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Crystallization and crystal properties of squid rhodopsin

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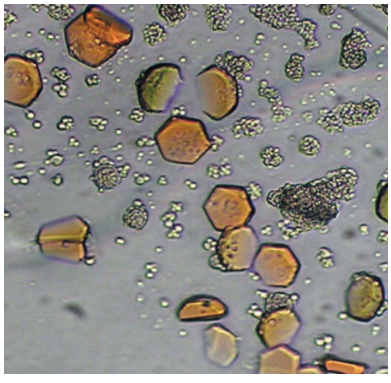
Rhodopsin, a photoreceptor membrane protein in the retina, is a prototypical member of the G-protein-coupled receptor family. In this study, rhodopsin from the retina of the squid *Todarodes pacificus* was treated with V8 protease to remove the C-terminal extension. Truncated rhodopsin was selectively extracted from the microvillar membranes using alkyl glucoside in the presence of zinc ions and was then crystallized by the sitting-drop vapour-diffusion method. Of the various crystals obtained, hexagonal crystals grown in the presence of octylglucoside and ammonium sulfate diffracted to 2.8 Å resolution. The diffraction data suggested that the crystal belongs to space group $P6_2$, with unit-cell parameters $a = b = 122.1$, $c = 158.6$ Å. Preliminary crystallographic analysis, together with linear dichroism results, suggested that the rhodopsin dimers are packed in such a manner that their transmembrane helices are aligned nearly parallel to the c axis.

1. Introduction

Rhodopsin is the primary molecule in the visual signalling cascade in vertebrate and invertebrate photoreceptor cells (Hubbard & St George, 1958; Yarfitz & Hurley, 1994; Shichida & Imai, 1998). Upon light absorption, the retinal chromophore in rhodopsin undergoes isomerization from the 11-*cis* to the all-*trans* configuration, which initiates the photoactivation process. In vertebrate retinas, the final photoproduct, meta-rhodopsin, activates a subtype of GTP-binding protein (G-protein), transducin (G_t), which in turn activates phosphodiesterase, an enzyme that degrades the second messenger molecule cyclic GMP (cGMP). In invertebrate retinas, on the other hand, acid meta-rhodopsin activates another subtype of G-protein, G_q, which is known to activate phospholipase C, an enzyme that hydrolyzes phosphatidyl inositol into the second messengers inositol triphosphate (IP_3) and diacyl glycerol (Terakita *et al.*, 1998).

Rhodopsin is categorized into subfamily A of the G-protein-coupled receptors (GPCRs), which comprises ∼90% of all GPCRs. These receptors have evolved to receive extremely diverse signals including visual light, odorant molecules, neurotransmitters and hormones. Most of them trigger IP_3 signalling cascades, as found for squid rhodopsin. Recently, the crystal structure of bovine rhodopsin was determined at high resolution (Palczewski *et al.*, 2000; Okada *et al.*, 2004; Li *et al.*, 2004). Its coordinates have been utilized as a structural template to estimate the properties of a ligand-binding site and a G-protein-binding region in other GPCRs. It has been shown that bovine meta-rhodopsin is unable to activate G_q-type G-proteins (Shichida & Yamashita, 2003). It is widely believed that vertebrate photoreceptor cells, which utilize the retina-specific cGMP signalling pathway, have evolved to acquire very specialized machineries for signal amplification (Terakita *et al.*, 1998). To date, little information has been available on the structural motifs required for activation of G_q-type G-proteins. It would therefore be expected that structural determination of squid rhodopsin should bring about better understanding of the molecular mechanism of IP_3-mediated signalling cascades.

Squid rhodopsin has been investigated intensively using various biochemical and spectroscopic techniques. Rhodopsin from the squid *Todarodes pacificus* contains 448 amino acids with a molecular weight



of ~50 kDa (Hara-Nishimura *et al.*, 1993). Its polypeptide chain is 100 residues longer than those of vertebrate counterparts, owing to a unique 10 kDa C-terminal extension composed of six repeats of the consensus sequence Pro-Pro-Gln-Gly-Tyr followed by proline-rich sequences. A low-resolution structure of C-terminally truncated rhodopsin from the squid *Loligo opalescens* was derived from cryo-electron micrographs of its two-dimensional crystals (Davies *et al.*, 2001). These previous studies showed that the overall structure of squid rhodopsin is similar to that of bovine rhodopsin. To reveal the distinct features of squid rhodopsin, however, high-resolution diffraction data from its three-dimensional crystals are highly desirable. Here, we report a hexagonal crystal of *T. pacificus* rhodopsin that was grown using octylglucoside as a detergent.

2. Materials and methods

Fresh squid (*T. pacificus*) were purchased from Yanagibashi fish market in Nagoya, Japan. All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), except for the endo-proteinase Glu-C from *Staphylococcus aureus* V8 (V8 protease), which was purchased from Sigma–Aldrich Japan Ltd (Tokyo, Japan).

All manipulations were performed under dim red light (>640 nm) at 277 K unless specified otherwise. Microvillar membranes from rhabdomeric photoreceptor cells of squid retina were purified according to the method reported by Kito *et al.* (1982). Briefly, crude microvillar membranes were isolated in phosphate buffer (50 m*M* sodium phosphate pH 6.8, 4% NaCl, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* DTT, 0.2 µg ml^{-1} leupeptin, 0.01% NaN_3) by the sucrose-flotation method. After sucrose step-gradient [20 and 32%(*w*/*v*)] centrifugation of the microvillar membranes, rhodopsin-rich membranes were collected from the interface between the 20 and 32% sucrose layers and were then washed with water at least three times to remove peripheral components. The purified microvillar membranes (5 mg ml^{-1} rhodopsin) were treated with the enzyme V8 protease (100:1 rhodopsin:enzyme by weight) at 277 K for 12 h in order to cleave a specific site in the C-terminal extension of rhodopsin, as previously reported (Venien-Bryan *et al.*, 1995). The digestion process was monitored every 4 h by SDS–PAGE to assess the completeness of the enzymatic digestion. The reaction was terminated by repeated washing of the microvillar membranes. The washed microvillar membranes were suspended in a minimal volume of water. The concentration of rhodopsin was determined from the absorbance at 480 nm using the reported absorption coefficient of 35 000 mol^{-1} cm^{-1} (Suzuki *et al.*, 1976). The membrane suspension containing ~10 mg ml^{-1} truncated rhodopsin was stored at 193 K until further purification.

Selective extraction of C-terminally truncated rhodopsin from the microvillar membranes was performed according to the method used for the purification of bovine rhodopsin (Okada *et al.*, 1998) with minor modifications. Truncated squid rhodopsin was extracted selectively from the microvillar membranes under various conditions by mixing the membrane suspension with stock solutions of buffer, detergent and salt for a few minutes. After incubation at 277 K overnight, the samples were centrifugated at 30 000 rev min^{-1} for 1 h.

The truncated rhodopsin was crystallized at 277–283 K by the sitting-drop vapour-diffusion method using 15-well crystallization plates (ICN Biomedicals Inc., Ohio). The protein solution (3 µl, ~8 mg ml^{-1}) was mixed with an equal volume of crystallization solution and the drop was equilibrated at 277 K against 0.5 ml crystallization solution. In initial trials, platelet crystals of the truncated rhodopsin were obtained using 10% PEG 400 as a precipitant. This type of crystal diffracted to 9 Å resolution. Higher quality crystals were obtained when ammonium sulfate was used as a precipitant: hexagonal crystals grew reproducibly when the purified rhodopsin solution was mixed with crystallization solution containing 3–3.4 *M* ammonium sulfate, 30 m*M* MES buffer pH 6.0–6.7, 30–50 m*M* EDTA, 1 m*M* β-mercaptoethanol. This type of crystal appeared in one month and continued to grow to a maximum size of 200 µm.

For X-ray diffraction, a single crystal was transferred into crystallization solution supplemented with 20% trehalose as a cryoprotectant. After soaking for 10 min, the crystal was flash-frozen in liquid propane and stored in liquid nitrogen. X-ray diffraction measurements were performed at SPring-8 BL41XU, where a frozen crystal kept at 100 K was exposed to a monochromatic X-ray beam at a wavelength of 1.0 Å with an X-ray flux rate of ~10^{15} photons mm^{-2} s^{-1}. Diffraction data were collected using a CCD detector (ADSC Quantum 315), with an oscillation range of 1° and an X-ray flux of 1–2 × 10^{14} photons mm^2 per image. Indexing and integration of diffraction spots were carried out using *MOSFLM* v.6.1 (Steller *et al.*, 1997). Scaling of the data was performed using *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Molecular replacement was performed using *CNS* v.1.1 (Brünger *et al.*, 1998).

Transmission electron microscopy was performed using a JEM2010 electron microscope (Jeol, Tokyo, Japan) operated at 200 kV. For cryogenic electron microscopy, a suspension of rhodopsin vesicles was mounted on a carbon-coated grid that had been rendered hydrophilic by glow discharge. Excess liquid was removed with a filter paper and the grid was plunged into liquid propane and transferred to a cryo-transfer stage (Oxford CT3500). A frozen sample was exposed to an electron beam at ~10 e Å^{-2} for 1 s and micrographic images were recorded using a CCD camera (Gatan Model 780). For the preparation of replicas of rhodopsin vesicles, rapidly frozen samples were fractured at 123 K in a freeze-etching apparatus (Jeol JFD-9010) and the fractured surface was replicated by evaporating platinum at an angle of 45°, followed by carbon coating to strengthen the replica. After washing with 50% ethanol, the replica was placed on a copper grid and its electron micrographs were recorded using

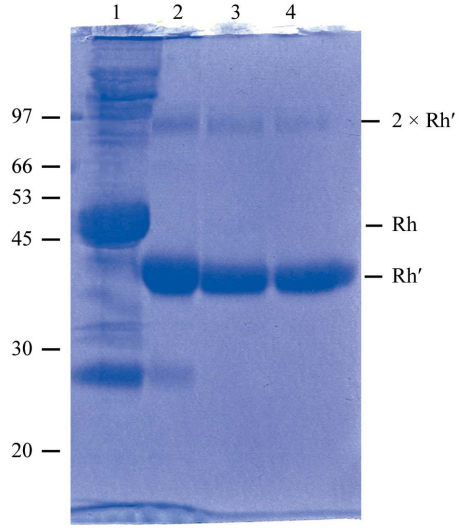


Figure 1
SDS–PAGE analysis of the purification of squid rhodopsin (Rh). Lanes 1 and 2, squid photoreceptor microvillar membranes before (lane 1) and after (lane 2) treatment with V8 protease for 20 h. Lanes 3 and 4, extraction of truncated rhodopsin (Rh′) from microvillar membranes with nonylglucoside (lane 3) and octylglucoside (lane 4). Molecular weights (kDa) are marked on the left.

SO-163 films (Kodak) and digitized with a scanner (Minolta AF-5000).

Absorption spectra were recorded with a Hitachi U-3300 spectrophotometer. SDS–PAGE was performed according to the method of Laemmli (1970).

3. Results and discussion

3.1. Specific truncation of the C-terminal extension of squid rhodospin

In a typical preparation, microvillar membranes containing ~40 mg rhodopsin were isolated from 80 squid eyeballs. SDS–PAGE showed three opsin bands with apparent molecular weights ranging from 45 to 50 kDa, probably owing to partial degradation in the C-terminal region (lane 1 in Fig. 1). Since the protein heterogeneity seemed to prevent the growth of a high-quality crystal, microvillar membranes were treated with V8 protease in order to cleave a specific peptide bond in the extension region of the C-terminus. After digestion by V8 protease, a single band of opsin was observed at the 40 kDa position on the gel (lane 2 in Fig. 1). V8 protease specifically hydrolyses the peptide bond on the carboxyl side of a glutamic acid residue under the solvent conditions used in this study (Breddam & Meldal, 1993). As judged from the molecular weight of the truncated opsin, it was suggested that the cleavage most likely occurred at Glu373, *i.e.* at the last glutamic acid in the C-terminus.

3.2. Selective extraction of truncated rhodopsin

Truncated squid rhodopsin was extracted selectively from the microvillar membranes by inducing a phase separation in the presence of alkyl glucoside and zinc ion. Firstly, the membrane suspension was mixed with MES buffer pH 6.4 and octylglucoside in various detergent:protein ratios. Zinc acetate was then added to a final concentration of 30 m*M*. This mixing procedure caused a significant increase in the turbidity and, after incubation at 277 K for >12 h, a phase separation took place in such a manner that a transparent orange phase floated on a turbid white phase. Rhodopsin molecules moved to the upper phase, while other proteins moved to

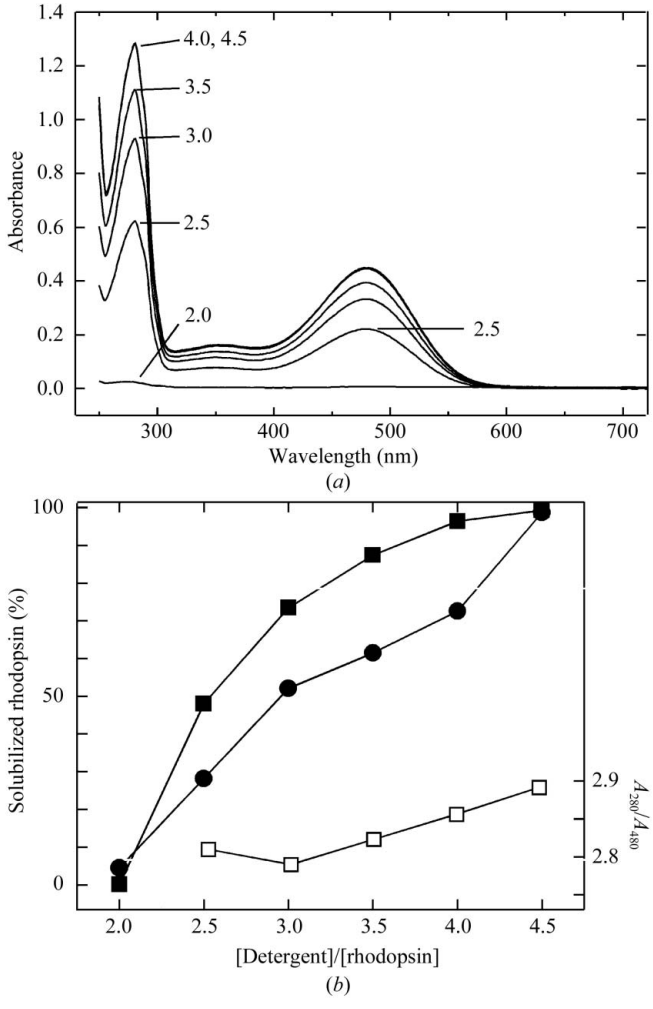


Figure 2
Extraction of rhodopsin from the microvillar membranes at various detergent:protein ratios. (*a*) Absorption spectra of the supernatant in the two-phase solutions generated after addition of octylglucoside and zinc acetate to the microvillar membranes. The numbers in this figure represent the detergent:protein weight ratio. (*b*) The percentage of rhodopsin extracted with octylglucoside (closed squares) and nonylglucoside (closed circles) against fully extracted rhodopsin is plotted against the detergent:protein weight ratio. Open squares represent the optical purity as expressed by A_{280}/A_{480}.

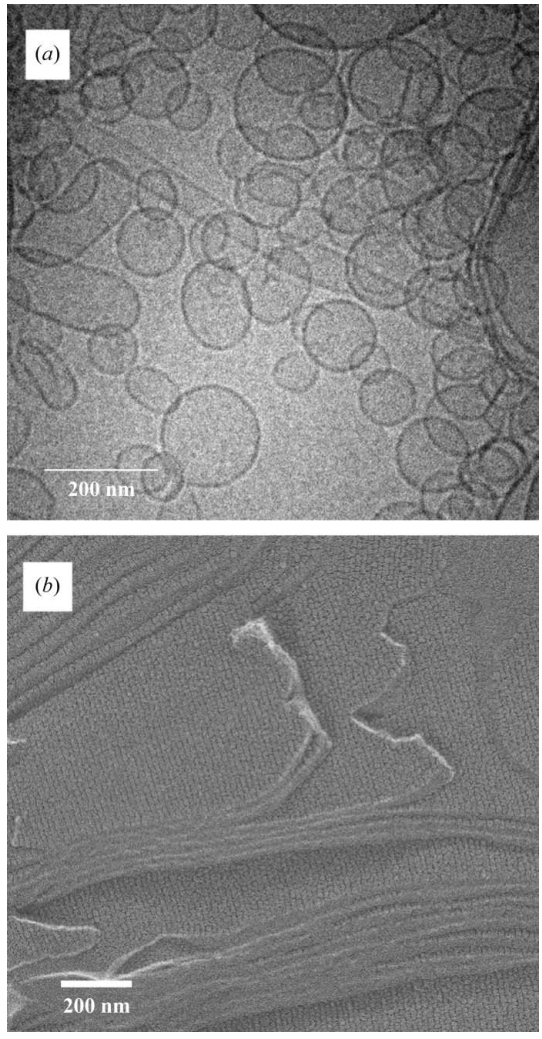


Figure 3
Membranous structures formed upon dialysis of purified rhodospin against an aqueous solution containing 20 m*M* MES pH 6.5 and 1 m*M* EDTA. (*a*) Transmission electron micrograph of vesicules. (*b*) Replica image of stacked membranes.

the lower phase. Centrifugation of the turbid phase at 30 000 rev min^{-1} for 1 h yielded a supernatant containing truncated rhodopsin.

When the concentration of detergent was adequately adjusted, ~80% rhodopsin was selectively extracted from the membranes. The extraction efficiency of rhodopsin increased with increasing amounts of detergent, but other proteins were also extracted at high detergent concentrations (Fig. 2). At the optimal detergent:rhodopsin ratio (3.5:1 by weight), a highly purified sample of squid rhodopsin with a homogeneous molecular weight can be obtained by a single-step extraction procedure (lane 4 in Fig. 1). The optical purity of the extracted rhodopsin, expressed as the ratio of the absorbance at 280 nm to that of 11-*cis* retinal, A_{280}/A_{480}, was 2.82. A similar result was obtained using nonylglucoside as detergent (lane 3 in Fig. 1).

The phase separation mentioned above had previously been utilized for the selective extraction of bovine rhodopsin from membranes of rod outer segments (ROS; Okada *et al.*, 1998). Since the lipid and protein compositions of squid microvillar membranes are different from those of bovine ROS membranes, it is likely that the selective extraction of rhodopsin is a consequence of specific interactions between rhodopsin and detergent (and possibly zinc ions). A recent crystallographic study of cytochrome *c* oxidase has shown that well defined detergent head groups are associated with aromatic residues in a manner similar to phospholipid head groups (Qin *et al.*, 2006). In a current model of bovine rhodopsin, several detergent molecules as well as native lipids are placed near the hydrophobic surface of the protein (Okada *et al.*, 2004). It is possible that the detergent molecules bound to squid rhodopsin mimic the lipid molecules originally associated with the protein, leading to a significant increase in the apparent solubility of rhodopsin.

It still remains unclear how the rhodopsin molecules complexed with detergents are organized in the extracted solution. Nonetheless, it should be noted that large vesicular assemblies and/or stacked membranes formed spontaneously when detergent molecules were dialysed out from the extracted solution of rhodopsin (Fig. 3). These observations suggested that some fraction of the lipid molecules remain attached to the protein even after the selective extraction of rhodopsin. It is possible that the lipid molecules bound strongly to the protein confer stability on the purified rhodopsin, which was able to maintain its original colour at 277 K for over a month. An increasing number of studies have shown that incorporation of native lipids in crystallization solution is effective in improving the stability and quality of membrane-protein crystals (Liu *et al.*, 2004; Okumura *et al.*, 2005).

3.3. Hexagonal crystal

Hexagonal crystals of truncated rhodopsin were obtained using either octylglucoside or nonylglucoside as a detergent. In investigations to date, octylglucoside was more effective in producing large crystals. The hexagonal crystals grown with octylglucoside exhibit strong linear dichroism; *i.e.* they become almost transparent when the polarization plane of the measuring light is parallel to the sixfold axis of the crystal (Fig. 4). This result suggested that the absorption dipole moment of the retinal chromophore is oriented nearly perpendicular to the sixfold axis.

3.4. Preliminary structural analysis

The hexagonal crystals diffracted X-rays to 2.8 Å resolution (Fig. 5). The diffraction data were fitted well by unit-cell parameters

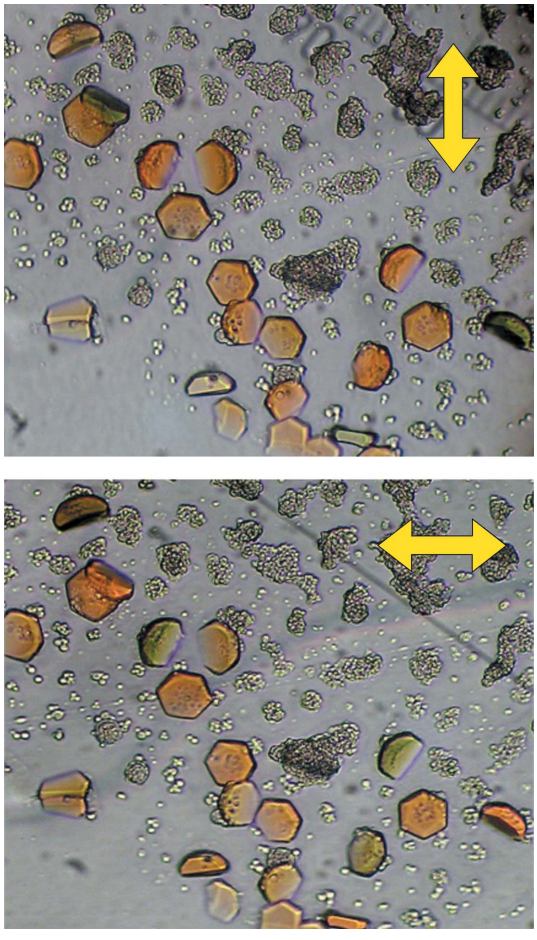


Figure 4
Hexagonal crystals of squid rhodopsin. The arrow in each panel indicates the direction of the polarization plane of the incident light.

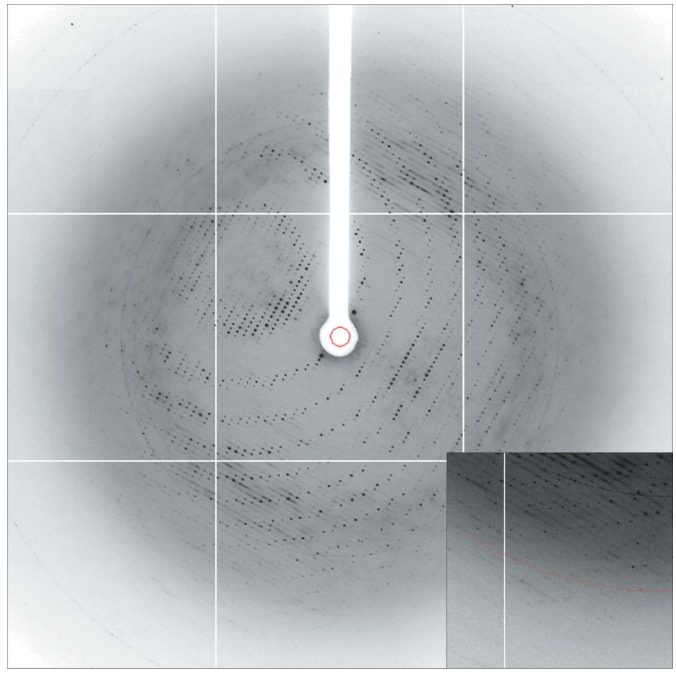


Figure 5
A diffraction image from a hexagonal crystal of squid rhodopsin. This diffraction pattern was recorded with a 1° oscillation at beamline BL41XU of SPring-8. An enlarged image (insert) shows diffraction spots extending beyond 2.8 Å resolution (the red line).

Table 1
Data-collection statistics.

Values in parentheses are for the outer shell.

Resolution (Å)	50–2.8 (2.96–2.80)
Space group	$P6_2$
Unit-cell parameters (Å)	$a = b = 122.1$, $c = 158.5$
R_{merge}†	0.065 (0.545)
No. of observations	89473
No. of unique reflections	32115
$\langle I\rangle/\sigma(I)$	12.6 (1.5)
Completeness (%)	98.1 (99.2)
Multiplicity	2.8
Mosaicity (°)	0.458

† $R_{merge} = \sum_{hkl}\sum_i |I_i - \langle I\rangle| / \sum_{hkl}\sum_i I_i$, where I_i is the intensity of an individual reflection and $\langle I\rangle$ is the mean intensity obtained from multiple observations of symmetry-related reflections.

$a = b = 122.1$, $c = 158.6$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The systematic absence of diffraction spots along the c^* axis indicated the presence of a threefold screw axis. From the histogram of the diffraction intensities, the crystal was suggested to be merohedrally twinned; the twinning fraction varied from 20 to 45% (Yeates, 1997). Crystal parameters and data-collection statistics are summarized in Table 1.

Structural analysis was performed by the molecular-replacement method using the coordinates of the transmembrane region (*i.e.* helices I–VII) of bovine rhodopsin (PDB codes 1u19 and 1gzm) as a search model. When the twinning was taken into consideration, the most likely space group was suggested to be $P6_2$. Taking into account the size of truncated rhodopsin, it was suggested that two rhodopsin molecules are packed in an asymmetric unit; in this case, the Matthews coefficient was calculated to be 4.07 Å^3 Da^{-1}. Together with the linear dichroism results, the structural analysis suggested that the proteins are aligned in such a manner that the transmembrane helices are oriented nearly parallel to the c axis. Further refinement is now in progress.

In contrast to vertebrate rhodopsins, in which the photo-isomerized retinal is eventually released, the retinal chromophore in invertebrate rhodopsins remains bound to the protein moiety under prolonged illumination by light. We observed that the hexagonal crystal of squid rhodopsin was stable under illumination at physiological temperatures. A trigonal crystal of bovine rhodopsin was reported to have similar stability (Salom *et al.*, 2006), but was not suitable for high-resolution structural analysis. Thus, the hexagonal crystal of squid rhodopsin may prove to be useful for structural investigation of the photoactivation processes of rhodopsin.

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