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X-Ray Diffraction Pattern from Internal Structure of Bovine Rhodopsin

Takeshi Kawaguchi,*,[1] Toshiaki Hamanaka,† and Yuji Kito‡

*Department of Electrical and Computer Engineering, Nagoya Institute of Technology, Showa-ku, Nagoya 466; and †Department of Biophysical Engineering, Faculty of Engineering Science, and ‡Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560

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Pure bovine rhodopsin pellets were prepared by removal of cholic acid from rhodopsin-cholic acid complex, and X-ray diffraction patterns from the internal structure of rhodopsin were obtained for the first time using wet and dry pellet samples. The peaks at around 10 and 4.3 Å spacings observed for both samples can be attributed to α-helices in rhodopsin molecule, providing direct evidence of the helical structure of rhodopsin. A shoulder peak at around 34 Å spacing was also observed for the dry pellet, which can be explained by the first-neighbor distance between rhodopsin molecules in the sample.

Key words: membrane protein, protein-detergent complex, protein structure, rhodopsin, X-ray diffraction.

Structural studies of rhodopsin-nonionic detergent complex by small-angle X-ray or neutron scattering were performed to elucidate the shape and size of the rhodopsin molecule. From an X-ray study (*1*) a dumbbell model was proposed for rhodopsin with hydration water, while from a neutron study (*2*) a cylinder model was proposed for unhydrated rhodopsin. Both models agree that rhodopsin is a transmembrane protein, but no information on the internal structure of rhodopsin was obtained from these studies. Structural studies of disk membranes oriented artificially (*3*) or in an intact retina (*4*) were also performed by X-ray or neutron diffraction, but these afforded less structural information on the rhodopsin molecule than small-angle scattering studies of rhodopsin-detergent complex (*5*).

The complete amino-acid sequence of rhodopsin has been determined and the existence of transmembrane helices (7-α-helices) was predicted from the secondary structure of the seven hydrophobic fragments (*6, 7*). The proportion of α-helices in rhodopsin was suggested to be about 50% from the circular dichroism spectrum of rhodopsin in disk membranes or detergent micelles (*8*). However, the helical structure has not been directly confirmed by X-ray or neutron diffraction, since neither a three-dimensional crystal nor a highly dense sample of rhodopsin has yet been obtained.

Recently, two-dimensional crystals of rhodopsin were prepared in frog (*9*) and bovine (*10*) disk membranes and a lipid membrane (*11*). The projection density map of the crystals perpendicular to the membrane plane was obtained from analysis of the electron micrograph. The existence of transmembrane helices (7-α-helices) is actually reported in Ref. *11*. However, there seem to be differences in the projections reported in these references. The problem may be related to preparation of the crystals and/or a damage by electron-beam irradiation. Therefore, we tried to prepare a highly dense sample of native rhodopsin for direct observation of X-ray diffraction from the internal structure of rhodopsin.

Makino *et al.* (*12*) previously reported that bovine rhodopsin solubilized with cholic acid could be purified by salting-out fractionation. By this procedure, we obtained a dense solution of rhodopsin-cholic acid complex (50-100 mg/ml rhodopsin). The X-ray diffraction pattern of the sample was measured using X-ray toroidal focusing optics. Several diffuse rings in the large-angle region, which do not appear with low-concentration samples, were observed in the X-ray film with the naked eye. However, these rings could not be detected with an optical microdensitometer because of the low signal intensity.

Cholic acid is a small ionic detergent (with a molecular weight of 410 and a critical micellar concentration [15-50 mM (*13*)] far higher than that of conventional nonionic detergents such as Triton X-100 [0.24 mM (*14*)]. Therefore, we removed cholic acid from the rhodopsin-cholic acid complex by dialysis. The removal of cholic acid was sufficient for the aggregation of rhodopsin molecules, and a pure rhodopsin pellet was easily obtained by mild centrifugation. Using the pellet sample, we obtained the first clear X-ray diffraction patterns from the internal structure of native rhodopsin.

MATERIALS AND METHODS

Purification of Rhodopsin—Rod outer segments (ROS) of fresh bovine retinae were purified by flotation in 40% (w/v) sucrose solution and sedimentation in 20% sucrose solution. The procedure was repeated at least four times, then 11-*cis*-retinal was added to the purified ROS solution to regenerate opsin in the ROS membrane. The ROS membrane was solubilized with 20 mg/ml cholic acid solution (50 mM sodium phosphate buffer, pH 8.0) saturated with ammonium sulfate (20 %, w/v).

[1] To whom correspondence should be addressed.

The rhodopsin extracted by cholic acid was purified by ammonium sulfate salting-out fractionation, according to the procedure of Makino *et al.* (*12*). The precipitate of the purified rhodopsin was obtained between 50 and 55% (v/v) saturation of ammonium sulfate. The precipitate was dissolved in 50 mM phosphate buffer (pH 8.0). The optical purity (A_{280}/A_{498}) of the rhodopsin solution was 1.62.

Preparation of Rhodopsin Pellet—To remove cholic acid from the rhodopsin solution, 10 ml of the solution was dialyzed using a visking tube against 1,000 ml of 50 mM phosphate buffer (pH 8.0) at 4°C for 2 days (*15*). The buffer solution was changed four times, which was sufficient to allow aggregation of rhodopsin. The aggregated rhodopsin was easily precipitated by mild centrifugation (6,000 × *g*, 30 min). The precipitate was washed twice with 50 mM phosphate buffer (pH 7.0), then the pellet of pure rhodopsin was prepared by centrifugation (20,000 × *g*, 30 min).

The residual phospholipid and cholic acid in the pellet were estimated by the methods of Bartlett (*16*) and Kier (*17*), respectively, to be less than 3 mol of phospholipid and less than 2 mol of cholic acid per mol of rhodopsin.

The pellet was sealed in a thin-walled quartz-glass capillary for X-ray experiments (wall thickness less than 10 μm). Another sample was prepared by drying the pellet overnight in a vacuum of about 10^{-2} Torr No significant denaturation of rhodopsin was observed as a result of the drying process. These samples are referred to respectively as "wet pellet" and "dry pellet."

X-Ray Diffraction Experiment—X-rays were generated by a rotating-anode microfocus generator (Rigaku Denki, RU-100) with a copper target. The generator was operated at 40 kV and 25 mA. The focal size was about 0.1 × 0.1 mm. Ni-filtered CuKα radiation (wavelength 1.542 Å) was used. The thickness of the Ni filter was 10 μm. Elliott toroidal focusing optics (*18*) was used with a set of double sector apertures. The parasitic scattering from the optics, the scattering from the capillary, and the scattering from the beam stop were checked by a blank test with an empty capillary.

The diffraction patterns were recorded on Sakura X-ray films. The photographic density was measured with a microdensitometer (Narumi, type C). The absorbance was converted to the scattered X-ray intensity using a standard scale. The diffraction spacings were calibrated with sodium myristate powder. Most of X-ray path between the sample and the film was evacuated to eliminate air scattering. The sample temperature was maintained at about 4°C using cooling equipment. All experiments were done in the dark or under dim red light.

X-Ray Small-Angle Experiment—We also examined the structure of rhodopsin-cholic acid complex by X-ray small-angle scattering, using the salted-out sample. The result is described in "APPENDIX."

RESULTS AND DISCUSSION

Peaks of 10 and 4.3 Å Spacings—Figure 1 shows the X-ray diffraction photographs of pure rhodopsin pellets [(a) wet pellet, (b) dry pellet]. The patterns are isotropic, suggesting that both samples are in an amorphous state. In both photographs, a diffuse ring is seen at around 10 Å spacing, as shown by an arrow. This is clearer in (b) than in (a), since the dry pellet has a higher density of rhodopsin molecules, and the distance from sample to X-ray film is shorter in (b) than in (a). In photograph (b), another broad ring (4.3 Å spacing) is seen outside the 10 Å ring. This ring was also observed for the wet pellet. Note the small-angle region for photograph (a) and the large-angle region for photograph (b).

Figure 2 shows the X-ray intensity curve of the dry pellet

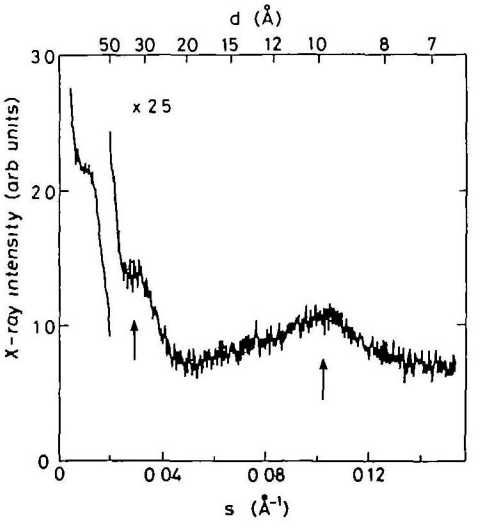

Fig. 2 **X-ray diffraction pattern of the dry pellet.** The intensity curve was obtained by microdensitometer tracing of X-ray film. Two arrows show the shoulder peak at around 34 Å spacing and the peak at around 10 Å spacing.

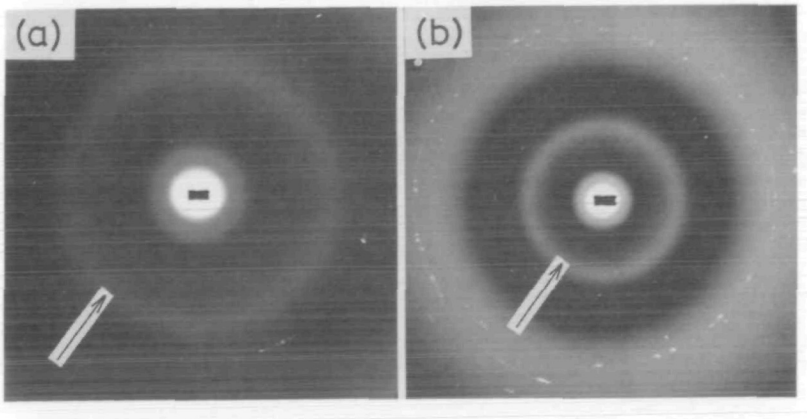

Fig 1. **X-ray diffraction photographs of pure rhodopsin pellets: (a) in a wet state; (b) in a dry state.** In both photographs, the arrow shows a diffraction ring at around 10 Å spacing. The distance from the sample to the X-ray film was (a) 6 cm and (b) 3 cm. The white spots outside the 10 Å ring are due to crystallites of the sodium phosphate salt used in the pH buffer

from about 200 to 6.5 Å spacing. The 10 Å peak shown by the arrow at right is clear but broad. No difference in the profiles of 10 and 4.3 Å peaks was observed between the wet and dry pellets. Therefore, it is suggested that these peaks are attributable to the internal structure of the rhodopsin molecule.

The 10 Å peak has been observed for dense samples of water-soluble proteins such as bovine serum albumin (*19*) and membrane proteins such as bacterio-opsin in brown membranes (*20*). In these reports, the 10 Å peak is explained by interference between X-rays from α-helices in the proteins. That is, the 10 Å spacing represents the average distance between the α-helices. On the other hand, the 4.3 Å peak is explained by interference between X-rays from amino-acids in an α-helix, *i.e.*, the ordering within the polypeptide chain. Therefore, the appearance of both peaks is direct evidence that α-helices are present in the rhodopsin molecule.

Peak of 34 Å Spacing—In Fig. 2, two shoulder peaks are seen at around 100 and 34 Å spacings. The 100 Å shoulder is due to parasitic scattering from the toroidal focusing optics used in this study. The 34 Å shoulder (indicated by the arrow at left) is attributable to interference between X-rays from rhodopsin molecules in the dry pellet, since the shoulder was hardly observed for the wet pellet. If the shoulder were due to interference between X-rays from rhodopsin and residual phospholipid or residual cholic acid, the 34 Å shoulder should also be observed for the wet pellet.

In the case of disk membranes, it is known that the X-ray equatorial diffraction pattern from an oriented disk membrane shows a broad peak centered at around 55 Å spacing (*3*). The equatorial peak has been explained by two-dimensional distribution of rhodopsin in the disk membrane. That is, the 55 Å spacing represents the first-neighbor average distance between rhodopsin molecules (*5*).

According to the cylinder model of rhodopsin reported by Osborne *et al.* (*2*), the cross-section of rhodopsin perpendicular to the membrane plane is approximately represented by a circle with a radius of 17 Å. Therefore, the closest distance between two rhodopsin molecules can be estimated as 34 Å.

If the rhodopsin molecule is spherical, the rhodopsin molecules in the dry pellet may form a hexagonal close-packed structure shown in Fig. 3(a). In this case, the volume of rhodopsin can be estimated from the 34 Å spacing as 2.1×10^4 Å^3. However, this value is too small to be consistent with the molecular weight [39,100 (*21*)] and partial specific volume [0.735 cm^3/g (*22*)] or the volume (about 6×10^4 Å^3) reported by Sardet *et al.* (*1*) and Osborne *et al.* (*2*).

If the shape of rhodopsin is modeled by a prolate ellipsoid of revolution, for example, the close-packed structure shown in Fig. 3(b) can reasonably be expected. In this case, the length of the short axis (a) is estimated from the 34 Å spacing as 15-17 Å. Then, the length of the long axis (γa) is expected to be 50-60 Å from the volume of 6×10^4 Å^3. This size is consistent with that reported for rhodopsin in detergent micelles or disk membranes. For other elongated shape models, such as a cylinder or a dumbbell, a similar size is obtained in the same manner. Therefore, the 34 Å peak of the dry pellet can be explained by the close-packed structure of rhodopsin molecules.

APPENDIX

No structural study of rhodopsin-ionic detergent complex has yet been performed, since the structure of the complex depends on the detergent concentration, pH, ionic strength, and so on (*23*). However, it is worthwhile to verify the structure even under limited solvent conditions of pH or ionic strength. Therefore, we also examined the structure of the rhodopsin-cholic acid complex by small-angle X-ray scattering.

The salted-out rhodopsin was dialyzed against 50 mM phosphate buffer solution (pH 8.0) containing 20 mg/ml cholic acid at 4°C for 2 days. The buffer solution after the dialysis was used as the back-scattering sample. A small-angle camera with three-slits system (Rigaku Denki) and a linear-position-sensitive counter of the delay-line read-out type (Rigaku Denki) were used in the X-ray experiments. The data were analyzed by the method of Kawaguchi *et al.* (*24*).

Figure 4 shows Guinier plots of the small-angle scatter-

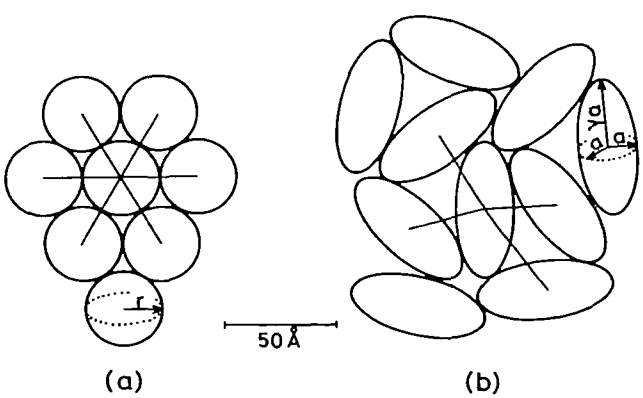

Fig. 3. **Illustration of packing mode of rhodopsin molecules in the dry pellet.** Two models were considered for the shape of rhodopsin: (a) sphere (radius, r); (b) prolate ellipsoid of revolution (semiaxes; a, a, γa).

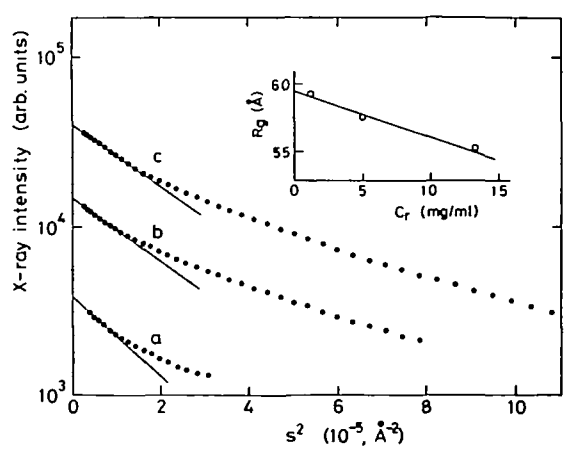

Fig. 4. **Guinier plots of small-angle X-ray scattering curves of rhodopsin-cholic acid complex.** The rhodopsin concentration of samples is (a) 1, (b) 5, and (c) 13 mg/ml. The inset shows the concentration dependence of the radius of gyration.

ing curves. The radius of gyration was 59.3, 57.5, and 55.5 Å for the samples with rhodopsin concentrations of 1, 5, and 13 mg/ml, respectively. The value extrapolated to zero concentration was 59.5 Å, as shown in the inset. The scattering data of the complex in 10 and 25% (w/v) sucrose solvents were also collected, but no significant change in the radius of gyration was observed for the electron-density contrasts. This result suggests that the electron-density distribution of the rhodopsin-cholic acid complex is approximately homogeneous, *i.e.*, the electron density of the cholic acid part is close to that of rhodopsin. From these facts, it follows that the complex involves a polymer of rhodopsin.

If the complex is assumed to be spherical, its volume is determined to be roughly 2×10^6 Å^3. In this case, since the complex contains about 60 molecules of cholic acid per rhodopsin molecule (*12*), one complex would be composed of about 15 molecules of rhodopsin and about 900 molecules of cholic acid (volumes of rhodopsin and cholic acid are about 6×10^4 and 1×10^3 Å^3, respectively).

However, as shown in Fig. 4, the Guinier region seems to be too limited to be consistent with a spherical shape. Furthermore, the plots outside the Guinier region deviate upward from the straight line. These are due to polydispersity in size and/or deviation in shape from the spherical. However, the details of the polydispersity and shape remain unknown. Therefore, the above numbers of rhodopsin and cholic acid molecules in one complex seem to have errors of at least ±20%.

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