Transglutaminase-Catalyzed Insertion of a Fluorescent Probe into the Protease-Sensitive Region of Rhodopsin†

J. S. Pober,‡ V. Iwanij, E. Reich, and L. Stryer*,§

ABSTRACT: Transglutaminase from guinea pig liver inserts dansylcadaverine and putrescine into bovine rhodopsin in retinal disk membranes. The stoichiometry of labeling is one amine per rhodopsin molecule. Labeling with putrescine prevents the subsequent insertion of dansylcadaverine, suggesting that the same glutamine residue is labeled by these amines. The site of labeling was determined by taking advantage of the fact that rhodopsin in disk membranes is cleaved by a variety of proteolytic enzymes of differing specificity into two fragments, called F1 and F2 [Pober, J. S., and Stryer, L. (1975), *J. Mol. Biol. 95,* 477]. Three lines of evidence indicate that the site labeled by transglutaminase is located in the protease-sensitive region between the F1 and F2 fragments. First, prior enzymatic proteolysis inhibits transglutaminase-catalyzed labeling. Second, prior transglutaminase-catalyzed labeling inhibits enzymatic proteolysis. Third, dansyl fluorescence is exhibited by F1 during the initial stage of proteolysis of labeled rhodopsin by subtilisin. The dansyl label is then excised from F1 with little change in the electrophoretic mobility of this fragment. This excised F1 fragment retains the oligosaccharide units which are known to be near the amino terminus of rhodopsin. Thus, the site labeled by dansylcadaverine is near the carboxy terminus of F1. The distance between dansylcadaverine and 11-*cis*-retinal in labeled rhodopsin in the disk membrane was estimated by energy-transfer spectroscopy. The apparent distance of 61 Å calculated from a transfer efficiency of 31% and an R_0' of 53 Å shows that rhodopsin has an elongated shape in its native membrane environment. The nanosecond emission anisotropy kinetics of the dansyl fluorescence showed that the probe rotates very rapidly (ϕ_1 = <1 ns) within a cone of half-angle 32°, followed by a slower rotational motion (ϕ_2 = 140 ns). In Ammonyx LO detergent solution, ϕ_2 was 5 ns, indicating that the region between the F1 and F2 units is much more flexible in detergent solution than in the disk membrane.

Transglutaminase from guinea pig liver catalyzes the insertion of primary amines into glutaminyl residues in peptides and proteins (Clarke et al., 1959; Folk and Chung, 1973; Connellan et al., 1971). This enzyme has been used to label membrane proteins and, in the absence of exogenous amines, to catalyze the formation of ε-(γ-glutamyl)-lysyl cross-links between them (Brewer and Singer, 1974; Dutton and Singer, 1975; Dutton et al., 1976; Iwanij, 1977). The specificity of the labeling reaction and the mildness of the reaction conditions stimulated us to explore the use of transglutaminase to insert spectroscopic probes into rhodopsin in the intact disk membrane. The probes used are not reactive in the absence of transglutaminase. Furthermore, only proteins can be labeled because this enzyme requires a glutamine side chain as one of its substrates. Only residues of rhodopsin located in the aqueous phase on the exposed side of the disk membranes were expected to be labeled because transglutaminase is unlikely to enter or traverse lipid bilayers, in contrast to many reactive fluorescent probes. Another advantage of using transglutaminase is that a very wide range of spectroscopic probes can be inserted, the only requirement being an aliphatic amino terminus. In fact, we found that rhodopsin is labeled in a highly specific way by transglutaminase when putrescine and dansylcadaverine were used as substrates. We report here proteolytic studies which identify the site of labeling and nanosecond fluorescence studies which provide an estimate of the distance between the dansylcadaverine label and 11-*cis*-retinal. The effects of bleaching and of extracting rhodopsin into detergent solution on the rotational mobility of the labeled site were also investigated.

Materials and Methods

Disk membranes of rod outer segments from bovine retinas (G. A. Hormel Co.) were isolated by a modified sucrose flotation method (McConnell, 1965; Bownds, 1967; Pober, 1976). Transglutaminase was purified from guinea pig livers by the method of Connellan et al. (1971). Thermolysin (three times recrystallized) was purchased from Calbiochem and subtilisin Carlsberg from Sigma. All radioisotopes and Aquasol liquid scintillation cocktail were obtained from New England Nuclear. Putrescine, dansylcadaverine, and bovine serum albumin (essentially fatty acid free) were purchased from Sigma. Sodium dodecyl sulfate ($NaDodSO_4$)[1] was obtained from B.D.H. Poole (Gallard-Schlessinger), and acrylamide, bisacrylamide, and hydroxylapatite were from Bio-Rad. Ammonyx LO (70% dodecyldimethylamine oxide and 30% tetradecyldimethylamine oxide) was a gift of the Onyx Chemical Co. All other chemicals were reagent grade.

Enzymatic Modification of Membrane-Bound Rhodopsin. Transglutaminase-catalyzed labeling of disk membranes with putrescine or dansylcadaverine was carried out by adding one part transglutaminase to ten parts rhodopsin by weight in a suspension of disk membranes containing 5 to 10 mg/mL rhodopsin. The buffer contained 100 mM Tris-acetate (pH

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520 (J.S.P. and L.S.), and the Rockefeller University, New York, New York 10021 (V.I. and E.R.). *Received November 30, 1977.* This work was supported by research grants from the National Institutes of Health. A preliminary account of this work was presented at the 1976 Biophysical Society meeting.

‡ Present address: Department of Pathology, Yale Medical School, New Haven, Conn. 06510.

§ Present address: Department of Structural Biology, Sherman Fairchild Center, Stanford Medical School, Stanford, Calif. 94305.

[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid; Con A, concanavalin A.

0006-2960/78/0417-2163$01.00/0

7.4), 5 mM dithiothreitol, 10 mM calcium chloride, and 10–20 mM amine substrate. All steps were conducted under dim red light. The membranes were labeled at 37 °C for up to 8 h with shaking. The labeling was stopped by adding 50–100 mM NaEDTA (pH 7.4) to chelate Ca^{2+} and by centrifuging the membranes at 37 000*g* for 10 min to remove transglutaminase.

Disk membranes were subjected to proteolysis with thermolysin or subtilisin by adding protease to a suspension of membranes containing 5 mg of rhodopsin/mL of 10 mM Tris–acetate buffer (pH 7.4). Thermolysin reactions were supplemented with 5 mM calcium chloride. All steps were carried out under dim red light at 37 °C. The weight ratio of protease to transglutaminase-modified rhodopsin was 1:10 for thermolysin and 1:50 for subtilisin. These enzyme concentrations were five- to tenfold greater than required for proteolysis of unmodified rhodopsin in disk membranes. Proteolysis was terminated by adding a specific enzyme inhibitor and by centrifuging the membranes at 37 000*g* for 10 min to remove the protease. The inhibitors were 10 mM EDTA for thermolysin and 1 mM phenylmethanesulfonyl fluoride for subtilisin.

Analysis of Enzymatically Modified Rhodopsin. Con A–agarose chromatography of cleaved rhodopsins was conducted as described previously (Pober and Stryer, 1975). Samples of disk membranes for $NaDodSO_4$ gel electrophoresis were prepared by the method of Papermaster and Dryer (1974). Analysis of [^{3}H]putrescine-labeled rhodopsin was carried out by gradient polyacrylamide gel electrophoresis and subsequent fluorography (Iwanij, 1977). The kinetics of [^{3}H]putrescine incorporation into rhodopsin were analyzed by electrophoresis on $NaDodSO_4$ slab gels using the buffer system of Laemmli (1970). The gels were sliced, and each slice was dissolved and assayed for radioactivity by liquid scintillation counting (Ward et al., 1970). Disk membranes, modified with dansylcadaverine, were analyzed by electrophoresis on cylindrical $NaDodSO_4$–acrylamide gels prepared by the method of Weber and Osborn (1969). The gels were fixed in 25% 2-propanol and 10% acetic acid and scanned for the position of fluorescent bands using an apparatus designed and constructed by Mark Yeager and Gerald Johnson. The gel was excited with monochromatic light of wavelength 360 nm, and emission was measured through a Corning 3-71 glass filter. The gels were subsequently stained with Coomassie blue (Fairbanks et al., 1971).

The stoichiometry of amine incorporation into rhodopsin was ascertained by purifying the labeled rhodopsin from disk membranes. Following 8 h of transglutaminase-catalyzed labeling with [^{14}C]putrescine of known specific activity, disk membranes were dissolved in Ammonyx LO solution and subjected to hydroxylapatite chromatography by the method of Applebury et al. (1974), as modified by Yeager (1976). This procedure separates rhodopsin from opsin and phospholipids. The rhodopsin content of each eluted fraction was determined by measuring the absorbance at 500 nm, and the [^{14}C]putrescine content of each fraction was determined in duplicate by adding a 50-μL aliquot to 10 mL of Aquasol and measuring the radioactivity by liquid scintillation counting. The stoichiometry of dansylcadaverine incorporation into rhodopsin was determined from the dansyl fluorescence of a purified sample of dansylcadaverine-labeled rhodopsin in 1% Ammonyx LO solution, using free dansylcadaverine as a standard.

Preparation of Dansylcadaverine-Labeled Rhodopsin for Spectroscopy. Disk membranes containing dansylcadaverine-labeled rhodopsin were prepared for spectroscopy under dim red light at 4 °C as follows: the membranes, labeled with dansylcadaverine, were suspended in 50 volumes of 1% bovine serum albumin in 0.1 M sodium acetate buffer (pH 5.8) and centrifuged at 37 000*g* for 15 min. This process was repeated five or six times until the albumin solutions no longer extracted noncovalently bound dansylcadaverine from the membranes. The membrane pellet was centrifuged three times at 37 000*g* for 10 min in 100 mM sodium phosphate buffer (pH 7.4), 5 mM dithiothreitol, and three times in deionized water to remove residual albumin. The final pellet was suspended in 5 volumes of the sodium phosphate buffer and sonicated five or six times in 2-min bursts of 100 W with a Model W 185 D sonifier cell disrupter (Heat Systems, Ultrasonics, Inc.) to disperse the membranes. The dispersion of sonicated membranes was briefly centrifuged (37 000*g* for 1 min) to remove large particles and was diluted with sodium phosphate buffer so that the optical density of the final solution was less than 0.3 for nanosecond spectroscopy and less than 0.03 for steady-state emission spectroscopy. Hydroxylamine (0.03 M) was added to the samples shortly before spectroscopic experiments were carried out to ensure that bleaching was rapid upon illumination.

Measurements of Fluorescence Lifetimes and Emission Anisotropy Kinetics. Fluorescence lifetimes and emission anisotropy kinetics were measured with a single-photon counting apparatus (Yguerabide et al., 1970; Yguerabide, 1973). The finite duration of the light pulse was taken into account by a convolution procedure. The sample was excited through a Corning 7-37 glass filter and emission was detected through a 3-71 filter. Fluorescence emission spectra were recorded at room temperature and corrected for the wavelength-dependent sensitivity of the detection system (Stryer, 1965). 8-Anilino-1-naphthalenesulfonate in absolute ethanol was used as a quantum yield standard of 0.37 (Stryer, 1965). The emission anisotropy $A(t)$ is defined as $A(t) = [y(t) - x(t)]/[y(t) + 2x(t)]$, where $y(t)$ and $x(t)$ are the vertically and horizontally polarized components of the fluorescence at time t, when the sample is excited with vertically polarized light.

Results

Transglutaminase Inserts One Amine per Rhodopsin in Disk Membranes. Transglutaminase catalyzes the insertion of putrescine or dansylcadaverine into bovine rhodopsin in retinal disk membranes (Figure 1). No labeling occurs in the absence of enzyme. Transglutaminase treatment also produces aggregates of rhodopsin that cannot be dissociated by $NaDodSO_4$, probably through the formation of interchain glutamyl–lysyl isopeptide bonds. These aggregates range in size from dimers of rhodopsin to oligomers too large to enter the $NaDodSO_4$–acrylamide gel. The aggregates are also labeled with putrescine or dansylcadaverine. The incorporation of putrescine reached a plateau value after 3 to 4 h of incubation (Figure 2A). The amount of putrescine inserted could not be increased by adding either fresh enzyme or amine, indicating that the reaction stops because the susceptible sites are fully modified. No appreciable difference was observed in the rate, extent, or products of the transglutaminase-catalyzed modification of disk membranes when rhodopsin was bleached by light prior to labeling with putrescine (Figure 2B).

After 8 h of transglutaminase catalysis, rhodopsin purified by hydroxylapatite chromatography in 1% Ammonyx LO solution contained 1.0 ± 0.1 mol of [^{14}C]putrescine/mol of rhodopsin (Figure 3). Similarly, the amount of dansylcadaverine incorporated was also about 1.0 mol of label/mol of rhodopsin, but there is some uncertainty in the stoichiometry because the quantum yield of dansylcadaverine attached to

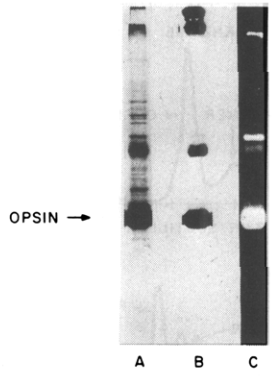

FIGURE 1: NaDodSO$_4$-acrylamide gels showing the transglutaminase-catalyzed labeling of rhodopsin in disk membranes. (A) Photograph of the Coomassie blue staining pattern and (B) autoradiogram of the tritium radioactivity of the same gel following electrophoresis of disk membranes that were labeled with [^{3}H]putrescine. (C) Photograph of a gel showing the ultraviolet-excited dansyl fluorescence following electrophoresis of disk membranes that were labeled with dansylcadaverine. Disk membranes were incubated with transglutaminase and amine substrate for 8 h. The high-molecular-weight species which do not enter the gels are oligomers of opsin (or of transglutaminase) produced by transglutaminase-catalyzed cross-linking.

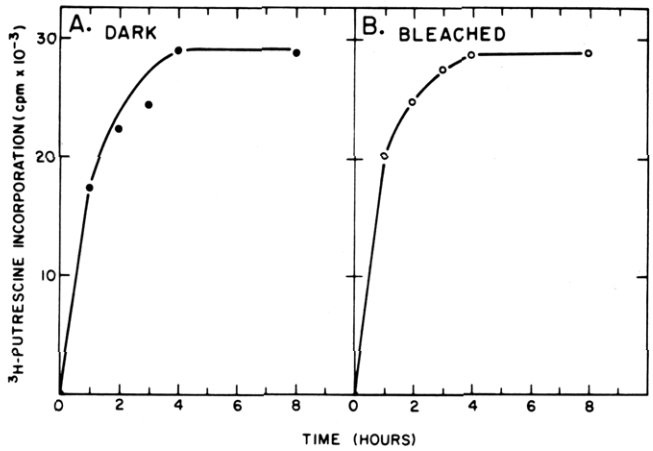

FIGURE 2: Kinetics of the transglutaminase-catalyzed labeling of rhodopsin in disk membranes with [^{3}H]putrescine (A) in the dark and (B) after bleaching.

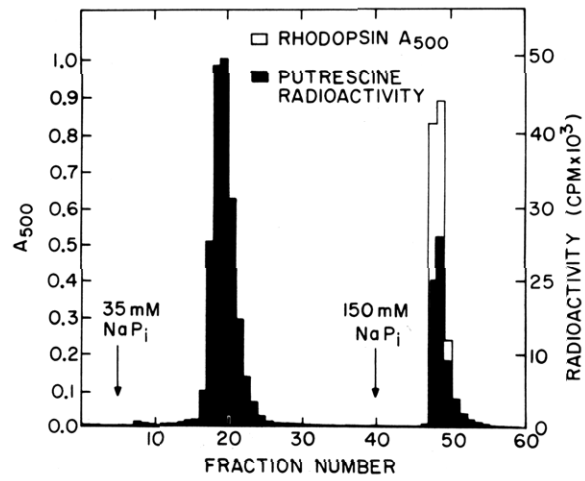

FIGURE 3: Elution profile of putrescine-labeled rhodopsin from a hydroxylapatite column showing the 500-nm absorbance and [^{14}C]putrescine radioactivity. Disk membranes were incubated for 8 h with transglutaminase and [^{14}C]putrescine. The labeled membranes were solubilized in Ammonyx LO, loaded on the column in the dark, and washed with 35 mM sodium phosphate buffer until the level of radioactivity reached a baseline value. Rhodopsin was eluted with 150 mM sodium phosphate buffer. The putrescine specific activity was 19.5 mCi/mmol, and the extinction coefficient of rhodopsin at 500 nm was taken to be 40 000 cm^{-1} M^{-1}. The stoichiometry of the peak fractions calculated from the ratio of the radioactivity to the 500 nm absorbance was 1.0 ± 0.1 mol of putrescine incorporated/mol of rhodopsin.

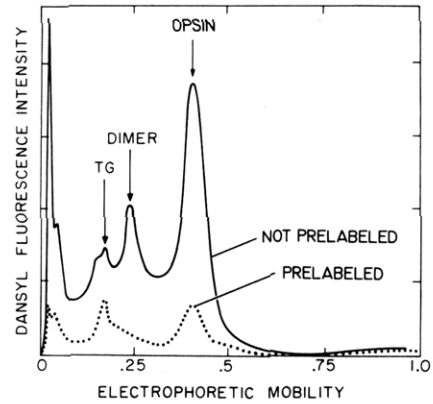

FIGURE 4: Putrescine and dansylcadaverine compete in labeling rhodopsin. One aliquot of disk membranes was prelabeled with putrescine for 3 h in a transglutaminase-catalyzed reaction. The other aliquot was incubated with putrescine in the absence of transglutaminase as a control. Each aliquot was then incubated with dansylcadaverine and transglutaminase for 8 h and electrophoresed on NaDodSO$_4$ gels. The dansyl fluorescence of the gels was scanned by exciting them at 360 nm and recording their emission through a Corning 3-71 filter. The amount of dansyl label incorporated by the prelabeled membranes (···) was less than 10% of that of the control (—).

rhodopsin is not precisely known. The stoichiometry of about one inserted label per rhodopsin molecule suggested that putrescine and dansylcadaverine become attached to the same site on rhodopsin. This possibility was tested by carrying out a competition experiment. Rhodopsin prelabeled with putrescine incorporates less than 10% of the amount of dansylcadaverine taken up by unmodified rhodopsin (Figure 4). The simplest interpretation of the observed labeling stoichiometry and this competition experiment is that a single glutamine residue on rhodopsin in disk membranes is susceptible to the transglutaminase-catalyzed insertion of primary amines.

The Site Modified by Transglutaminase Is in the Protease-Sensitive Region. The site of labeling was determined by taking advantage of the fact that rhodopsin in disk membranes is cleaved by a variety of proteolytic enzymes of differing specificity, such as thermolysin, subtilisin, and papain, into two fragments, called F1 and F2 (Pober and Stryer, 1975). F1 and F2 are, in fact, sets of fragments differing slightly in molecular weight, as shown by electrophoresis on polyacrylamide gradient gels having high-resolving power (e.g., the gel system of Laemmli, 1970). The set of larger fragments (F1) produced by cleaving rhodopsin with thermolysin retains the blocked amino terminus of intact rhodopsin, as shown by end-group analyses using the microdansyl technique, whereas the set of smaller fragments (F2) has a reactive amino terminus (Pober, 1976). Hence, F1 comes from the amino-terminal side of rhodopsin and F2 from the carboxy-terminal side. Our initial proteolysis experiments yielded a striking result: thermolysin does not cleave dansylcadaverine–rhodopsin to any appreciable extent. Furthermore, the rate of formation of F1 and F2 fragments using subtilisin and papain was tenfold slower for dansylcadaverine–rhodopsin than for unmodified rhodopsin. We then ascertained the effect of prior proteolysis by thermolysin on the extent of incorporation of dansylcadaverine. The dansyl fluorescence of NaDodSO$_4$ gels showed that

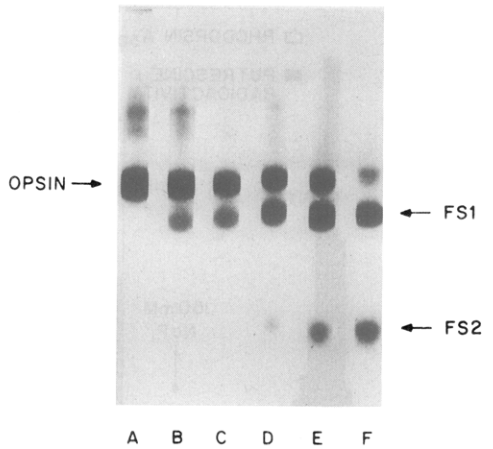

FIGURE 5: Cleavage of dansylcadaverine labeled rhodopsin by subtilisin. Dansylcadaverine-labeled disk membranes were incubated with subtilisin, (weight ratio to rhodopsin of 1:200) for (A) 15 min, (B) 30 min, (C) 1 h, (D) 2 h, (E) 5 h, and (F) 24 h and electrophoresed. The Coomassie blue staining pattern of the $NaDodSO_4$ gels shows that opsin, the major band prior to proteolysis, is replaced by two bands having higher electrophoretic mobilities, called FS1 and FS2.

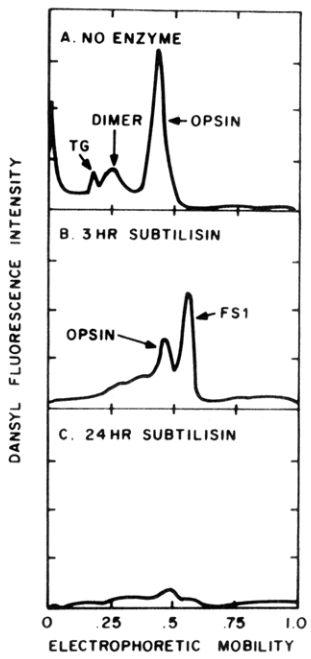

FIGURE 6: Fluorescence scans showing the localization of dansylcadaverine following cleavage of labeled rhodopsin by subtilisin. Dansylcadaverine-labeled membranes were incubated with subtilisin (as in Figure 6) for (A) 0, (B) 3, and (C) 24 h and electrophoresed. The dansyl fluorescence shifts from opsin and its oligomers to FS1 after 3 h. Almost no dansyl fluorescence remains after 24 h. A comparison with the Coomassie blue staining pattern (Figure 6F) shows that dansylcadaverine was excised from FS1 with little change in the electrophoretic mobility of this fragment.

thermolysin-cleaved rhodopsin incorporated less than 20% of the amount of dansylcadaverine incorporated uncleaved rhodopsin. Thus, prior enzymatic *proteolysis inhibits transglutaminase labeling,* and, conversely, *prior transglutaminase-catalyzed labeling inhibits enzymatic proteolysis.* The most likely interpretation of these results is that *the site of labeling is located in the protease-sensitive region between the F1 and F2 regions.*

Additional evidence for this proposed localization of the labeled site comes from an analysis of the proteolysis of dansylcadaverine–rhodopsin by subtilisin (Figures 5 and 6). During the initial stage of proteolysis, dansyl fluorescence is associated with FS1, the set of large fragments produced by cleavage with subtilisin (Figure 6B). At longer times, the dansyl label was excised from FS1 (Figure 6C) without any appreciable decrease in the amount of FS1 or its electrophoretic mobility, as seen by Coomassie blue staining (Figure 5E,F), Similar results were obtained when dansylcadaverine–rhodopsin was cleaved by papain.

The large fragment (FS1) produced by subtilisin cleavage of rhodopsin was isolated by Con A–agarose affinity chromatography. Disk membranes, labeled with dansylcadaverine, were subjected to proteolysis with subtilisin for varying times. The membranes were then dissolved in Triton X-100 solution and applied to a Con A–agarose column in the dark. The column was illuminated to photodissociate the cleaved rhodopsin, thereby eluting FS2. The FS1 fragment, which remained bound, was subsequently eluted by adding 0.1 M α-methyl mannoside, which competes for binding to Con A. Most of the FS1 purified in this way from labeled membranes that were incubated with subtilisin for 3 h contained dansylcadaverine. In contrast, FS1 isolated from a sample subjected to 24 h of proteolysis by subtilisin was devoid of dansyl fluorescence. *Thus, subtilisin excises the dansylcadaverine label from cleaved rhodopsin without removing the specific binding site for Con A.*

The Dansylcadaverine Label Is Far from 11-cis-Retinal. Fluorescence energy-transfer spectroscopy was used to estimate the distance between the attached dansylcadaverine and the 11-*cis*-retinal group of rhodopsin in disk membranes. This experiment was feasible because no component of the disk membrane other than rhodopsin was covalently labeled by dansylcadaverine to an appreciable extent. The noncovalently bound dansylcadaverine was removed by washing the membranes with a 1% solution of bovine serum albumin. The amount of residual noncovalently bound dansylcadaverine was less than 5% of the amount covalently linked to rhodopsin.

The fluorescence emission spectrum of the dansyl group, peaked at 510 nm, effectively overlaps the 500-nm absorption band of the 11-*cis*-retinal moiety of rhodopsin. The R_0 distance for 50% efficient Förster transfer is given by (Förster, 1966; Stryer and Haugland, 1967):

$$R_0 = (Q_0 J n^{-4} K^2)^{1/6} \times 9.7 \times 10^3 \text{ Å} \quad (1)$$

where Q_0 is the quantum yield of the donor in the absence of acceptor, J is the spectral overlap integral, n is the refractive index of the intervening medium, and K^2 is the dipole–dipole orientation factor. For energy transfer from dansylcadaverine to 11-*cis*-retinal in labeled rhodopsin in disk membranes, $Q_0 = 0.85 \pm 0.1$, $J = 1.8 \times 10^{-13}$ cm^3 M^{-1}, and n is taken to be 1.4. Assuming $K^2 = 2/3$, $R_0 = 53$ Å.

The efficiency of energy transfer was determined from steady-state and nanosecond kinetic measurements of the dansyl fluorescence of labeled rhodopsin before and after bleaching. The transfer efficiency E calculated from

$$E = 1 - (F_d/F_b) \quad (2)$$

where F_d and F_b are the dansyl fluorescence intensities before and after bleaching, respectively, was 39%. The transfer efficiency is also given by

$$E = 1 - (\tau_d/\tau_b) \quad (3)$$

where τ_d and τ_b are the dansyl excited-state lifetimes before and after bleaching, respectively. The nanosecond emission kinetics of the dansyl group are shown in Figure 7. The fluorescence kinetics of the unbleached labeled membranes contain more than one component and have an average lifetime of 13.7

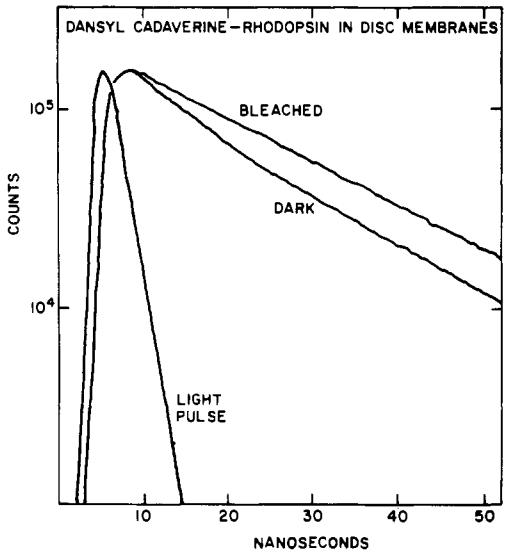

FIGURE 7: Nanosecond fluorescence kinetics of dansylcadaverine-rhodopsin in disk membranes before and after bleaching.

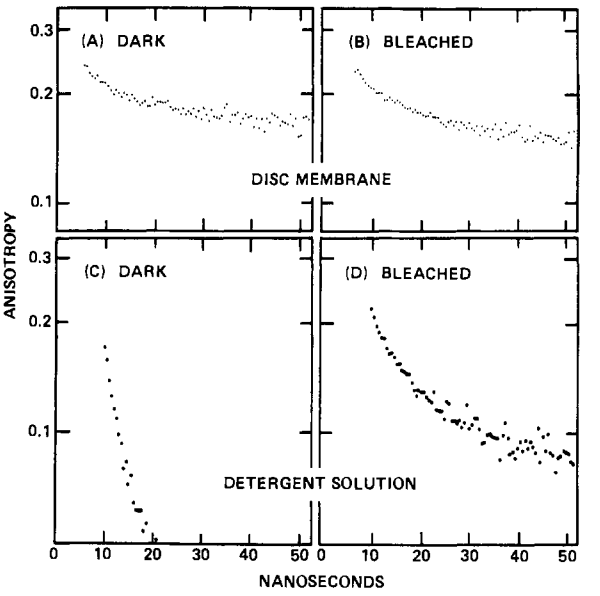

FIGURE 8: Nanosecond fluorescence emission anisotropy kinetics of dansylcadaverine-rhodopsin in disk membranes (A) before and (B) after bleaching and in 1% Ammonyx LO detergent solution (C) before and (D) after bleaching. The time of the light pulse was the same as in Figure 7. The average excited-state lifetimes of the dansyl group in detergent solution before and after bleaching were 10.1 and 16.4 ns, respectively.

ns. On bleaching, the emission kinetics approximate a single-exponential decay with a lifetime of 18.5 ns. The simplest interpretation of the fluorescence kinetics of the unbleached sample is that it consists of two components, one from dansyl-labeled rhodopsin with a lifetime of 12.8 ns and a relative amplitude of 0.8, and the other from dansyl-labeled opsin with a lifetime of 18.5 ns and a relative amplitude of 0.2. This explanation is plausible because the proportion of opsin in disk membranes isolated from commercially-obtained frozen retinas is typically 15 to 20%. The transfer efficiency calculated from eq 3 using τ_b = 18.5 ns and τ_d = 12.8 ns is 31%, which agrees quite well with the value of 39% determined from the change in fluorescence intensity on bleaching. The transfer efficiencies calculated in this way assume that bleaching erases the 11-*cis*-retinal energy acceptor but does not alter Q_0, the quantum yield of the dansyl donor in the absence of transfer. This assumption is supported by the finding that the dansyl emission spectra and emission anisotropy kinetics (Figure 8A,B) are virtually unaffected by bleaching. The distance between the donor and acceptor is then given by

$$r = R_0(E^{-1} - 1)^{1/6} \quad (4)$$

For an R_0 of 53 Å based on $K^2 = 2/3$, the apparent distance r is 61 Å for E = 31% and 57 Å for E = 39%. The dansyl emission anisotropy kinetics indicate that r', the apparent distance, calculated in this way is a good approximation to r, the actual distance. As discussed below, the dansyl group rotates very rapidly within a cone of half-angle 32°. This rotational mobility of the energy acceptor markedly diminishes the uncertainty concerning the choice of K^2. For convenience, we define α as the ratio of the actual distance to the apparent distance:

$$\alpha = r/r' \quad (5)$$

The effect of rapid rotational motion on α has been calculated. For rapid rotation within a cone of half-angle 32°, α can range from 0.69 to 1.3 (Dale and Eisinger, 1974). Furthermore, there is a 90% probability that α lies between 0.71 and 1.18 (Stryer, 1978). This analysis shows that for r' = 61 Å there is a 90% probability that the actual distance r between the dansyl group and 11-cis-retinal is between 43 and 72 Å. This range will be narrowed if the emission of the donor or the absorption of the acceptor is not purely along one axis (Stryer, 1978). We conclude that the site labeled by transglutaminase is far from 11-*cis*-retinal.

The Rotational Mobility of the Labeled Site Changes When Dansylcadaverine-Rhodopsin Is Extracted into Detergent Solution. The nanosecond emission kinetics of the dansyl group of labeled rhodopsin were measured to provide information about the orientation factor in energy transfer and to monitor the effects of bleaching and of extraction into detergent solution on the conformation of the protein. The decay of the emission anisotropy of dansylcadaverine-rhodopsin in disk membranes (Figure 8A) exhibits at least two components. The emission anisotropy decreases from 0.36, the value expected for the dansyl group in the absence of rotational motion (as measured for dansylcadaverine in glycerol), to 0.21, the initial observed value, in a time short compared to the duration of the light pulse. This rapid decrease in the emission anisotropy can be interpreted in terms of a very rapid rotational motion within the volume of a cone of half-angle θ_c, given by the expression (Kawato et al., 1977)

$$r_1/r_0 = \cos^2\theta_c(1 + \cos\theta_c)^2/4 \quad (6)$$

where r_1 is the initial observed emission anisotropy and r_0 is the value in the absence of any rotational motion. For r_1 = 0.21 and r_0 = 0.36, the cone half-angle θ_c is 32°. The rotational correlation time ϕ_1 of this motion is less than 1 ns. This very fast, restricted motion is followed by a slow rotation of the dansyl group with a ϕ_2 of about 140 ns. There is almost no change in the emission anisotropy curve when the sample is bleached (Figure 8B), suggesting that the conformation of the protease-sensitive region is not appreciably altered by the isomerization of retinal. The constancy of the dansyl emission maximum on bleaching labeled rhodopsin in disk membranes is consistent with this finding.

A quite different picture emerges from our emission anisotropy studies of labeled rhodopsin in 1% Ammonyx LO detergent solution. First, the slow (ϕ_2 = 140 ns) rotation of the dansyl group in the disk membrane is absent. Instead, there is a much faster rotation, with a ϕ_2 of about 5 ns (Figure 8C).

Thus, the dansyl label is much more mobile in detergent solution than in the intact disk membrane. Second, the emission anisotropy characteristics of labeled rhodopsin change markedly on bleaching in detergent solution (Figure 8D) but not in the disk membrane. The rotational freedom of the dansyl group becomes markedly hindered on bleaching in detergent solution. Complementary information about these conformational differences is provided by the dansyl emission spectra. The emission maximum in detergent solution shifts from 490 to 475 nm on bleaching, whereas no shift was observed in the disk membrane. The efficiency of energy transfer in detergent solution could not be determined because the environment of the dansyl energy donor changes on bleaching.

Discussion

This study shows that rhodopsin in disk membranes can be specifically labeled with an amine probe such as dansylcadaverine by using transglutaminase from guinea pig liver to catalyze a transamidation reaction. The stoichiometry of labeling at saturation is one amine per rhodopsin. The labeled site is located in the protease-sensitive region of rhodopsin between its F1 and F2 units (Pober and Stryer, 1975) as shown by three lines of evidence. First, prior proteolysis inhibits transglutaminase-catalyzed labeling. Second, prior transglutaminase-catalyzed labeling inhibits proteolysis. Third, the dansyl fluorescence is associated with the FS1 fragment produced during the initial stage of proteolysis by subtilisin. Subsequently, subtilisin excises the dansyl label from FS1 without removing its carbohydrate units or substantially altering its molecular weight. Since the carbohydrate units are located near the amino terminus of rhodopsin, specifically at positions 2 and 15 (Hargrave, 1977), it is evident that dansylcadaverine is linked near the carboxy terminus of FS1. Hence, the labeled site is near the protease-sensitive region connecting FS1 and FS2. The F2 fragment contains three markers: the site A reactive sulfhydryl group, the *N*-retinyl group formed by borohydride reduction (Pober and Stryer, 1975), and phosphate transferred from ATP to opsin in the presence of a kinase (Saari, personal communication; Hargrave and Fong, 1977). The sequence of these labels on F2 has not yet been defined.

The fluorescence energy transfer experiments demonstrate that dansylcadaverine in labeled rhodopsin in disk membranes is far from 11-*cis*-retinal, the apparent distance being 61 Å. The reactive site A sulfhydryl is about 75 Å from 11-*cis*-retinal (Wu and Stryer, 1972; Steinemann et al., 1973). These proximity relationships indicate that rhodopsin in the disk membrane must have an elongated shape because the diameter of a 40 000 dalton sphere would be 45 Å. The finding that both site A and the transglutaminase-labeled site are far from 11-*cis*-retinal suggests that they might be near each other. Preliminary energy-transfer experiments indicate that this is, in fact, the case.

The emission anisotropy kinetics of the dansyl fluorescence demonstrate that this probe becomes much more mobile when labeled rhodopsin is extracted from disk membranes into 1% Ammonyx LO detergent solution (Figure 8A,C). The longer rotational correlation time ϕ_2 decreases from about 140 to 5 ns. The detergent could produce this change in ϕ_2 by unfolding the region between the F1 and F2 units or by bringing the dansyl group out of a niche of the rhodopsin molecule. Ammonyx LO has virtually no effect on the 500-nm absorption band (Ebrey, 1971) or resonance Raman spectrum (Mathies et al., 1976) of rhodopsin, but it does alter the strength of the induced circular dichroism of the 11-*cis*-retinal chromophore (Waddell et al., 1976). Our emission anisotropy data suggest that Ammonyx LO alters the conformation of a portion of rhodopsin far from retinal. They caution that this detergent should not be assumed to be a nonperturbing solubilizing agent for membrane proteins even though a particular region of the solubilized protein is unaffected by the detergent. The large change in the emission anisotropy kinetics on bleaching in detergent solution (Figure 8) is consistent with previous studies showing that opsin in 1% Ammonyx LO detergent solution has a quite different conformation than in the disk membrane, as evidenced by the loss of regenerability, changes in the polypeptide circular dichroism spectrum, and by its marked tendency to aggregate (Stubbs et al., 1976; Yeager, 1976). In contrast, the labeled site does not appear to sense the isomerization of retinal when rhodopsin is located in the disk membrane. The shape of the emission spectrum and the emission anisotropy kinetics (Figure 8) exhibit no appreciable change on bleaching, nor is the rate of transglutaminase-catalyzed labeling affected (Figure 2).

We plan to use transglutaminase to study other aspects of the structure of rhodopsin. The attractive features of the transglutaminase-catalyzed labeling reaction are its high degree of specificity, its completeness in intact disk membranes, and its versatility in terms of the range of labels that can be introduced. This combination of important characteristics, which was not previously attained in the labeling of rhodopsin, affords opportunities to carry out some interesting experiments. For example, it will be informative to ascertain some additional proximity relationships by energy-transfer spectroscopy. The elucidation of the distance between the transglutaminase-labeled site and the carbohydrate moieties of rhodopsin, which can be enzymatically labeled using galactosyl transferase and galactose oxidase (Shaper and Stryer, 1978), is one such possibility. Transglutaminase can also be used to insert haptens into macromolecules for ferritin–antibody localization by electron microscopy (Dutton et al., 1976). In this way, it should be feasible to ascertain whether the site labeled by transglutaminase is on the same or opposite side of the membrane as the carbohydrate units of rhodopsin.

References

Applebury, M. L., Zuckerman, D.M., Lamola, A. A., and Jovin, T. M. (1974), *Biochemistry 13,* 3448.

Bownds, D. (1967), Ph.D. Thesis, Harvard University.

Brewer, G., and Singer, S. J. (1974), *Biochemistry 13,* 3580.

Clarke, D. D., Mycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79,* 338.

Connellan, J. M., Chung, S. I., Whetzel, N. K., Bradley, L. M., and Folk, J. E. (1971), *J. Biol. Chem. 246,* 1093.

Dale, R. E., and Eisinger, J. (1974), *Biopolymers 13,* 1573.

Dutton, A., Rees, E. D., and Singer, S. J. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1532.

Dutton A., and Singer, S. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2568.

Ebrey, T. (1971), *Vision Res. 11,* 1007.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10,* 2606.

Folk, J. E., and Chung, S. I. (1973), *Adv. Enzymol. 38,* 109.

Förster, T. (1966) *Mod. Quantum Chem. Lect. Istanbul Int. Summer Sch. 3,* 93.

Harf e, P. A. (1977), *Biochim. Biophys. Acta 492,* 83.

Hargrave, P. A., and Fong, S.-L. (1977), *J. Supramol. Struct.,* in press.

Heller, J., and Lawrence, M. A. (1970), *Biochemistry 9,* 864 (1970).

Iwanij, V. (1977), *Eur. J. Biochem. 80,* 359.
Kawato, S., Kinosita, K., and Ikegami, A. (1977), *Biochemistry 16,* 2319.
Laemmli, N. K. (1970), *Nature (London) 227,* 680.
McConnell, D. G. (1965), *J. Cell Biol. 27,* 459.
Mathies, R., Oseroff, A. R., and Stryer, L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1.
Papermaster, D. S., and Dreyer, W. J. (1974), *Biochemistry 13,* 2438.
Pober, J. S. (1976), Ph.D. Thesis, Yale University.
Pober, J. S., and Stryer, L. (1975), *J. Mol. Biol. 95,* 477.
Pontus, M., and Delmelle, M. (1975), *Exp. Eye Res. 20,* 599.
Shape, J., and Stryer, L. (1977), *J. Supramol. Struct. 6,* 291.
Shichi, H. (1971), *Photochem. Photobiol. 13,* 499.
Steinemann, A., and Stryer, L. (1973), *J. Supramol. Struct. 1,* 348.
Stryer, L. (1965), *J. Mol. Biol. 13,* 482.
Stryer, L. (1978), *Annu. Rev. Biochem. 47,* 819.
Stryer, L., and Haugland, R. P. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 719.
Stubbs, G. W., Smith, H. G., and Litman, B. J. (1976), *Biochim. Biophys. Acta 425,* 46.
Waddell, W. H., Yudd, A. P., and Nakanishi, K. (1976), *J. Am. Chem. Soc. 98,* 238.
Waggoner, A. S., and Stryer, L. (1971), *Biochemistry 10,* 3250.
Ward, S., Wilson, D. I., and Gilliam, J. J. (1970), *Anal. Biochem. 38,* 90.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Wu, C.-W., and Stryer, L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1104.
Yeager, M. (1976), *Brookhaven Symp. Biol. 27,* 3.
Yguerabide, J. (1972), *Methods Enzymol. 26,* 498.
Yguerabide, J., Epstein, H. F., and Stryer, L. (1970), *J. Mol. Biol. 51,* 573.

Sugar Binding Properties of Various Metal Ion Induced Conformations in Concanavalin A†

A. D. Sherry,* A. E. Buck, and C. A. Peterson

ABSTRACT: Concanavalin A is known to undergo a first-order conformational transition when metals are added to the demetallized protein at pH 5.6 (Brown, R. D., III, et al. (1977) *Biochemistry 16,* 3883–3896). The rate constants for this process, which we have measured using a polarographic technique, are identical when zinc, cobalt, or manganese occupies S1 and calcium occupies S2. The reducible sugar, *p*-nitrophenyl α-D-mannopyranoside, binds only to the locked conformational structure which is formed upon the addition of metals. The affinity of the protein for sugars is dependent upon occupancy of S1 and S2 and quite sensitive to the identity of the metal in S2. The metals may be removed from the locked protein structure and the protein temporarily retains its ability to bind with sugars but with a considerably lower affinity. The locked form of concanavalin A is unstable at a pH near 2 and unfolds to the unlocked structure with a half-life of 25 min resulting in simultaneous loss of metal and sugar binding.

The sugar binding and cell agglutination properties of concanavalin A[1] (Con A) are thought to be mediated by specific metal ions. The native crystalline protein contains one divalent manganese and one divalent calcium ion per protein monomer located near the established sugar binding region (Brewer et al., 1973; Hardman & Ainsworth, 1973; Villafranca & Viola, 1974). The function of these metal ions appears to relate to the stabilization of a polypeptide loop on the surface of the molecule to form the specific sugar recognition site. Thus, these metals must be added to apo-Con A before the protein will bind with simple monosaccharides and dextrans or agglutinate whole cells. Recently, however, other investigators have questioned the calcium requirement in maintaining the structural integrity of the active protein. Brewer et al. (1974) have presented evidence that calcium may be dialyzed away from the intact manganese-calcium-Con A ternary complex leaving a calcium deficient protein which retains all properties of the native system while Grimaldi & Sykes (1975) have suggested that calcium plays a catalytic role in the conversion of the apo-protein conformation to an active sugar binding conformational state. Results from various experiments in our laboratory did not agree with these earlier reports and have led us to search for a convenient method to examine more definitively the function played by the metals in establishing the sugar recognition site in Con A. Brown et al. (1977) have recently presented a comprehensive examination of the different conformational states of Con A by following the time and frequency dependence of the proton relaxation rates of the divalent manganese derivatives. They show Mn^{2+} and Ca^{2+} form a metastable ternary complex with apo-Con A which subsequently undergoes a first-order transition to a more stable ternary complex. The latter ternary complex binds calcium so tenaciously that it takes days to dialyze away the bound Ca^{2+}.

† Contribution from The University of Texas at Dallas, P.O. Box 688, Richardson, Texas 75080. *Received December 7, 1977.* Financial support from The National Institute of Arthritis, Metabolism and Digestive Diseases, Department of Health, Education and Welfare, Grant No. 1 RO1 AM16947, and The Robert A. Welch Foundation, Grant No. AT-584, is gratefully acknowledged.

[1] Abbreviations used: Con A, concanavalin A; EDTA, ethylenediaminetetraacetate; PNPM, *p*-nitrophenyl α-D-mannopyranoside; DME, dropping mercury electrode; ESR, electron spin resonance; P and PL refer to the "unlocked" and "locked" conformations of Con A, respectively (see Brown et al., 1977); C, divalent calcium; M, divalent manganese (or any other divalent first-row transition metal ion where noted).

0006-2960/78/0417-2169$01.00/0