Rhodopsin Mobility, Structure, and Lipid–Protein Interaction in Squid Photoreceptor Membranes†

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ABSTRACT: Treatment of outer segment membranes from *Loligo forbesi* with endoprotease-V8 from *Staphylococcus aureus* results in cleavage of the C-terminal extension of the squid rhodopsin, with accompanying reduction of the apparent molecular weight from 47 000 to 36 000 on sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Negative-stain electron microscopy of the intact membranes shows that small clusters of the rhodopsin C-termini form structures extending from the membrane surface and that these are absent after protease treatment. Fourier transform infrared spectra of the amide I band of the protein indicate that removal of the C-terminal extension increases the relative α-helical content of squid rhodopsin to a level comparable to that for bovine rhodopsin in disk membranes, and to an extent which suggests that the α-helical structure lies mainly in the M_r 36 000 (transmembrane) section of the protein. Saturation-transfer electron spin resonance (ESR) spectroscopy of the spin-labeled protein reveals that the rotational diffusion of squid rhodopsin in outer segment membranes that have been extensively washed with urea to remove peripheral proteins is much slower than that of bovine rhodopsin in rod outer segment disk membranes. This reduction in rotational mobility is also found with purified squid rhodopsin reconstituted in egg phosphatidylcholine and in urea-washed outer segment membranes which have been treated with endoprotease-V8 to remove the C-terminal extension of squid rhodopsin. In the latter case, the saturation-transfer ESR spectra are virtually identical to those of the nonproteolyzed membranes. Conventional ESR spectra of spin-labeled phosphatidylcholine incorporated in squid outer segment membranes contain a motionally restricted component corresponding to lipids associated at the intramembranous surface of rhodopsin. The size of this restricted lipid population remains unchanged after removal of the C-terminal extension of squid rhodopsin by V8 protease treatment, consistent with a surface location of the C-terminus. It is concluded that squid rhodopsin is aggregated to a limited extent both in native membranes and when reconstituted in egg phosphatidylcholine but that the C-terminal extension is not directly responsible for the stability of the aggregates, although it may be involved in their formation.

The cephalopod photoreceptors differ from those of other species investigated to date in that the rhodopsins from both squid (Hall et al., 1991) and octopus (Ovchinnikov et al., 1988) contain an unusual proline-rich extension of the C-terminal domain. The proline-rich region of squid rhodopsin contains a 10-fold tandem repeat of the consensus sequence Pro-Pro-Gln-Gly-Tyr. So far, it has not been possible to assign any functional role to this C-terminal extension of cephalopod rhodopsin. However, the presence of similar proline-rich repeats in other proteins has been suggested to play a role in their interaction with components of the cytoskeleton or other peripheral proteins (Noegel et al., 1990; Südhof et al., 1987). It is found, for instance, that polyproline and polyglycine form crystalline aggregates composed of hexagonally packed open helices where the latter are stabilized by interchain hydrogen bonding (Cowan & McGavin, 1955; Crick & Rich, 1955). Such structures may form likely candidates for the mediation of protein–protein interactions.

Unlike the case of the mammalian rhodopsins, the rotational motion of cephalopod rhodopsins must be very restricted in order to explain their high degree of selectivity for plane-polarized light [for a review, see Saibil (1990)]. Therefore, it is possible that the role of the C-terminal extension of the cephalopod rhodopsins may be involved in organization of their membrane distribution, through self-association, and/or by interaction with the microvillar cytoskeleton (Saibil & Hewat, 1987).

In order to investigate whether the C-terminal region of squid rhodopsin mediates its aggregation, we have measured the rotational mobility of intact squid rhodopsin and of squid rhodopsin from which the proline-rich C-terminal extension was removed by proteolysis in outer segment membranes. It is found from saturation-transfer electron spin resonance (STESR)[1] spectroscopy of the spin-labeled protein that the rotational motion of squid rhodopsin in outer segment

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[1] Abbreviations: ESR, electron spin resonance; STESR, saturation-transfer ESR; V_1, first-harmonic ESR absorption signal detected in-phase with respect to field modulation; V_2', second-harmonic absorption ESR signal detected 90° out-of-phase with respect to field modulation; FTIR, Fourier transform infrared; 5-InVSL, 2-[(1-oxy-2,2,5,5-tetramethylpyrrolin-3-yl)methenyl]indane-1,3-dione; 14-PCSL, 1-acyl-2-[14-(4,4-dimethyl-*N*-oxyoxazolidin-2-yl)stearoyl]-*sn*-glycero-3-phosphocholine; egg PtdCho, phosphatidylcholine from egg yolk; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; ROS, rod outer segment.

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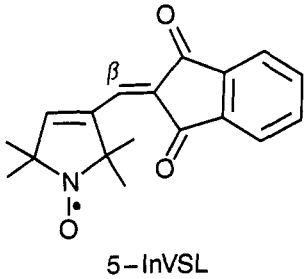
membranes is considerably slower than that of bovine rhodopsin in ROS disks. However, no change in spectral line shape was detected on cleavage of the repetitive proline-rich C-terminal domain. Consistent with this result, conventional ESR spectra of spin-labeled phosphatidylcholine showed that, on removal of the C-terminal domain, there was no change in the amount of lipid that was motionally restricted by interaction with the membrane-spanning domain of squid rhodopsin. Therefore, though in this work we have not examined in detail its interaction with the cytoskeleton, it appears that the unusual C-terminus of cephalopod rhodopsins is not the only determinant involved in the restriction of the motion of squid rhodopsin in photoreceptor membranes.

MATERIALS AND METHODS

Materials. Squid (*Loligo forbesi*) were obtained from the Marine Biological Association, Plymouth, U.K. Eyes from freshly killed animals were dissected, washed with saline buffer (600 mM NaCl, 5 mM EDTA, 1 mM EGTA, and 5 mM Hepes, pH 7.4), and rapidly frozen in isopentane cooled with liquid nitrogen. The animals were not dark-adapted, but processing was carried out in dim red light, and treatment with alkaline hydroxylamine indicated that more than 90% of rhodopsin was in the form unexposed to light with this type of processing. Bovine ROS disks in the unbleached form were prepared as described by Smith and Litman (1982). Endoprotease-V8 from *Staphylococcus aureus* was obtained from Boehringer-Mannheim (FRG). The indanedione vinyl nitroxide derivative 5-InVSL, used for covalent spin-labeling of



5-InVSL

rhodopsin, was synthesized according to Hankovszky et al. (1989) and was a gift from Prof. K. Hideg (University of Pécs, Pécs, Hungary). Spin-labeled phosphatidylcholine, 14-PCSL, was synthesized as described in Marsh and Watts (1982). All buffer solutions were saturated with argon (on ice) prior to use. The buffer used was 5 mM Hepes, pH 7.2, 1 mM EDTA, and 1 mM DTT (hypotonic buffer), which also contained 6 M urea for the urea wash or 150 mM NaCl for the measurements.

Cleavage and Spin-Labeling of Rhodopsin. All procedures were carried out at 4 °C in the dark or under dim red illumination (Osram 4563) unless otherwise stated. The isolation and purification of the photoreceptor outer segments from *Loligo forbesi* were carried out as described previously (Saibil & Hewat, 1987; Ryba et al., 1991). The squid outer segments were then washed extensively to remove peripheral components from the membranes containing rhodopsin. The following sequence of buffers was used for the washes: 2× hypotonic buffer; 3× 6 M urea; 3× hypotonic buffer; 2× 100 mM $NaHCO_3$. Enzymatic cleavage of rhodopsin in the membrane was accomplished by incubating membranes (50 μM rhodopsin) with endoprotease-V8 (100:1 w/w rhodopsin: endoprotease-V8 ratio) at 20 °C for 30 min. The reaction was terminated by 20-fold dilution with ice-cold hypotonic buffer, followed by centrifugation. Both cleaved and uncleaved membranes were then subjected to a further series of washes: 3× 6 M urea; 2× hypotonic buffer. For spin-labeling, membranes (100 μM rhodopsin) as treated above were incubated with a 5-fold molar excess of 5-InVSL for 30 min. Unreacted spin-label was then removed by a series of washes: 2× in hypotonic buffer containing 20 mM DTT; 5× in hypotonic buffer; and 3× in the measuring buffer. After ESR measurement, spin-labeled samples were extracted using 2% sucrose monolaurate (Novabiochem AG, Läufelfingen, Switzerland), and it was found that less than 2% of the ESR signal intensity was associated with the detergent-insoluble fraction, i.e., bound to non-rhodopsin proteins. The degree of incorporation of the spin-label into the protein was not determined, but from the overall intensity of the ESR spectra, it seems likely that it is in the region of one (or more) labels per protein.

The 5-InVSL and similar labels react, as does maleimide, with sulfhydryl groups by a Michael addition across the double bond (Esmann et al., 1990). Determinations with a model thiol compound (Hankovszky et al., 1989) indicate that the addition takes place at the β-position of the vinyl bond (cf. 5-InVSL structure). This implies a maximum restriction in mobility of the spin-label relative to the point of attachment to the protein, making such labels particularly suitable for determination of the overall rotational diffusion of the protein by saturation-transfer ESR spectroscopy (Esmann et al., 1989; Horváth et al., 1990). Both the conventional and saturation-transfer ESR spectra of the squid system indicate that there is no rapid segmental motion of the spin-label, in agreement with this expectation. At the pH used for labeling, it is likely that only cysteine residues on the protein are labeled, in common with studies on other proteins using this label (Esmann et al., 1989, 1990; Horváth et al., 1990; Esmann & Marsh, 1992). Comparison of the ESR spectra from the photoreceptor membrane with those from purified rhodopsin reconstituted in phosphatidylcholine confirms, as expected, that only the protein is labeled.

Lipid Spin-Labeling. For incorporation of spin-labeled lipid, 14-PCSL (ca. 1.5 mol % relative to the membrane lipid) was added in a small volume (<0.2% of total) of 5 mg/mL ethanolic solution to a suspension of urea-washed squid outer segment membranes in measurement buffer on ice. The combined dispersion was vortex-mixed immediately and then incubated on ice for 5 min. Unincorporated spin-label was resolved from the membranes by using five washes with the urea buffer, followed by two washes with measurement buffer.

Reconstitution of Rhodopsin in Phosphatidylcholine. Membranous rhodopsin, which was spin-labeled as above, was extracted with 2% octyl glucoside in measuring buffer. The rhodopsin (2 mL, 50 μM) was then purified by ion-exchange chromatography using DEAE-cellulose (DE-52; bed volume, 2 mL; flow rate, 1 mL min^{-1}). Under these conditions, rhodopsin was not retained by the ion-exchange resin and was collected in 2.5 column volumes. Further purification and concentration of rhodopsin were achieved by using affinity chromatography (concanavalin A–Sepharose; bed volume, 2 mL; flow rate, 1 mL min^{-1}). Squid rhodopsin was applied to the affinity column in 2% octyl glucoside in measuring buffer, washed with 5 column volumes of detergent solution, and then eluted with 100 mM methylmaltose in 2% octyl glucoside. The purified rhodopsin was then concentrated to 250 μM by using ultrafiltration.

Reconstitution of rhodopsin in lipid membranes was achieved by mixing an 80:1 molar ratio of egg PtdCho (20 mM, in 2% octyl glucoside) with purified spin-labeled rhodopsin. The detergent-solubilized mixture was then diluted

20-fold and dialyzed against measuring buffer (2.5 L; with six changes at ca. 12-h intervals). Reconstituted rhodopsin, recovered by centrifugation (50000*g* for 10min), was then applied to a linear 10–40% sucrose gradient in measuring buffer. The majority of the reconstituted protein was recovered as a single tight band which was harvested and washed 3 times to remove sucrose.

Protein and Lipid Assays. The phospholipid content was determined by phosphate analysis (Eibl & Lands, 1969); protein was determined by a modified Lowry assay (Markwell et al., 1981) using purified squid rhodopsin as standard. The rhodopsin content was determined from the absorbance at 494 nm, using an extinction coefficient of ϵ_{494} = 40 600 M^{-1} cm^{-1} (Hubbard & St. George, 1954). The phospholipid: rhodopsin ratio of the squid outer segment membranes was found to be approximately 90:1 mol/mol.

ESR Spectroscopy. ESR spectra were recorded on a Varian Century Line Series 9-GHz spectrometer equipped with a nitrogen gas flow temperature regulation system. Membrane samples were packed in 1-mm i.d. capillaries to a height of 5 mm to minimize the effects of microwave and modulation field inhomogeneities (Fajer & Marsh, 1982; Hemminga et al., 1984). The sample capillaries were accommodated within standard 4-mm quartz ESR tubes containing light silicone oil for thermal stability. Temperature was measured by a fine-wire thermocouple located at the top of the microwave cavity within the silicone oil. Conventional, in-phase ESR spectra (V_1-display) were recorded at a modulation amplitude of 1.6 G p–p and a modulation frequency of 100 kHz. Saturation-transfer, quadrature-phase ESR spectra (V_2'-display) were recorded at a modulation amplitude of 5 G p–p and a modulation frequency of 50 kHz with second-harmonic detection. The phase of the detection was set by the self-null method at subsaturating microwave power [see, e.g., Marsh (1982)]. The microwave power was adjusted so as to give an average microwave field at the sample of $\langle H_1^2 \rangle^{1/2}$ = 0.25 G for recording the STESR spectra (Fajer & Marsh, 1982; Hemminga et al., 1984). Calibrations of the effective rotational correlation times in terms of the STESR spectra of spin-labeled hemoglobin in isotropic solution were taken from Horváth and Marsh (1988).

FTIR Spectroscopy. Infrared spectra were recorded on a Bruker IFS 25 FTIR spectrometer with DTGS detector, at a resolution of 4 $cm,^{-1}$ apodized with a triangular function and Fourier transformed after one level of zero-filling. Samples prepared as for ESR spectroscopy were washed 3 times in D_2O and were then dissolved in 2% sucrose monolaurate in D_2O. The detergent-soluble fraction was introduced into a thermostated cuvette with CaF_2 windows and a 50-μm Teflon spacer for recording spectra. Peak positions in the amide I band were resolved by Fourier self-deconvolution (Kauppinen et al., 1981) and quantitated by band-fitting of the deconvoluted spectra, using programs kindly provided by Dr. H. H. Mantsch.

Electron Microscopy. Membrane samples containing 0.1 mg/mL rhodopsin, treated as described above, with and without C-terminal cleavage by endoproteinase-V8, were negatively stained with 2% ammonium molybdate on carbon support films. Photographs were taken on a JEOL 200CX electron microscope operated at 80 kV.

RESULTS AND DISCUSSION

Rhodopsin Cleavage. Incubation of squid outer segment membranes with endoproteinase-V8 was found to reduce the apparent molecular weight of the squid rhodopsin from 47 000 to 36 000, as judged by SDS/PAGE (see Figure 1). Electroelution and sequence determination of the polypeptide with M_r 36 000 demonstrated that its N-terminus corresponded to that predicted for rhodopsin (Hall et al., 1991). Therefore, cleavage of rhodopsin must have occurred toward the C-terminus of the protein. Both the specificity of endoproteinase-V8 and the size of the fragment suggest that cleavage occurred in the acidic domain on the C-terminal side of the seventh putative transmembrane helix. Cleavage was determined reproducibly to be >90% effective by densitometry of the electrophoresis gels. The efficiency of cleavage of squid rhodopsin in its native form at residues in this region of the protein is consistent with this region forming a surface loop. The visible absorption spectrum of the cleaved squid rhodopsin with M_r 36 000 was identical to that of the native M_r 47 000 protein in 2% sucrose monolaurate. After the spectrum was bleached, a similar pH dependence was observed for the spectral characteristics of the M_r 47 000 and 36 000 meta-rhodopsins (data not shown).

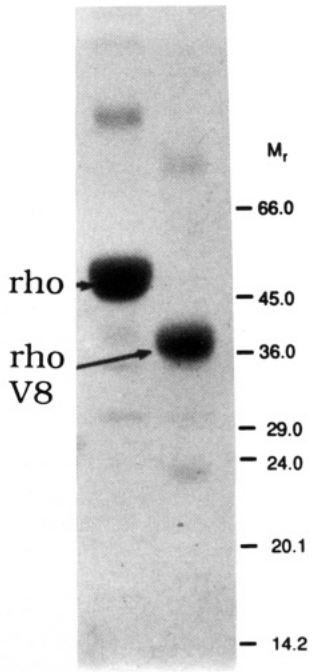


FIGURE 1: SDS–PAGE of squid rhodopsin from urea-washed outer segment membranes with and without treatment with V8 endoprotease. Squid outer segment membranes were prepared as described for the samples for ESR, except that no spin-label was added. Membranes were solubilized in 2% sucrose monolaurate; the membrane protein was denatured by adding the SDS–PAGE mixture, fractionated by electrophoresis on a 5–20% acrylamide gradient, and visualized by staining with Coomassie blue. Abbreviations: rho, rhodopsin; rho V8, protease-cleaved rhodopsin. Some dimers of both forms of rhodopsin are present, along with some minor components at lower molecular weight.

Electron Microscopy. The negative-stain electron micrographs of urea-washed membranes, before and after treatment with V8 endoprotease, are shown in Figure 2. Membranes containing intact rhodopsin exhibited distinctive projections which were no longer visible after protease treatment. These are likely to represent small clusters of rhodopsin molecules with intact C-terminal regions, as they are too large (6–10 nm in diameter) and too widely separated (15–20 nm center-to-center) to be single rhodopsin molecules. Presumably, the additional extramembranous bulk (ca. 12–15 nm^3 per monomer) contributed to the clusters by the C-terminal regions renders them visible as stain-excluding regions in the electron micrographs. Assuming a diameter for rhodopsin of 3–3.5 nm, the area of a cluster is 4–11 times that of a single rhodopsin

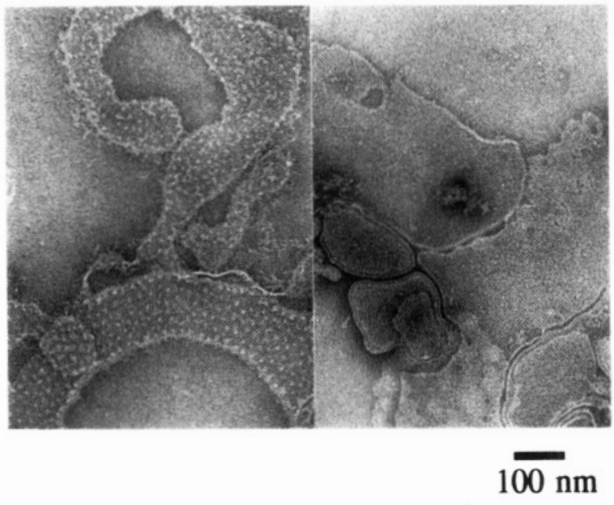


FIGURE 2: Negative-stain electron micrographs of urea-washed squid outer segment membranes: (left) before treatment with V8 endoprotease; (right) after treatment with V8 endoprotease. The membrane projections of the uncleaved protein are seen as light dots evenly spread over the membrane surface.

molecule. Considering molecular close-packing arrangements, an aggregate of six to seven approximately cylindrical rhodopsin monomers would have a diameter (ca. 8–10 nm) in the range observed for the clusters. The size of the projections is therefore consistent with an aggregate of approximately seven rhodopsin molecules.

FTIR Spectroscopy and Protein Structure. The FTIR spectra of squid rhodopsin were recorded in detergent so as to eliminate any residual microvillar cytoskeletal proteins [which are insoluble in sucrose monolaurate; see Pottinger et al. (1991)] that may still be associated with the outer segment membranes. The amide I region of the infrared absorption spectra of squid rhodopsin solubilized in sucrose monolaurate in D_2O buffer is shown in Figure 3a. The peaks of the various component bands have been resolved in this spectrum by Fourier self-deconvolution as described in the figure legend. The major band at 1654 cm^{-1} corresponds to the α-helical part of the protein. The peaks in the region of 1635, 1625, and 1675 cm^{-1} lie in the region expected for extended structures, that at 1665 cm^{-1} for turns, and random structures would be expected to lie in the region of 1645 cm^{-1} (Byler & Susi, 1986). The other peaks correspond to unassigned structures. The corresponding deconvoluted FTIR spectra for the cleaved squid protein in sucrose monolaurate and for bovine rhodopsin in ROS disks are given in panels b and c, respectively, of Figure 3.

The relative intensities of the peaks revealed by Fourier self-deconvolution in Figure 3 were determined by band-fitting of the deconvoluted spectra [cf. Byler and Susi (1986)]. Band-fitting of the original spectra was not possible because of the overlap of the strong lipid (and detergent) carbonyl band with the amide I band in the original FTIR spectra of the squid rhodopsin (data not shown). A constraint on the fitting, in order to ensure strict comparability between the different spectra, was that all three spectra should be fitted by the same peaks (as indicated by the deconvolutions) and that the widths of all the peaks should be comparable (ca. 10–15 cm^{-1}), consistent with the effects of deconvolution. It was found that similar results were obtained irrespective of whether the α-helical band at 1654 cm^{-1} was fitted by a single peak or by two closely positioned peaks which would allow for any residual protonated amide structures.

Results of band-fitting with the uncleaved squid rhodopsin of M_r 47 000 (Figure 3a) indicated an intensity of approx-

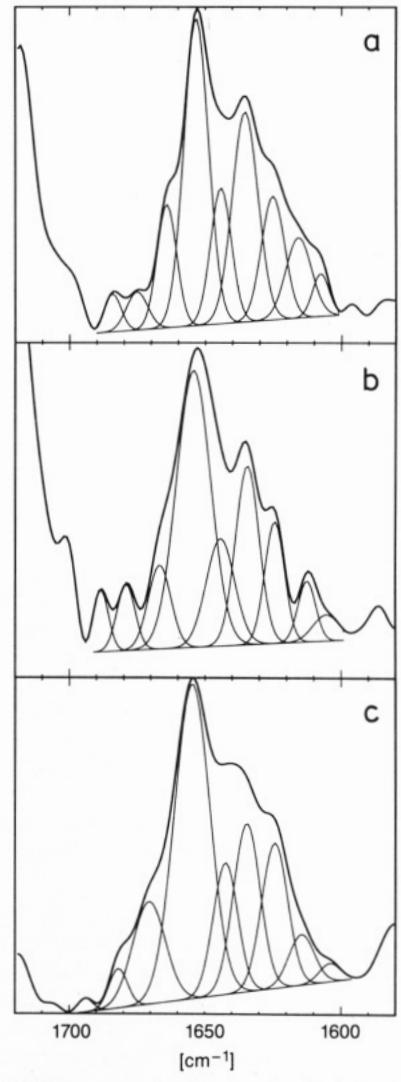


FIGURE 3: FTIR spectra (amide I region) of rhodopsins in D_2O buffer after Fourier self-deconvolution with a Lorentzian line width of 17 cm^{-1} and a band-narrowing factor of 2. (a) Intact squid rhodopsin (M_r 47 000) solubilized in sucrose monolaurate; (b) squid rhodopsin (M_r 36 000) cleaved by treatment with V8 protease and solubilized in sucrose monolaurate; (c) bovine ROS disks in suspension. The deconvoluted spectra are fitted by single Gaussian bands as indicated, with residuals in the regions of the individual band positions of <0.02–0.1%.

imately 30% (relative to the total amide I band) for the band at 1654 cm^{-1}, corresponding to α-helical structure and a relatively large proportion of extended chain structure, ≥30%, as indicated by the relative intensities of the bands in the region 1625–1635 cm^{-1}. For the cleaved form of squid rhodopsin with M_r 36 000 (Figure 3b), the relative intensity of the 1654-cm^{-1} band was considerably greater, corresponding to an α-helical content of approximately 40%, and the bands in the extended chain region at 1625–1635 cm^{-1} were correspondingly diminished (ca. 25%). This increase in the percentage α-helical content corresponds rather closely to the decrease in the molecular weight for the cleaved protein, suggesting that α-helical structure is confined to the M_r 36 000, i.e., transmembrane, section of the protein.

Comparison of the FTIR spectrum of bovine rhodopsin (Figure 3c) with that of cleaved squid rhodopsin (Figure 3b) revealed a rather similar content of the α-helical band at 1655 cm^{-1} (approximately 40%) for this protein which is also of lower molecular weight and lacks the C-terminal extension of the intact squid protein. This suggests that the overall

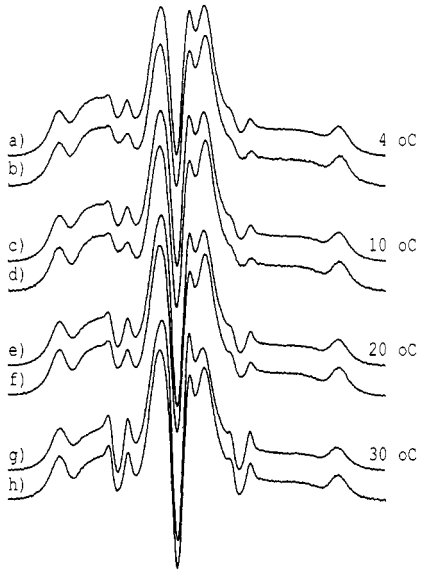


FIGURE 4: Second-harmonic, 90° out-of-phase, absorption STESR spectra (V_2'-display) of rhodopsin covalently spin-labeled with 5-InVSL in urea-washed squid outer segment membranes, recorded at the temperatures indicated. The upper spectrum of each pair corresponds to the native membranes and the lower spectrum to the membranes treated with V8 protease. Total scan width = 100 G.

secondary structure of the N-terminal portion of squid rhodopsin may be rather similar to that of bovine rhodopsin, which is in agreement with the sequence homologies and rather similar hydropathic profiles of the two proteins (Hall et al., 1991).

Whereas the criteria used for band-fitting were designed to enable a quantitative comparison between the spectra from the three different forms of rhodopsin, the absolute values deduced for the proportions of α-helical structure may be subject to a systematic error. This is because a band at 1643–1645 cm^{-1}, which corresponds to the spectral region expected for unordered peptide structures, was included in the fit, although a band was not resolved at this position in the deconvoluted spectra of either bovine rhodopsin or the cleaved squid rhodopsin. This unresolved band corresponded to approximately 11–12% of the total amide I intensity in all three cases. If this extra intensity were attributed to the band at 1654 cm^{-1}, the estimates of the α-helical content of the proteins would be revised to ca. 40% for the uncleaved squid rhodopsin and ca. 50% for the cleaved squid rhodopsin and the intact bovine rhodopsin. The latter value is in closer agreement with that found for bovine rhodopsin by other means (Shichi & Shelton, 1974; Stubbs et al., 1976).

The FTIR spectra, and hence the secondary structure, of both forms of squid rhodopsin were essentially unchanged on exposure of the samples to light. This insensitivity to photoexcitation indicates that, as for the vertebrate protein in ROS disks (Shichi et al., 1969), large-scale changes in secondary structure do not contribute to signal transduction. Small, localized changes in secondary structure cannot be excluded, however.

STESR Spectra of Spin-Labeled Rhodopsin. Squid rhodopsin was spin-labeled covalently with 5-InVSL after extensive urea washing of the outer segment membranes to remove peripheral proteins. The second-harmonic, out-of-phase, absorption STESR spectra of the 5-InVSL-labeled membranes, before and after cleavage of the rhodopsin with V8 endoprotease, are given in Figure 4. Although there are systematic changes in the relative intensities in the low-field, central, and high-field regions of the spectra with increasing

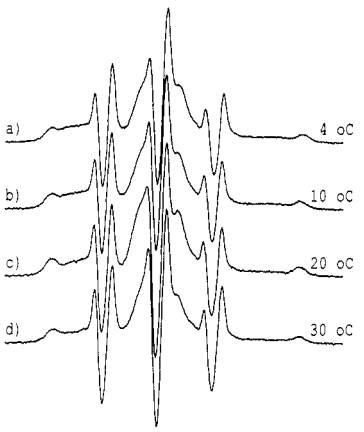


FIGURE 5: Second-harmonic, 90° out-of-phase, absorption STESR spectra (V_2'-display) of bovine rhodopsin covalently spin-labeled with 5-InVSL in ROS disk membranes, recorded at the temperatures indicated. Total scan width = 100 G.

temperature, the overall line shape is indicative of a high degree of restriction in the rotational mobility of the labeled protein in the squid outer segment membrane. This is seen clearly by comparison with the STESR spectra of bovine rhodopsin spin-labeled with 5-InVSL in ROS disk membranes that are given in Figure 5. The relative intensities in the low- and high-field diagnostic regions of the spectra [see Thomas et al. (1976) for a definition] are considerably lower in the bovine system (Figure 5) than in the squid system (Figure 4), indicative of the greater rotational mobility of rhodopsin in the former case. Although the STESR spectra of bovine rhodopsin spin-labeled with 5-InVSL indicate a clear difference in the rotational mobility, they serve only as a qualitative comparison and are not suitable for quantitative analysis. This is because the conventional ESR spectra (data not shown) indicate that spin-labeled components of higher mobility are present in the diagnostic regions normally used for defining the STESR line-height ratios.

In order to check whether the unusual cytoplasmic C-terminus of squid rhodopsin, which is absent in bovine rhodopsin, was responsible for the restriction of rotational mobility in squid outer segments, comparison was made with spin-labeled membranes that had been treated with V8 endoprotease. The lower spectrum of each pair in Figure 4 corresponds to the proteolyzed membranes. It is seen that over the entire temperature range the STESR line shapes are practically unchanged after removal of the C-terminal section of rhodopsin to produce the M_r 36 000 form of the protein. This indicates that the unique C-terminal extension of squid rhodopsin is not essential in maintaining the reduced rotational mobility of the protein.

To determine whether either the lipid composition or the residual peripheral membrane components were responsible for the rotational restriction of squid rhodopsin in the urea-washed outer segment membranes, the affinity-purified protein was reconstituted with egg PtdCho. The STESR spectra of spin-labeled squid rhodopsin in egg PtdCho vesicles with a comparable lipid:protein ratio to that of the native urea-washed membranes are given in Figure 6. At corresponding temperatures, the spectra from the reconstituted lipid–protein complexes are rather similar to those from the native urea-washed outer segment membranes. This indicates that the restriction in rotational mobility of the squid rhodopsin is comparable in the two systems. Hence, it is likely that aggregation of the protein is responsible for the low rotational rate of the squid protein, although this is not directly related to the presence of the C-terminal extension.

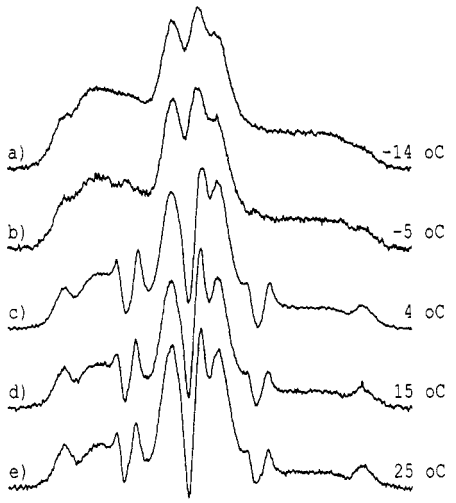


FIGURE 6: Second-harmonic 90° out-of-phase, absorption STESR spectra (V_2'-display) of squid rhodopsin covalently spin-labeled with 5-InVSL and reconstituted in egg phosphatidylcholine (lipid:protein ratio 70:1 mol/mol). Spectra were recorded at the temperatures indicated. Total scan width = 100 G.

Table I: Diagnostic Line-Height Ratios in the Low-Field (L''/L) and High-Field (H''/H) Regions and Normalized Integral Intensities (I_{ST}) Deduced from STESR Spectra of Squid and Bovine Rhodopsin Spin-Labeled with 5-InVSL in ROS Membranes

membrane/treatment	T (°C)	L''/L	H''/H	I_{ST}/ (×10²)
squid/urea wash	4	1.29	0.90	0.54
	10	1.22	0.87	0.48
	20	1.05	0.79	0.39
	30	0.96	0.76	0.38
squid/urea wash + V8 protease	4	1.32	0.91	0.49
	10	1.26	0.86	0.43
	20	1.07	0.80	0.36
	30	1.00	0.76	0.29
squid/reconstituted (+egg PtdCho)	−14	1.50	1.62	0.94
	−5	1.43	1.39	0.75
	4	1.34	0.92	0.82
	15	1.13	0.83	0.48
	25	1.04	0.74	0.37
bovine/none	4	1.12	0.74	0.84
	10	1.06	0.72	0.73
	20	0.89	0.60	0.41
	30	0.82	0.57	0.60

The quantitative features of the STESR spectra are summarized in terms of the diagnostic line-height ratios, L''/L and H''/H, and the normalized spectral integrals [see Thomas et al. (1986) and Horváth and Marsh (1988) for definitions] in Table I. These parameters are related directly to the rotational mobility of the spin-labeled protein, and the temperature dependence of the effective rotational correlation times deduced from them by using isotropic calibrations from spin-labeled hemoglobin (Horváth & Marsh, 1988) is given in Figure 7. These latter values illustrate the similarity between the three different membrane systems containing squid rhodopsin, and show an increasing rotational mobility with increasing temperature in all three cases. Some differences are seen between the effective rotational correlation times deduced from the L''/L and H''/H line-height ratios, possibly attributable to the anisotropic rotation of the spin-labeled rhodopsin. Nevertheless, the values deduced from both are of a similar order of magnitude and indicate relatively low mobility in all three cases.

The effective rotational correlation times, τ_R^{eff}, deduced from STESR for squid rhodopsin at 20 °C are in the region of 7×10^{-5} s (Figure 7). The uniaxial rotational correlation

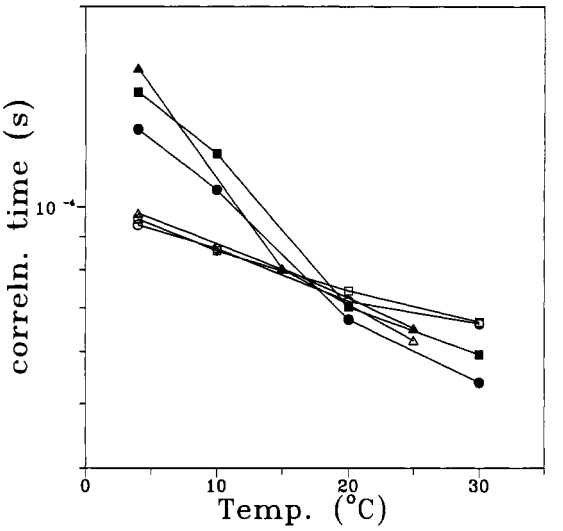


FIGURE 7: Temperature dependence of the effective rotational correlation time of rhodopsin spin-labeled with 5-InVSL, deduced from the low-field (L''/L, filled symbols) and the high-field (H''/H, open symbols) diagnostic line-height ratios in the STESR spectra. (○) Squid urea-washed outer segment membranes; (□) squid urea-washed outer segment membranes treated with V8 protease; (△) squid rhodopsin reconstituted in egg phosphatidylcholine at a lipid:protein ratio of 70:1 mol/mol.

time of the spin-labeled protein, $\tau_{R\parallel}$, is related to this effective correlation time by [see, e.g., Marsh and Horváth (1989)]

$$\tau_{R\parallel} = 1/6D_{R\parallel} = {}^1/_2\tau_R^{eff} \sin^2\theta \qquad (1)$$

where $D_{R\parallel}$ is the rotational diffusion coefficient of the protein and θ is the orientation of the spin-label z axis relative to the rotational axis ($\parallel$), i.e., to the membrane normal. Uncertainties in the latter have a strong influence on estimates of the rotational correlation time of the protein, but assuming that $\theta \approx 90°$ will yield an upper limit for $\tau_{R\parallel}$. For bovine rhodopsin in ROS disk membranes, a rotational relaxation time of $1/D_{R\parallel} = 2 \times 10^{-5}$ s (corresponding to an effective STESR rotational correlation time of 7×10^{-6} s, when $\theta = 90°$) has been measured by optical means (Cone, 1972), and an effective rotational correlation time of 2×10^{-5} s has been measured by STESR spectroscopy (Baroin et al., 1977). Since the rotational correlation time is directly proportional to the cross-sectional area of the rotating species [see, e.g., Marsh and Horváth (1989)], this would imply that the squid rhodopsin consists of aggregates composed of approximately 4–10 monomers in the membrane, where the higher value is an upper estimate, corresponding to $\theta = 90°$.

Estimation of the intramembranous diameter of the rotating unit for squid rhodopsin from the rotational correlation time can be made by using hydrodynamic theory [cf. Marsh and Horváth (1989)]:

$$\tau_{R\parallel} = 2\pi\eta a^2 h/3kT \qquad (2)$$

where a is the intramembranous radius. This yields an upper estimate for the diameter of the aggregate of $2a \approx 11$ nm, assuming a membrane viscosity of $\eta = 5$ P, a membrane thickness of $h = 4.5$ nm, and that $\theta \approx 90°$. Taking a monomer diameter of ca. 3.5 nm would again yield an effective cross-sectional area corresponding maximally to ca. 10 monomers, or a close-packed arrangement of 7 cylindrical monomers, for the aggregates of squid rhodopsin. These values are consistent with the size of the extensions of the uncleaved protein that are seen in electron microscopy. Any segmental motion of the spin-label relative to the protein, if present, would cause an underestimate of the rotational correlation time for the

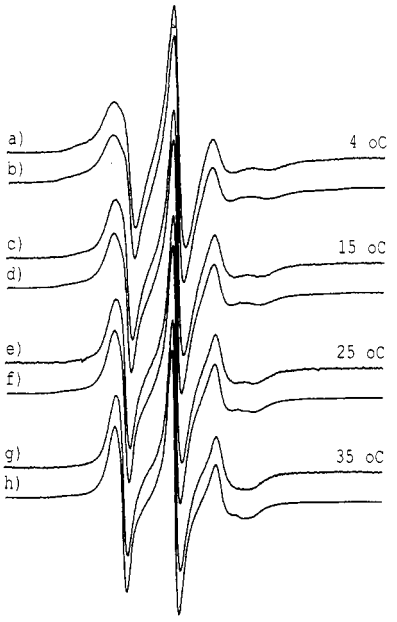


FIGURE 8: Conventional ESR spectra (V_I-display) of spin-labeled phosphatidylcholine (14 PCSL) in urea-washed squid outer segment membranes, recorded at the temperatures indicated. The upper spectrum of each pair corresponds to the native membranes and the lower spectrum to the membranes treated with V8 protease. Total scan width = 100 G.

protein deduced from the STESR measurements. This would therefore serve to offset the tendency to overestimation caused by the assumption that $\theta = 90°$.

STESR experiments with squid outer segment membranes that had not been subjected to washes with urea revealed a considerably higher degree of rotational immobility of the spin-labeled membrane protein, with effective rotational correlation times in the range of $(1–3) \times 10^{-4}$ s at 25 °C (data not shown). Although peripheral membrane components probably also were labeled in this case, it appears that all major labeled components (including rhodopsin) had a rather low rotational mobility. Thus, the shorter effective correlation time found with the urea-washed membranes might imply an important contribution from the cytoskeleton in maintaining the rotational immobility of squid rhodopsin. Indeed, earlier work has suggested that an axial alignment of the rhodopsin chromophores exists in the native structure of certain microvillar photoreceptors [arthropod and cephalopod; discussed in Saibil and Hewat (1987)], implying a severely restricted mobility of rhodopsin in these intact systems relative to that found here in squid photoreceptor membranes from which cytoskeletal elements have been removed by treatment with urea.

ESR of Spin-Labeled Lipids. Phosphatidylcholine spin-labeled on the 14–C atom of the *sn*-2 chain was used to study lipid–protein interactions with the intramembranous surface of squid rhodopsin in outer segment membranes. The conventional ESR spectra of 14-PCSL in urea-washed outer segment membranes, both before and after cleavage of the squid rhodopsin with V8 endoprotease, are given in Figure 8. As in many different membrane systems and reconstituted lipid–protein complexes [see, e.g., Marsh (1985)], the spectra consist of two components. The anisotropic component in the main part of the spectrum is very similar to the spectrum of 14-PCSL in aqueous dispersions of the extracted membrane lipids (data not shown), and corresponds to the fluid bilayer regions of the membrane. The second, broader component visible in the wings of the membrane spectra, but not present in the spectra from the extracted membrane lipids, corresponds to spin-labeled lipids in direct contact with the intramembranous surface of the integral membrane proteins. With increasing temperature, the spectral component corresponding to the fluid lipid regions of the membranes narrows due to increasing motional amplitudes. The temperature dependence of the protein-interacting component, on the other hand, is less steep due to its lower mobility, and therefore, because of the absence of appreciable line-narrowing, it becomes less easy to identify relative to the fluid component at the higher temperatures.

The spectra of the 14-PCSL label are seen to be very similar for the native squid membranes and for those in which the rhodopsin has been cleaved with the V8 protease. This demonstrates that the proportion of motionally restricted lipid does not change, implying conservation of the intramembranous surface of the protein on enzymatic cleavage. These results regarding the insensitivity of the lipid–protein interactions are consistent with the cytoplasmic location of the cleaved C-terminus and the lack of change in the aggregation state of the protein as indicated by the STESR measurements of the protein rotational diffusion.

Spectral subtraction [cf. Marsh (1982)] reveals that the motionally restricted (i.e., protein-interacting) component corresponds to approximately 15% of the total intensity of the spectra from the spin-labeled lipid in squid outer segment membranes that are given in Figure 8. For comparison, ca. 37% of the lipids are found to be motionally restricted in bovine ROS disk membranes that have a comparable lipid: protein molar ratio (Watts et al., 1979). This reduction in the population of motionally restricted lipids can be attributed to a decrease in the hydrophobic protein surface accessible to lipids resulting from aggregation of squid rhodopsin in the membrane, as evidenced by the protein rotational diffusion measurements described above. Geometrical considerations show that a close-packed arrangement of seven cylindrical monomers would be expected to reduce the protein surface available to lipid to ca. 40% of that for an isolated monomer. This is similar to the reduction observed in the motionally restricted lipid component in the squid membranes, relative to that in bovine ROS disk membranes, and is in reasonable agreement with estimates of the size of the squid rhodopsin aggregates based on the rotational diffusion measurements given above. However, it cannot be excluded absolutely that some lipid may be trapped within the aggregates of the squid rhodopsin protein.

Conclusions. Squid rhodopsin differs from its mammalian counterparts in possessing a C-terminal extension of unique, proline-rich structure. Cleavage of this extra C-terminal sequence leaves a protein whose overall secondary structure appears similar to that of bovine rhodopsin, but does not alleviate the restriction in protein rotational mobility relative to that of bovine rhodopsin. It is possible that in the native membranes, the C-terminus is involved in interactions with the cytoskeleton and that these interactions are disrupted by the extensive washing stages in sample preparation used here. Alternatively, the C-terminus may be required to mediate specific association of the protein, producing the membrane projections that are seen by negative-stain electron microscopy. However, once formed, other interactions, perhaps between polar residues in the membrane-spanning helices, may maintain the association of squid rhodopsin even in the absence of the additional cytoplasmic domain.

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