Removal of the Carboxyl-terminal Peptide Does Not Affect Refolding or Function of Bacteriorhodopsin As a Light-dependent Proton Pump*

Mei-June Liao‡ and H. Gobind Khorana

From the Departments of Biology and Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

(Received for publication, September 6, 1983)

Treatment of the purple membrane with carboxypeptidase A, Pronase, or papain, results in the cleavage of amino acids from the carboxyl terminus of bacteriorhodopsin, a maximum of about 17 amino acids being released with papain. Protease-treated bacteriorhodopsin, after denaturation, refolds to the native structure, binds retinal as tightly as the intact protein and, on reconstitution into vesicles, gives full proton translocating activity.

The CD spectrum of papain-treated purple membrane shows exciton coupling characteristic of the intact purple membrane. The trimeric bacteriorhodopsin in papain-treated purple membrane dissociates into monomers in Triton X-100 which, after removal of the detergent, reassociate to form the oligomeric structures. Chymotrypsin cleaves papain-treated bacteriorhodopsin between amino acids 71 and 72 as has been previously found for intact bacteriorhodopsin. The resulting fragments C-1 (amino acids 72–231) and C-2 (amino acids 1–71) reassociate, bind retinal, and regenerate the native chromophore, as previously demonstrated for the corresponding fragments from the intact protein. We conclude that the COOH-terminal peptide in bacteriorhodopsin is not required for the correct refolding of denatured bacteriorhodopsin to the native tertiary and quaternary structure, for chromophore regeneration or for light-driven proton translocation.

The purple membrane of Halobacterium halobium is a specialized membrane that carries out light-dependent proton translocation (1–4). BR, the sole protein in this membrane, has been the subject of extensive structural investigations and the secondary structure model shown in Fig. 1 has been proposed on the basis of amino acid sequence (5–7), electron and neutron diffraction data (8–10), proteolysis (5, 11), and the results of cross-linking studies using a photosensitive retinal analog (12). It was shown early (13–15) that the peptide segment at the carboxyl terminus is susceptible to proteolytic cleavage and that different proteolytic enzymes remove different lengths of this segment. It was also concluded that these cleavages do not affect proton pumping (13–15). However, recently, new reports have appeared on the structure and possible functional role of the carboxyl-terminal segment. Wallace and Henderson (16) concluded from an electron diffraction study that the carboxyl terminus is almost entirely disordered. On the other hand, fluorescence studies by Renthal et al. (17) indicated that the carboxyl-terminal segment (amino acids 230–240) is immobilized and may be rigidly held at the membrane surface. Conflicting results have also been reported on the role of the carboxyl terminus in proton pumping function. Gerber et al. (15), Huang et al. (14), Abdulaev et al. (13) and their respective coworkers reported that proteolysis by Pronase or papain did not affect the proton pumping activity in phospholipid vesicles reconstituted with the proteolyzed BR. However, Govindjee et al. (18) observed that, after proteolysis, the yield of light-induced proton release from digested purple membrane sheets decreased by 50–70% and the half-time of proton uptake increased almost 2-fold.

In view of the above reports, we have now carried out a further study of the possible effect of removal of the carboxyl-terminal segment on structure and function of BR. This study has been made possible especially because a number of new structural criteria for BR are now available. Thus, it is known that completely denatured BR can refold to a native structure in lipid/detergent mixtures (19, 20) and upon reconstitution into vesicles, the renatured BR displays full proton-translocating activity (20–22). Furthermore, the oligomeric structure of BR as observed in purple membrane (1, 2) can also be regenerated from the detergent-solubilized BR monomers (19, 23, 24). It has also been shown that the two fragments, C-1 (amino acids 72–248) and C-2 (amino acids 1–71), produced by chymotryptic cleavage of BR associate to generate the native BR chromophore (25, 26). These structural studies have now been carried out with BR proteolyzed at the COOH terminus and we find that the behavior of BR lacking the carboxyl-terminal peptide is virtually identical in every way that we have studied to that of intact BR. We therefore conclude that the carboxyl-terminal peptide of BR is not important in protein refolding, in reassociation of fragments to native structure, in intermolecular interactions leading to the formation of the trimers, or in proton translocation.

EXPERIMENTAL PROCEDURES

Materials

Purple membrane was isolated from Halobacterium halobium (strain S9) as previously described (27). Carboxypeptidase A (diisopropylfluorophosphate-treated) was obtained from Sigma. SDS (electroforesis grade) and Bio-Beads SM-2 were from Bio-Rad. CHAPS, DMPC, and Pronase were purchased from Calbiochem-Behring. Papain (mercuri-papain) was obtained from Worthington. Triton X-100 was from Rohm and Haas Co. All-trans-retinal was purchased from Calbiochem-Behring.
Bacteriorhodopsin Lacking COOH-terminal Peptide

Fig. 1. A proposed model of BR structure. BR consists of one polypeptide chain of 248 amino acids, whose sequence was determined both by amino acid and gene sequencing. The polypeptide is believed to traverse the membrane seven times in the form of α-helical rods. A single retinal molecule linked as a Schiff's base to the ε-amino group of Lys-216 serves as the chromophore. The β-ionone ring of retinal was shown approaching helix 6 with predominant cross-linking sites on Ser-193 and Glu-194 by using photocleavable analog of retinal. Chymotrypsin cleaved BR to two fragments, C-1 (72–248 amino acids) and C-2 (1–71 amino acids). Papain removed about 17 amino acids, Pronase removed 10 amino acids, and carboxypeptidase A removed three to four amino acids from the COOH terminus of BR.

from Eastman. Soybean phospholipids were obtained from Associated Concentrates, Woodside, NY.

Methods

Proteolysis of Purple Membrane—With papain, purple membrane (1.2 mg/ml) was incubated with cysteine-activated papain in 10 mM Tris buffer (pH 7) at a BR to papain ratio of 120:1 (w/w) at 37 °C for 3.5 h. With Pronase, purple membrane was incubated with Pronase in 10 mM Tris buffer (pH 8) containing 0.15 M NaCl at a BR to Pronase ratio of 100:1 at 37 °C for 4.5 h. With carboxypeptidase A, purple membrane was digested with LiCl-activated carboxypeptidase A in the above buffer (pH 8) at a BR to enzyme ratio of 50:1 at 37 °C for 2.5 h. In each case, after digestion, the purple membrane was pelleted by centrifugation (100,000 × g, 30 min) and washed twice with distilled water.

Preparation of Delipidated Bacterio-opsin and of Chymotryptic Fragments, C-1 and C-2—These were prepared, respectively, from purple membrane and from papain-digested purple membrane as described previously (20, 28). The final protein or the fragments solutions (20–25 μM) contained 0.2% (w/v) SDS and 10 mM NaP, (pH 6). Since no aromatic amino acid was removed by proteolysis, protein concentration for protease-treated delipidated bacterio-opsin preparation and the fragments C-1 and C-2 was determined from absorbance at 280 nm in SDS using the same extinction values as previously described (26). t

Denaturation of Purple Membrane in SDS—Protease-treated or untreated purple membrane (0.4 mg) was denatured in 20 μl of 10% (w/v) SDS at 37 °C for 1 h as judged by the disappearance of purple chromophore and subsequent appearance of yellow color (free retinal). 0.98 ml of 10 mM NaP buffer (pH 6.0) containing 0.025% (w/v) NaN₃ was then added to obtain a final solution containing 15 μM bacterio-opsin in 0.2% (w/v) SDS.

Regeneration of BR Chromophore—BR chromophore was regenerated from denatured purple membrane (3 nmol of protein in 0.2% SDS), from delipidated bacterio-opsin (3 nmol) or from a mixture of C-1 (3 nmol) and C-2 (3 nmol) in 0.4 ml of DMPC/CHAPS/SDS (2% (w/v) DMPC, 0.75% (w/v) CHAPS, 0.1% (w/v) SDS, 50 mM NaP, 0.025% (w/v) NaN₃) at pH 6 as previously described (26).

Regeneration of Oligomers of BR—Papain-treated purple membrane (3 mg) was suspended in 0.5 ml of 5% (w/v) Triton X-100 in 0.1 M NaP at pH 6 and kept at room temperature in the dark for 3 days. The solution was diluted to 3 ml with water containing 0.025% (w/v) NaN₃ and centrifuged at 100,000 × g for 2 h to remove any unsolubilized purple membrane. No precipitate was detected after centrifugation. To form BR oligomers, the sample was treated with Bio-Beads SM-2 to remove Triton X-100 as described (19) except that the final solution contained 5 mM NaP (pH 6).

Reconstitution of BR or Protease-treated BR into Phospholipid Vesicles and Proton Pumping Assays—Purple membrane or protease-treated purple membrane (3.8 nmol of protein) denatured in 0.3 ml of 0.2% (w/v) SDS was mixed with trans-retinal (1 μl of 4.5 mM in ethanol). To the mixture was added 0.4 ml of 2% (w/v) soybean phospholipid/2% (w/v) CHAPS in 10 mM NaP, (pH 6). Vesicles were then reconstituted from the above mixture by the dialysis method.
Bacteriorhodopsin Lacking COOH-terminal Peptide

(20, 26), Vesicles from delipidated bacterio-opsin in SDS (3.5 nmol of protein) were prepared by the same procedure (20).

**Absorbance Measurements**—Absorbance measurements were made in 1-cm light path quartz cells using a Cary 15 spectrophotometer at 21 ± 2 °C as described (26).

**Circular Dichroism Measurements**—Visible CD spectra of samples containing 1 mg of protein/ml and buffer blanks in 1-cm light path quartz cells were measured at 29 ± 2 °C in a Cary 60 instrument with a 6002 CD attachment. UV-CD spectra of samples containing 0.2 mg of protein/ml were taken in 1-mm light path quartz cells. Instrument calibration has been described previously (20), [θ] in UV-CD spectrum is reported in units of degrees.cm²/dmol of amino acid residues and in visible CD spectrum as units of degrees.cm²/dmol of chromophore. The experimental error of [θ] is within 3%.

**RESULTS**

**Proteolytic Cleavage of Peptides from the Carboxyl Terminus of Bacteriorhodopsin**—Purple membrane was digested with papain, Pronase, and carboxypeptidase A and the products were examined by SDS-polyacrylamide (15%) gel electrophoresis. As seen in Fig. 2, BR was cleaved to different extents. Papain cleaved approximately 17 amino acids (13), Pronase removed about 10 amino acids (14), while digestion with carboxypeptidase A resulted in the removal of only three to four amino acids (15). In each case, a single band was observed (Fig. 2), showing that proteolysis occurred only at or near the carboxyl terminus. Furthermore, the band corresponding to the intact BR was absent in every case. Thus, the digestion went to completion to give products of essentially uniform size.

**Renaturation of Protease-digested Bacterio-opsin and Regeneration of Native Chromophore with Retinal**—Protease-treated purple membrane samples prepared as described above, were denatured in SDS as described under "Methods." Renaturation was then performed by the addition of DMPC/CHAPS and retinal ("Methods"). Fig. 3 shows the kinetics of regeneration of the intact BR chromophore in the different samples. The half-times of the fast phase are similar for the proteolyzed BR samples and untreated proteins, although the extents of regeneration after 1 day are somewhat different. The Papain- and papain-digested BR gave 80% of chromatophore regeneration. Carboxypeptidase A-digested protein, on the other hand, gave virtually quantitative regeneration (96%). It should be noted that renaturation of BR and proteolyzed BR using higher initial protein concentration (37 μM instead of 17 μM) gave lower yield of the chromophore (data not shown). This effect of initial protein concentration is particularly striking with papain- and pronase-treated BR samples (50% yield). We ascribe it to the increased tendency of the protein to aggregate following removal of a substantial portion of the hydrophilic COOH-terminal segment. Thus, in a separate experiment, when papain-treated delipidated bacterio-opsin, which had been well solubilized in SDS by the dialysis method (20), was used for regeneration, the extent of chromatophore regeneration increased to 88% (see below).

**Binding of Retinal to Bacterio-opsin Lacking COOH-terminal Peptide**—Solutions of delipidated bacterio-opsin and papain-treated delipidated bacterio-opsin in SDS were incubated overnight with different amounts of all-trans-retinal in DMPC/CHAPS. The titration curves obtained (Fig. 4) showed similar retinal binding constants (about 10⁷ M⁻¹) for delipidated bacterio-opsin and papain-digested delipidated bacterio-opsin. The data of Fig. 4 also show the same chromatophore extinction coefficient, (ε₅₂₀ = 52,000) at pH 6, as calculated from the initial slope of the titration curve. It is also seen in Fig. 4 that the extent of regeneration of the chromatophore from papain-treated bacterio-opsin was about 88% of that from untreated bacterio-opsin.

**Association of the Fragments C-1 and C-2 to Regenerate the Native Chromophore Is Not Affected by the Absence of the Carboxyl-terminal Peptide from C-1**—As shown previously, the chymotryptic fragments C-1 and C-2 associate to form a bacterio-opsin-like complex which binds retinal as tightly as the intact protein (26). A similar experiment was carried out with C-1 prepared from the proteolyzed purple membrane and C-2. The behavior of C-1 lacking the carboxyl-terminal pep-

---

2 The kinetics of chromatophore formation can be described by a predominant (78% of protein) fast phase (t½ = 2.5 min) and (a) much slower phase(s) (26).
opsin 0.2 ml of papain-treated or -untreated delipidated bacterio-opsin (3 nmol of protein in 0.2% (w/v) SDS, pH 6) was mixed with 0.2 ml of DMPC (4% w/v)/CHAPS (1.5% (w/v)) and various amounts of retinal. Absorbance was recorded after 1 day of incubation in the dark.

The regenerated membrane showed adaptation and 565 nm after light adaptation. These values of the trimeric state of BR. In Fig. 6A are shown the CD spectral profiles obtained for purple membrane and the papain-digested BR. In the purple membrane, BR is present as trimers and these form a two-dimensional hexagonal lattice. Solubilization of the purple membrane in Triton X-100 produces BR trimers. Although the nature of interactions between BR monomers is not known, it was of interest to determine whether the hydrophilic carboxyl-terminal segment is required for the regeneration of the oligomeric structure of BR in the above type of experiment.

CD spectroscopy was employed to distinguish between the oligomeric and monomeric state of BR (24, 29–31) since the exciton coupling in the visible CD spectrum is characteristic of the trimeric state of BR. In Fig. 6A are shown the CD spectral profiles obtained for purple membrane and the papain-digested purple membrane. It is seen that both profiles are identical with respect to exciton coupling effect, showing that the two-dimensional crystal lattice is completely unaffected after papain digestion of the purple membrane. When the proteolyzed purple membrane was solubilized in Triton X-100, the spectrum (Fig. 6A) showed only a weak single peak, similar to that of intact BR monomers in Triton X-100 (19). Upon subsequent removal of Triton X-100 by Bio-Beads (“Methods”), regeneration of oligomeric form was observed. The regenerated membrane showed \( \lambda_{max} = 557 \text{ nm} \) after dark adaptation and 565 nm after light adaptation. These values are similar to those shown by the purple membrane. Fig. 6B shows that the regenerated membrane regains the exciton coupling effects characteristic of their trimeric form. The \( \theta \) between the two extrema (at 520 and 595 nm) indicates a restoration of the intensity. The wavelength of the extrema and crossover point are also close to that originally observed in the purple membrane.

Protein pumping activities of protease-digested BR reconstituted with soybean phospholipids

Reconstitution of proteins in vesicles was carried out as described under “Methods.” Aliquots used per assay contained 0.14 nmol of the proteins in 25-\( \mu \)l vesicles solution which was mixed with 2 ml of 2 M NaCl (14, 20).

![FIG. 4. Binding of all-trans-retinal to renatured bacterio-opsin (○) and to renatured papain-treated bacterio-opsin (□). 0.2 ml of papain-treated or -untreated delipidated bacterio-opsin (3 nmol of protein in 0.2% (w/v) SDS, pH 6) was mixed with 0.2 ml of DMPC (4% w/v)/CHAPS (1.5% (w/v)) and various amounts of retinal. Absorbance was recorded after 1 day of incubation in the dark.](http://www.jbc.org/)

![FIG. 6. CD spectra of BR and papain-treated BR at visible wavelengths. A, BR in purple membrane (pm) (0.8 mg/ml in 10 mM NaPi, pH 6); ○, BR in papain-digested purple membrane (0.9 mg/ml in the same buffer); X, solubilized papain-treated BR (0.5 mg/ml in 0.8% Triton X-100, 17 mM NaPi, pH 6). B, ○, CD of regenerated “purple membrane” (0.27 mg/ml in 5 mM NaPi, pH 6) from solubilized BR; ○, from solubilized papain-treated BR. Regeneration procedures were described under “Methods.”](http://www.jbc.org/)
Boxyl peptide segment contains five different carboxyl groups that are possible of a structural role for the carboxyl-terminal peptide. Since monomers seem to be arranged in head to tail fashion within the trimer, the carboxyl-terminal segment could be involved in interactions between the monomers. Third, the carboxyl-terminal peptide may be important in the determination of orientation of BR molecules in the membrane. In the Halobium cells, the carboxyl terminus is on the cytoplasmic side, while in the reconstituted vesicles it is exclusively on the external side. Since the endogenous phospholipids in purple membrane are highly asymmetric, it is possible that specific interaction between carboxyl-terminal peptide and phospholipids are important in orienting BR in the membrane.

The present results show, however, that none of the properties characteristic of BR are affected by treatment of the latter with papain, Pronase, or carboxypeptidase A. Thus, proteolyzed BR after denaturation in SDS can refold to a native conformation and bind retinal as tightly as intact BR, and, upon reconstitution into phospholipid vesicles, it translocates protons as efficiently as intact BR. Furthermore, the direction of proton pumping in reconstituted vesicles by BR lacking the carboxyl-terminal peptide is the same as that of reconstituted intact BR, being opposite to that in the whole cells. Thus, the inside out orientation of BR in reconstituted vesicles is not influenced by the presence or absence of the carboxyl-terminal peptide. The CD spectrum of the papain-digested purple membrane shows the same exciton coupling effect as the intact purple membrane, indicating that removal of the carboxyl-terminal peptide does not affect the trimeric structure of BR. This is in agreement with the conclusion of Wallace and Henderson (16) drawn from their diffraction studies. We also find that BR monomers lacking the carboxyl-terminal peptide interact to regenerate the oligomeric structure, with properties similar to those of the original purple membrane (CD exciton coupling, λMAX, after light and dark adaptation and the sedimentation behavior). Since, as now shown, the formation of the BR trimers does not require carboxyl-terminal peptide, the nature of interaction between monomers remains unknown. Finally, fragment C-1, prepared by chymotryptic cleavage of BR lacking the carboxyl-terminal peptide also binds to C-2 and retinal to form the native BR-like chromophore. This result further confirms that the interaction between helices during refolding as well as the interaction between the two fragments does not require the carboxyl-terminal peptide.

The above results on proton pumping by vesicles reconstituted from BR lacking the carboxyl-terminal peptide are at variance with those obtained by Govindjee et al. (18). However, it should be noted that the system and the experimental method used by these workers are different from those used in the present work. Govindjee et al. (18) measured the light-induced proton release or uptake from the proteolyzed purple membrane sheets by monitoring the proton concentration using a pH-sensitive dye. The time scale in their measurements was in the millisecond range while the time scale in our proton pumping measurements is in the second range. It could be argued that the rate-determining step in proton pumping is the entry of the counterions such as chloride ions into the reconstituted vesicles. Nevertheless, we believe that our initial pumping rates are not limited by the transport of counterions across the vesicular membrane because the initial rates are found to be approximately proportional to the amount of protein present in the vesicles. Furthermore, Govindjee et al. (18) used the crystalline purple membrane lacking the terminal peptide while we have used the reconstituted vesicles that contained monomers. The removal of the above-mentioned negative charges present in the carboxyl-terminal peptide may affect the purple membrane sheets differently.

**DISCUSSION**

The exposed hydrophilic segments in polytopic integral membrane proteins may serve specific functions. For example, rhodopsin, which like BR is believed to traverse the membrane seven times, has an exposed carboxyl-terminal segment. This contains the sites of light-dependent phosphorylation (32). Similarly, one could envisage a number of possibilities for a function of the carboxyl-terminal segment in BR. The carboxyl peptide segment contains five different carboxyl groups (terminal serine, 3 glutamic acid residues and 1 aspartic acid residue) and one could postulate that one of these groups serves as the proton donor in the early events in the photochemical cycle, presumably, involving transfer of a proton from the cytoplasm to the Schiff's base at Lys-216. Next, there is the possibility of a structural role for the carboxyl-terminal peptide. In the purple membrane, BR is present as trimers. Since monomers seem to be arranged in head to tail...
from the proteolyzed BR in our reconstituted vesicles. Absence of the negative charges from the purple membrane surface may cause adherence of the purple membrane sheets at the cytoplasmic face. This aggregation may hinder equilibration of protons between the sheets and the bulk aqueous solution. Indeed, we have observed that renaturation shows dependence on initial protein concentration (see “Results”) and similarly, Wallace and Henderson (16) have suggested that the less ordered diffraction pattern of the proteolyzed purple membrane than that of the native membrane may be due to the aggregation of the membrane.

REFERENCES

Removal of the carboxyl-terminal peptide does not affect refolding or function of bacteriorhodopsin as a light-dependent proton pump.

M J Liao and H G Khorana


Access the most updated version of this article at http://www.jbc.org/content/259/7/4194

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/7/4194.full.html#ref-list-1