases. One such aldehyde is *o*-salicylaldehyde which can form a stabilized Schiff base with the $\epsilon$-amino group of lysine residues because of the ionizable ortho-hydroxyl group

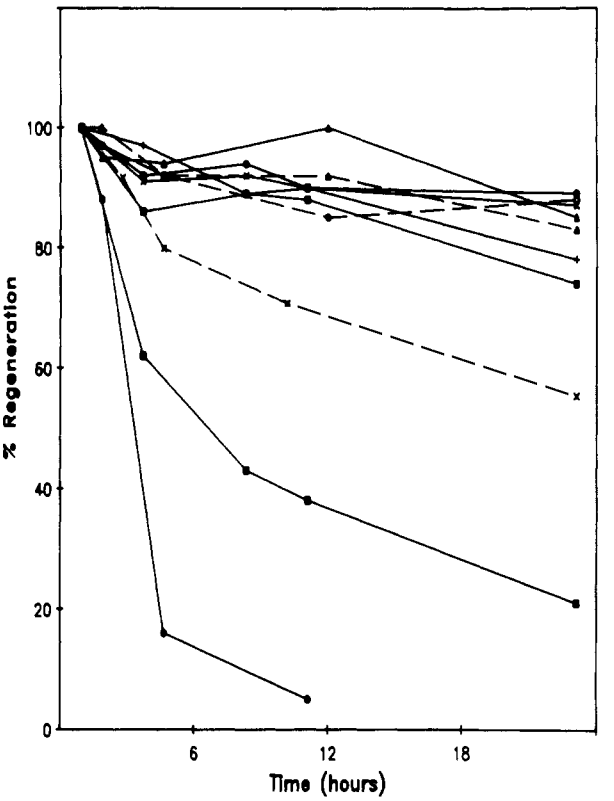



FIGURE 2: Attempted reaction of various reagents with the active-site lysine of opsin, measured by regeneration with 11-*cis*-retinal. Reagents were 10 mM chloral hydrate (●, solid line), 10 mM chloral hydrate and 20 mM pyridine/borane (●, dashed line), 10 mM acetaldehyde (×, solid line), 10 mM acetaldehyde and 20 mM pyridine/borane (×, dashed line), 10 mM acetonylacetone (▲), 10 mM formaldehyde (□), 20 mM pyridine/borane (▲, dashed line), 0.75 mM trinitrobenzenesulfonate (+), 10 mM salicylaldehyde (■), and saturated salicylaldehyde solution (●). All reactions were carried out at 4 °C in the PIPES/dodecyl maltoside buffer, pH 6.5.

Table III: Reductive Ethylation of the Active-Site Lysine of Bleached PM-Rh[a]

| modification | observed result |
|---|---|
| [$^{14}$C]ethyl groups incorpd per opsin molecule | 0.46 |
| regeneration of active-site ethylated opsin compared to nonethylated control | 0.55 |

[a] Labeling of the active-site lysine of bleached PM-Rh with [$^{14}$C]-acetaldehyde and pyridine/borane was carried out as described under Methods. The 45% loss of regeneration after 24 h is due to ethylation of the active-site lysine, which is thus unable to take part in Schiff base formation with 11-*cis*-retinal.

(Means & Feeney, 1971b). Indeed, *o*-salicylaldehyde reacts readily with the active-site lysine of bleached PM-Rh (Figure 2). A structurally similar aromatic aldehyde, OPA, proved also to be a potent inactivator of bleached PM-Rh. This reagent can only react with primary amines and forms a highly fluorescent adduct (Trepman & Chen, 1980). OPA has proved to be invaluable in our studies concerned with the preparation of active-site monomethylated rhodopsin (Longstaff & Rando, 1985).

In Figure 3, the reaction of OPA with bleached PM-Rh was studied at three different pH values. The reaction with OPA was slow and not strongly pH dependent (Figure 3). It should be noted, however, that at pH 6.5 virtually complete >95% blockade of the active-site lysine will occur over a 4-day period (unpublished experiments). Even after 4 days at 4 °C, substantial activity (80–90%) of the control opsin toward regeneration remained (unpublished experiments). The control

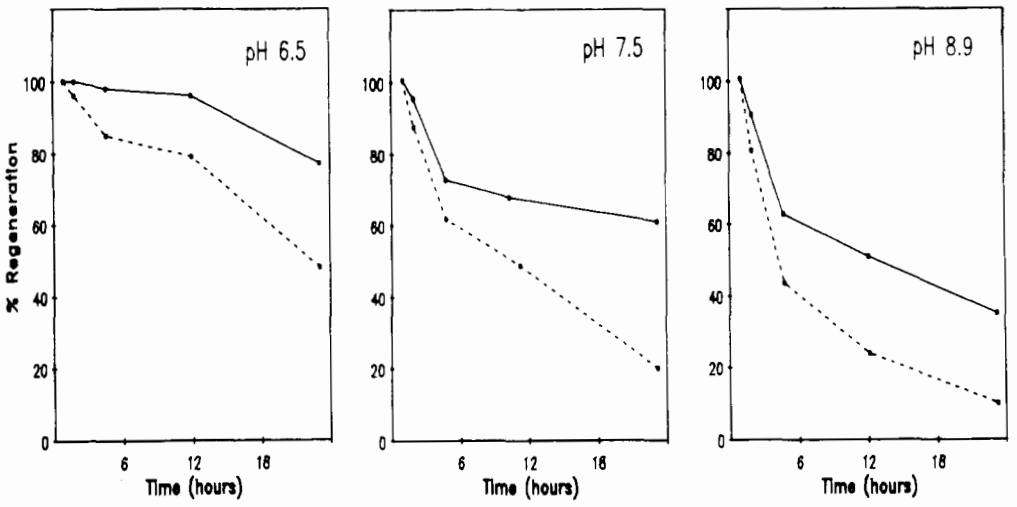



FIGURE 3: Effect of pH on the reactivity of the active-site lysine of opsin and on protein stability. Percentage regeneration (of initial value) of opsin with 11-*cis*-retinal is shown over 24 h, for three different pH values, either without OPA and mercaptoethanol (solid line) or with OPA and mercaptoethanol (dashed line). All reactions were carried out at 4 °C in the PIPES/dodecyl maltoside buffer, pH 6.5, or in this buffer after the addition of 0.5 M disodium phosphate (100 μL/mL of reaction mixture, final pH 7.5) or 0.5 M sodium borate buffer, pH 9.5 (100 μL/mL of reaction mixture, final pH 8.9).

opsin at pH 6.5 shown here in Figure 3 is somewhat more deactivated than usually observed under these conditions.

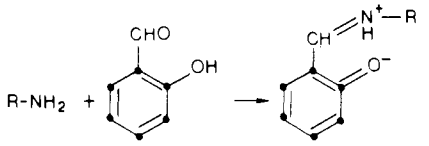
## DISCUSSION

The non-active-site lysine residues of rhodopsin can be modified by a variety of chemical means. Previous studies had shown that the protein could be acetimidated with methyl acetimidate (deGrip et al., 1973) and reductively methylated with formaldehyde and pyridine/borane (Longstaff & Rando, 1985). The non-active-site lysine residues are relatively reactive to nucleophilic reactions as shown by the ready reaction with OPA (Figure 1). The rates of reaction of rhodopsin with OPA were similar to that of $N^{\alpha}$-acetyllysine and considerably faster than the OPA reaction with alanine (Figure 1). The $\epsilon$-amino group of lysine is expected to be more reactive than the $\alpha$-amino group of alanine due to the lower p$K_a$ of the conjugate acid of the former. In addition, steric factors favor reaction at the $\epsilon$-amino group compared to that at an $\alpha$-amino group.

Given the ease with which the lysine residues of rhodopsin can be modified, it was of interest to determine whether these modifications would affect the ability of the modified rhodopsin to activate the G protein as measured by its GTPase activity. It is known that the interaction of R* with the G protein catalyzes the rate of GTP for GDP exchange by a factor of approximately $10^7$ (Stryer, 1984). This requires that R* stabilizes the G protein transition state for this exchange by approximately 10 kcal/mol, requiring that significant interaction take place between the two proteins. Interestingly, incorporation of close to 20 methyl groups per rhodopsin molecule, which completely dimethylated all non-active-site lysine residues, had no apparent effect on the ability of the modified rhodopsin to activate the G protein after photolysis (Table I). Although dimethylation increases the steric bulk of the modified lysines, the charge would remain the same at physiological pH values. Similar results were obtained by reductive ethylation, although only four monoethyl groups were incorporated (Table I). Indeed, even modification with methyl acetimidate, which under the conditions of the experiments incorporated approximately six to seven acetimidate moieties per rhodopsin molecule, did not alter the ability of the modified protein to activate the G protein (Table I). Here again, the net charge on the lysine residues is not altered. These experiments strongly suggest that direct and critical interaction between photochemically activated rhodopsin (R*) and the G protein does not involve direct participation of the lysine residues. Most surprisingly, however, is the finding that acetylation of a substantial number of lysine residues of rhodopsin (approximately nine) also does not have a measurable effect on the ability of the protein to activate the G protein (Table I). In this case, the positive charges borne by the lysines, assuming that they are protonated, are neutralized by the acetylation process. Interestingly, the cytoplasmic loop of rhodopsin between helices 5 and 6 which could be involved in interacting with the G protein contains three lysine residues (Hargrave et al., 1983). These results are also consistent with a mechanism in which the lysine residues per se are not involved in direct interaction with the G protein, either by steric interactions or by salt bridges, and can be interpreted to mean that the net charge of these lysine residues does not play an important role in maintaining the normal conformations of rhodopsin. Of course, this is only true to a certain extent because major alterations of the lysine residues do lead to modified rhodopsin molecules incapable of activating the G protein. Treatment of rhodopsin with succinic anhydride leads to the incorporation of approximately eight succinates per rhodopsin molecule with a concomitant large change in net charge (Table I). Each succinate moiety incorporated converts a positive charge to a negative charge. Not surprisingly, this modification leads to a rhodopsin molecule incapable of activating the G protein after photolysis although regeneration can still significantly occur (Table I). The incorporation of five of the bulky hydrophobic picryl moieties per rhodopsin molecule led to a protein incapable of activating the G protein and incapable of being regenerated (Table I). It is interesting that only with these extreme alterations, either in charge or in bulk and hydrophobicity, is an effect on the activation of the G protein observed. It is noteworthy that none of the modified procedures substantially affected the $A_{278}/A_{500}$ ratios of the rhodopsin and furthermore substantial regeneration with 11-*cis*-retinal after bleaching was observed in all cases except with trinitrobenzenesulfonate (Table I).

In view of the remarkable similarities between the photoreceptor G protein and the GTP binding protein of hormone-activated adenylate cyclase systems (Wheeler & Bitensky, 1977; Dop et al., 1984; Gilman, 1984), especially the ability of irradiated rhodopsin to activate erythrocyte adenylate

Scheme III



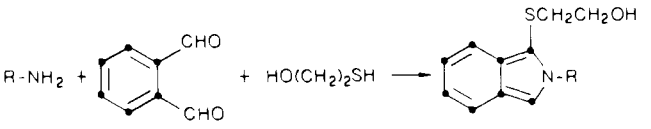
cyclase (Bitensky et al., 1982), it seems reasonable to suggest that the G protein binding site on hormone receptors may be similar to the binding site of rhodopsin. In particular, one might predict that lysine is not stringently required for binding and that the mechanism of binding is not ionic.

Experiments similar to those described above were carried out using carboxyl group modifying reagents. In this instance, three different amines were conjugated to free glutamate and/or aspartate residues of rhodopsin using a water-soluble carbodiimide. The three amines were chosen to (1) retain the same charge (taurine), (2) neutralize the charge (glycine methyl ester), or (3) convert the negative charge to a positive charge (ethylenediamine), all when conjugated to the free carboxyl groups. It is assumed here that the nonburied carboxyl groups of rhodopsin are ionized. Interestingly, none of the modifications prevented the activation of the G protein, but they all prevented regeneration of the modified opsin after it was bleached (Table II). Given the nature of the group-specific chemical modification procedures used, it would be difficult to determine if any one, or a few, of the carboxyl-containing amino acids is critical in the activation of the G protein. However, certain of the hydrophilic carboxyl-containing amino acids must be important for the proper refolding of the apoprotein when treated with 11-*cis*-retinal.

Very little is known about the reactivity of the active-site lysine residue of opsin and what types of reagents might be used to modify it. It might be predicted that its p$K$ may be unusually low in order for it to form a Schiff base with 11-*cis*-retinal readily. This would not be unprecedented as the active-site lysine of acetoacetate decarboxylase, which forms a Schiff base with acetoacetate, has been reported to have a p$K$ of approximately 6 (Kokesh & Westheimer, 1971). In the studies reported here, none of the simple aliphatic aldehydes formed a stable Schiff base with previously non-active-site permethylated opsin at 4 °C (Figure 2). Acetonylacetone, a reagent which can irreversibly react with primary amines, also proved not to irreversibly block the active-site lysine (Figure 2). That readily reversible Schiff base formation must occur between the active-site lysine of opsin and aliphatic aldehydes could be shown by adding the reducing agent pyridine/borane along with the aldehyde. For example, the reductive methylation of bleached PM-Rh with formaldehyde and pyridine/borane led to the incorporation of mono- and dimethyl groups at the active-site lysine (Longstaff & Rando, 1985). Purified active-site monomethylated opsin formed a pigment absorbing at 520 nm when incubated with 11-*cis*-retinal (Longstaff & Rando, 1985). Interestingly, reductive ethylation of the active-site lysine with acetaldehyde and pyridine/borane led to the incorporation of approximately 0.5 ethyl group/opsin molecule, but this modified opsin failed to regenerate with 11-*cis*-retinal (Figure 2 and Table III). It is known that the reductive ethylation of amino groups does not lead to diethylation due to steric interactions (Means & Feeney, 1968). These steric interactions which prevent diethylation from occurring must also prevent the monoethyllysine residue from engaging in Schiff base formation with 11-*cis*-retinal.

Although simple aliphatic aldehydes cannot react irreversibly with primary amines, several aromatic aldehydes can. For example, *o*-salicylaldehyde can react with amines to form

Scheme IV



the stabilized Schiff base adduct shown in Scheme III. Ionization of the ortho-hydroxyl group stabilizes the protonated Schiff base, making the complex a stable one (Scheme IV). In fact, treatment of bleached PM-Rh with *o*-salicylaldehyde led to the apparent irreversible inhibition of the protein (Figure 2). No attempt was made to determine if this inhibition could be reversed by hydrolysis. Even at 10 mM *o*-salicylaldehyde, the reaction was not pseudo first order, suggesting relatively weak binding between the aldehyde and the opsin molecule (Figure 2). A structurally similar aromatic aldehyde, *o*-phthalaldehyde, plus mercaptoethanol (OPA) can react irreversibly with a primary amine (Scheme IV) (Trepman & Chen, 1980). Indeed, OPA reacts readily and irreversibly with non-active-site lysine residues of rhodopsin (Figure 1). The rate of the reaction is similar to the observed rate with the $\epsilon$-amino group of lysine. OPA also reacts with the active-site lysine; only the reaction rate is much slower than with the peripheral lysine residues (Figures 1 and 3). The slower reaction at the active-site lysine is probably a ramification of the restricted steric selectivity at this site. Both *o*-phthalaldehyde and mercaptoethanol must be present at the active site in order for a reaction to ensue. Nevertheless, the selective reaction of OPA with the unmodified lysine of reductively methylated bleached PM-Rh proved to be invaluable in the preparation of purified permethylated active-site methylated rhodopsin (Longstaff & Rando, 1985; Longstaff et al., 1986). Finally, the fact that the sterically similar OPA and *o*-salicylaldehyde both react with the active-site lysine of rhodopsin suggests that rhodopsin possesses an active site which can accommodate a benzene ring. This is not unexpected given the hydrophobic, polyene nature of 11-*cis*-retinal.

In summary, the chemical modification of rhodopsin leads to four classes of alterations in the protein. The most disruptive class (trinitrophenylation of peripheral lysine residues and most active-site lysine modifications) leads to a protein that can neither activate the G protein after bleaching nor regenerate with 11-*cis*-retinal. Another class of alterations (succinylation of non-active-site lysine residue) leads to a regenerable protein incapable of activating the G protein. Carboxyl group modification does the reverse. Finally, permethylation, acetylation, and acetimidation of non-active-site lysine residues yield rhodopsin derivatives capable of both G protein activation and regeneration.

## ACKNOWLEDGMENTS

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**Registry No.** Lysine, 56-87-1; aspartic acid, 56-84-8; glutamic acid, 56-86-0; 11-*cis*-retinal, 564-87-4; methyl acetimidate, 14777-29-8; succinic anhydride, 108-30-5; trinitrobenzenesulfonic acid, 2508-19-2; taurine, 107-35-7; glycine methyl ester, 616-34-2; ethylenediamine, 107-15-3.

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# Evidence for a Single Steroid-Binding Protein in the Rabbit Progesterone Receptor†

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ABSTRACT: The rabbit uterine progesterone receptor copurifies as two molecular weight ($M_r$) forms of about 105 000 and 78 000. To investigate whether these are different proteins, we have used protease digestion, reversible denaturation, and photoaffinity labeling in studies on the steroid-binding domain of the receptor. Digestion of the $M_r$ 105 000 and 78 000 forms, photoaffinity labeled with [$^3$H]R5020, with *Staphylococcus aureus* V8 protease revealed identical peptide fragments of $M_r$ 43 000, 39 000, and 27 000–30 000. When receptor in cytosol was denatured, separated by electrophoresis, and then reconstituted, [$^3$H]progesterone bound specifically to a single form at about $M_r$ 105 000. After partial purification, the reversible denaturation procedure revealed both the larger and the smaller progesterone-binding species similar to the photoaffinity-labeled species in this preparation. Receptor in uterine cytosol prepared under mild conditions appeared as a predominant large molecular weight form on photoaffinity labeling with [17$\alpha$-*methyl*-$^3$H]R5020, [6,7-$^3$H]R5020, or [$^3$H]RU27987. Further purification of this cytosol showed the generation of a smaller labeled species. These results from three different approaches reinforce the view that the rabbit progesterone receptor contains a single steroid-binding protein.

Most steroid receptors consist of a single hormone-binding subunit, which may be present in a homo- or heterodimer or tetramer (Greene et al., 1979; Wilson & French, 1979; Katzenellenbogen et al., 1983; Vedeckis, 1983; Chang et al., 1984; Miesfeld et al., 1984; Monsma et al., 1984; Rowley et al., 1984; Sakai & Gorski, 1984; Sherman & Stevens, 1984; Wrange et al., 1984). The subunit structure of the progesterone receptor, however, is less clear. For the chicken oviduct progesterone receptor, two dissimilar hormone-binding subunits have been identified with molecular weights of 79 000 and 108 000; these subunits exhibit differential binding to DNA and chromatin (Grody et al., 1982). Other workers have

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