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LIGHT-ENHANCED CROSS-LINKING OF RHODOPSIN IN ROD OUTER SEGMENT MEMBRANES AS DETECTED BY CHEMICAL PROBES

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Summary

Bovine rod outer segment membranes were treated with cross-linking reagents before and after light exposure. Bleached membranes showed enhanced cross-linking with difluorodinitrobenzene or methyl acetimidate compared to dark-adapted membranes. The light-induced enhancement of cross-linking may be due to increased association of rhodopsin monomers in the light and/or due to increased reactivity of amino and sulfhydryl groups of bleached rhodopsin. In some instances, the band ascribed to the rhodopsin monomer in gel electrophoresis appears as a partially resolved doublet. Treatment of bleached rod outer segment membranes with methyl acetimidate improved the resolution of the doublet into two closely migrating bands.

Introduction

Rhodopsin, the major membrane protein of the rod outer segment membrane [1], undergoes a series of light-initiated changes which are coupled to the cell's transduction of light energy into electrical signals. The structure and function of rhodopsin have been studied extensively in recent years. It is now considered that rhodopsin spans the membrane, based on evidence from energy-transfer measurements [2], freeze-fracture electron microscopy [3], light-induced permeability increases [4—7] and chemical labelling experiments [8]. The transmembrane rhodopsin molecule undergoes rapid rotational [9] and lateral movement in the plane of the membrane [10,11]. Equatorial X-ray diffraction measurements on rod outer segment membranes [12—14]

show a broad diffraction peak which is consistent with a random distribution of cylindrical rhodopsin monomers. Although the available evidence indicates that rhodopsin in the rod outer segment is monomeric on a time average, the possibility has been suggested by Montal et al. [5] that rhodopsin monomers interact upon light exposure to form transiently a multimeric channel. At least two laboratories have examined rod outer segment membranes for evidence of light-induced association of rhodopsin. Downer and Cone [15] reported no evidence of rhodopsin aggregation in rotational diffusion measurements on bullfrog retinae. Brett and Findlay [16] studied the organization of rhodopsin in sheep rod outer segment disk membranes with several cross-linking agents, and they observed rhodopsin cross-linking patterns were similar in both dark-adapted and bleached membranes. They concluded that rhodopsin was monomeric in the dark and in the light.

In this paper, we have used cross-linking agents to detect light-induced changes in bovine rod outer segment membranes. Under appropriate conditions, we find a marked difference in the cross-linking patterns of rhodopsin in dark-adapted and bleached rod outer segment membranes. High molecular weight cross-linked species of rhodopsin are enhanced upon illumination of the membranes. Moreover, in some instances, gel electrophoresis of rod outer segment membranes after reaction with chemical probes shows heterogeneity in the band normally ascribed to the rhodopsin monomer. Heterogeneity in the band associated with rhodopsin has been reported by Siebert et al. [17] and by Uhl et al. [18].

Methods and Reagents

Bovine rod outer segment and rod outer segment disk membranes were prepared from frozen bovine retinae by using the method of Hong and Hubbell [19] and that of Smith et al. [20], respectively. The rod outer segment membranes were washed twice with double-distilled H_2O to remove sucrose, then suspended in buffer. The spectral ratio, A_{280}/A_{500}, was 2.4—2.8 for these preparations. The rod outer segment disk membranes were water-washed twice to remove Ficoll, then suspended in buffer. The spectral ratio of the disk preparations was 2.1—2.3. 2,4-Dinitro-1,5-difluorobenzene (DFDNB) and methylacetimidate, were obtained from Pierce Chemical Co. Acrylamide was obtained from Bio-Rad. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by using the method of Laemmli [21]. The rod outer segment membranes were dissolved in 1% SDS buffer at 21°C. Electrophoresis was carried out on a Bio-Rad slab gel Model 220 unit using a Bio-Rad Model 500 power supply. For tube gels, a Savant unit was used with the Bio-Rad power supply. The gels were fixed for 1—2 h in 12.5% trichloroacetic acid and stained for 2 h with 0.1% Coomassie brilliant blue R250 (Index Co., Greenwood, IL) in 25% isopropanol/1% acetic acid in water. Destaining was performed in a Bio-Rad diffusion destainer using methanol/acetic acid/water (5 : 1 : 5, v/v). Gels were scanned at 550—600 nm on a Gilford spectrophotometer equipped with a gel scanner and recorder. Pyronin Y dye was used as a marker.

Results

The time course of cross-linking of rhodopsin in rod outer segment membranes with 100 μM DFDNB at 37°C, as followed using SDS-acrylamide gel electrophoresis, is shown in Fig. 1. The control gels are shown in Fig. 1A and the membranes treated with 100 μM DFDNB at 37°C are shown in Fig. 1B. The DFDNB/rhodopsin molar ratio was 36 : 1 based on a molecular weight of 38 000 for rhodopsin. Bleached rod outer segment membranes show a more rapid cross-linking of rhodopsin into dimers (band 6), trimers (band 4) and high molecular weight aggregates (band 1) than do unbleached rod outer segment membanes. The cross-linking of rhodopsin is detected at the earliest time (a few seconds) it takes to stop the reaction by addition of excess glycine. After 30 min, most of the rhodopsin is cross-linked to high molecular weight aggregates (band 1) that do not enter the 8% gel. Although not shown, some

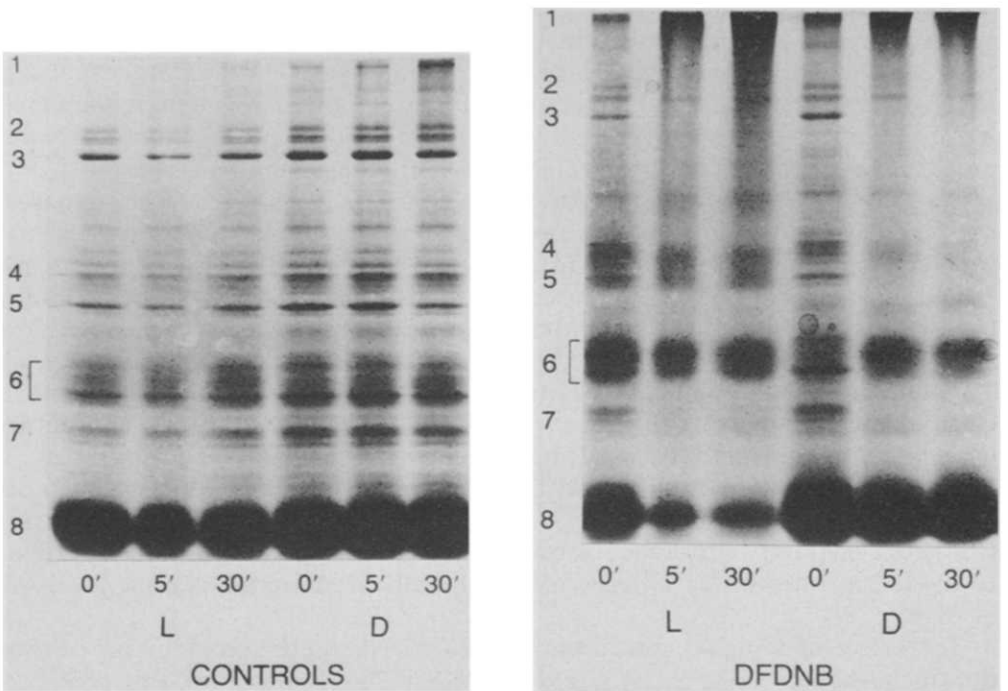

Fig. 1. SDS-polyacrylamide gel profiles of control and DFDNB-treated rod outer segment membranes. Rod outer segment membranes were treated as follows. (A) Control gels: 12-μl aliquots containing 19 μg of protein, were kept in the dark (gels D) or were bleached at 21°C for 30 min (gels L). The samples were then diluted with 168 μl of buffer A (containing 50 mM NaCl, 150 mM $NaHCO_3$, 3 mM KCl and 1 mM EDTA), pH 8.3. (B) DFDNB-treated rod outer segment membranes: Other 12-μl aliquots of rod outer segment membranes were treated as above, diluted to 168 μl with buffer A and DFDNB was added to give a final concentration of 100 μM DFDNB. The molar ratio of probe to rhodopsin was 36 : 1. Unbleached rod outer segment membranes were reacted in the dark (labeled D) and the bleached rod outer segment membranes were reacted in the light (labeled L) at 37°C for 0, 5 or 30 min. The reactions were stopped by addition of 0.5 ml of 2 M glycine, allowed to stand for 5 min at 21°C and centrifuged at 25 000 × *g* for 30 min. The pellets were dissolved in 20 μl of buffer B (40 mM Tris-acetate buffer, pH 7.4, containing 1% SDS, 40 mM dithiothreitol, 4 mM EDTA and 5% glycerol). The samples containing 50 μg protein per tube were run on slab gels according to the method of the Laemmli [21] using a 3% stacking gel and 8% running gel.

aggregates are so large that they do not enter the 3% stacking gel. The stacking gels are fragile and often break off during the handling. The control gels not treated with DFDNB show a small amount of bands moving in the area of dimers (band 6) and trimers (band 4) of rhodopsin, but very little high molecular weight aggregates are observed. The somewhat lower intensity of the bands in the light samples is believed to be due to decreased loading, which occurs because exposure to light enhances aggregation of the membranes so that it is difficult to remove quantitatively aliquots for gel electrophoresis.

The cross-linking reaction with DFDNB is rapid at 37°C. In some experiments, which were performed as described in the legend to Fig. 1, the reaction was quenched at 0.5, 1 or 2 min. Even at these reaction times, the major products were high molecular weight aggregates of rhodopsin. Both dimers and trimers were also produced with the dimer in greater yield.

The formation of high molecular weight aggregates can be reduced by incubating the rod outer segment membranes and DFDNB at 0 rather than 37°C. These conditions facilitate the formation of dimers and trimers of rhodopsin. When the cross-linking incubation is carried out at 0°C, the product distribution depends on the temperature of the rod outer segment membranes during the light exposure of the membranes. If the rod outer segment membranes are bleached at 21°C then cooled to 0°C for DFDNB incubation, more dimers and trimers of rhodopsin are formed than if the bleaching temperature is 0°C prior to the 0°C incubation with DFDNB. In each case, the light-exposed samples yield more cross-linking products than the corresponding dark membranes. Conditions favorable for formation of dimers and trimers of rhodopsin include a low incubation temperature for the DFDNB reaction, short reaction times, and bleaching of the rod outer segment membranes at room temperature rather than at 0°C.

The light enhancement of cross-linking of rhodopsin with DFDNB, which is clearly demonstrated in Fig. 1 and in several similar experiments, appears to depend on the concentrations of the reagents and the reactant ratios. Most of our data were obtained with a DFDNB concentration of 100 μM and a ratio of DFDNB to rhodopsin of 30—50 : 1. At both higher and lower concentrations, the light-dark difference is less pronounced. At 10 μM DFDNB (probe-to-rhodopsin ratio of 3 : 1), very little light-enhanced cross-linking is observed at 37°C.

The effect of a higher concentration of DFDNB on the cross-linking of rhodopsin is shown in Fig. 2. At 5 mM DFDNB (121 : 1 molar ratio of probe to rhodopsin) nearly all the rhodopsin and other membrane proteins become cross-linked into large aggregates which enter neither the 8% gel nor the 3% stacking gel. A difference in the light-treated as compared to the dark-adapted sample is still apparent, but is less clear than in Fig. 1 because of the high conversion of rhodopsin to polymer products. Also visible on the gels are stained cross-linked lipids which move at the front of the gel [22]. Incubation in the light or dark after rod outer segment bleaching does not effect the cross-linking reaction.

Crain and Marinetti [23] have shown previously that methyl acetimidate, a monofunctional probe, can cross-link membrane proteins and membrane phospholipids. The effect of this water-soluble reagent on the cross-linking

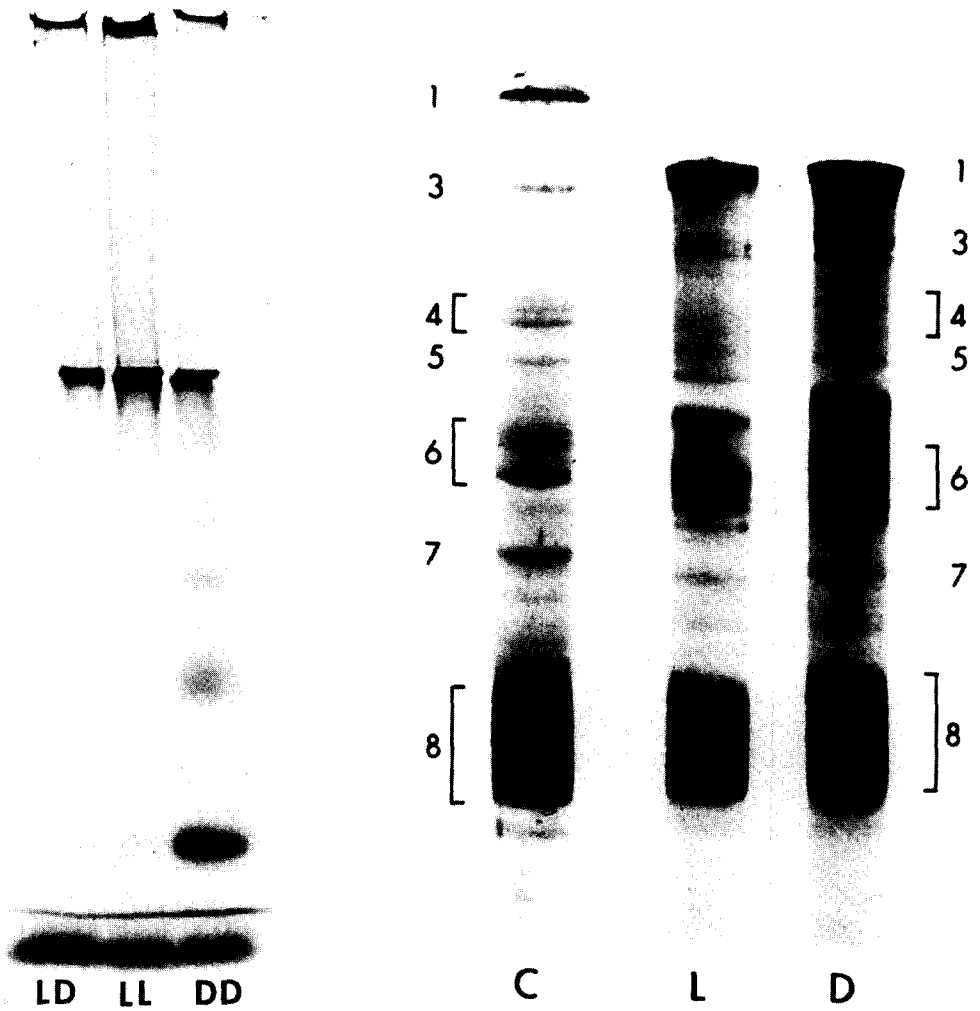

Fig. 2. SDS-polyacrylamide gel profiles of rod outer segment membranes treated with high concentration of DFDNB. Rod outer segment membranes (110 μg rhodopsin) were reacted with 5 mM DFDNB in 70 μl of buffer A (See Fig. 1) at 37°C for 30 min, then stopped as in Fig. 1. This represents a DFDNB-to-rhodopsin molar ratio of 121 : 1. Aliquots containing 30 μg of rhodopsin were dissolved in buffer B and subjected to electrophoresis on tube gels as described in the text [21], using a 3% stacking gel and 8% running gel. LL signifies that the samples were first incubated in the light for 30 min prior to reacting with DFDNB in the light. LD denotes that the samples were first incubated for 30 min in the light and then reacted with DFDNB in the dark. DD indicates that the samples were first incubated for 30 min in the dark and reacted for 30 min in the dark.

Fig. 3. SDS-polyacrylamide gel profiles of control and methyl acetimidate-treated rod outer segment membranes. Rod outer segment membranes (500 μg of protein) in 0.5 ml of buffer A (see Fig. 1) were incubated at 20°C in the dark or in the light. To control samples were added 4.5 ml of buffer A containing methyl acetimidate. The final concentration of methyl acetimidate was 50 mM. The molar ratio of probe to rhodopsin was 19 : 1. The membranes were incubated at 37°C for 30 min and 1 ml aliquots (100 μg protein) were removed and treated for 2 min with 0.5 ml of 2 M glycine to stop the reaction. The samples were centrifuged at 14 000 rev./min in an Eppendorf microcentrifuge. The pellets were dissolved in 50 μl of buffer B and 20 μl (50 μg protein) were layered on tube gels (5 mm diameter) and subjected to electrophoresis according to the method of Laemmli [21] using a 9% polyacrylamide gel without the 3% stacking gel. D, rod outer segment disk membranes reacted in the dark. L, rod outer segment disk membranes reacted in the light. C, control rod outer segment disk membranes.

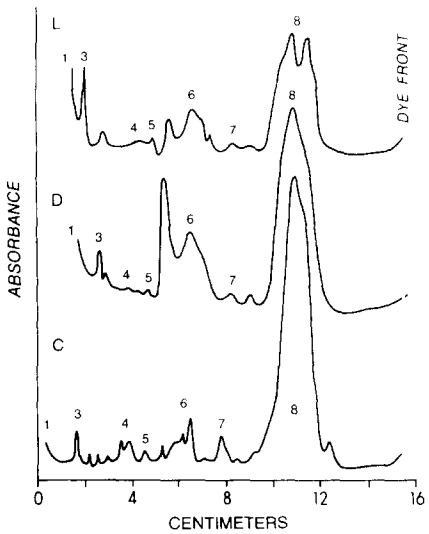

Fig. 4. Tracings of SDS-polyacrylamide gels of control and methyl acetimidate-treated rod outer segment disk membranes. The gels shown in Fig. 3 were scanned at 600 nm. L, rod outer segment disk membranes reacted in the light; D, rod outer segment disk membranes reacted in the dark; and C, control rod outer segment disk membranes.

of rhodopsin in isolated rod outer segment membranes is shown in Figs. 3 and 4. The reaction using 50 mM methyl acetimidate is shown at one time interval of 30 min. This represents a molar ratio of methyl acetimidate to rhodopsin of 19 : 1. More total cross-linking of rhodopsin occurs in bleached membranes (gel L). This is evident by the decrease in intensity of the rhodopsin monomer band 8 and an increase in the cross-linked aggregates (band 1) and in the aggregates which remain in the stacking gel. The amount of this high molecular weight aggregate is increased in the light. In the dark, bands which migrate like rhodopsin dimers and trimers are seen to accumulate (bands 4—6).

Another significant observation is the resolution of the rhodopsin monomer band 8 into a doublet or triplet band in the light-exposed sample. Tracings of the SDS gels in Fig. 3 are shown in Fig. 4. The splitting of the band normally ascribed to rhodopsin monomer band 8 into two components in gel L is clearly visible. The slower moving component has a shoulder which indicates the possible presence of a third component. Bands migrating like rhodopsin dimers and trimers are also seen in the gels of the reacted membranes (gels L and D, bands 4—6).

Discussion

Our studies have shown a light-enhanced cross-linking of rhodopsin by cross-linking agents in bovine rod outer segment membranes. This work is contrary to the results of Brett and Findlay [16] who used sheep rod outer segment membranes. We can only surmise why our results differ from those of Brett and Findlay. Factors such as the source of rod outer segment membranes,

probe concentration, time of reaction and buffer type may play a role. Brett and Findlay used slow dialysis against ammonium acetate to stop the reaction whereas we stopped the reaction rapidly with excess glycine. They used DFDNB at 5 mM and from their data we estimate a DFDNB-to-rhodopsin molar ratio of 191 : 1. We generally used DFDNB at 100 μM which represents a 30 : 50 molar ratio of probe to rhodopsin. At higher molar ratios, the light-enhanced cross-linking is less evident. They dissolved the rod outer segment disks in 10% SDS and ran gels in 5% acrylamide. We dissolved the membranes in 1% SDS and ran gels in 8—9% acrylamide. Thus, different experimental conditions between our studies and the work of Brett and Findlay exist which may explain the different results.

The rod outer segment membranes are highly organized with a rhodopsin-to-lipid ratio of about 70 : 1 [24]. The dynamic nature of the membranes is demonstrated by the rapid rotational [9] and lateral movement [10,11] of rhodopsin in the membrane. The addition of DFDNB or methyl acetimidate to the rod outer segment membranes results in rapid cross-linking of rhodopsin to itself and, possibly, to other proteins available in the preparations. As seen in Fig. 1, the rod outer segment membrane preparations contain several minor proteins in addition to the major band 8. It has been reported that highly washed and purified rod outer segment disk membranes contain two major protein components, rhodopsin (95 wt%) and a 240 000 dalton protein (1—3 wt%) [25,26]. Our preparations were not rigorously purified to remove traces of minor proteins, and it is possible that some of the peripheral and soluble proteins [27] are involved in the cross-linking. The major reaction decreases the amount of band 8, with the formation of products which have molecular weights characteristic of dimers, trimers and polymers of rhodopsin. The high yield of high molecular weight aggregates at 37°C is not surprising considering the concentration of rhodopsin in the membranes and the lateral mobility of the monomers. Presumably, the chemical probe reacts with an amino or sulfhydryl group on the rhodopsin and proceeds to react with a nearest neighbor rhodopsin in a similar fashion. This dimer now provides a site for more rapidly moving monomers to accumulate and react to produce polymers.

The reasons and possible physiological significance of the light-enhanced cross-linking remain to be determined. One possibility is that light exposure increases the probability of cross-linking by a natural light-induced association of rhodopsin monomers. Such an aggregation of exposed rhodopsin was suggested by Montal et al. [5]. They proposed that bleached rhodopsin transiently interacts to form a channel for ion transport. Since single proton events produce electrical events in single rods [28], and one bleached rhodopsin per rhodopsin-phospholipid vesicle yields an increase in permeability of the membrane vesicle [29], the light-induced association would have to be between a bleached rhodopsin and one or more unbleached rhodopsin monomers. Although our conditions are not physiological, the observed light enhancement of cross-linking reopens the possibility that light exposure leads to association of bleached rhodopsin with other rhodopsin molecules.

The light enhancement of cross-linking may also be due to increased reactivity of amino and sulfhydryl groups of rhodopsin. Rhodopsin molecules in

membranes have six sulfhydryl groups with different degrees of reactivity to SH reagents [30,31]. Two SH groups are available in the dark to react with 4,4′-dithiopyridine. The rate of reaction is increased on bleaching rhodopsin in the membrane, and the number of reactive SH groups is increased from two to four or five [30]. The bleaching produces conformational changes in rhodopsin which make some SH groups more available for reaction. It has also been reported that bleaching rhodopsin in the presence of methyl acetimidate yields amidination of rhodopsin lysine groups more completely than the same reaction in the dark [32]. Therefore, it is reasonable to expect increased reactivity of DFDNB with rhodopsin in the light. The present data do not allow a distinction between these two possible explanations of the light enhancement of cross-linking. It is also possible that opsin and rhodopsin react differently with DFDNB and this in part may explain the light-enhanced aggregation.

The effect of temperature during bleaching on the extent of cross-linking is interesting. When the bleaching was performed at 0°C, followed by DFDNB reaction at 0°C, less cross-linking was observed than if the bleaching was carried out at 21°C, followed by DFDNB incubation at 0°C. Since the reaction pH is 8.3, it is likely that at 0°C, the predominant rhodopsin intermediate available for reaction is metarhodopsin I. Matthews et al. [33] demonstrated that bleached rhodopsin in detergent at 3°C exists in equilibrium between metarhodopsin I and metarhodopsin II. At pH 8, the equilibrium strongly favors metarhodopsin I, therefore at pH 8.3 and 0°C, we expect the chief bleached species to be metarhodopsin I. When the bleaching occurs at room temperature, metarhodopsin II and later intermediates are readily formed and are therefore available to react with DFDNB. Thus, the conditions which favor metarhodopsin II formation also favor cross-linking to form dimers, trimers and some high molecular weight aggregates.

Our present experiments also reveal heterogeneity in the band normally associated with rhodopsin monomers on gel electrophoresis. Heterogeneity of the rhodopsin band has been reported previously [17,18]. In our experiments, the heterogeneity manifests itself as a closely running doublet or triplet band which is occasionally seen in control samples, but which is accentuated in rod outer segment membranes treated with methyl acetimidate (Fig. 3). Assuming that rhodopsin is a homogeneous polypeptide chain containing carbohydrate residues, the splitting of the rhodopsin monomer band into two or three closely moving bands of slightly slower mobility may be due to a chemical modification of either or both of the polypeptide chain and sugar residues by the chemical probes. Methyl acetimidate or DFDNB also may cause intramolecular cross-linking of rhodopsin monomers at different points on the peptide backbone or sugar residues such that the packing density and interaction of rhodopsin with SDS is modified. Another possibility is that rhodopsin monomers represent a heterogeneous population of molecules with similar molecular weights, but which may differ slightly in their carbohydrate residues, and that reaction with chemical probes allows these differences to be expressed. This effect also may be due in part to cross-linking of neighboring aminophospholipid molecules such as phosphatidylserine or phosphatidylethanolamine to rhodopsin. Crain et al. [22] have published

previously the results of the cross-linking of these aminophospholipids to rhodopsin.

The hydrophobic neutral probe DFDNB is more effective in cross-linking rhodopsin than is the hydrophilic neutral probe methyl acetimidate. This we believe is due to DFDNB penetrating the hydrophobic membrane and reacting with more buried amino groups or SH groups of rhodopsin. Methyl acetimidate, although it can penetrate cell membranes [34], reacts with amino groups primarily on the parts of rhodopsin which are exposed to the aqueous environment and which may not be able to get as close for cross-linking as can the more buried parts of rhodopsin.

Acknowledgement

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