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Methylation of the Active-Site Lysine of Rhodopsin†

Colin Longstaff and Robert R. Rando*
Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: Purified bovine rhodopsin was reductively methylated with formaldehyde and pyridine/borane with the incorporation of approximately 20 methyl groups in the protein. Rhodopsin contains 10 non-active-site lysines, which account for the uptake of the 20 methyl groups. The permethylated rhodopsin thus formed is active toward bleaching, regeneration with 11-*cis*-retinal, and the activation of the GTPase (G protein) when photolyzed. The critical active-site lysine of permethylated rhodopsin can be liberated by photolysis. This lysine can be reductively methylated at 4 °C. Methylation under these conditions leads to the incorporation of approximately 1.5 methyl groups per opsin molecule using radioactive formaldehyde, with the ratio of ϵ-dimethyllysine:ϵ-monomethyllysine:lysine being approximately 5:4:1. The modified opsin(s) can regenerate with 11-*cis*-retinal to produce a mixture of active-site methylated and unmethylated rhodopsins having a λ_{max} = 512 nm. Using [^{14}C]formaldehyde and [^{3}H]retinal followed by reduction of the Schiff base, digestion, and chromatography showed that the active-site *N*-methyllysine was bound to the retinal. Treatment of the methylated opsin mixture (containing 1.5 active-site methyl groups) with *o*-phthalaldehyde/mercaptoethanol to functionalize the opsin bearing unreacted lysine, followed by regeneration with 11-*cis*-retinal and chromatographic separation, led to the preparation of the pure active-site ϵ-lysine monomethylated rhodopsin with a λ_{max} = 520 nm, significantly shifted bathochromically from rhodopsin or permethylated rhodopsin. Thus, the active site of rhodopsin can accommodate a methyl group attached to the active-site lysine, and the λ_{max} of this pigment supports the hypothesis that the Schiff base of rhodopsin bears a full formal positive charge. Furthermore, since this new pigment can be bleached, it must mean that deprotonation of the Schiff base must not be obligate for this conversion to proceed. With this methyl reporter group attached to the active-site lysine of rhodopsin, spectroscopic and biochemical experiments on the role of charge and charge movement in the mechanism of action of rhodopsin can be performed.

Rhodopsin is an integral rod outer segment disk protein containing 11-*cis*-retinal bound to active-site lysine-296 via a protonated, or partially protonated, Schiff base (Hargrave et al., 1983). Photolysis of rhodopsin results in the isomerization of the 11-cis chromophore to its all-trans congener, with the subsequent hydrolysis of the Schiff base linkage, to form the protein opsin and *all-trans*-retinal (Wald, 1968). One of the spectroscopically identifiable intermediates on the way to *all-trans*-retinal formation, probably metarhodopsin II, is responsible for initiating the cascade of biochemical events which leads to the hyperpolarization of the rod outer segment, and hence to visual signal transduction (Parkes et al., 1979; Calhoon et al., 1981). It should be noted that metarhodopsin II may be comprised of several distinct conformers. The biochemical events affected by the photochemical activation of rhodopsin are now at least partially understood. Activated rhodopsin (metarhodopsin II) catalyzes the exchange of GTP for GDP in a G protein (GTPase), also called transducin, which can then in turn activate a phosphodiesterase specific for cGMP (Shinozawa et al., 1979; Fung et al., 1981). There

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* Correspondence should be addressed to this author.

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is now good evidence to show that cGMP opens the plasma membrane sodium channels and hence its hydrolysis would, of course, cause them to close (Fesenko et al., 1985). It has been noted that the initial biochemical events in vision, notably the rhodopsin-catalyzed exchange of GDP for GTP, are shared by many drug/receptor complexes and, indeed, notable sequence homology exists within the family of G proteins (Hurley et al., 1984).

An important question that arises in determining the mechanism of the metarhodopsin II mediated activation of the GTPase is that of the nature of the activated rhodopsin complex. Although this question is multifaceted, one aspect of it concerns the structure of the chromophore as it relates to the mechanism of the activation process. Of great interest here is how the photochemical isomerization of the chromophore leads to the energization of rhodopsin. Much of the light energy captured by rhodopsin is transformed into the chemical potential energy of the holoprotein (Cooper, 1979). The role of the potentially charged Schiff base in this energization step and in visual transduction is likely to be considerable. Charge movement is probably the key to the conversion of light energy into chemical potential energy by rhodopsin (Honig et al., 1979a). Furthermore, at the critical metarhodopsin I $\rightarrow$ metarhodopsin II conversion, a proton is taken up from the medium at the same time that spectroscopic evidence suggests that the Schiff base is either deprotonated or hydrolyzed (Cooper & Converse, 1976; Doukas et al., 1978). In addition, the protonated Schiff base is thought to be of paramount importance in determining the positions of maximal absorption for the rod and cone pigments (Honig et al., 1979a). It should also be noted that the bacterial counterpart of mammalian rhodopsin, bacteriorhodopsin, may pump protons via its protonated Schiff base (Stoeckenius et al., 1979). For these reasons, it was considered important to place a reporter group at the active-site lysine of rhodopsin, which would allow one to assess the importance of charge and charge movement during bleaching, signal transduction, and regeneration. To these ends, we have prepared active-site lysine-methylated rhodopsin and report here on some of its biochemical properties.

Materials and Methods

Materials. Dodecyl maltoside and methyl α-mannopyranoside were obtained from Behring Diagnostics. Pyridine/borane was a product of Aldrich Chemical Co. [^{3}H]-Formaldehyde and [^{14}C]formaldehyde were obtained from New England Nuclear. N^{ϵ}-Methyllysine and N^{ϵ}-dimethyllysine were obtained from Vega Chemicals, Tucson, AZ. Ammonyx LO was provided by Onyx Chemical Co. *all-trans*-[^{3}H]Retinol was a product of Amersham Corp. Thin-layer chromatography (TLC) plastic sheets of silica gel 60 (without fluorescent indicator), 0.2-mm thickness, were products of E. Merck, West Germany. Sep-Pak cartridges were obtained from Waters Associates. Concanavalin A–Sepharose 4B, *o*-phthalaldehyde, and 2-mercaptoethanol were obtained from Sigma Chemical Co. Hydroxylapatite, DNA grade, was a product of Bio-Rad Laboratories. Scintillation fluid (Hydrofluor) was obtained from National Diagnostics. All other chemicals and solvents used were of the highest grade commercially available.

Preparation of Proteins. Rod outer segments and solubilized rhodopsin were prepared as described previously (Calhoon & Rando, 1985). Unless otherwise stated, rhodopsin modifications were carried out in a buffer of 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 6.5, containing 6 mM dodecyl maltoside. All procedures involving the handling of nonbleached rhodopsin were conducted under dim red light. Rhodopsin and modified rhodopsins were stored at −70 °C between experiments.

Methylation Procedures. Detergent-solubilized rhodopsin was methylated prior to bleaching to produce permethylated rhodopsin in which >95% of all available, i.e., non-active-site, lysines were dimethylated. This was accomplished by carrying out two rounds of methylation using 2 mM formaldehyde and 20 mM pyridine/borane. A stock solution of 0.2 M formaldehyde was prepared by hydrolyzing paraformaldehyde, and this was stored at 4 °C and used over a period of several months (Jentoft & Dearborn, 1983). Stock solutions of pyridine/borane (2 M) were made up in 2-propanol and stored at −70 °C in aliquots of 1–2 mL. Rhodopsin concentrations varied between 10 and 40 μM, on the basis of the absorbance at 500 nm using $\epsilon = 40\,000$ M^{-1} cm^{-1} (Applebury et al., 1974). Each round of methylation of rhodopsin was allowed to proceed for 20–24 h at 20–25 °C, whereupon modified protein was isolated from the reaction mixture by using a desalting column of G-25 Sephadex.

Methylation of the active-site lysine was carried out following bleaching of the permethylated rhodopsin under strong white light, in the presence of 10 mM hydroxylamine. Bleached protein was isolated on a small column of concanavalin A–Sepharose 4B (ca. 1-mL gel for each 100 nmol of protein) and subsequently eluted with 0.5 M methyl α-mannopyranoside. All reactions and manipulations of the bleached protein were performed at 4 °C because opsin was found to be much more susceptible to thermal denaturation than rhodopsin. As above, reagent concentrations were 2 mM formaldehyde and 20 mM pyridine/borane. Protein concentrations were routinely 3–15 μM, on the basis of the absorbance at 278 nm, using $\epsilon = 64\,000$ M^{-1} cm^{-1} (Applebury et al., 1974). For greater levels of active-site methylation, reactions were allowed to proceed for 48 h, and a further addition of both reagents at 24 h was made (final concentrations 4 mM formaldehyde and 40 mM pyridine/borane). On completion of this step, modified protein was isolated by using a desalting column of G-25 Sephadex, and modified rhodopsin was regenerated by adding ca. a 5-fold excess of 11-*cis*-retinal in 2-propanol [volume of 2-propanol added being <1% (v/v) of the protein solution]. Regeneration was complete in 30 min at 20–25 °C. Protein spectra (1 mL of solution) were recorded with a Perkin-Elmer Lambda 3B UV/Vis spectrophotometer.

The extent of methylation of protein lysine groups in rhodopsin was measured by using [^{14}C]formaldehyde (1 Ci/mol). Prior to scintillation counting, radiolabeled protein samples were dialyzed for at least 3 days against 10 mM HCl containing 0.5% Ammonyx LO (three exchanges of 500 mL for up to 10 mL of protein solution). This extra dialysis step was included to remove dissociable protein/formaldehyde products which are known to form readily (Means & Feeney, 1971).

Analysis of Active-Site-Methylated Protein Samples. After rhodopsin was bleached, further methylation at 4 °C of previously permethylated rhodopsin gave a mixture of proteins having dimethyllysine, monomethyllysine, or free lysine at the active site. The proportions of these three species were determined by methylating with [^{14}C]formaldehyde, digesting the protein, and separating the radiolabeled amino acids by TLC. Following dialysis of the labeled protein, an aliquot (1 mL, 3–15 nmol) was lyophilized and hydrolyzed with 6 M HCl in vacuo at 110 °C for 20 h. The hydrolysate was lyophilized and redissolved in 10 mM HCl (50 μL). Samples of this solution were applied directly to TLC plates as 4-cm streaks along with nonlabeled N^{ϵ}-methyllysine and N^{ϵ}-dimethyllysine

to act as carriers. The TLC solvent system found to give good separation of methylated lysines was MeOH/$CHCl_3$/NH_4OH (8:6:5). TLC plates were developed for ca. 15 cm, then dried, and sprayed with ninhydrin [0.2% (w/v) in acetone] to locate the methylated lysine standards, which were run alongside the digest. The plates were then cut into 1-cm strips, and these were placed in water (1 mL) in scintillation vials for at least 3 h before scintillation fluid (Hydrofluor) was added and the vials were counted. From the known incorporation of moles of [^{14}C]CH_3 per mole of protein and the distribution of counts in the dimethyllysine and monomethyllysine strips, the ratio of dimethyllysine, monomethyllysine, and unreacted lysine at the active site could be calculated.

As a further check, samples of [^{14}C]CH_3-labeled protein could be dual labeled by using a second round of methylation with 2 mM [^{3}H]formaldehyde (7.8 Ci/mol) and 20 mM pyridine/borane at 37 °C for 24 h to maximize methyl group incorporation. This step was performed prior to the acid dialysis treatment.

Dual Labeling of the Active-Site Lysine with [^{14}C]Formaldehyde and all-trans-[^{3}H]Retinals. A mixture of 9-*cis*- and 11-*cis*-[^{3}H]retinal was prepared from *all-trans*-[^{3}H]retinol by using the method of Hubbard et al. (1971). No attempt was made to separate these isomers. Regeneration with opsin was allowed to proceed as with 11-*cis*-retinal to give a mixture of rhodopsin and isorhodopsin. The specific activity of the [^{3}H]retinals prepared was 60 Ci/mol.

A standard of N^{ϵ}-retinyllysine was synthesized following the method of Akhtar et al. (1968). N^{ϵ}-Methylretinyllysine was synthesized in the same way, using N^{ϵ}-methyllysine in place of lysine.

From a batch of permethylated rhodopsin, two samples (1 mL, ca. 5 nmol of each) of bleached protein were prepared either with unmodified active-site lysine or with the mixture of ^{14}C-methylated lysines and free lysine obtained following a 48-h methylation reaction (with two additions of reagents). Both samples were regenerated with the [^{3}H]retinal mixture (10–20-fold excess). When regeneration had gone to completion, 1 M hydroxylamine solution, pH 6.5, was added to both samples to give a final concentration of 10 mM, thus forming retinal oxime with the excess [^{3}H]retinal. The Schiff base linkage between retinal and the protein was then reduced by using $NaBH_4$ (1 mg/mL solution) with illumination of the sample (DeGrip & Daemen, 1982). Octanol (ca. 3 drops) was added to prevent excessive foaming. After 30 min of illumination, acetone (1–2 drops/mL) was added to the mixture to destroy excess $NaBH_4$, and both samples were lyophilized. Excess [^{3}H]retinal oximes were removed from the lyophilized solids by extraction with absolute ethanol (10 mL, four washes). Basic hydrolysis of the washed proteins was then carried out by using 5 M NaOH, as previously described (Bownds, 1967). After neutralization and lyophilization of the hydrolysates, N^{ϵ}-retinyllysines were extracted by using $CHCl_3$/MeOH (6:5) (2 × 5 mL). These washings were pooled and evaporated to dryness (Rotavap). The residues were redissolved in the same solvent (0.5 mL), and to this was added N^{ϵ}-retinyllysine or N^{ϵ}-methylretinyllysine (ca. 0.5 μmol in methanol) as appropriate. Each solution was then passed through a Sep-Pak cartridge to remove salts and some of the amino acids taken up during extraction of the hydrolysates. After solutions were loaded on the Sep-Pak, impurities were eluted with methanol (3 mL); then N^{ϵ}-retinyllysines were eluted with $CHCl_3$/MeOH/NH_4OH (6:6:4) (3 mL). Eluates were evaporated to dryness (Rotavap) and redissolved in methanol (0.5 mL). Standards of N^{ϵ}-retinyllysine and N^{ϵ}-methylretinyllysine (ca. 0.5 μmol) were added at this stage, also in methanol (50 μL), and the solutions were reduced in volume (to ca. 100 μL) under a stream of N_2 for spotting on a TLC plate. An aliquot of the ^{3}H-labeled sample containing a total of ca. 35 000 dpm was spotted alongside the ^{3}H/^{14}C dual-labeled sample containing ca. 135 000 ^{3}H dpm and 8000 ^{14}C dpm. The solvent system used was 1-butanol/acetic acid/water (3:1:1) which was found to give good separation of N^{ϵ}-retinyllysines from free lysines and retinal or retinol. TLC plates were then cut into 1-cm strips which were placed in scintillation vials with methanol (1 mL) for 3 h before counting.

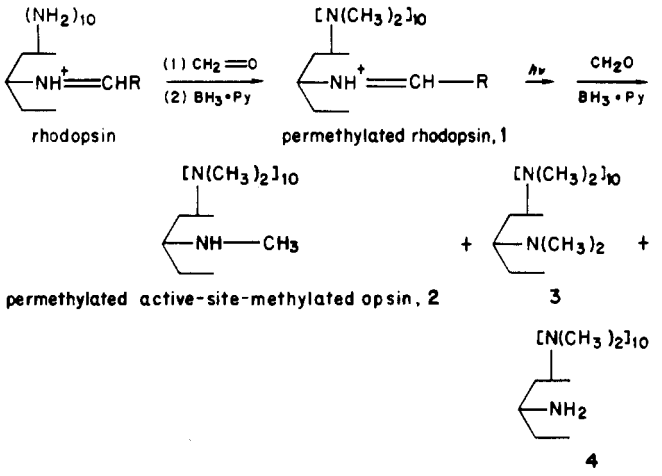
Reaction of Unmethylated Active-Site Lysine with o-Phthalaldehyde. From a batch of permethylated rhodopsin, two protein samples were prepared having either the mixture of methylated and unmethylated active-site lyzines following a 48-h methylation at 4 °C (two additions of reagents) or unmethylated active-site lysine. By use of the absorance at 278 nm and $\epsilon = 64\,000$ M^{-1} cm^{-1}, protein concentrations were estimated to be 7.6 μM for the unmethylated sample (2.5 mL) and 7.2 μM for the active-site-methylated sample (5.5 mL). Both these solutions were treated identically for the reaction with *o*-phthalaldehyde and mercaptoethanol. *o*-Phthalaldehyde was added as a methanolic solution (80 mg/mL) to give a final concentration of 0.8 mg/mL. Mercaptoethanol was present in the reaction mixture at a concentration of 15 mM (Trepman & Chen, 1980). The reaction was allowed to proceed for 44 h at 4 °C. Aliquots (0.5 mL) were removed after 20 and 44 h of reaction, made up to a volume of 1 mL, and regenerated with 11-*cis*-retinal as described above. Aliquots (0.3 mL) of the reaction mixture were diluted 10-fold for fluorescence measurements. Fluorescence spectra were recorded by using a Perkin-Elmer 512 double-beam fluorescence spectrophotometer (excitation and emission maxima were 340 and 440 nm, respectively).

Isolation of Regenerated Protein. Following reaction of active-site lysine with *o*-phthalaldehyde/mercaptoethanol and regeneration with 11-*cis*-retinal, excess reagents were removed from the protein by using a desalting column of G-25 Sephadex preequilibrated and run in 10 mM PIPES, pH 6.5, containing 6 mM dodecyl maltoside. The pooled protein-containing fractions from this column were applied directly to a column of hydroxylapatite (10 × 1 cm) which had been preequilibrated with 10 mM potassium phosphate, pH 6.5, containing 6 mM dodecyl maltoside and 0.02% (w/v) NaN_3. Protein was eluted by using a linear gradient formed between this buffer and 0.5 M potassium phosphate, pH 6.5, containing 6 mM dodecyl maltoside and 0.02% NaN_3, at a flow rate of ca. 15 mL/h.

RESULTS

Reductive Methylation of Bovine Rhodopsin. Rhodopsin possesses 11 free amino—the active-site lysine as well as 10 others (Hargrave et al., 1983). Its N-terminus is acetylated and hence blocked. Since there would be no obvious way of methylating the active-site lysine specifically without affecting the remaining lysines, a method was sought to permethylate rhodopsin, to be followed by bleaching and active-site methylation. To these ends, reductive methylation of rhodopsin and bleached permethylated rhodopsin was studied (Scheme I). It was important that permethylated rhodopsin be biochemically active for the further studies to yield meaningful data. The non-active-site lysine residues of rhodopsin can be methylated in the dark by using formaldehyde and pyridine/borane at 4 and at 25 °C (Figure 1). The data are shown here for the pyridine/borane reduction. Similar kinetics of reduction were obtained with $NaCNBH_3$ as the reductant.

Scheme I



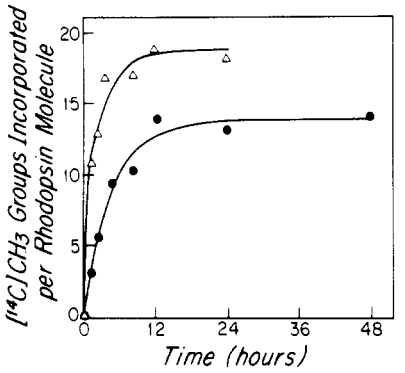


FIGURE 1: Time course of the methylation reaction of non-active-site lysines in rhodopsin with 2 mM [^{14}C]formaldehyde and 20 mM pyridine/borane. The two curves show the progress of methyl group incorporation by rhodopsin at 4 (●) or 22 °C (△).

A single methylation at 20–25 °C under these conditions leads to the incorporation of approximately 18–19 methyl groups into the rhodopsin, as judged by ^{14}C incorporation. A second round of methylation leads to the incorporation of approximately one additional methyl group. The UV/visible spectrum of this permethylated rhodopsin is shown in Figure 2. Indeed, it is identical with that of rhodopsin itself (λ_{max} = 498 nm) (Wald, 1968). The permethylated pigment bleaches, regenerates ≥90% (Figure 2) with 11-*cis*-retinal, and, most importantly, activates the GTPase (G protein), the only known biochemical entity directly affected by the bleaching of rhodopsin (Fung et al., 1981; Shinozawa et al., 1979). When compared to rhodopsin, permethylated rhodopsin was 105% ± 7% as active (R. D. Calhoon, personal communication). These results show that methylation of the non-active-site lysines is not important for the activation of the GTPase.

Reductive Methylation of Bleached Permethylated Rhodopsin. To methylate the active-site lysines by reductive methylation, the 2× permethylated rhodopsin was first bleached and methylated with [^{14}C]formaldehyde at 4 °C (Figure 3). After 48 h at 4 °C, 1.17 methyl groups were incorporated into the protein. As a control, permethylated rhodopsin was reductively methylated under the same conditions, with the introduction of only 0.09 methyl group per rhodopsin molecule. A decrease in the ability of the methylated opsin to regenerate was found to occur concomitant with the extent of methylation of the active site (Figure 3 insert). To determine the relative proportions of active-site dimethyllysines, monomethyllysines, and unmethylated lysines, the labeling experiments described below were performed. Under the conditions described in Figure 3, permethylated

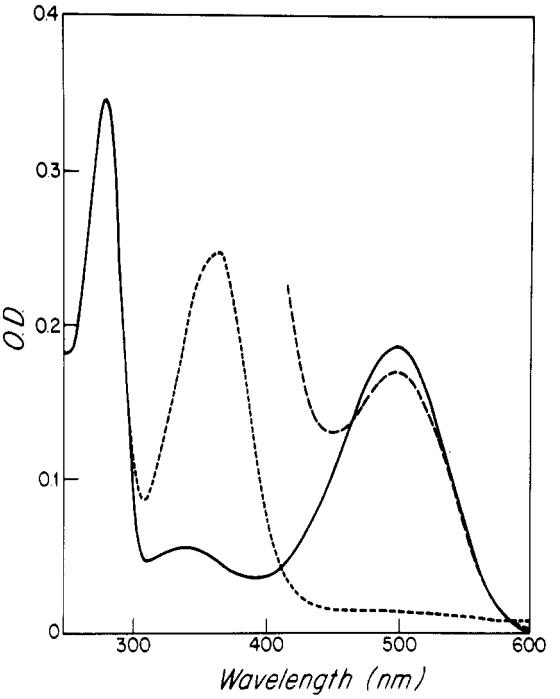


FIGURE 2: UV/visible spectrum of permethylated rhodopsin following two rounds of methylation at 20–25 °C in the dark (with the incorporation of 19–20 methyl groups per rhodopsin molecule) (solid line). The dotted line shows the spectrum of bleached, permethylated rhodopsin. Also shown is the partial spectrum of this protein after regeneration (dashed line) with 11-*cis*-retinal as described in the text. The level of regeneration in this case was 90%. Bleaching and regeneration were carried out as in Wong & Rando (1984).

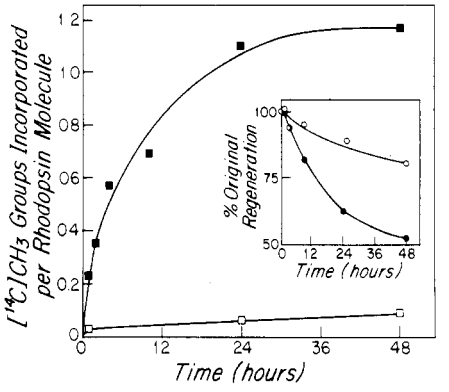


FIGURE 3: Time course of the methylation of the active-site lysine of bleached permethylated rhodopsin using 2 mM [^{14}C]formaldehyde and 20 mM pyridine/borane at 4 °C. Rhodopsin was permethylated with the incorporation of 19–20 methyl groups (two rounds of methylation) and then bleached to expose the active-site lysine. Shown is the incorporation of [^{14}C]methyl groups by bleached protein (■) and nonbleached protein (□). The insert shows loss of regeneration of bleached permethylated rhodopsin during an active-site methylation reaction (●) and a control sample not undergoing active-site methylation (○) (regeneration at zero time of permethylated rhodopsin being taken as 100%).

rhodopsin was bleached and treated with [^{14}C]formaldehyde and pyridine/borane at 4 °C for 24 h, leading to the incorporation of 0.91 methyl group per opsin molecule. When this protein sample was hydrolyzed, it was found that the radioactivity coeluted with both N^{ϵ}-monomethyllysine and N^{ϵ}-dimethyllysine (Figure 4). At 4 °C (Figure 4A), the production of both ε-monomethyl- and ε-dimethyllysines is apparent. When a sample identical with the one methylated at 4 °C was further methylated with [^{3}H]formaldehyde and pyridine/borane at 37 °C for 24 h, an additional methyl group was incorporated into the protein, and, indeed, hydrolysis of the

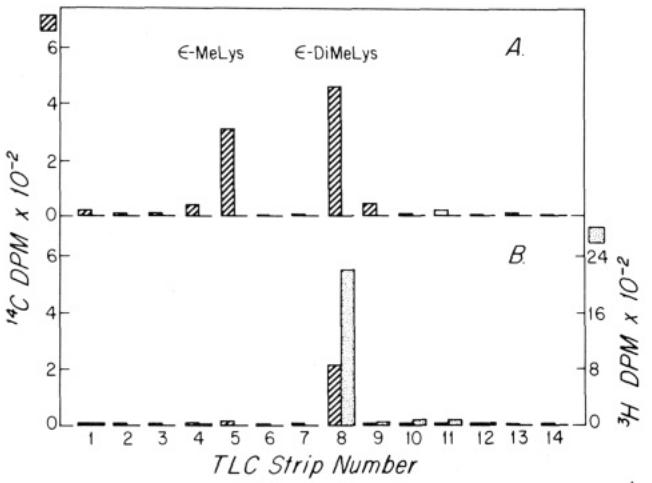


FIGURE 4: Analysis of $^{14}C/^{3}H$-labeled monomethyllysine and dimethyllysine from a digest of active-site methylated, bleached, permethylated rhodopsin. Shown in panel A is the distribution of dpm from monomethyllysine (strips 4 and 5) and from dimethyllysine (strips 8 and 9) following a 24-h methylation at 4 °C using 2 mM [^{14}C]-formaldehyde and 20 mM pyridine/borane. The TLC analyzed for panel B was of a sample of the protein used for panel A which had undergone a further round of active-site methylation using 2 mM [^{3}H]formaldehyde and 20 mM pyridine/borane at 37 °C for 24 h. In this case, all the dpm in the digest coelute with the dimethyllysine standard (strip 8). Recovery of applied dpm from TLC plates was typically 40–50% following the procedure described under Materials and Methods.

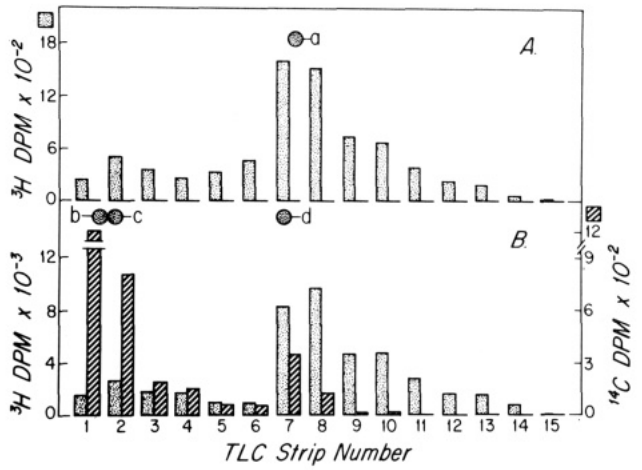


FIGURE 5: TLC analysis of a digest of [^{3}H]retinylopsin produced from either bleached, permethylated rhodopsin (A) or bleached, permethylated rhodopsin which had also been methylated at the active site by using [^{14}C]formaldehyde (see text) (B). N^{ϵ}-Retinyllysine is shown to elute with strips 7 and 8. In panel B, ^{14}C dpm coelute with strips 7 and 8, showing the N^{ϵ}-retinyllysine to be methylated. The dotted circles indicate the positions of standards on the same TLC plate: (A) N^{ϵ}-retinyllysine (a); (B) N^{ϵ}-methylretinyllysine (d) and methyllysine and dimethyllysine (c and b, respectively).

modified protein showed the presence of only ϵ-dimethyllysine (Figure 4B). Given that complete blockage of the ϵ-amino groups of the active-site lysine could be achieved, it was desired to achieve maximal monomethylation at the active site with as little denaturation as possible. To this end, permethylated rhodopsin was bleached and methylated over 48 h at 4 °C with two additions of [^{14}C]formaldehyde and pyridine/borane to achieve extensive active-site methylation. Again, the total amount of labeling of the active-site lysine could be determined as well as the proportion of unmethylated, monomethylated, and dimethylated species as in Figure 4. It could be determined that 1.42 active-site methyl groups were incorporated and that the *N*-dimethyllysine:*N*-monomethyllysine:lysine ratios was estimated to be 5:4:1. In this particular experiment, 1.53 methyl groups were incorporated into the bleached permethylated rhodopsin and 0.11 into the permethylated rhodopsin. When a sample treated in this way was allowed to regenerate with 11-*cis*-retinal, 42% of the protein was regenerable. This must mean that the methylated lysine can engage in Schiff base formation with 11-*cis*-retinal. The regenerated, modified rhodopsin can be repeatedly bleached and regenerated as shown later.

Active-Site Labeling Studies with [^{3}H]Retinal. The experiments described above are all consistent with an active-site methylation of bleached permethylated rhodopsin by formaldehyde and pyridine/borane. To gain further evidence to support this contention, direct active-site labeling experiments were performed with [^{3}H]retinaldehyde and [^{14}C]formaldehyde. Permethylated rhodopsin was bleached and reductively methylated at 4 °C for 48 h (two additions) with [^{14}C]formaldehyde and pyridine/borane. After the excess formaldehyde was removed, the pigments were regenerated with [^{3}H]retinals. After regeneration had occurred, the pigments were reduced with $NaBH_4$ in the light to irreversibly fix the active-site lysine to the retinoid (DeGrip & Daemen, 1982). The protein was then hydrolyzed by base (Bownds, 1967), and the mixture was applied to a thin-layer chromatographic system used to separate *N*-methylretinyllysine from the other amino acids. In Figure 5A, a control is shown where [^{3}H]retinal bound to permethylated rhodopsin was reduced and processed as recorded under Materials and Methods. The major bands of radioactivity, strips 7 and 8, coeluted with standard retinyllysine. When the same procedure was performed with ^{14}C-labeled active-site-methylated, permethylated rhodopsin, the results shown in Figure 5B were obtained. Significant ^{3}H and ^{14}C counts eluted with the standard *N*-methylretinyllysine (strips 7 and 8). These results demonstrate that the active-site lysine must have been methylated by formaldehyde and pyridine/borane and that this modified amino acid must be competent to form a Schiff base with added 11-*cis*-retinal and hence be competent to form a visual pigment.

Spectral Studies on the Active-Site-Methylated Permethylated Rhodopsins. Given the assumption that the ϵ-monomethylated lysine can form a visual pigment with 11-*cis*-retinal, it was of some interest to determine its spectral properties. In Figure 6 is shown the UV/visible spectrum of a permethylated rhodopsin bleached and active-site-methylated with two additions of reagents over 48 h at 4 °C and then regenerated with 11-*cis*-retinal. As can be seen, the composite spectrum is bathochromically shifted approximately 12 nm over that of permethylated rhodopsin (Figure 2). Of course, the spectrum of Figure 6 is a composite of approximately 4 parts active-site lysine-methylated permethylated rhodopsin to approximately 1 part non-active-site-methylated lysine-permethylated rhodopsin. The mixture can be bleached and regenerated with 11-*cis*-retinal (Figure 6).

Purification of Permethylated Active-Site-Methylated Rhodopsin. To determine the spectrum of the pure active-site-monomethylated rhodopsin, a method of separating the unmethylated and methylated adducts was sought. To this end, the methylated opsin mixture was treated with *o*-phthalaldehyde, a reagent which can only react with primary amino groups and in so doing generates a fluorescent adduct (Trepman & Chen, 1980). Under the conditions shown in Table I, treatment of the methylated opsin mixture with *o*-phthalaldehyde led to the formation of an adduct and a decrease of approximately 40% of the regenerable activity of the methylated opsins. Bleached permethylated rhodopsin when

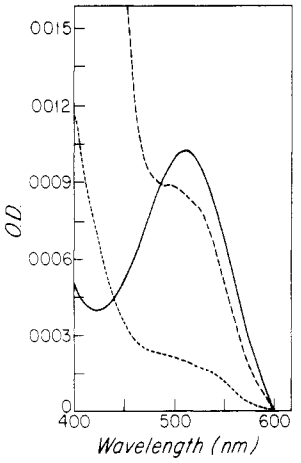


FIGURE 6: Partial visible spectrum of regenerated protein from a 48-h active-site methylation (using two additions of reagents, 2 mM formaldehyde and 20 mM pyridine/borane at zero time and 24 h) of bleached, permethylated rhodopsin (solid line). The position of λ_{max} is at 512 nm. Also shown (dotted line) is the spectrum after bleaching of this sample with intense light filtered through running water and an orange filter (Corning 3-68, cutoff 540 nm). The spectrum after regeneration with 11-*cis*-retinal is shown by the dashed line. Regeneration was estimated to be between 60% and 70% in this case.

Table I: Reaction of *o*-Phthalaldehyde/Mercaptoethanol with the Active-Site Lysine of Permethylated Rhodopsin[a]

time of incubn (h)	active-site treatment	% regenertion with 11-*cis*-retinal	rel fluorescence
0	methylated	42	0
	control	87	0
20	methylated	27	52
	control	18	101
44	methylated	25	63
	control	~8	117

[a] One batch of bleached protein had undergone a 48-h methylation (in this case incorporating 1.5 methyl groups per rhodopsin at the active site, measured by ^{14}C incorporation; see text for details); the other was not active-site-methylated. Data for percent regeneration (value for permethylated rhodopsin before active-site modification being taken as 100%) and relative fluorescence demonstrate that active-site methylation affords some protection from reaction with these reagents, by virtue of the monomethyllysine which is still able to form a rhodopsin-like pigment with 11-*cis*-retinal.

treated in the same way almost completely lost (91%) its ability to regenerate with 11-*cis*-retinal (Table I). The relative fluorescence of the adducts increased with the amount of unmethylated active-site lysines, as expected (Table I). The methylated opsin/*o*-phthalaldehyde mixture was treated with 11-*cis*-retinal, and the regenerated active-site-monomethylated permethylated rhodopsin was chromatographed on hydroxylapatite (Figure 7). A purified, active-site-methylated regenerated derivatized rhodopsin band was obtained in the first major peak from the column with a spectrum shown in Figure 8. This material would be expected to contain <5% permethylated rhodopsin having an unmodified lysine at the active site. In addition, a small amount of nonregenerable protein might still be contaminating the pool of active-site-methylated regenerated rhodopsin. The λ_{max} of 520 nm is considerably shifted over that of permethylated rhodopsin itself, also shown in Figure 8.

Discussion

A protonated, or partially protonated, Schiff base formed between opsin and 11-*cis*-retinal looms large in the biochemical functioning of rhodopsin. On the one hand, the absorption

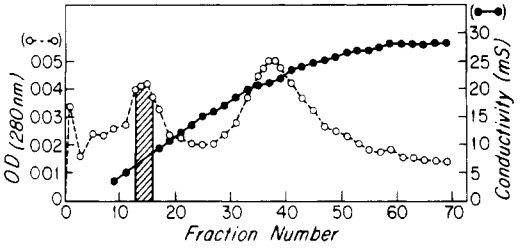


FIGURE 7: Elution profile showing the isolation of regenerated, active-site-methylated rhodopsin by hydroxylapatite column chromatography. Bound protein was eluted by using a gradient of potassium phosphate as outlined in the text. The hatched area shows those protein-containing fractions which were pooled and used for further studies. The later eluting peak contained nonregenerable protein and excess 11-*cis*-retinal.

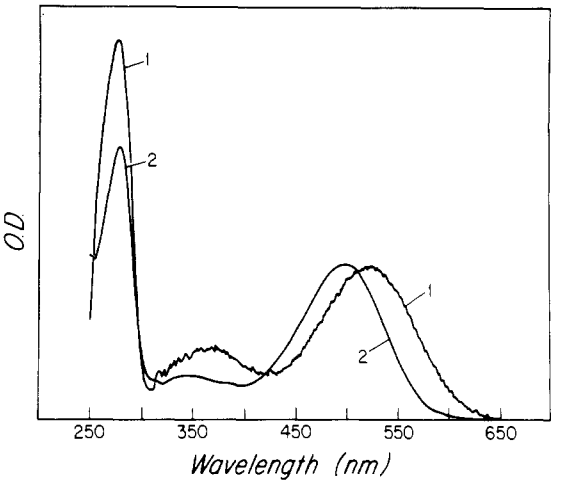


FIGURE 8: Spectrum of purified, active-site-methylated, permethylated rhodopsin isolated by hydroxylapatite column chromatography (1). The chromophore peak is shifted from 498 to 520 nm. Shown for comparison (2) is a spectrum of permethylated rhodopsin (no active-site modification) also chromatographed on a hydroxylapatite column, leading to an improvement in the A_{278}:A_{498} ratio. This ratio was 2.06 for applied protein and 1.76 for protein isolated from the column. Spectra 1 and 2 have been corrected to have the same peak heights at 520 and 498 nm, respectively (full scale expansion was 0.025 for spectrum 1 and 0.21 for spectrum 2).

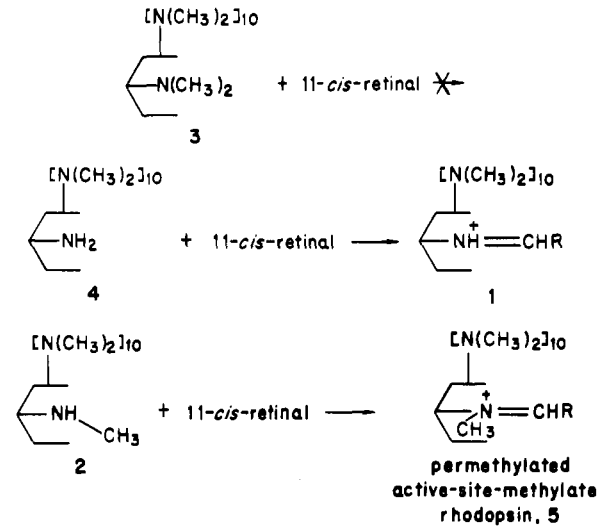
spectra of the various rod and cone pigments are critically dependent on the presence of a charged Schiff base nitrogen (Honig et al., 1979b). The opsin shift, upon which the λ_{max} values of the various visual pigments depend, is predicated on the presence of a protonated Schiff base interacting through space with point negative charges in the opsin backbone (Honig et al., 1979b). A further important role for a charged Schiff base appears to be in the visual transduction mechanism itself. Like any other receptor, rhodopsin must be energized before it can act. Most drug receptors utilize the binding energies of the drug for this purpose, whereas rhodopsin utilizes light energy for its activation. In fact, early studies demonstrated that much of the light energy absorbed by rhodopsin is stored in the potential energy of the protein at the bathorhodopsin stage, which is the first discernible intermediate formed past photolysis (Cooper, 1979). Plausible mechanisms for energy storage involve separation of the protonated Schiff base from its amino acid counterion as a consequence of photochemical excitation [for example, see Honig et al. (1979a)]. Of additional interest as noted before is the notion that a proton may be lost from the putative protonated Schiff base during the critical metarhodopsin I to metarhodopsin II conversion (Cooper & Converse, 1976). A proton is gained from the medium at this time coinciding with a visible spectral shift of 478–380 nm (Abrahamson & Ostroy, 1967). The latter

spectral change is consistent with a deprotonation of the Schiff base, as is hydrolysis or simple hydration (Allan & Cooper, 1980). These and other experiments suggested that it would be of great interest to prepare and study an opsin molecule with a methyl group attached to its active-site lysine. First, this methyl group could serve as a spectroscopic ([^{13}C]methyl NMR/Raman spectroscopy) reporter group to monitor the mechanism of rhodopsin activation. Furthermore, the methyl-labeled ϵ-amino group of lysine also allows for a test of whether or not a full charge on the Schiff base is important, and whether or not it is obligated to move during photochemical excitation. Indeed, it is of significant interest to determine whether an active-site-methylated opsin would even form a pigment with 11-*cis*-retinal given that the methyl ketone analogue of 11-*cis*-retinal will not form a pigment with opsin (Ebrey et al., 1972). Similar kinds of experiments with bacteriorhodopsin can also be entertained. Thus, the preparation of active-site-methylated (N^{ϵ}-methyllysine) rhodopsin was undertaken.

One approach to selective active-site amino acid substitutions is by site-specific mutagenesis of a cloned gene which can later be expressed. This approach was of no immediate interest here. Since there is no obvious way to selectively monomethylate the active-site lysine group, a more global chemical approach was required. Reductive methylation using formaldehyde and a reducing agent is an obvious approach since the conditions for this modification procedure are mild and selective for amino groups under appropriate conditions (Jentoft & Dearborn, 1979). Even though this was the reaction of choice, neither kinetic data on the reaction nor clear data on the reaction's ability to discriminate between the introduction of mono- and dimethylamines were available in the literature. Nevertheless, the reductive methylation of rhodopsin was studied in detail here. In the experiments reported in this paper, pyridine/borane was utilized as the reducing agent. Sodium cyanoborohydride was also used, but its use was discontinued because the results obtained with it were more variable than with pyridine/borane (Wong et al., 1984). In the reductive methylation procedures developed here, it was necessary to block all of the non-active-site lysine residues first or else there would be no way of determining the extent of methylation later. Rhodopsin was methylated, with the expected incorporation of approximately 20 methyl groups, prior to studies on the labeling of the active-site lysine. Importantly, rhodopsin modified in such a fashion was bleachable, regenerable, and still fully active toward the activation of the GTPase when illuminated (Figure 2). Since the net charge on the lysines is not altered by this modification, this result may not be overly surprising. It should be noted that lysine modifications by acetamidation also lead to a bleachable and regenerable protein (DeGrip et al., 1973). We have found that this modification, although not as complete as methylation, also provides pigment capable of activating the GTPase (C. Longstaff and R. Calhoon, unpublished results).

Permethylated rhodopsin was then bleached and further methylated at low temperatures (4 °C) with formaldehyde and pyridine/borane. These experiments had to be performed at 4 °C because of the thermal lability of opsin. Nevertheless, at 4 °C over a 48-h period with two additions of reagents, 1.4–1.5 methyl groups could be incorporated per rhodopsin molecule. An upper limit of approximately 2.0 methyl groups could be incorporated at 37 °C. The latter protein is, of course, not regenerable with 11-*cis*-retinal. When the reductive methylation procedure was carried out at 4 °C for 48 h, the 1.5 methyl groups incorporated were partitioned between the

Scheme II

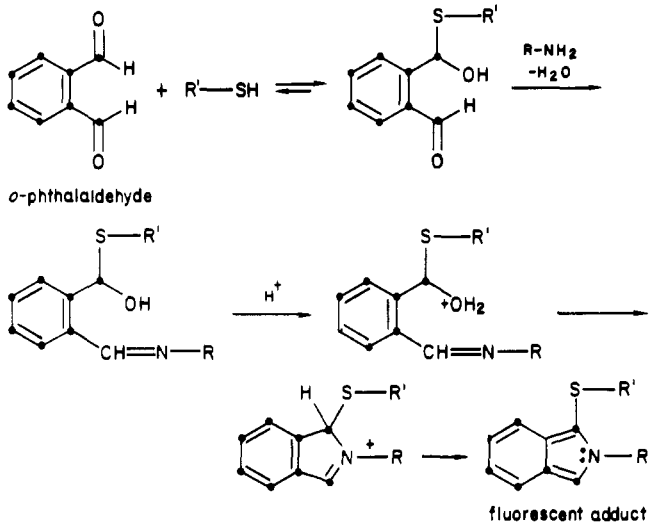


formation of N^{ϵ}-monomethyled and N^{ϵ}-dimethylated lysine. Longer periods of methylation using higher concentrations of methylating agents were not fruitful for eliminating unmethylated lysines, because increased denaturation of the protein was observed. Such reactions carried out over 48 h at 4 °C always led to incomplete methylation of the active-site lysine, with an estimated 10% rhodopsin retaining nonmethylated lysine. The dimethyllysine:monomethyllysine ratio was calculated to be 5:4. These values were determined by digesting the radioactivity-labeled protein, separating the dimethyl- and monomethyllysines by TLC, and determining the amounts of the two amino acids (Figure 4). These experiments also demonstrated that, indeed, active-site lysines were N^{ϵ}-methylated, as expected, rather than another amino acid. In addition, it can be concluded that the reductive methylation procedure shows little selectivity with respect to the extent of N-methylation, a point not made clear in the literature. Of the methylated protein, 50% could not regenerate with 11-*cis*-retinal because of dimethylation. When this modified protein mixture was treated with 11-*cis*-retinal, 42% regeneration was obtained. This means that the active-site-monomethylated opsin must have been capable of regeneration, because only 10% of the total protein contained unmethylated active-site lysines. The fact that 42% regeneration was observed, rather than the expected 50%, it doubtless due to some denaturation of the opsin produced during the methylation procedure. Further evidence for the idea that monomethylated material can regenerate comes from the position of the λ_{max} of the modified protein, which appeared at 512 nm rather than 500 nm for the permethylated rhodopsin (Figure 6). This modified rhodopsin could be bleached and regenerated (Figure 6).

To further confirm that the monomethylated lysine-derivatized permethylated opsin could partake in Schiff base formation, permethylated opsin was reductively methylated with [^{14}C]formaldehyde and then regenerated with a mixture of 11-*cis*-[^{3}H]retinal and 9-*cis*-retinal. The Schiff base was reduced with sodium borohydride in the light and digested with base (Bownds, 1967). Both ^{3}H and ^{14}C dpm coeluted with the *N*-methylretinyllysine standard, showing that the active site was attached to the ϵ-amino group of lysine (Figure 5).

The spectrum displayed in Figure 6 is of a mixture of the active-site N-monomethylated and active-site unmethylated rhodopsins (Scheme II). It was desired to separate permethylated active-site-methylated rhodopsin (**5**) from the mixture of the permethylated rhodopsin (**1**) and permethylated

Scheme III



opsin (**3**) (Scheme II). To carry this out, opsins **2**, **3**, and **4** (Scheme II) were treated with *o*-phthalaldehyde and mercaptoethanol, reagents that specifically react with primary amino groups to form a fluorescent adduct (Scheme III) (Trepman & Chen, 1980; Sternson et al., 1985). This derivatization led to a loss of approximately 40% of the regenerability of the mixture of **2**, **3**, and **4** (Table I). Of the total regenerable protein, 80% had a monomethyl group at its active-site lysine and 20% did not. Therefore, it might have been predicted that only a 20% loss of regeneration ability should have been suffered during the reaction with *o*-phthalaldehyde and mercaptoethanol. However, the derivatization reaction was carried out over a 44-h period where some loss of regeneration ability is to be expected. On the other hand, bleached permethylated rhodopsin was almost completely prevented from regenerating with 11-*cis*-retinal by prior treatment with *o*-phthalaldehyde/mercaptoethanol, which is an expected result, since the unmodified lysine active site should all be available for derivatization. Since the *o*-phthalaldehyde/mercaptoethanol/primary amine adduct is fluorescent, the magnitude of the fluorescent peak recorded should be related to the amount of underivatized active-site lysine of the starting opsin, as was found (Table I). Careful chromatography of the active-site-methylated permethylated rhodopsin/*o*-phthalaldehyde/mercaptoethanol mixture on hydroxylapatite led to the elution of two main protein peaks (Figure 7), one of which contained **5** with a λ_{max} of 520 nm. The approximately 20-nm bathochromic shift of this pigment over that of permethylated rhodopsin is of some interest. Model studies on Schiff bases formed between *n*-butylamine and *all-trans*-retinal (followed by protonation with HCl) on the one hand and *N*-methylbutylamine hydrochloride/*N*-methylbutylamine and *all-trans*-retinal on the other did not show this shift. In $CHCl_3$, the protonated primary amine Schiff base absorbed at 462 nm and the secondary amine Schiff base also at 462 nm. The reason why the active-site-methylated rhodopsin is bathochromically shifted with respect to rhodopsin could stem from the point charge effect described by Honig and Nakanishi (Honig et al., 1979b). Small displacements between the negatively charged amino acid counterion or point charge and the positively charged Schiff base head group in the rhodopsin could lead to significant spectral shifts. This could mean that the counterion is slightly displaced and further away from the *N*-methyl-charged Schiff base than it is from the protonated Schiff base. It will be of interest to determine the λ_{max} of the bathorhodopsin intermediate formed from active-site-methylated rhodopsin.

In summary, we have prepared a functional opsin containing a methyl group attached to its active-site lysine group. This modified opsin can form a pigment with 11-*cis*-retinal having a λ_{max} = 520 nm. This pigment can be bleached and regenerated with 11-*cis*-retinal, which shows that the active site of rhodopsin can accommodate a methyl group and a formal positive charge. Furthermore, neutralization of the Schiff base of rhodopsin is apparently not important for bleaching. The spectroscopic and biochemical properties of this new pigment are currently under investigation.

ACKNOWLEDGMENTS

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Registry No. 11-*cis*-Retinal, 564-87-4; lysine, 56-87-1; *o*-phthalaldehyde, 643-79-8; *N*-monomethyllysine, 1188-07-4; *N*-dimethyllysine, 2259-86-1.

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Mechanism of Inhibition of Protein Glycosylation by the Antiviral Sugar Analogue 2-Deoxy-2-fluoro-D-mannose: Inhibition of Synthesis of Man(GlcNAc)$_2$-PP-Dol by the Guanosine Diphosphate Ester†

William McDowell, Roelf Datema,* Pedro A. Romero, and Ralph T. Schwarz
Institut für Virologie, Justus Liebig-Universität, Giessen, D-6300 Giessen, FRG
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ABSTRACT: 2-Deoxy-2-fluoro-D-mannose (2FMan), an antiviral mannose analogue, inhibited the dolichol cycle of protein glycosylation. To specifically inhibit oligosaccharide–lipid synthesis, and not (viral) protein synthesis in influenza virus infected cells, the addition of guanosine to the 2FMan-treated cells was required. Under these conditions an early step in the assembly of the oligosaccharide–lipid was inhibited, and as a consequence, the glycosylation of proteins was strongly inhibited. Low-molecular-weight, lipid-linked oligosaccharides accumulated in cells treated with 2FMan *plus* guanosine, although dolichol phosphate (Dol-P) and GDP-Man were still present in the treated cells, and membranes from these cells were not defective in assembly of lipid-linked oligosaccharides. Thus, the presence of a soluble inhibitor of oligosaccharide–lipid assembly in these cells was postulated, and GDP-2FMan and UDP-2FMan, two metabolites found in 2FMan-treated cells, were synthesized and used to study in cell-free systems the inhibition of oligosaccharide–lipid assembly. GDP-2FMan inhibited the synthesis of Man(GlcNAc)$_2$-PP-Dol from (GlcNAc)$_2$-PP-Dol and GDP-Man, and in addition, it caused a trapping of Dol-P as 2FMan-P-Dol, whereas UDP-2FMan only inhibited Glc-P-Dol synthesis. However, it is probable that neither trapping of Dol-P nor inhibition of Glc-P-Dol synthesis by UDP-2FMan contributed to inhibition of protein glycosylation in cells treated with 2FMan. Incorporation of 2FMan from GDP-2FMan or UDP-2FMan into dolichol diphosphate linked oligosaccharides and interference of GDP-2FMan with the latter steps of assembly of the dolichol diphosphate linked oligosaccharide could not be shown. It is concluded that 2FMan, via GDP-2FMan, inhibits protein glycosylation by blocking formation of Man(GlcNAc)$_2$-PP-Dol and, thus, further assembly of the oligosaccharide–lipid.

The mechanism of protein glycosylation and the biological roles of glycosylation of glycoproteins have received widespread attention (Hubbard & Ivatt, 1981; Kornfeld, 1982; Schwarz & Datema, 1982a). Thus, it has been firmly established that the biosynthesis of the asparagine-linked oligosaccharides of glycoproteins occurs in two discrete stages: (1) the assembly of the tetradecasaccharide Glc$_3$Man$_9$(GlcNAc)$_2$ on Dol-PP[1] (see Figure 1) and, following transfer to protein, (2) the processing of the protein-linked oligosaccharide to the complex-type and nonglucosylated, high-mannose-type oligosaccharides.

Several analogues of mannose and glucose interfere with glycosylation of proteins. They can be inhibitors of the assembly of the dolichol diphosphate linked oligosaccharide (2-deoxy-D-glucose, 2-deoxy-2-amino-D-glucose, 2-deoxy-2-fluoro-D-glucose, or 4-deoxy-4-fluoro-D-mannose; Schwarz & Datema, 1982a; Grier & Rasmussen, 1984), interfere with the processing of protein-linked oligosaccharides (bromoconduritol, *N*-methyl-1-deoxnojirimycin; Schwarz & Datema, 1982a; Elbein, 1984), or interfere with both stages (nojirimycin, 1-deoxynojirimycin; Datema et al., 1984).

The mannose analogue 2-deoxy-2-fluoro-D-mannose (2FMan) shows antiviral effects, probably because of inhibiting the glycosylation of viral glycoproteins (Schmidt et al., 1976). In chick embryo and yeast cells 2FMan is converted to both GDP-2FMan and UDP-2FMan (Schmidt et al., 1978). In

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* Correspondence should be addressed to this author at the Department of Antiviral Chemotherapy, Research & Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje, Sweden.

[1] Abbreviations: Dol-P, dolichol phosphate; Dol-PP, dolichol diphosphate; EDTA, ethylenediaminetetraacetic acid; 2FMan, 2-deoxy-2-fluoro-D-mannose; Glc, glucose; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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