Regeneration of Native Bacteriorhodopsin Structure from Fragments*

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The previously described chymotryptic fragment of bacteriorhodopsin, C-2 (amino acids 1–71), is cleaved by 70% formic acid to two fragments, A-1 (amino acids 1–36) and A-2 (amino acids 37–71), which have been separated by high pressure liquid chromatography. The fragments A-1 and A-2, separately or together, are not able to replace C-2 in forming a stable bacteriorhodopsin-like complex with the fragment C-1 (amino acids 72–248) and all-trans-retinal. A second set of bacteriorhodopsin fragments, B-1 (amino acids 1–155) and B-2 (amino acids 156–248), have been prepared by sodium borohydride cleavage of bacteriorhodopsin. Following denaturation, fragments B-1 and B-2 reassociate in the presence of retinal to regenerate the native bacteriorhodopsin chromophore (~40%). Fragment B-1 also interacts with fragment C-1 and all-trans-retinal to form a complex with spectral properties and secondary structure similar to those of bacteriorhodopsin. Vesicles prepared from the reconstituted fragment complexes (B-1 + B-2) or (B-1 + C-1), show proton pumping activities comparable to the previously described activity fragments C-1 and C-2.

BR†, the only protein present in the specialized purple membrane of Halobacterium halobium, carries out light-dependent proton translocation (1–4). Based on diffraction data provides opportunities for studies of a number of important aspects of BR structure and function. Thus, they could provide insights into the topography and interhelical interactions within the BR molecule. It may also be possible to learn how many helices are indeed essential to form the retinal binding pocket and for light-dependent proton translocation. Furthermore, modifications or labeling of specific amino acids in chosen fragments provide a general approach for studying BR structure and function. For example, we have used fragment reconstitution to confirm our earlier conclusion regarding the site of attachment of retinal within the protein (15, 16). Since only two defined fragments, C-1 and C-2, have been available so far, we have now undertaken the preparation and characterization of additional fragments and to study their ability to complement each other in forming BR-like complexes. The present paper reports results of these studies.

**EXPERIMENTAL PROCEDURES**

**Materials**

Purple membrane was isolated as previously described (17) from H. halobium (strain S9, originally obtained from W. Stoeckenius, University of California, San Francisco). DMPC, CHAPS, cholic acid, SDS, soybean phospholipids, and all-trans-retinal, were obtained as described (14). Dimyristoylphosphatidylglycerol and diphytanoylphosphatidylcholine were purchased from Avanti Polar-Lipids, Inc. (Birmingham, AL). Dipalmitylphosphatidylcholine was obtained from Calbiochem-Behring. Egg yolk phosphatidylcholine was isolated as described (18).

**Buffers**

Buffer A used for reconstitution consisted of 0.2% (w/v) SDS, 10 mM NaPi (pH 6.0) and 0.025% (w/v) NaN3.

**Methods**

**Cleavage of the Fragment C-2 by Formic Acid**—The preparation of the fragment C-2 has previously been described (14). C-2 (5 mg) was dissolved in 1.2 ml of 50% formic acid and water (0.2 ml) was added dropwise such that the final formic acid concentration was 70–75%. The solution was flushed with nitrogen, the test tube sealed under vacuum, and the solution incubated in the dark at 37 °C for 3–4 days. Absolute ethanol (3 ml) was then added and the solvent was removed for the vacuum and the residue was stored under ethanol at −20 °C.

**Preparation of B-1 and B-2 by Treatment of Purple Membrane with NaBH4**—Purple membrane (20 mg in 10 ml of H2O) was mixed with 10 ml of 6% (w/v) NaBH4 in 0.1 M NaCO3 (pH 10) in the dark and the mixture was stirred at 4 °C for 2 days. The reaction was stopped by the addition of 4 ml of 1 M NaPi (pH 6) and the digested membrane was collected by centrifugation, washed twice with distilled water, and lyophilized. The dry membrane was dissolved in 1.5 ml of 88% formic acid followed by the addition of 3.5 ml of absolute ethanol. To hydrolyze the Schiff’s base, the formic acid/ethanol solution was treated with 50 μl of 10 M NH2OH/HCl in water. The reaction was complete in 3 h at room temperature as judged by the change in the color from orange (protonated Schiff’s base, λmax = 440 nm) to light yellow (oxime, λmax = 360 nm). The mixture was then chromatographed on a Sephadex LH-20 column (1.5 × 180 cm) in formic acid/ethanol (3:7) at room temperature. Fractions corresponding to B-1 and B-2 were pooled, solvents removed, and the fragments transferred.

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1 The abbreviations used are: BR, bacteriorhodopsin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; SDS, sodium dodecyl sulfate; dnasf, 5-dimethylaminonaphthalene-1-sulfonfyl; DMPC, dimyristoylphosphatidylcholine; HPLC, high pressure liquid liquid chromatography.
into Buffer A by dialysis as described (13).

**Protein Determination**—Protein concentration was determined from absorbance at 280 nm in SDS solution (12). Values of ε_{280} were calculated from the literature values for extinction coefficients of tryptophan and tyrosine (19) using the tryptophan and tyrosine content of each fragment. The calculated values were then normalized to a total ε_{280} of 66,000 cm^{-1} M^{-1} which is the previously determined extinction coefficient of delipidated bacterio-opsin in SDS solution (12). The resulting values are ε_{280} = 51,070 for B-1, ε_{280} = 13,890 for B-2, ε_{280} = 15,000 for A-1, and ε_{280} = 3,000 for A-2.

**Regeneration of BR Chromophore**—The procedures for regeneration of the chromophore from intact protein, as well as from C-1 and C-2 have been described in detail (14). The same procedure was used for regeneration of BR chromophore from (B-1 + B-2), (B-1 + C-1), or (A-1 + A-2 + C-1).

**Reconstitution of the Fragments into Phospholipid Vesicles and Assay for Proton Pumping Activity**—The procedures for preparation of reconstituted vesicles from BR or from fragments have been described (13), except that the initial lipid/detergent mixture contains soybean phospholipids/CHAPS or H. halobium phospholipid/CHAPS (2/2%, w/v) in 50 mM NaPi (pH 6) buffer. Proton pumping activity of reconstituted vesicles was assayed as described previously (15).

**Amino Acid Analysis**—Hydrolysis of peptide samples was performed in 6 N HCl at 110 °C for 24 h. Amino acid analysis was performed on a Beckman 119CL amino acid analyzer.

**Sequence Analysis**—Automated amino acid sequence analyses were performed on an unmodified Beckman 890C sequencer as previously described (21) and a solid phase sequencer (sequemat Mini 15) by the procedure of Takenaka et al. (22).

**Circular Dichroism Measurements**—UV-CD spectra of samples containing 0.2 mg of protein/ml were taken in 1-mm light path quartz cells using a Cary 15 spectrophotometer at 21 °C as described (13).

**RESULTS**

**Cleavage of C-2 to Fragments A-1 and A-2**—Previously, Walker et al. (23) reported that the peptide bond Pro-37 to Asp-38 in C-2 was acid-labile. According to the proposed BR model (10), this linkage is in the loop region between the first and the second helices. A study of the interaction of these two helices, separately or together, with C-1 was clearly of interest and their preparation was undertaken. When C-2 was dissolved in 70% formic acid and the solution incubated at 37 °C under vacuum for 4 days, 70% of C-2 was cleaved to form two main fragments, A-1 and A-2. Separation by reverse phase HPLC gave the elution profile shown in Fig. 1. Five peaks with absorption at 280 nm were observed. The fractions under each peak were pooled and identified by gel electrophoresis using the method of Swank and Munkres (24). Peaks III and IV corresponded to fragments A-2 and A-1, respectively, and were well resolved from uncleaved C-2 (peak V). Small amounts of the sample found in peaks I and II also corresponded, respectively, to A-1 and A-2, as judged by amino acid analyses. The reason for the elution of parts of A-1 and A-2 ahead of the major peaks may be due to overlapping or possible modification of residues by formic acid and consequent changes in their mobilities.

Amino acid analyses of the peptides in peak III and IV are shown in Table I. The compositions agree well with those expected for the peptide sequences. Furthermore, the number of Pro and Asp residues found in A-1 and A-2 confirmed that C-2 was cleaved between Asp-36 and Pro-37, this site being different from that concluded by Walker et al. (23) (see above).

To obtain further support for the present conclusion, the NH₂ terminus and amino acid sequence of A-2 were determined. Proline was found to be the only NH₂-terminal amino acid residue as determined by dansylation. The amino acid sequence of A-2 using solid phase sequencer also gave the unique sequence, H₂N-Pro-Asp-Ala-Lys-Lys-Phe-Tyr-Ala-Ile... We concluded that formic acid cleaves C-2 between Asp-36 and Pro-37 to form fragments A-1 (amino acids 1-36) and A-2 (amino acids 37-71).

**Attempted Regeneration of the Bacteriorhodopsin Chromophore from Fragments A-1, A-2, and C-1 with Retinal**—Our previous results have shown that fragments C-1 and C-2 can reassociate and bind retinal to regenerate the native BR chromophore (14). We thus used the protocol developed previously for renaturation of A-1, A-2, and C-1. Denatured A-1, A-2, and C-1 at 1:1:1 molar ratio in SDS solution was mixed with 1 eq retinal and DMPC/CHAPS mixture at pH 6. Only 5-10% of the expected chromophore was regenerated. Furthermore, no chromophore was observed when mixtures of A-1 and C-1 or A-2 and C-1 with retinal were used. As a control, the uncleaved C-2 (peak V, Fig. 1) was mixed with C-1 and retinal under the same conditions. Fifty per cent of the expected chromophore was regenerated.

**Cleavage of BR to Fragments B-1 and B-2 by NaBH₄ (pH 10)**—When purple membrane was incubated with NaBH₄ (pH 10) in the dark for 2 days, BR was cleaved to two fragments, a large fragment B-1 and a small fragment B-2. The two fragments were separated by gel permeation column chromatography, the elution profile being shown in Fig. 2A. The fragments were well resolved and free from uncleaved intact protein as judged by SDS-polyacrylamide gel electrophoresis (Fig. 2B). The size of the B-1 fragment is smaller than the chromotryptic fragment C-1 while B-2 is larger than C-2. Under the reaction conditions, a small amount (7%) of B-2 fragment containing reduced retinyl Schiff's base (absorption at 330 nm; ε = 45,000 in 0.2% SDS) was also observed.

**Determination of the amino acid sequence of B-2**—by both solid and liquid phase sequencing showed the unique sequence: H₂N-Phe-Thr-Ser-Lys-Ala-Glu-Ser-Met-Arg-Pro-Glu-Val-Ala... Thus, BR was cleaved between Gly-155 and Phe-156. B-1 contains the amino acid residues 1-155, and B-2 contains residues 156-248.

**Regeneration of BR Chromophore from B-1, B-2, and Retinal**—B-1 and B-2 in formic acid/ethanol (3:7) (Fig. 2) were transferred into SDS solution and were mixed in a 1:1 molar ratio with 1 eq retinal in the presence of DMPC/CHAPS/SDS (pH 6). The BR-like chromophore was regenerated to the extent of 40% (Table II). The regenerated chromophore showed the same λ_max (550 nm) and light-dark adaptation as the native BR chromophore.

The treatment of BR with NaBH₄ did not significantly affect the function of BR as evidenced by the ability of uncleaved bacterio-opsin to bind retinal (78% of activity recovered). However, we cannot rule out the possibility of chemical modification on certain amino acid residues.

The effect of pH on renaturation of the purple chromophore

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3 The yield was lower than that obtained from C-1 and C-2 complex that C-2 had not been treated with formic acid. One explanation is that some amino residues were modified after prolonged acid treatment.
FIG. 1. Characterization of fragments formed from chymotryptic fragment C-2. A, HPLC separation of fragments formed by formic acid treatment of the chymotryptic fragment C-2. Digestion of C-2 and separation by HPLC have been described under "Methods." B, SDS/urea/polyacrylamide (12%) gel electrophoresis of fractions eluted from HPLC by the method of Swank and Munkres (24). The gel was stained with Coomassie blue.

Table I

Amino acid analysis of A-1 and A-2 fragments

|   | A-1 (calc) | A-1 (obs) | A-2 (calc) | A-2 (obs) |
|---|------------|-----------|------------|-----------|
| Asp | 1          | 0.9 (1)   | 1          | 1.1 (1)   |
| Thr | 3          | 2.9 (3)   | 4          | 4.3 (4)   |
| Ser | 1          | 1.0 (1)   | 1          | 1.2 (1)   |
| Gly | 3          | 2.1 (2)   | 0          | 0.4 (0)   |
| Pro | 1          | 1.4 (1)   | 3          | 2.9 (3)   |
| Gly | 6          | 4.8 (5)   | 2          | 2.5 (3)   |
| Ala | 3          | 3.0 (3)   | 4          | 3.5 (4)   |
| Val | 2          | 2.1 (2)   | 2          | 2.3 (2)   |
| Met | 2          | 1.8 (2)   | 3          | 2.8 (3)   |
| lle | 2          | 1.2 (1)   | 2          | 1.9 (2)   |
| Leu | 6          | 5.6 (6)   | 5          | 5.8 (6)   |
| Tyr | 1          | 1.2 (1)   | 3          | 3.0 (3)   |
| Phe | 1          | 1.3 (1)   | 3          | 3.4 (3)   |
| Lys | 1          | 1.1 (1)   | 2          | 2.9 (3)   |
| Arg | 1          | 1.4 (1)   | 0          | 0.2 (0)   |
| Trp | 2          |           |            |           |
| Total | 36        | 35       |

Table II

Chromophore regeneration from fragment complexes

| Sample      | % of chromophore regenerated in DMPC/CHAPS/SDS (pH 6) |
|-------------|-----------------------------------------------|
| B-1 + B-2 + retinal | 40                             |
| B-1 + C-1 + retinal | 74                             |
| C-1 + C-2 + retinal | 95                             |
| Bacterio-opsin + retinal | 100                          |

from B-1 and B-2 was investigated. Maximum regeneration was observed at pH 5.5–6.0 (data not shown), a result that is similar to that obtained previously for renaturation of intact BR and of fragments C-1 and C-2 (14).

Renaturation of B-1 and B-2 was investigated using a variety of phospholipids: dimyristoylphosphatidylglycerol, dipalmitoylphosphatidylcholine, soybean phospholipids, egg yolk phosphatidylcholine, diphytanoylphosphatidylcholine, and H. halobium lipids. DMPC/CHAPS at pH 6 gave the highest yield (40%). This yield is much lower than that from fragments C-1 and C-2.

The binding of B-1 to B-2 was studied by regeneration of BR-like chromophore as a function of B-1/B-2 and B-2/B-1 molar ratios (Fig. 3, A and B). These experiments showed that the extent of regeneration increased as the molar ratios increased above. Both titration curves also indicated a weak binding between B-1 and B-2. The fact that both curves plateaued at about 70% regeneration rather than 100% is possibly due to the presence of some denatured B-1 and B-2.

Regeneration of the BR-like Chromophore from Fragments B-1, and C-1, and Retinal—As shown in Table II, a mixture of B-1 and C-1 binds retinal to regenerate BR-like chromophore. This regeneration which occurs in the extent of 74% is more efficient than that from B-1 and B-2. The regenerated chromophore is as stable as that from C-1 and C-2 and can be stored at room temperature for at least 3 months.

We know that neither C-1 nor B-1 alone binds retinal and the latter binds only to the complex formed from the two fragments (14). Therefore, we determined the stoichiometry of this complex by measuring the chromophore regeneration as a function of C-1 to B-1 molar ratio in the presence of excess retinal (Fig. 3C). The equivalence point for the formation of C-1, B-1, and retinal complex was found to be at C-1/B-1 ratio of 0.97. We also calculated, from the plateau of the curve (Fig. 3C), that at least 70% of B-1 molecules were active. Similarly, we have determined from the titration experiment between C-1 and C-2 (data not shown) that at least 80% of C-1 molecules were active. Therefore, it seems likely that C-1 binds B-1 at a 1:1 molar ratio.

Secondary Structure of B-1 and C-1 in the Ternary Complex
the presence of excess retinal in DMPC/cholate/SDS (pH 7).

...generated from renatured C-1, C-2, and retinal (14) and to the renatured intact BR (12). It suggests that both C-1 and C-2 are renatured to their native secondary structures.

**Reconstitution of Vesicles from Bacterio-opsin and Fragment Complex**—Regeneration of the chromophore was carried out from (B-1 + B-2), (B-1 + C-1), (C-1 + C-2) and intact bacterio-opsin by treatment with retinal and soybean phospholipids or *H. halobium* lipids. These preparations were then reconstituted into vesicles by dialysis, and their proton pumping activities were measured (Table III). Thus, the pumping activities of the complexes reconstituted from the above sets of fragments were comparable, both, in soybean and *H. halobium* lipid vesicles. However, the pumping activities were smaller than that obtained with the reconstituted intact protein under the same conditions.

**DISCUSSION**

With a view to increasing the scope of BR structure-function studies by fragment reconstitution, we have described the preparation of new fragments of bacterio-opsin. The results obtained on their reassociation to form the native structure are of interest. Fragments B-1 and B-2 have been shown to associate, bind retinal, and generate the BR-like chromophore. However, the extent of chromophore regeneration was much less (40%) than that previously observed with C-1 and C-2 (14). The regenerated B-1, and B-2 complex, on reconstitution into phospholipid vesicles showed light-dependent proton translocating activity.

Interestingly, the fragments B-1 and C-1, containing considerable overlap in their primary structure, form a stable 1:1 complex with a secondary structure similar to that of intact BR. Also, the spectral properties of the chromophore regenerated from B-1 and C-1 suggest that the complex possesses a native retinal binding pocket. This complex is more stable than that formed from B-1 and B-2 in the presence of retinal. Conceivably, the presence of extra polypeptide chain in the fragments, B-1 and C-1, promotes the refolding to the native structure and/or stabilizes the folded structure. It is difficult to visualize the tertiary structure of the complex at this stage. According to the model (10, 11), B-1 contains the first five helices from the NH2-terminal end. C-1 also contains five helices, helices 3–7. If, as is probable, both fragments refold to form a native 7-helical structure, three helices (helices 3–5) would be redundant. It is not known at present whether

**TABLE III**

Proton pumping activities of reconstituted vesicles in soybean phospholipids and in *halobium* lipids

| Proteins in vesicles | Initial rate H+/fragment complex/s or H+/BR/s | Total protons pumped H+/fragment complex or H+/BR |
|----------------------|---------------------------------------------|-----------------------------------------------|

Reconstituted in soybean lipids
- B-1 + B-2
- C-1 + B-1
- C-1 + C-2
- BR

| | 0.19 | 21 |
| | 0.20 | 10 |
| | 0.33 | 30 |
| | 0.9 | 44 |

Reconstituted in halobium lipids
- B-1 + B-2 + valinomycin
- C-1 + C-2
- C-1 + C-2 + valinomycin
- BR + valinomycin

| | 0.47 | 6 |
| | 0.61 | 17 |
| | 0.54 | 6 |
| | 0.73 | 13 |
| | 1.53 | 17 |
| | 1.5 | 30 |

*Valinomycin (5 μl of 1 mM in ethanol) was added to reconstituted vesicles (50 μl) in 2 ml of 2 M KCl.*
these three extra helices present in the complex come from B-1 or C-1.

Regeneration of the native BR structure from C-1 and C-2 is efficient (14). The large fragment, C-1, seems to contain most of the retinal binding pocket (25). However, free retinal does not stably bind to C-1. This suggests that for stabilization of the retinylidene complex with C-1, an additional polypeptide fragment is required. To investigate this point, the two helices in C-2 were cleaved and the separated helices, A-1 and A-2, were studied for their ability to regenerate the BR chromophore in combination with retinal. This possibility can be tested when tested in the presence of A-2. However, the possibility remains that a BR derivative could form a stable complex with retinal and form BR-like chromophore in combination with A-2, C-1, and C-2. Together, they would be able to form a stable complex with retinal and form BR-like chromophore and pump protons.

Furthermore, A-1 and A-2 together gave only insignificant (5-10%) renaturation when tested in the presence of C-1. Thus, a single covalent cleavage in C-2 seems to have a striking effect on the capacity of the resulting helices to complement C-1 in the renaturation process. However, the possibility remains that a BR derivative containing six continuous helices (helices 2–7) would be able to form a stable complex with retinal and form BR-like chromophore and pump protons. This possibility can be tested by introducing suitably placed chain terminating mutations in the BR gene by the use of recombinant DNA methods (26).

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