Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5426–5430, December 1977
Biochemistry

Orientation of bacteriorhodopsin in *Halobacterium halobium* as studied by selective proteolysis

(purple membrane/apomembrane/retinal/proton pump/reconstituted vesicles)

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ABSTRACT **The orientation of bacteriorhodopsin in the purple membrane of *Halobacterium halobium* has been studied by proteolytic degradation of purple membrane sheets, reconstituted vesicles, and whole cells, with the following results: (*i*) Bacteriorhodopsin in purple membrane sheets is cleaved at a single site by Pronase or trypsin; a polypeptide segment of about 15 amino acids is lost from the carboxyl end. Carboxypeptidase A sequentially releases amino acids from the carboxyl end; the tetrapeptide sequence -Ala-Ala-Thr-Ser(COOH) was tentatively deduced for this terminus. (*ii*) The apomembrane, which lacks retinal, undergoes a second cleavage with trypsin releasing a fragment of approximately 6300 molecular weight from the amino terminus. (*iii*) Vesicles reconstituted from the purple membrane sheets and synthetic lecithins, in which the direction of proton pumping is opposite to that in the whole cells, have the carboxyl terminus of bacteriorhodopsin accessible to proteolysis. (*iv*) In envelope vesicles, which largely pump protons in the same direction as the whole cells, the carboxyl terminus is largely protected against proteolysis. (*v*) Treatment of whole cells with proteinase K hydrolyzes the cell wall proteins but has no effect on bacteriorhodopsin. However, the same treatment after lysis of the cells results in degradation of the hydrophilic region at the carboxyl terminus. The results show that the carboxyl terminus as well as the additional cleavage site near the amino terminus observed in apomembrane are on the cytoplasmic side of the purple membrane.**

The purple membrane of a number of extremely halophilic bacteria, e.g., *Halobacterium halobium*, functions as a light-driven proton pump (1–4). It contains a single protein, bacteriorhodopsin, of molecular weight approximately 26,000 (1, 5) with one molecule of retinaldehyde (1, 2) covalently bound to a lysine residue. Bacteriorhodopsin constitutes 75% of the total weight of the purple membrane; the remainder is a special set of phospholipids (6). Because the protein forms highly regular arrays in the purple membrane, it has been possible to deduce the electron density map at 7-Å resolution by electron microscopy (7). Thus, a model has been proposed in which the bacteriorhodopsin molecule forms a continuum of seven α helixes, each of which spans the membrane and is largely embedded in it. No further information is available at present regarding the orientation or chemical structure of bacteriorhodopsin in the purple membrane, although some initial work on the primary amino acid sequence has been reported by Keefer and Bradshaw (8).

In view of our interest in the structure and function of bacteriorhodopsin (9), we have investigated selective proteolysis of the purple membrane in sheets as well as in vesicles and whole cells. In addition, the apomembrane, which lacks retinaldehyde, has been studied. The following results, which are of interest in regard to the topology of the protein, have been obtained. The protein is largely embedded in the membrane; only a small region [~1,500 molecular weight (M_r)] at the carboxyl terminus is accessible to the proteolytic enzymes. The retinal-free apomembrane shows susceptibility at an additional site; this cleavage results in a fragment of M_r approximately 6300 from the amino terminus. The possibility is considered that this cleavage occurs at or near the retinal binding site. Finally, the results of proteolysis obtained with purple membrane sheets, reconstituted vesicles, and whole cells are all consistent with the conclusion that the carboxyl terminus as well as the additional cleavage site in the apomembrane are on the cytoplasmic side of the purple membrane. A preliminary account of these findings has appeared (10).

MATERIALS AND METHODS

Materials

Trypsin from porcine pancreas was purchased from Miles Laboratory and Pronase from Calbiochem; deoxyribonuclease, ovalbumin, cytochrome *c*, soybean trypsin inhibitor, basic pancreatic trypsin inhibitor, and carboxypeptidase A (di-isopropylfluorophosphate-treated) were all from Sigma Chemical Co.; proteinase K was from Boehringer-Manheim, Germany. Myoglobin was obtained from Beckman.

Methods

Preparation of Purple Membrane. *Halobacterium halobium* cells were grown, and the purple membrane was isolated by sucrose density gradient as described (9, 11). The concentration of the purple membrane was determined by using the molar extinction coefficient at 560 nm of 6.3×10^4 (1, 5); this value was confirmed by amino acid analysis.

Preparation of Apomembrane. The purple membrane was suspended (2 mg/ml) in 4.0 M NaCl containing 1.0 M NH_2OH·HCl (pH 7.0) and the stirred suspension was irradiated at 25° with a 500-W quartz halogen lamp with a Schott 530 filter until the purple color had completely disappeared (12). The apomembrane was collected by centrifugation, washed twice with distilled water, and used immediately for proteolysis.

High-Voltage Paper Electrophoresis. The supernatant obtained on centrifugation of the trypsin digest of the purple membrane was lyophilized, the residue was redissolved in water, and the solution was applied to Whatman 3MM paper (up to 0.1 μmol/cm). The paper was wetted with pyridine acetate (pH 6.1) (pyridine/acetic acid/water, 100:4:900) and

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Abbreviations: M_r, molecular weight; $NaDodSO_4$, sodium dodecyl sulfate.

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electrophoresis was carried out at 20 V/cm for 2 hr. The peptides were identified by staining guide strips with ninhydrin reagent. The peptides were recovered by chromatographic elution with 1% pyridine.

Polyacrylamide Gel Electrophoresis. This was carried out in 18% polyacrylamide gels containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) as described by Laemmli (13). The gels were fixed and stained as described by Fairbanks *et al.* (14).

Amino Acid Analysis. Peptide or protein samples, dried in Pyrex glass tubes, were dissolved in constant-boiling HCl (0.5 ml). The tubes were evacuated, sealed, and heated at 110° for 24 hr. Amino acid analyses were performed on the hydrolysates in a Beckman 119C amino acid analyzer.

Determination of the Amino Terminus by Dansylation Procedures. This was performed by the procedure of Weiner *et al.* (15).

Preparation of vesicles

Reconstituted Vesicles with Synthetic Phospholipids. These were prepared by sonication of the purple membrane and synthetic dimyristoyl or other phosphatidylcholine in 0.15 M KCl (16). The proton pumping activity from outside to inside was in the range of 160 neq/mg of the protein.

Envelope Vesicles. These were prepared essentially by the published procedure (17, 18), except that sonication was done with a Branson sonifier W 185 (50 W, four times at 15 sec). After centrifugation at 46,000 × *g* for 30 min, the pellet was resuspended in 4 M NaCl/50 mM Tris·HCl (pH 8)/5 mM $CaCl_2$.

RESULTS

Release of a water-soluble peptide from carboxyl terminus

The purple membrane sheets were treated with trypsin and degradation was followed by the formation of water-soluble peptide(s) as well as by electrophoresis on a $NaDodSO_4$/polyacrylamide gel (Fig. 1). The reaction leveled off with the release of about 15 mol equivalents of amino acids per mole of the protein in about 5 hr and the concurrent formation of a single new large product identified by polyacrylamide gels. The latter product corresponded to M_r approximately 24,500, i.e., 1500 less than that of bacteriorhodopsin (Fig. 1). The cleavage at the above site (designated site I) was inhibited by 0.15 M NaCl (the activity of trypsin on synthetic substrates is not inhibited under these conditions).

The water-soluble peptide formed was purified by high-voltage paper electrophoresis and showed the following amino acid composition: Asp, 2.1; Thr, 1.9; Ser, 1.7; Glu, 1.1; Gly, 3.0; and Ala, 5.1. The fact that no lysine or arginine was present in this fragment, which was produced by trypsin digestion, showed that it was derived from the carboxyl terminus of the protein. Treatment of the peptide with carboxypeptidase A resulted in the sequential release of serine (1.0), threonine (1.0), and alanine (2.0). The degradation with carboxypeptidase A was also followed kinetically by using the purple membrane directly; the results shown in Fig. 2 were obtained. Again, the amino acids serine, threonine, and alanine were released sequentially, suggesting that the sequence at the carboxyl terminus of the protein is -Ala-Ala-Thr-Ser(COOH).

Failure to detect an NH_2 group in bacteriorhodopsin and in products formed after cleavage by trypsin

Results of attempts to detect a free amino group in bacteriorhodopsin by the dansylation procedure were negative, in agreement with the conclusion of others (8, 19). Further, no free amino group was detected after cleavage by trypsin as described in Fig. 1. This result showed that the cleavage by trypsin released a fragment only from the carboxyl terminus.

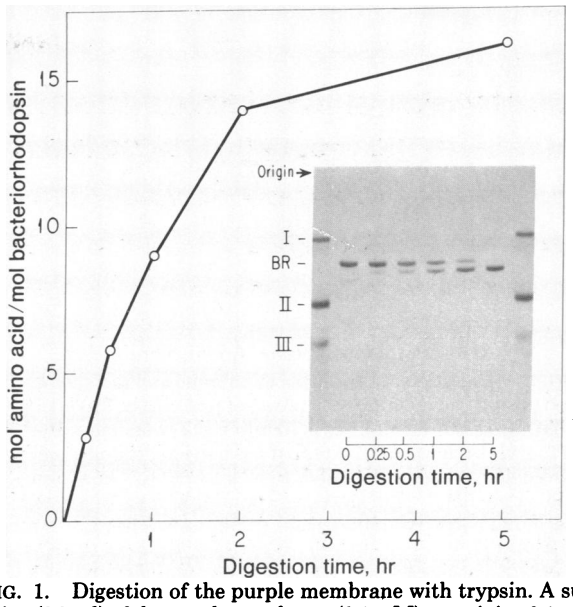

FIG. 1. Digestion of the purple membrane with trypsin. A suspension (0.5 ml) of the purple membrane (0.1 mM) containing 0.1 mM norleucine in 10 mM Tris/KOH (pH 8) buffer was treated with porcine trypsin (final concentration, 25 g/ml). At intervals aliquots were removed and treated with one-third volume 0.1 M HCl and the precipitate that formed was sedimented by centrifugation at 8000 × *g* for 2 min. Aliquots of the supernatants were subjected to amino acid analysis; the values were corrected for the enzyme and substrate blanks. The pellets of precipitated membrane were dissolved in 1% $NaDodSO_4$ solution and aliquots (5 μg of protein) were subjected to electrophoresis on a $NaDodSO_4$/polyacrylamide gel. The markers used and their molecular weights are: I, carboxypeptidase A (34,600); II, soybean trypsin inhibitor (21,500); and III, lysozyme (14,400). BR, bacteriorhodopsin.

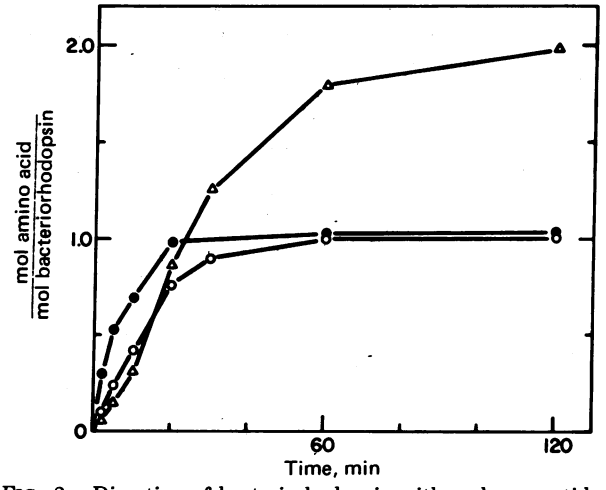

FIG. 2. Digestion of bacteriorhodopsin with carboxypeptidase A. The purple membrane (0.1 mM) in 20 mM Tris/KOH buffer (pH 8.5), 0.15 M KCl, and 0.1 mM norleucine was incubated at 37° with carboxypeptidase A (final concentration, 40 μg/ml). At different time intervals, aliquots were removed and the reaction was terminated by the addition of 4 volumes of 0.2 M sodium citrate (pH 2.2). The precipitate was pelleted by centrifugation at 8000 × *g* for 2 min, and an aliquot of the supernatant was subjected to amino acid analysis. The recovery was corrected by norleucine, the internal standard. The enzyme blank was negligible in all cases. △, Alanine; ●, serine; ○, threonine.

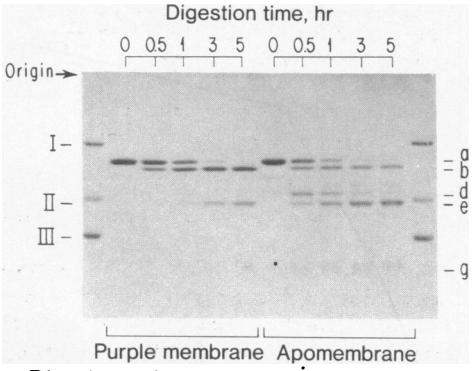

FIG. 3. Digestion of the purple membrane and the apomembrane with trypsin. The membranes (120 μg of protein) in 120 μl of 50 mM Tris·HCl (pH 7.5)/30 mM NaCl/10 mM $CaCl_2$ were incubated at 37° with 1 μg of porcine trypsin. At the times indicated, 20-μl aliquots were removed and treated with 2 μl of 10% $NaDodSO_4$. The samples were boiled immediately and portions containing 5 μg of protein were subjected to polyacrylamide gel electrophoresis. The protein markers were as in Fig. 1.

Digestion of apomembrane with trypsin, proteinase K, and Pronase

In Fig. 3 are shown the results of treatment of the purple membrane and of the apomembrane with trypsin. (Although not shown, the results with Pronase were similar to those in Fig. 3.) As is seen, the result with the purple membrane was as described in Fig. 1. However, with the apomembrane an additional site (designated as site III) sensitive to trypsin (or Pronase) was observed. Cleavage at this site alone resulted in the formation of two fragments of M_r 19,700 and M_r 6300 (fragments d and g, Fig. 3). Further cleavage of the large fragment at site I gave fragment e (M_r 18,200). The rates of attack at the two sites in the apomembrane were comparable under the conditions used since intermediate products resulting from cleavage at either site were observed. Further, the cleavage at site III in apomembrane occurred to a small extent with the purple membrane as well. This result is ascribed to the presence of some retinal-free protein in the purple membrane preparation since the extent of cleavage did not increase either when the ratio of enzyme to purple membrane was increased to 1:1, instead of the usual 1:20, or when the digestion was prolonged to 18 hr instead of 3 hr, in which time the cleavage at site III in apomembrane was essentially complete.

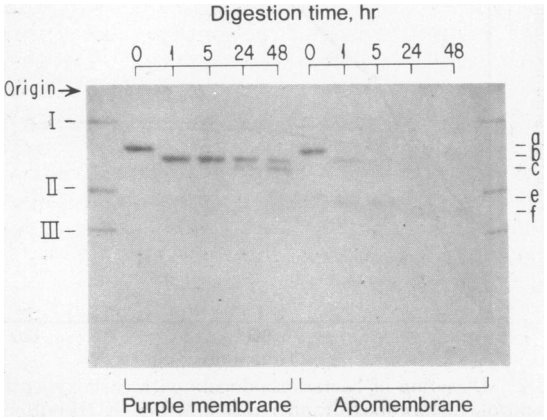

FIG. 4. Digestion of the purple membrane and apomembrane with proteinase K. The membranes (200 μg of protein) were incubated at 37° with 40 μg of proteinase K in 1 ml of 50 mM Tris·HCl (pH 8.0)/4 M NaCl/10 mM $CaCl_2$. At the time intervals indicated, 100-μl aliquots were removed. The reaction was terminated with 1 μl of 0.1% phenylmethylsulfonyl fluoride and 10 μl of 10% $NaDodSO_4$, and the samples were boiled immediately. Aliquots containing 5 μg of protein were subjected to polyacrylamide gel electrophoresis. The protein markers were as in Fig. 1.

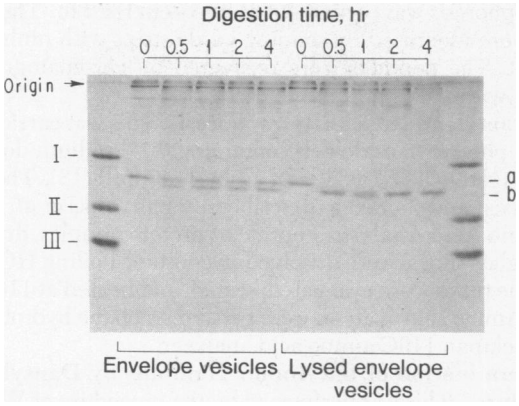

FIG. 5. Kinetics of digestion of "envelope" vesicles with proteinase K. "Envelope" vesicles containing 60 μg of bacteriorhodopsin were suspended in 240 μl of 50 mM Tris·HCl (pH 8.0)/4 M NaCl/5 mM $CaCl_2$. The suspension was incubated at 37° with 3 μg of proteinase K. At the time intervals indicated, aliquots (40 μl each) were withdrawn and the reaction was terminated by the addition of 1 μl of 0.1% phenylmethylsulfonyl fluoride. After addition of 16 μl of 10% $NaDodSO_4$, each sample was diluted to a final volume of 160 μl and boiled, and a portion (30 μl) was subjected to polyacrylamide gel electrophoresis. An identical amount of "envelope" vesicles was sedimented by centrifugation at 45,000 × *g* for 30 min and the pellet was suspended in 36 μl of H_2O at 25° for 30 min. The lysed vesicles were then digested with proteinase K and aliquots were subjected to polyacrylamide gel electrophoresis. Markers I–III were as in Fig. 1.

Fig. 4 shows the results of treatment of the purple membrane and the apomembrane with proteinase K. In addition to the cleavage noted above with trypsin or Pronase, a cleavage at a new site (designated site II) was observed in both cases. As shown by the kinetics in Fig. 4, the proteinase K-specific cleavage occurred more slowly than the cleavages described above (Fig. 3). Thus, with purple membrane, the large fragment first formed (M_r 24,500) corresponded to the cleavage at site I, and subsequent cleavage at site II gave the large fragment of M_r 23,500. With the apomembrane, the large fragment, ultimately formed as a result of the three cleavages, corresponded to M_r 17,200. As described below, the proteinase K-specific cleavage probably occurs at a site adjacent to site I, which in turn is proximal to the carboxyl terminus. In addition to the above cleavages, prolonged incubation of the apomembrane or of the purple membrane in the absence of 4 M NaCl with proteinase K resulted in complete degradation.

Orientation of bacteriorhodopsin in vesicles and *H. halobium* cells

Vesicles Reconstituted from Purple Membrane Sheets and Synthetic Lecithins. In these vesicles, the direction of proton pumping is opposite that in the whole cells, and the carboxyl terminus of bacteriorhodopsin is largely (85–95%) accessible to proteolysis. Thus, treatment of the reconstituted vesicles with carboxypeptidase A released terminal amino acids (Fig. 2) in the expected amount and treatment with Pronase resulted in cleavage at site I. Removal of the fragment with M_r 1500 from the carboxyl terminus had no effect on the proton pumping activity of bacteriorhodopsin in the reconstituted vesicles.

Envelope Vesicles. Sonication of whole *H. halobium* cells yields "envelope" vesicles that have largely the correct sidedness since they pump protons in the same direction as do the whole cells (17, 18). Fig. 5 shows the kinetics of digestion of the en-

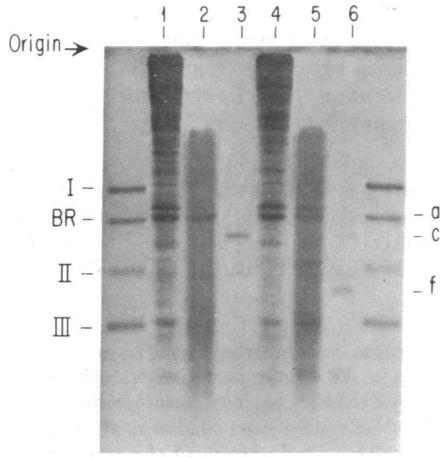

FIG. 6. Digestion of *H. halobium* cells with proteinase K. (*1*) Control: Intact, purple *H. halobium* cells (~5 × 10^7) were collected by centrifugation at 8000 × *g* for 2 min, and the pellet was suspended in 380 μl of 50 mM Tris·HCl (pH 8)/4 M NaCl/10 mM $CaCl_2$. The suspension was incubated at 37° for 70 hr. The reaction was terminated by the addition of 1 μl of 0.1% phenylmethylsulfonyl fluoride. The suspension was again centrifuged as above and the pellet was solubilized by boiling with 100 μl of 1% $NaDodSO_4$ and then subjected to polyacrylamide gel electrophoresis. (*2*) The procedure was as in *1* except that 12 μg of proteinase K was added prior to the incubation at 37°. (*3*) Lysed *H. halobium* cells pelleted as above were suspended in 40 μl of H_2O for 30 min at 25° and then treated with proteinase K as described above. After termination of the reaction with 1 μl of 0.1% phenylmethyl sulfonyl fluoride, the suspension was centrifuged at 45,000 × *g* for 30 min and the pellet was analyzed as described above. (*4*) Control: *H. halobium* cells containing apomembrane were prepared by reaction with hydroxylamine. An aliquot (containing ~10^8 cells) was centrifuged and the pellet was treated as described in *1*, execpt that the incubation at 37° was for 40 hr. (*5*) An aliquot of the above (*4*) apomembrane cells was digested with proteinase K at 37° for 40 hr as described in *2* above. (*6*) Apomembrane cells were digested with proteinase K at 37° for 40 hr and the membranes were isolated as described in *3*.

velope vesicles by proteinase K. As is seen, the carboxyl terminus (site I) is now largely protected from proteolysis. Lysis of the vesicles by suspension in water confers susceptibility to the proteinase since the carboxyl terminus is now accessible.

***H. halobium* Cells.** Attempted proteolysis of bacteriorhodopsin in whole cells would require the accessibility of the purple membrane surface to the proteolytic enzymes. Mescher and Strominger (20) have shown that digestion of *H. salinarium* cells with Pronase results in the degradation of the outer wall glycoprotein and in the concomitant conversion of rod-shaped cells to spheres, which retain viability. Since a similar glycoprotein has been reported to be present in *H. halobium* (21), proteolytic digestion should expose the plasma membrane and, consequently, bacteriorhodopsin should be accessible from outside. Fig. 6 shows the results of proteinase K digestion of whole cells containing the purple membrane or apomembrane. Channels 1 and 4 show the control, in which the normal and apomembrane cells were subjected to electrophoresis without proteinase K treatment. Channels 2 and 5 show the patterns after digestion with proteinase K; the digestions were continued until the cells had attained spherical shape. Channels 3 and 6 show the patterns obtained with membranes isolated after treatment of lysed cells with proteinase K. Bacteriorhodopsin in whole cells remained unaffected‡ while bacteriorhodopsin in the lysed cells underwent complete cleavages at sites I and III and almost complete cleavage at site II. The results thus show that in whole cells the cleavage sites are located on the cytoplasmic side.

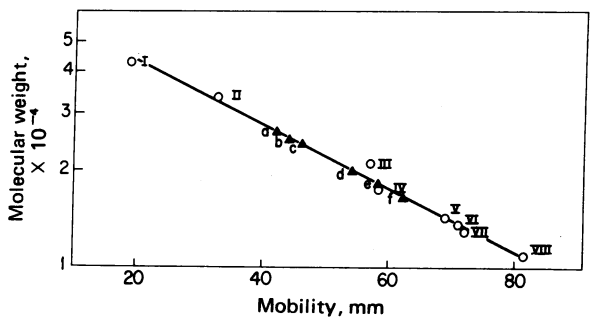

FIG. 7. Determination of molecular weights of the proteolytic fragments of bacteriorhodopsin by polyacrylamide gel electrophoresis. Each protein marker (5 μg) was dissolved in 20 μl of 1% $NaDodSO_4$ containing 1% 2-mercaptoethanol. The solution was heated to 100° and subjected to gel electrophoresis. Aliquots of samples from Figs. 3 and 4, processed as in the legend to Fig. 3, were run on the same slab gel. The mobility of the markers was measured from the top of the running gel to the middle of the protein band. The markers (○), I–VIII (M_r shown in parentheses) used were as follows: I, ovalbumin (43,000); II, carboxypeptidase (34,600); III, soybean trypsin inhibitor (21,500); IV, myoglobin (17,500); VI, cytochrome *c* (23,500); and VII and VIII, chymotrypsin (23,000 and 11,000, respectively).

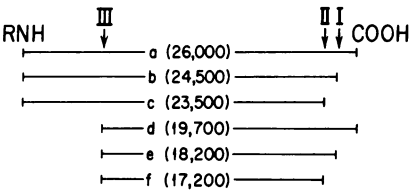

FIG. 8. Proposed arrangement of the proteolytic fragments.

DISCUSSION

Molecular Weights and Ordering of Proteolytic Fragments. The present work has described an initial study of the topology of bacteriorhodopsin in the purple membrane. The molecular weights of the fragments obtained by proteolysis are shown in Fig. 7, while their arrangement along the primary sequence of bacteriorhodopsin is shown in Fig. 8. First, the molecular weight (26,000) of bacteriorhodopsin as found in the present work by using terminal degradation with carboxypeptidase is consistent with the conclusion of other workers (1, 5). Second, in all, three proteolytic cleavages were observed (Fig. 8). Treatment of bacteriorhodopsin in purple membrane with trypsin or Pronase gave a single cleavage (site I), producing a membrane-bound fragment (b in Fig. 7) of M_r ~24,500 and, with trypsin, a hydrophilic polypeptide of about 15 amino acids. When proteinase K was used in this experiment, cleavage at site I was followed by a second slower cleavage at site II, which further decreased the M_r of the large membrane-bound fragment by ~1000 (fragment c, Figs. 7 and 8).

With the apomembrane, cleavage at an additional site (site III) was observed. Thus, cleavage now occurred at sites I and III with trypsin and Pronase and at sites I, II, and III with proteinase K. The fact that products from cleavage at site III alone were detected as intermediates [fragment d, M_r 19,700, and a second membrane-bound fragment (g)] showed that site III must be near the opposite end of the molecule from site I. Since

‡ There could be the possibility that the failure to obtain proteolysis at site I in whole cells or envelope vesicles could be due to protection by one or more cell wall proteins despite the prolonged proteinase K treatment. This seems unlikely, although further independent evidence on the present conclusion would be desirable.

the latter is clearly at the carboxyl terminus, site III must be near the amino terminus. Further, while the molecular weight of the fragment d (Figs. 7 and 8) could be assigned from the mobility in the $NaDodSO_4$/polyacrylamide gel, that of the fragment (g) released from the amino terminus could only be deduced by difference to be ~6300 since the gel method does not resolve polypeptides with M_r below 11,000. Further, the slow cleavage at site II described above for purple membrane was also observed with the large fragment (M_r 18,200) that was obtained from apomembrane by cleavages at sites I and III. Site II must therefore be at an end of the molecule unaffected by site III, i.e., near the carboxyl terminus of bacteriorhodopsin.

Some other observations made in the present work cannot be uniquely interpreted at this stage but could no doubt be of interest in ultimately understanding the structure of bacteriorhodopsin in the purple membrane. Cleavage at site I by trypsin was inhibited by the presence of salt in as low as 0.15 M concentration. On the other hand, cleavage at site III by trypsin was not detectably affected at this salt concentration. With proteinase K, the presence of 4 M NaCl was necessary for selective cleavages; complete digestion of bacteriorhodopsin occurred in the absence of salt. One interpretation of these results is that salt alters the protein conformation or brings about tightening of the structure. Hsia and coworkers (22) have reported on salt-dependent conformational changes in the cell membrane of *H. salinarium*. Further, cleavage at site III occurred only in the absence of retinal. (The apomembrane was also much more susceptible to proteinase K.) This result could mean either that site III is located at or near the retinal-binding site or that the binding of retinal induces a conformational change in the protein so that site III is no longer accessible to the proteolytic enzymes. (See *Note Added in Proof.*)

Orientation of Bacteriorhodopsin in Purple Membrane. The results obtained with the reconstituted vesicles and whole cells all consistently showed that the carboxyl terminus of bacteriorhodopsin is on the cytoplasmic side of the membrane. Thus, in vesicles reconstituted with synthetic phospholipids, the carboxyl terminus of bacteriorhodopsin was accessible to the proteolytic enzymes. Previously such vesicles have been found to pump protons from outside to inside, i.e., in a direction the reverse of that in whole cells. Therefore, in the synthetic vesicles the orientation of bacteriorhodopsin must be inverted and in the whole cells the carboxyl terminus must be on the cytoplasmic side. This conclusion was supported by experiments with envelope vesicles, which pump protons largely from inside to outside, and with whole cells. In both cases, the carboxyl terminus was protected from proteolysis and, in addition, in whole cells containing apomembrane, the cleavage at site III was also protected. These results all show that the natural orientation of bacteriorhodopsin in purple membrane is such that the carboxyl terminus is on the cytoplasmic side and, further, that site III is also on the inside.

The finding that the carboxyl terminus in bacteriorhodopsin is on the cytoplasmic side of the purple membrane may be of general significance in regard to the orientation of membrane proteins or at least those that span the bilayer. At least two other membrane proteins whose amino acid sequences and orientation in the membrane are known also have the carboxyl terminus on the cytoplasmic side. These are glycophorin (23) and bacteriophage M-13 coat protein (24). It seems reasonable to postulate that the proteins begin to be synthesized on the cytoplasmic side and the growing polypeptide chain begins to traverse the bilayer before the completion of the synthesis (25). The carboxyl terminus is synthesized last and remains on the inside of the membrane.

Note Added in Proof. Sequence analysis of the fragments e (M_r 18,200) and g (M_r 6300) has now shown that site III is not located within the previously determined (5) retinal-binding sequence. Furthermore, the latter sequence is contained in fragment g (G.E. Gerber, R.J. Anderegg, W.C. Herlihy, C.P. Gray, K. Biemann, and H.G. Khorana, unpublished data).

This investigation was supported by the National Cancer Institute (Grant CA11981) and the Institute of Allergies and Infectious Diseases (Grant AI11479) of the National Institutes of Health, U.S. Public Health Service, and the National Science Foundation, Washington, DC (Grant PCM73-06757). G.E.G. is the recipient of a postdoctoral fellowship from the Medical Research Council in Canada; D.W. was the recipient of a postdoctoral fellowship from NATO (1974–1976).

1. Oesterhelt, D. & Stoeckenius, W. (1971) *Nature* **233**, 149–152.
2. Oesterhelt, D. (1976) *Angew. Chem.* **88**, 16–24.
3. Oesterhelt, D. & Stoeckenius, W. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 2853–2857.
4. Danon, A. & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1234–1238.
5. Bridgen, J. & Walker, I. D. (1976) *Biochemistry* **15**, 792–798.
6. Kushwaha, S. C., Kates, M. & Martin, S. G. (1974) *Can. J. Biochem.* **53**, 284–292.
7. Henderson, R. & Unwin, P. N. T. (1975) *Nature* **257**, 28–32.
8. Keefer, L. M. & Bradshaw, R. A. (1977) *Fed. Proc.* **36**, 1799–1804.
9. Wildenauer, D. & Khorana, H. G. (1977) *Biochim. Biophys. Acta* **466**, 315–324.
10. Gerber, G. E., Wildenauer, D. & Khorana, H. G. (1977) *Fed. Proc.* **36**, 896.
11. Oesterhelt, D. & Stoeckenius, W. (1974) in *Methods in Enzymology*, eds. Fleischer, S. & Packer, L. (Academic Press, New York), Vol. 31, pp. 667–678.
12. Oesterhelt, D., Schumann, L. & Gruber, H. (1974) *FEBS Lett.* **44**, 257–261.
13. Laemmli, U. K. (1970) *Nature* **227**, 580–685.
14. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617.
15. Weiner, A. M., Platt, T. & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242–3251.
16. Racker, E. & Stoeckenius, W. (1974) *J. Biol. Chem.* **249**, 662–663.
17. Kanner, B. I. & Racker, E. (1975) *Biochem. Biophys. Res. Commun.* **64**, 1054–1061.
18. MacDonald, R. E. & Lanyi, J. K. (1975) *Biochemistry* **14**, 2882–2889.
19. Kishwaha, S. C., Kates, M. & Stoeckenius, W. (1976) *Biochim. Biophys. Acta* **426**, 703–710.
20. Mescher, M. F. & Strominger, J. L. (1976) *Proc. Natl. Acad. Sci. USA* **78**, 2687–2691.
21. Koncewicz, M. A. (1972) *Biochem. J.* **128**, 124.
22. Hsia, J. C., Wong, P. T. S. & MacLennan, D. H. (1971) *Biochem. Biophys. Res. Commun.* **43**, 88–93.
23. Wickner, W. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1159–1163.
24. Tomita, M. & Marchesi, V. (1976) *Proc. Natl. Acad. Sci. USA* **72**, 2964–2968.
25. Smith, W. P., Tai, P-C., Thompson, R. C. & Davis, B. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2830–2834.