Journal of Chromatography, 326 (1985) 243–250
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 555

ISOLATION OF DETERGENT-SOLUBILIZED MONOMERS OF BACTERIORHODOPSIN BY SIZE-EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Bacteriorhodopsin, an integral membrane protein of purple membranes, was solubilized with *n*-octylglucoside and isolated as intact monomeric micelles by high-performance size-exclusion chromatography. It was shown that separation was obtained between these micelles and either those containing bacterio-opsin or retinal as well as bacterio-opsin in the aggregated state. Estimates of the apparent molecular weights and Stokes radii were obtained by comparison with water-soluble proteins with known properties. Thermal denaturation of the native protein micelle induced the formation of a denatured species, which was similar to that found from denaturation at 4°C.

INTRODUCTION

Bacteriorhodopsin (bR) is the sole membrane protein (M_r = 25 kD) contained in purple membranes of halophilic bacterium[1]. A single retinal chromophore is attached to the ε-amino group of a lysine residue in a protonated Schiff base linkage. Highly specific, noncovalent interactions occur between this chromophore and the apoprotein[2]. This results in the retinal chromophore absorbing light in the visible region of the spectrum and is responsible for the intense purple color of the membrane. Absorption of light by the bR-bound retinal chromophore culminates in the generation of a proton electrochemical gradient across the membrane. This potential gradient is used to drive the formation of adenosine triphosphate in the red membrane of the bacterium, which is physically distinct from the purple membrane[1]. Thus, bR is an energy transducer, and *Halobacterium* is the simplest example of photosynthetic system.

A wide variety of detergents have been used to solubilize bR, which uniquely forms a two-dimensional crystal in the purple membranes. Two detergents, Triton X-100 and *n*-octylglucoside (OG), have been most successful in this endeavor[3,4] and

pump[5]. However, the solubilized protein is considerably less stable than bR in the crystalline arrays of the purple membranes[5]. A detectable amount of bacterio-opsin (bO) is formed upon solubilization of bR, and this amount increases with time, even at low temperatures. This is of particular consequence when it is necessary to have a homogeneous preparation of monomeric bR, such as in either nuclear magnetic resonance experiments[6] or in attempts to crystallize this protein[7]. Based on a recently developed procedure to purify bovine rhodopsin[8], a method for the isolation of OG micellar solutions of bR from those of bO by high-performance liquid chromatography (HPLC) is reported in this communication.

MATERIALS AND METHODS

Halobacterium halobium (S9) was grown at 37°C on a complex medium in a laboratory-built fermentor, equipped with fluorescent lights. Purple membranes were isolated and purified according to standard procedures[9]. As determined by SDS gel electrophoresis, this procedure yields a homogeneous preparation of bR. Solubilization of bR was accomplished with 30 m*M* solutions of OG (CalBiochem) and 100 m*M* sodium phosphate buffer (pH = 7.0) in the dark at 4°C. Monomeric bR/OG micelles were obtained by centrifugation (Beckman Model J-21) at 20000 rpm (45000 *g*). The pellets were resuspended in OG and resolubilized. The absorption spectra were recorded on a Beckman Model 26 spectrophotometer (Beckman Instruments, Palo Alto, CA, U.S.A.).

HPLC separations were obtained with a Beckman high-performance liquid chromatograph, equipped with a Model 165 variable-wavelength (Series 220) detector. Eluents were measured simultaneously at two wavelengths: typically, these were either 280 and 550 nm or 280 and 370 nm. Separations were made with an analytical TSK-2000 SW (30 cm × 7.5 mm I.D.) or with a preparative TSK-G-3000 SW (60 cm × 21.5 mm I.D.) column. The flow-rate was 0.4 ml/min at a pressure of 220 p.s.i. for the analytical column and 2.0 ml/min at a pressure of 500 p.s.i. for the preparative column. The eluent for the analytical column was 0.06 *M* sodium phosphate buffer (pH 6.5) with 0.15 *M* sodium chloride and 30 m*M* OG. This buffer was the same for the preparative column, except that sodium sulfate was used rather than sodium chloride. Chloride anions were avoided in an attempt to prevent corrosion. The buffer and protein solutions were filtered through Millex-GS 0.22-μm filters (Millipore). The aqueous protein standards (BioRad) were analyzed as described above, except that the eluting buffer contained no OG.

RESULTS AND DISCUSSION

OG-solubilized bR exhibits an intense absorbance band, centered at 548 nm, due to the π–π^* transition of the strongly perturbed retinal Schiff base chromophore[2]. Less intense bands occur in the 400-nm range, also due to this chromophore. The protein absorbance from the aromatic residues is centered at 278 nm. A typical ratio of these absorbances (A_{278}/A_{548}) is 2.10. Upon standing in the dark at 4°C for about two weeks, the 548-nm band decreases with a concomitant increase of a new absorbance, centered at 370 nm (curve T in Fig. 1). The A_{278}/A_{548} ratio increases and was 5.95 for the preparation used in Fig. 1. The new band at 370 nm is due to the

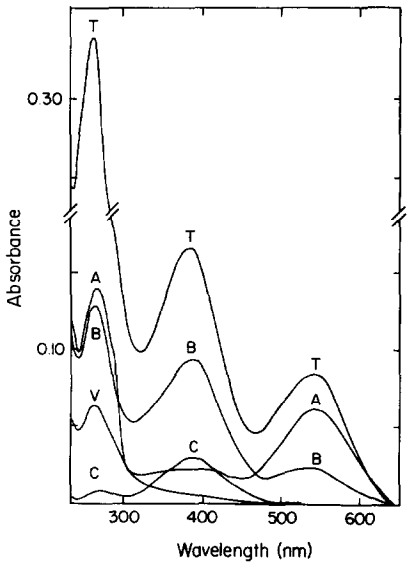

Fig. 1. The absorption spectra of bR at various stages of denaturation in 30 m*M* OG and 0.06 *M* sodium phosphate buffer (pH = 6.5) with 0.15 *M* sodium chloride. Curve T corresponds to partially denatured bR prior to HPLC analysis. Curves A, B, C, and V represent the spectra of fractions A, B, C and V from the HPLC elution (see Fig. 2). Fraction T was filtered prior to analysis. All spectra were obtained at room temperatures with 1-cm cells.

absorbance of the free retinal chromophore, no longer covalently bound to bR, and is indicative of a denaturation process. Protein aggregation also accompanies this process, as evidenced by the increase in turbidity of the solution. All samples were filtered, as described in the Materials and methods section, prior to absorption and HPLC analyses.

Injection of this sample on an analytical size-exclusion HPLC column results in the elution profile shown in Fig. 2. Four main fractions, labeled V, A, B, and C, are apparent in the chromatogram when the sample is monitored at 280 nm (bottom elution profile). These fractions are eluted at 8.6, 11.4, 12.6, and 13.8 ml, respectively. Simultaneous monitoring at 550 nm (dashed curve in top elution profile) shows that only fraction A absorbs at this wavelength. Reinjection of this sample, monitored at 370 nm (solid curve in top elution profile) rather than 550 nm, shows that fractions A, B, and C absorb at this wavelength, with fraction B displaying the most intense absorption.

The absorption spectrum of each fraction pooled from two injections, is displayed in Fig. 1. The spectrum of fraction A contains bands centered at both 548 and 278 nm. This spectrum is nearly identical to the spectrum of bR–OG, measured immediately after solubilization. The A_{278}/A_{548} ratio for the HPLC purified fraction was 2.01, which is slightly lower than the ratio found for bR initially solubilized (2.10). Furthermore, the elution profile of bR–OG, just after solubilization, shows essentially one major band consistent with band A in Fig. 1 (a minor band occurs at the elution time of fraction B). Since the spectrum indicates that the retinal chromophore is intact, fraction A seems to correspond to purified bR–OG micelles in the native state.

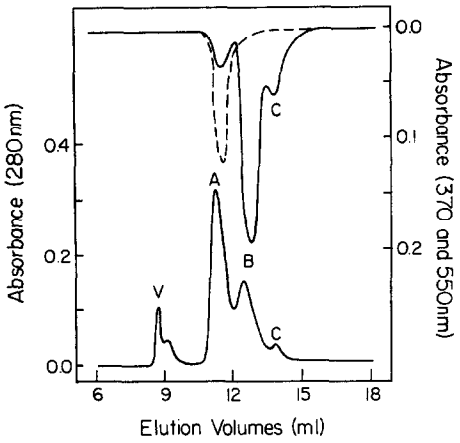

Fig. 2. HPLC chromatogram of 40-μl samples of partially denatured bR, corresponding to T in Fig. 1. Chromatographic conditions were: column TSK-2000 SW (30 cm × 7.5 mm I.D.); eluent, 60 m*M* sodium phosphate buffer (pH 6.5), 0.15 *M* sodium chloride and 30 m*M* OG; flow-rate, 0.4 ml/min. The bottom tracing was monitored at 280 nm and the top tracing was monitored at either 370 nm (solid curve) or 550 nm (dashed curve).

The absorption spectrum of fraction B displays intense bands at both 370 and 278 nm and a minor band at 548 nm. The 370-nm absorbance arises from retinal that is noncovalently attached to b0. The 278-nm band is due primarily to the aromatic residues of b0. This suggests that retinal is associated with b0 in the same micelle. Previous reports, which indicate that retinal oximes have a high affinity for a binding site in b0, are consistent with this suggestion[10]. The elution patterns of preparations, which have a variable amount of 370 nm absorbance, showed a direct correlation to the intensity of fraction B. Thus, it is expected that this fraction corresponds to b0 with retinal noncovalently attached. The 548-nm absorbance is probably due to native bR/OG micelles, which occur in this fraction due to trailing of fraction A (see dashed curve in top profile of Fig. 2).

The absorption spectrum of fraction C contains essentially one major band at 370 nm and a minor one at 278 nm. This spectrum is consistent with the absorbance of all-*trans* retinal in OG (spectrum not shown), which has a strongly allowed π–π^* transition at 370 nm and other π–π^* transitions at higher energies near 278 nm[11]. Since the absorption band at 278 nm is not intense, it is expected that the protein concentration is minimal in this fraction. Furthermore, injection of all-*trans* retinal in OG shows that this micelle is eluted together with fraction C. This indicates that fraction C represents free retinal–OG micelles.

The absorption spectrum of fraction V, which is the void fraction, has only one band, centered at 278 nm. Since this fraction has no absorbance at either 370 or 548 nm, it is expected that fraction V represents b0 without the retinal chromophore. It should be noted that the sum of the absorbances for fractions A, B, C, and V correspond closely to the spectrum of T. This indicates that the yield from the column is nearly quantitative.

The resolution of fractions A, B, and C in the elution profile was dependent on the amount of material injected, the age of the column, and the flow-rate. Nor-

mally, 1 nmol of protein was injected in a volume of 50 μl or less. At higher levels of protein or with large volumes, the resolution of fractions A and B decreased significantly. Decreased resolution was also apparent with prolonged use of the column and was most evident for the separation of fractions B and C. The flow-rate was adjusted between 0.2 and 0.5 ml/min to determine optimal conditions for the separation. While an increase in resolution occurred with a flow-rate of 0.2 ml/min, the effect was not dramatic enough to warrant the use of a decreased flow rate.

In order to separate a larger quantity of bR–OG micelles, it was necessary to use a preparative size-exclusion HPLC column. The qualitative features of the elution profile were similar to the profile yielded by the analytical column (Fig. 2), with fractions A, B, and C being eluted with 237.0, 247.8, and 268.8 ml, respectively (void volume was 132.0 ml). Separation could be obtained on 20 nmol of protein per injection with no apparent loss in resolution in comparison to the analytical column. One minor change occurred in the elution profile from the analytical column. The shoulder on fraction V was further separated from the void fraction and was eluted with 216.0 ml from the preparative column. Increased resolution of the higher-molecular-weight fractions was obtained by the use of a preparative column with an exclusion limit of 300 kD rather than 80 kD (analytical column).

The determination of the apparent molecular weight of the bR–OG micelle is important for studies involving solubilized membrane proteins. This can be estimated by standardizing the columns with proteins of known molecular weights (Fig. 3). Standards were chromatographed at flow-rates between 1.5 and 3.5 ml/min for the preparative column. It was determined that a 2.0 ml/min flow-rate was the fastest rate that could be used while still giving the linear correlation shown in Fig. 3b for the 10–100 kD molecular weight range. Fractions A, B, and C are eluted with apparent molecular weights of 40, 24 and 15 kD, respectively, as determined from the preparative column (Fig. 3b). The apparent molecular weights are higher for fractions

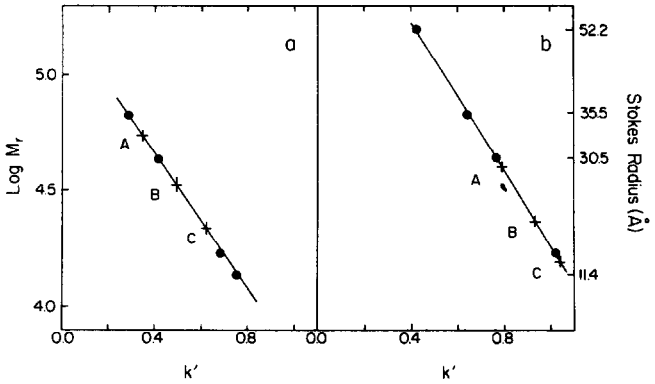

Fig. 3. Calibration curves of the analytical TSK-2000 SW (a) and preparative TSK-G-3000 SW (b) size-exclusion columns and fractions A, B, and C elution volumes (+). The analytical column was standardized with bovine serum albumin, ovalbumin, myoglobin, and ribonuclease (●). The preparative column was standardized with γ-globulin, bovine serum albumin, ovalbumin, and myoglobin (●). Thyroglobulin was used to mark the void volumes. Injections of 40 μl and 1.0 ml were made in the analytical and preparative columns, respectively. The k' represents capacity factor, $k' = (V_i - V_0)/V_0$, where V_i and V_0 are the sample and void elution volumes, respectively[12].

A, B, and C on the analytical column (Fig. 3a). This anomalous behavior may be due to the use of different anions in the eluting buffer (chloride *versus* sulfate) for each column[12]. However, the relative order of the fractions remains the same for each column.

A micellar size can be estimated from the effective molecular weights. Based on the Stokes radius of each of the protein standards, it is estimated that a bR–OG micelle has a radius of 29 Å. This is consistent with a previously reported value of 28 ± 5 Å, determined by standard gel-filtration studies[3] and supports the fact that the bR–OG micelles contain monomers of bR. Since the analytical column yielded a higher estimate for the Stokes radius, it is expected that the preparative results are quantitatively more accurate. The b0–OG micelle containing retinal is significantly smaller than the bR–OG micelle. Estimates of its Stokes radius are at 20 Å. In comparison, retinal–OG micelles are eluted as soluble proteins with a 14-Å radius. It has been reported from gel filtration studies that OG micelles are eluted at 8 kD[13], which would correspond to a Stokes radius of about 7 Å. Since the linear dimension of all-*trans* retinal is 15 Å[14], it is expected that a significant increase occurs in the apparent size of these micelles. Finally, the fraction eluted with 216.0 ml is consistent with a protein with a Stokes radius of 35.5 Å and probably represents an aggregate of b0.

The arguments made above must be viewed with the understanding that size-exclusion columns not only separate according to molecular size, but also are influenced by ionic and hydrophobic effects as well as adsorption to the column material[12,15,16]. In this study, the ionic effects are minimized by using a high concentration of salt. Furthermore, it is expected that, since a sufficient amount of detergent was present to maintain micellar formation (the critical micellar concentrations of OG is 25 m*M*), hydrophobic and adsorptive effects should be minimized or at least be the same. Thus, we suggest that the predominant effect in differentiating between bR and b0 micelles is one of size. This is supported by the fact that the retinal chromophore is located at the interior of the protein and, upon denaturation, must be excluded from this environment[1]. A decrease in size of the protein could accompany this process.

It is of interest to determine the effects of elevated temperatures on the denaturation pattern of bR–OG micelles. From a comparison of the spectrum of the HPLC purified bR–OG micelle (Fig. 1, curve A), it is apparent that even the initially solubilized protein contains a 370-nm band (Fig. 4, curve 1). HPLC analysis of this fraction shows a small band, corresponding to fraction B. As indicated by absorption spectroscopy (Fig. 4), the absorbance of the bR–OG micelles decreases in the 548-nm band with a concomitant increase of the 370-nm band for the temperature range of 25 to 45°C (curves 1–4). Substantial protein aggregation occurs above this temperature (Fig. 4, curve 5). The absorption spectrum of the filtered sample shows that the 370- and 548-nm bands are nearly equal in intensity (Fig. 4, curve 6). The elution pattern of this sample yields the same bands as displayed in Fig. 2, but with slightly different intensities. Complete thermal denaturation occurs at 60°C, as indicated by the loss of the 548-nm band (Fig. 4, curve 7). This fact is also confirmed by differential scanning calorimetry data, which show a protein denaturation transition of the bR–OG micelles at this temperature[17]. Injection of this sample yields two major fractions, which are consistent with fractions B and C in Fig. 2. Thus, it seems that denaturation at low or elevated temperatures results in similar species.

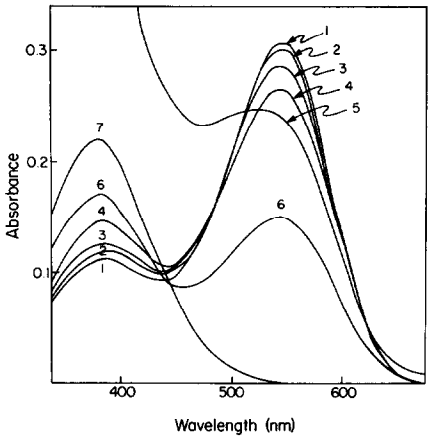

Fig. 4. The absorption spectra of bR in 30 m*M* OG and 0.100 *M* sodium phosphate (pH = 7) buffer. Curves 1–5 correspond to 25, 35, 41, 45, and 49°C. Curve 6 represents the bR spectrum at 49°C after filtration. Curve 7 represents the sample, heated to 60°C for 5 min, cooled, and filtered. Cell length was 1 cm.

CONCLUSION

This paper describes a method for the isolation of monomeric bR–OG micelles from those of the denatured protein and free retinal at both preparative and analytical levels. The main advantage of this method is the increase in both the resolving power of the HPLC technique and the speed at which the analyses can be performed. Additionally, this method is an accurate means of estimating the relative size of micellar systems.

ACKNOWLEDGEMENTS

This work was supported in part by NIH Grant CA-13148 and a UAB Graduate School Grant to DDM, and a Biomedical Research Support Grant RR05807 and UAB Graduate School Grant to LJD.

REFERENCES

1 W. Stoeckenius, R. H. Lozier and R. A. Bogomolni, *Biochim. Biophys. Acta*, 505 (1979) 215.
2 K. Nakanishi, V. Balogh-Nair, M. Arnaboldi, K. Tsujimoto and B. Honig, *J. Amer. Chem. Soc.*, 102 (1980) 7945.
3 N. A. Dencher and M. P. Heyn, *FEBS Lett.*, 96 (1978) 322.
4 R. Casadio, H. Gutowitz, P. Mowery, M. Taylor and W. Stoeckenius, *Biochim. Biophys. Acta*, 590 (1980) 13.
5 N. A. Dencher and M. P. Heyn, *FEBS Lett.*, 108 (1979) 307.
6 D. D. Muccio, W. G. Copan, E. W. Abrahamson and G. D. Mateescu, *Biophys. J.*, 37 (1982) 273a.
7 H. Michel, *Trends Biochem. Sci.*, 8 (1983) 56.
8 L. J. DeLucas and D. D. Muccio, *J. Chromatogr.*, 296 (1984) 121.
9 B. Becher and J. Y. Cassim, *Prep. Biochem.*, 5 (1975) 161.
10 B. Becher and J. Y. Cassim, *Biophys. J.*, 19 (1977) 285.
11 R. R. Birge, *Ann. Rev. Bioeng.*, 10 (1981) 315.

12 R. A. Jenik and J. W. Porter, *Anal. Biochem.*, 111 (1981) 184.
13 P. Rosevaer, T. Van Aken, J. Baxter and S. Ferguson-Miller, *Biochemistry*, 19 (1980) 4108.
14 T. Hamanaka and T. Mitsui, *Acta Cryst.*, B28 (1972) 214.
15 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
16 F. Hefti, *Anal. Biochem.*, 121 (1982) 378.
17 C. Brouilette and D. Muccio, unpublished results.