# Effect of the C-terminal proline repeats on ordered packing of squid rhodopsin and its mobility in membranes

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Received 1 December 1994

**Abstract Negative stain electron microscopy and saturation transfer electron spin resonance spectroscopy have been used to compare the lattice ordering and in-plane membrane mobility of full-length and C-terminally cleaved squid rhodopsin. The C-terminus of squid rhodopsin contains a negatively charged region followed by 9–10 repeats of a proline-rich sequence, not found in rhodopsins other than those of cephalopod invertebrates, but similar proline repeats are found in other, unrelated membrane proteins. We find that the proline repeats cluster the rhodopsins into small groups, interfering with two-dimensional crystallization and maintaining their mobility in the membrane.**

*Key words:* 2D crystal; Negative stain electron microscopy; Spin label ESR (spectroscopy); Rotational diffusion; Cephalopod rhodopsin

## 1. Introduction

In the photoreceptor membranes of the squid retina, rhodopsin is found in an ordered membrane–cytoskeleton network composed of microvilli [1,2]. Squid photoreceptor membranes can be separated into detergent-soluble and cytoskeletal protein fractions, and the rhodopsin purified and reconstituted into membrane vesicles. The proline-rich C-terminal extension, unique to squid and octopus rhodopsins, is readily cleaved by proteolysis to yield a truncated 'core' rhodopsin similar in size and topology to other known rhodopsins [3–5]. Similar proline repeat sequences in other proteins have been hypothesised to act as binding sites for recognition or for membrane fusion events [6]. In a previous spectroscopic study, the rotational mobility of squid rhodopsin in the plane of the photoreceptor membrane was shown to be lower than that of bovine rhodopsin, and removal of the C-terminus did not have a significant effect [5]. The rhodopsin was found to form clusters of 4–10 molecules. To test the possibility that the clusters would disperse if the cleaved rhodopsins were detergent-solublized and reconstituted, we performed electron spin resonance (ESR) spectroscopy on squid rhodopsin with and without the C-terminal extension, purified and reconstituted after proteolytic cleavage. Unexpectedly, the rotational correlation time of squid rhodopsin increased by a further 2-fold after cleavage and membrane reassembly. The mobility measurements agree well with electron microscopy (EM) studies showing ordered array formation and an increase in crystallinity with increasing purification.

This work shows that the 40 kDa 'core' of squid rhodopsin contains interactive regions that cause ordering of rhodopsin monomers into larger, less mobile assemblies. The presence of the C-terminal extension appears to limit the array formation.

## 2. Materials and methods

Endoproteinase Glu C from *Staphylococcus aureus* was from Boehringer-Mannheim. The spin label 4-maleimido-tempo, used for covalent spin labelling of rhodopsin, was from Sigma. All manipulations of the membranes were done with dim red illumination (Ilford safelight ISO 906).

Squid photoreceptor membranes were prepared as previously described [7]. Briefly, *Loligo forbesi* retinas were rapidly frozen in low-$Ca^{2+}$ artificial sea water immediately after dissection from freshly killed animals. The microvillar layers were detached by shaking and purified by flotation on a 0–40% (w/v) sucrose step. Rhodopsin concentration was measured by the change in $A_{493\,nm}$ after bleaching at pH 10 [8].

Membranes (0.5 ml, containing 2.5 mg rhodopsin·$ml^{-1}$) in 50 mM Tris, pH 7.4, and 1 mM dithiothreitol (DTT) were solubilized with an equal volume of 100 mM octyl glucoside (final concentration 50 mM) in the same buffer. The mixture was incubated on ice for 10 min to extract detergent-soluble proteins, and the insoluble, cytoskeletal fraction was pelleted by centrifugation (12,000 × *g*, 5 min, 4°C). The detergent supernatant was loaded onto a pre-equilibrated 1 ml column of DEAE-cellulose. The eluate was collected and the column washed 4 times with Tris/octyl glucoside buffer to elute unbound proteins (mainly rhodopsin) and co-purifying squid photoreceptor lipids [9]. The eluate and washes were pooled and the detergent removed by dialysis for 36 h at 4°C against 4 × 1 l changes of buffer containing 50 mM Tris, pH 7.4, 1 mM $MgCl_2$, 100 mM NaCl and 1 mM DTT, in order to reconstitute the purified rhodopsin with native retinal lipids. Samples for SDS-PAGE were taken at each step in the process. In some experiments, membranes were solubilized in nonyl glucoside instead of the octyl form. This did not appear to have any significant effect on the results.

Photoreceptor membranes containing 50 μM rhodopsin in hypotonic buffer (5 mM HEPES pH 7.8, 1 mM EGTA, 1 mM DTT) were incubated with endoproteinase Glu C (100:1 w/w rhodopsin:enzyme) at 20°C for 30 min. The reaction was terminated by 20-fold dilution with ice-cold hypotonic buffer, followed by centrifugation (50,000 × *g*, 20 min, 4°C). The membranes were washed twice more in hypotonic buffer by centrifugation, and a sample removed for SDS-PAGE prior to purification and reconstitution of the cleaved rhodopsin as described in the previous section.

Samples for negative stain EM were washed into low salt buffer

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*SSDI* 0014-5793(94)01449-3

(10 mM Tris, pH 7.4, 5 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT). 10 μl samples were applied to freshly glow-discharged carbon-coated copper EM grids and allowed to adhere for 1 min. Excess sample was blotted off before adding a drop of negative stain (2% ammonium molybdate, pH 7.4). After several seconds, excess stain was removed and the grids allowed to air dry in the dark before storage and observation. Electron micrographs were recorded at magnifications between × 30,000 and × 46,000, at voltages of 80 or 120 kV under low electron dose conditions, on a Philips 400 or JEOL 1200EX microscope.

Membrane areas were estimated by tracing around the areas occupied by all vesicles and by those with discernable order on 50 micrographs of several different samples, and measuring the percentage of ordered area, either by cutting out and weighing the paper or by digitising the images and using image analysis software. Diffraction patterns were calculated from 512 × 512 areas that were digitized with pixel size 5 or 6.7 Å on the specimen, using a CCD camera.

Squid rhodopsin was labelled covalently with 4-maleimido-tempo (added in a small volume of ethanol) in 5-fold molar excess for 30 min at 4°C. Unreacted spin label was removed by 3 washes with hypotonic buffer (5 mM HEPES, pH 7.2, 1 mM EDTA, 20 mM DTT) and 2 washes with measuring buffer (5 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). ESR spectra were recorded in a Bruker 200 ESR spectrometer equipped with a nitrogen gas flow temperature regulation system. Membrane samples were packed in 100 μl capillary tubes to a height of 5 mm. The capillaries were then sealed under argon in a standard 4 mm quartz ESR tube. Spectral reading and sample transfer were performed in the dark or under dim red light. Conventional spectra (ESR) were recorded as the in-phase first harmonic with a 100 kHz modulation field and a modulation amplitude of 1 G. Saturation transfer spectra (STESR) were recorded 90° out of phase at a modulation frequency of 50 kHz with second harmonic detection and the modulation amplitude was increased to 5 G. The microwave power setting was 63 mW (nominal). Rotational correlation times were deduced from the saturation transfer spectral parameters using the calibration of Horváth and Marsh [10] with a simple analytical fit [11].

20 μl samples of membranes containing 5 mg/ml rhodopsin were scanned in a Perkin Elmer DSC7 calorimeter, calibrated with indium and palmitic acid, at a scan speed of 5°C/min.

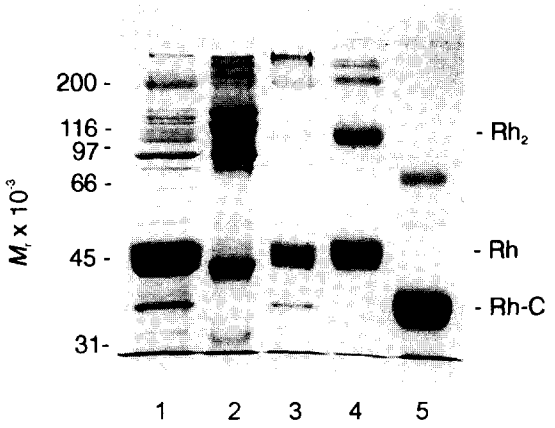


Fig. 1. 10% SDS-polyacrylamide gel showing (1) proteins of whole squid photoreceptor membranes, (2) detergent-insoluble fraction, (3) detergent-soluble fraction (sample loaded on column), (4) purified and reconstituted rhodopsin, (5) purified and reconstituted rhodopsin following pre-treatment with endoproteinase Glu C. The positions of $M_r$ markers are indicated. Rh, rhodopsin; $Rh_2$, rhodopsin dimer; Rh-C, proteolytically cleaved rhodopsin. Rhodopsin has a tendency to polymerize in SDS, and dimers and some higher oligomers of rhodopsin are present.

## 3. Results

### 3.1. Arrangement of rhodopsins in the membrane

Fig. 1 shows SDS-PAGE of the photoreceptor membrane and rhodopsin preparations used in the EM and ESR experiments. In Fig. 2a, a negative stain EM of isolated, native squid

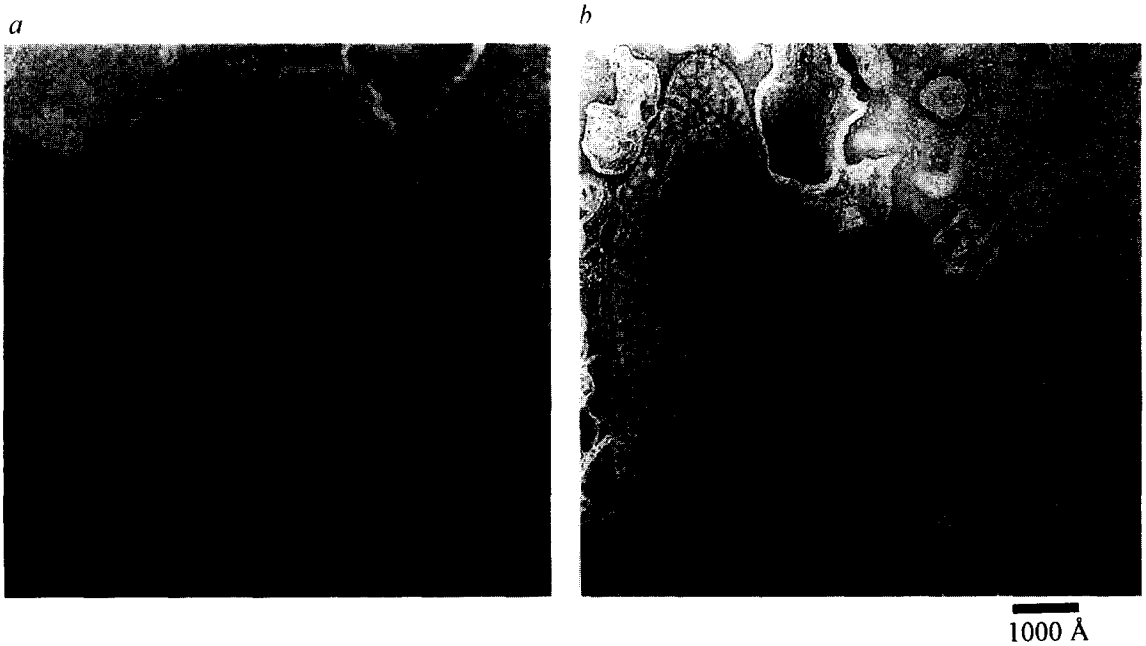


Fig. 2. (a) Negative stain EM of isolated, native squid photoreceptor membrane, showing the semi-ordered arrays that are occasionally seen with this preparation. The C-terminal extensions are seen as pale areas ~100 Å in diameter and spaced ~200 Å apart on the membrane sheet. (b) EM of the reconstituted, detergent-soluble fraction from which the cytoskeletal proteins have been removed. The rhodopsin lattices are more frequent and more ordered after this partial purification, and the membrane sheets have a stronger tendency to stack in overlapping layers. The individual clusters seen in the lower right of the image are also ~100 Å in diameter, spaced at about 150 Å separation.

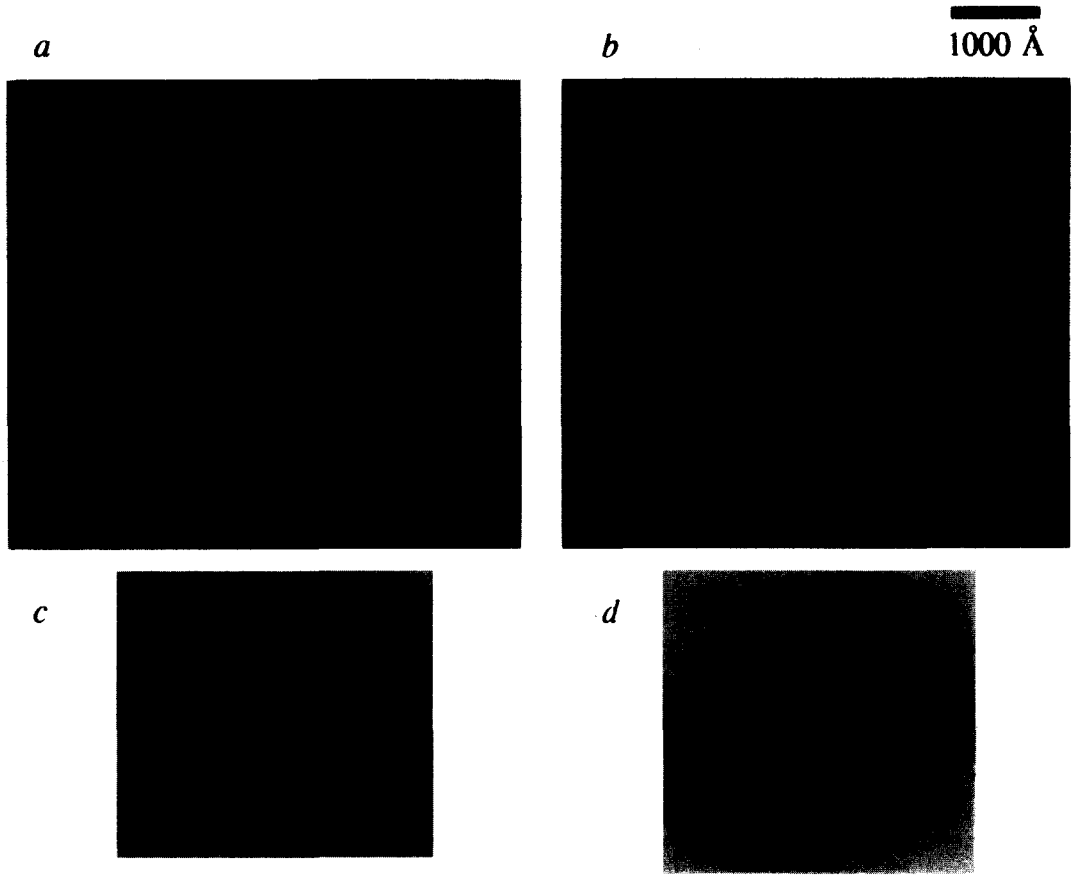


Fig. 3. (a) EM of purified, reconstituted rhodopsin prepared as for the ESR experiments, showing regions of regular packing. Such ordered regions are rarely seen in these samples. (b) Sample prepared as in (a) but with proteolytic cleavage of the proline-rich C-terminus before purification and reconstitution. Many of the vesicles show small regions of crystalline packing, barely visible as very fine striations. The magnification in (a) and (b) is the same as in Fig. 2. (c) Computed transform of the lattice in (a). A 64 Å repeat is present, indicating limited order in one direction. (d) Diffraction pattern of (b), showing good lattice order, with a rectangular unit cell of 44 × 66 Å.

photoreceptor membranes shows an area with clusters of rhodopsins (light dots) packed in quasi-ordered domains. The clusters are about 100 Å in diameter and spaced about 200 Å apart. Although such partially ordered regions do not form the majority of the membranes seen by negative staining, their presence indicates that the rhodopsins are capable of semi-ordered packing in membranes containing all the native microvillar membrane-associated proteins. In Fig. 2b, membranes reconstituted after detergent solubilization and removal of the detergent-insoluble fraction by centrifugation also show ordered arrays of the rhodopsin clusters. In this case the degree of ordering is somewhat higher, but the images are complicated by the membrane stacking into two or more overlapping layers. The region of single-layered membrane in the bottom right of the image shows a small group of clusters packing together more closely than in Fig. 2a. The visible cluster size is still about 100 Å, but the repeat distance is smaller, about 150 Å.

When the rhodopsin is purified by ion-exchange chromatography and reconstituted with native lipids, small, ordered regions are occasionally found. One of the best ordered regions from this type of sample is shown in Fig. 3a, and its computed transform is shown in Fig. 3c. The lattice area is small and only ordered in one direction, showing lines giving a single, 64 Å spacing. Along the direction of the lines, clusters are seen as in Fig. 2b. The percentage of ordered regions in the uncleaved rhodopsin membranes is less than 5%, using generous criteria to try to obtain an upper limit for the amount of lattice. Finally, when the membranes are reconstituted from purified and protease-cleaved rhodopsin, apparently featureless membrane vesicles are produced (Fig. 3b). However, closer inspection of these images reveals the presence of small regions of crystalline packing. These regions have very fine stripes, with a repeat distance of the order of 60–65 Å. The computed transform of the lattice region is shown in Fig. 3d. Sharp diffraction spots reveal a well-ordered lattice with a rectangular unit cell of 44 × 66 Å. In the absence of the C-terminus, there is no clustering of the rhodopsins. A fuller analysis of the underlying crystal lattice and its relationship to the superlattice formed by C-terminal clustering will be published elsewhere (A.D., C.V.-B. H.R.S., in preparation). The percentage of ordered regions for truncated rhodopsin membranes is much higher; a conservative lower limit for the percentage of lattice regions is 30%.

### 3.2. *Rhodopsin mobility*

STESR (saturation transfer electron spin resonance) spectra of spin-labelled rhodopsin are shown in Figs. 4 and 5. Reconstituted rhodopsin was covalently spin-labelled using the maleimide derivative. In general, the conventional ESR spectra indi-

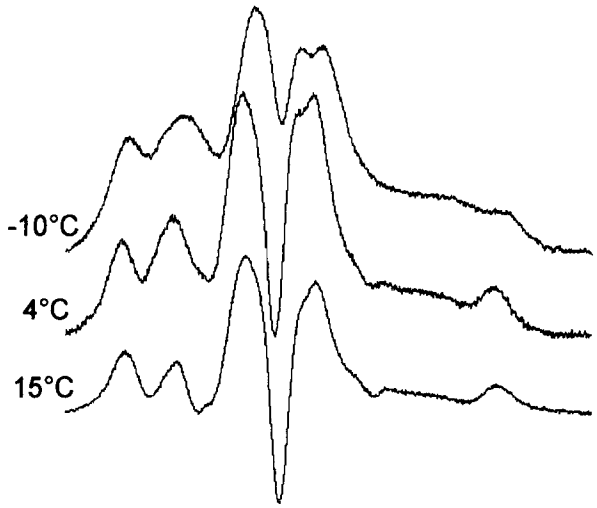


Fig. 4. Second harmonic, 90° out-of-phase STESR spectra of purified rhodopsin in native squid photoreceptor lipids. The main parameter determining the derived rotational correlation time is the height ratio of the low field peaks (the two peaks on the left of the trace) at 15°C.

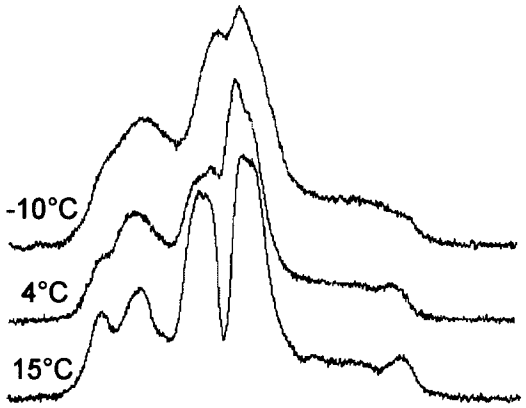


Fig. 5. Second harmonic, 90° out-of-phase STESR spectra of purified, C-terminally cleaved rhodopsin. The low field peaks in the 15°C spectrum are reversed in relative height compared to those in Fig. 4.

cated a high degree of immobilization of the spin label with very little contamination of the spectrum from labelling of highly mobile groups. STESR spectra of native rhodopsin reconstituted in native lipids at 15°C, 4°C, and −10°C are shown in Fig. 4. Although there are systematic changes in the relative intensities in the low-field, central and high-field regions of the spectra with decreasing temperature, the overall lineshape of the spectra of the labelled native protein is indicative of a high degree of restriction in the rotational mobility. An even lower mobility is recorded for C-terminally cleaved rhodopsin reconstituted in native lipids at 15°C, 4°C and −10°C (Fig. 5). Table 1 shows the apparent rotational correlation times for the native protein and for the cleaved protein reconstituted in native lipids. These values were obtained from the low-field (L″/L) and high field (H″/H) diagnostic line-height ratios, using calibrations for isotropic rotation [11]. The apparent rotational correlation time for squid rhodopsin purified and reconstituted in native lipids at 15°C deduced from L″/L is about $5 \times 10^{-5}$ s. STESR experiments with rhodopsin cleaved then purified and reconstituted in native lipids revealed a significantly lower degree of rotational mobility of the spin-labelled membrane protein, with an apparent rotational correlation time around $10^{-4}$ s at 15°C.

### 3.3. Thermal transition

DSC traces of the reconstituted, rhodopsin-containing membranes show an irreversible transition at 65°C, which is not affected by removal of the C-terminus, and is similar to that seen for bovine rhodopsin [12–14].

## 4. Discussion

Electron micrographs of the various membrane preparations allow a semi-quantitative comparison of the degree of ordered packing under different sample conditions. Examples of ordered packing were found for each type of sample, but the degree of order was generally greater with increasing purification. In particular, ordered regions were more readily observed when the proline-rich C-terminal extension was removed than with intact squid rhodopsin ($\geq 30\%$ vs. $\leq 5\%$). Unlike the squid protein, vertebrate rhodopsins do not spontaneously form lattices, but they have been crystallized and a projection map showing the arrangement of helices in bovine rhodopsin has been obtained [15].

Our results show that the rotational mobility of squid rhodopsin ($5 \times 10^{-5}$ s) is restricted compared with that of bovine rhodopsin ($2 \times 10^{-5}$ s; [16]), in agreement with the previous study [5] (and see also [17]). That study [5] indicated that removal of the C-terminus did not have a significant effect on mobility and that the unique C-terminal extension of squid rhodopsin is therefore not essential to maintaining the reduced rotational mobility of the protein. In those experiments, the cleaved rhodopsins were not solubilized in detergent. Instead, the cytoskeletal and peripheral membrane components were extracted with urea, having behind the protease-cleaved rhodopsin. The rhodopsin was found to form clusters of 4–10

Table 1
STESR diagnostic line height ratios and apparent rotational correlation times for squid rhodopsin preparations. Values are averages of 2 independent experiments.

| $T$ (°C) | L″/L | $\tau^{eff}$ (L″/L) (μs) | H″/H | $\tau^{eff}$ (H″/H) (μs) |
|---|---|---|---|---|
| *Full-length rhodopsin reconstituted in native lipids* | | | | |
| 15 | 0.88 ± 0.3 | 48 ± 4 | 0.73 ± 0.02 | 73 ± 7 |
| 4 | 1.33 ± 0.07 | 150 ± 33 | 0.95 ± 0.05 | 124 ± 15 |
| −10 | 1.25 ± 0.06 | 120 ± 18 | 1.43 ± 0.01 | 339 ± 9 |
| *Cleaved rhodopsin reconstituted in native lipids* | | | | |
| 15 | 1.17 ± 0.09 | 98 ± 20 | 0.89 ± 0.05 | 108 ± 13 |
| 4 | 1.72 ± 0.02 | 900 ± 177 | 1.05 ± 0.01 | 153 ± 4 |
| −10 | 1.60 ± 0.3 | 400 ± 40 | 1.41 ± 0.21 | 327 ± 135 |

molecules, and there was no significant effect of C-terminal cleavage. To test the possibility that the clusters would disperse if the cleaved rhodopsins were solubilized with detergent and reconstituted, we analyzed the mobility of full-length and cleaved spin-labelled rhodopsin, purified and reconstituted in native squid photoreceptor lipids. Our STESR results show that, unexpectedly, the cleaved rhodopsin is significantly less mobile than the full-length rhodopsin. The apparent rotational correlation time of the cleaved, reconstituted rhodopsin is $10^{-4}$ s, compared with $5 \times 10^{-5}$ s for the full-length rhodopsin. The low rotational diffusion rate of the cleaved squid rhodopsin appears to be due to a substantial amount of organised lattice regions as shown by EM. Lattice regions were rarely seen in membranes containing the full-length rhodopsin, and these were less well ordered.

The present data suggest that intermolecular interactions between rhodopsins provide the dominant structural feature of squid photoreceptor membranes rather than membrane–cytoskeleton interactions imposing membrane order.

*Acknowledgements:* We thank the Wellcome Trust for support, and Juliet Munn for EM support.

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