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TIME-DEPENDENT MONOMERIZATION OF BACTERIORHODOPSIN IN TRITON X-100 SOLUTIONS ANALYZED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Bacteriorhodopsin from *Halobacterium halobium* was monomerized in Triton X-100 solutions. The process of delipidation was monitored by size-exclusion high-performance liquid chromatography under conditions that preserved the native conformation of the protein. The effects on the process of monomerization of the concentration and pH of the Triton X-100 solutions were investigated. The monomeric bacteriorhodopsin separated was active in light-dependent proton translocation when incorporated into soy bean lecithin liposomes.

INTRODUCTION

Since mechanically highly stable chromatographic media based on modified silicic acid became available, the separation of proteins by high-performance liquid chromatography (HPLC) has been studied in many laboratories. Many of these studies were performed in organic solvents and on reversed-phase materials[1,2].

In this paper we describe the time-dependent delipidation (monomerization) of bacteriorhodopsin (BR) monitored by a size-exclusion HPLC system. As the aqueous BR solutions are separated with polyol silica materials in the presence of a non-denaturating detergent, the natural conformation of the membrane protein is preserved. Although an analytical HPLC column was used the yield of monomeric protein was in the micro-preparative range (about 0.4 mg).

The chromoprotein BR is an integral protein of the plasma membrane of the photosynthetic bacterium *Halobacterium halobium*. The protein occurs as a two-dimensional hexagonal arrangement of trimers in specialized membrane regions, the purple membrane patches (PM)[3,4]. Most of the primary[5] and secondary structure[6] of BR is known. The protein shows some structural analogy with the visual rhodopsin. Its structure comprises an α-helix and β-sheet conformation. The chromophoric group (retinal) is bound to a lysine residue via a Schiff-base linkage. The protein shows maximum light absorption at 568 nm. It has attracted interest as a model for lipid protein interaction in various membrane systems.

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BR was chosen for this study because it is very stable and easily purified[7]. As in the natural membrane, when incorporated into model membranes BR acts as a light-driven proton pump[8]. Investigations of the proton translocation activity are usually performed by incorporating PM fragments into model membrane systems (BLM, Liposomes)[9,10]. This leads, however, to a lipid mixture in reconstituted membranes because the PM contain tightly bound archaebacterial lipids.

In contrast to the lipids usually present in biological membranes, these lipids typically lack fatty acid residues. These are replaced by isoprenoid alcohols, such as dihydrophytol, bound to glycerol via ether linkages[11]. In order to avoid inhomogeneous reconstituted BR–lipid systems, delipidation and purification of BR prior to the insertion is required[12–14].

As shown by Dencher and Heyn[15], delipidation with the detergents Triton X-100 and β-D-octylglucoside yields the monomeric protein without significant loss in activity. In our HPLC experiments we preferred Triton X-100, because monomeric BR is more stable when attached to Triton X-100 micelles than to β-D-octylglucoside micelles. The low cmc (critical micelle concentration) and the polyether structure of Triton X-100, however, hinders its removal by dialysis[16]. In reconstitution experiments the presence of Triton X-100 leads to leaky membranes. This may be overcome by replacement of the detergent by ion-exchange chromatography[17–19].

EXPERIMENTAL

Halobacterium halobium S9 cells, a gift from Professor D. Oesterhelt (Martinsried), were grown as described[20]. PM patches were isolated according to the method in ref. 7, except that a 30–40% sucrose gradient was used in the final purification step. They were dialyzed overnight in sucrose solution against twice distilled water and collected by centrifugation at 20,000 rpm (40,000 *g*) in a WKF G 50 K centrifuge (WKF, Darmstadt, F.R.G.).

Delipidation of BR was performed with Triton X-100 (Serva, Heidelberg, F.R.G.) at concentrations from 0.1 to 5.0% (w/w) in 0.1 *M* Tris–acetate buffer (Merck, Darmstadt, F.R.G.). The pH of the detergent solutions was varied from 5.0 to 9.0 at the minimum concentration of Triton X-100 required for complete delipidation. The protein concentration was 1.0–1.5 mg BR/ml as revealed by spectroscopic measurements assuming an extinction coefficient of 58,000 l M^{-1} cm^{-1} (ref. 13). Delipidation was achieved by gentle mechanical shaking at 25°C in a RCS 20 Compact Kryostat (MGW, Lauda, F.R.G.) in the dark. Samples for HPLC were passed through 0.4-μm Nucleopore filters. Buffer solutions were passed through 0.45-μm Millipore filters and degassed for at least 1 h *in vacuo* before use. In delipidation experiments, the detergent concentrations of the incubation mixture and the corresponding HPLC buffer were identical.

HPLC measurements were carried out with a Si 200 Polyol 0.005-mm column (250 × 4.6 mm, Serva) connected to a Milton Roy Instrument Minipump (Milton Roy, Philadelphia, PA, U.S.A.). Prior to each delipidation experiment the column was equilibrated for at least 20 h with detergent buffer. The solvent flux of 0.07–0.1 ml/min resulted in a pressure of 10–15 bar in the HPLC system. Eluents were analyzed spectrophotometrically at 580 nm or 280 nm with an ISCO Type 6 dual-beam optical unit (ISCO, Lincoln, U.S.A.) equipped with a 10-μl high pressure cell and connected

to an ISCO UA-5 absorbance monitor. Elution profiles were collected with a CBM 4032 computer (Commodore Business Machines, Santa Clara, CA, U.S.A.) equipped with a 12-bit ADC interface[21] and a PET-Graphic (ELTEC, Mainz) high resolution graphics expansion and data aquisition program "DATENRECORDER". Data were stored with a CBM 8050 dual-drive floppy disk.

UV spectra of the collected HPLC fractions were measured with a 554 Perkin-Elmer spectrophotometer (Perkin-Elmer, Überlingen, F.R.G.).

Molecular weights were determined by calibration of the HPLC system with the marker proteins bovine serum albumin (BSA), albumin (egg) and chymotrypsinogen A (Serva).

The digitized HPLC elution profiles were standardized with a normalization program "NORM" using interactive high resolution graphics. The normalized profiles were then combined to yield three-dimensional plots[22] by use of a high resolution graphics program "DREIDIM" and plotted with a CBM 3022/2022 matrix printer. The programs will be published elsewhere.

RESULTS

In Fig. 1, the elution volumes, V_e, are plotted *versus* the absorption at 580 nm of the eluents at various amounts (%, w/w) of detergent in the HPLC buffer. The elution profiles were taken after incubation with Triton X-100 for 20 h. The three-dimensional plot reveals three different peaks, I–III. At a concentration of 0.2% (w/w) Triton X-100, peak III (BR monomers, see below) is still very small after 20

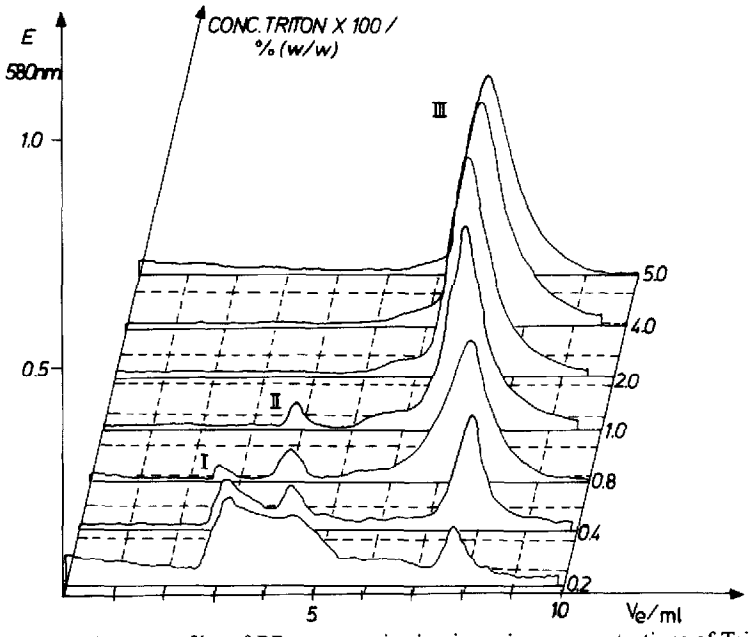

Fig. 1. Elution profiles of BR monomerization in various concentrations of Triton X-100 recorded after 20 h of incubation. The eluent flux varied from 0.95 to 1.07 ml/min. BR concentrations were between 1.0 and 1.5 mg/ml. I = BR aggregates; II = BR trimers; III = BR monomers.

h of incubation. Peaks I (larger PM fragments, see below) and II (trimeric BR molecules, see below) are not completely separated. With increasing amounts of Triton X-100 (0.2–0.8%), peaks I and II become better separated. On the other hand, the amount of protein in these peaks decreases whereas that in peak III increases.

For calibration (molecular weight determination), marker proteins were applied both separately and as a mixture and eluted under identical conditions to those in the BR delipidation experiments.

In Fig. 2 the elution volumes, V_e, of fractions I–III are plotted *versus* the molecular weights. The V_e values were taken from a series of experiments performed with 1% Triton X-100 after an incubation time of 6 h. At higher detergent concentrations peak II is not well separated. The elution volume of the marker proteins is linearly dependent on the logarithm of the molecular weight within the MW range studied. From the calibration curve the MWs of the protein fractions in Fig. 1 were determined to be 23,000 daltons (peak III), 86,000 daltons (peak II) and about 170,000 daltons (peak I).

A concentration of 0.1% (w/w) Triton X-100 causes such instability of the HPLC baseline that no elution profile was measurable (data not shown). Increasing amounts of detergent stabilize the baseline and increase the rate of delipidation. Within a concentration range of 2–5% (w/w) Triton X-100 the rate of delipidation is constant and the elution profiles obtained after 20 h of incubation show no marked differences. Thus, 2% Triton X-100 was considered to be optimal for further investigations on BR monomerization.

The time dependence of the delipidation of BR in 2% Triton X-100–0.1 *M* Tris–acetate (pH 7.0) in the course of about 22 h is shown in Fig. 3. The time of incubation preceding the HPLC analysis is given on the Z-axis. BR monomerization is generally completed within 6 h. The two significant protein fractions correspond

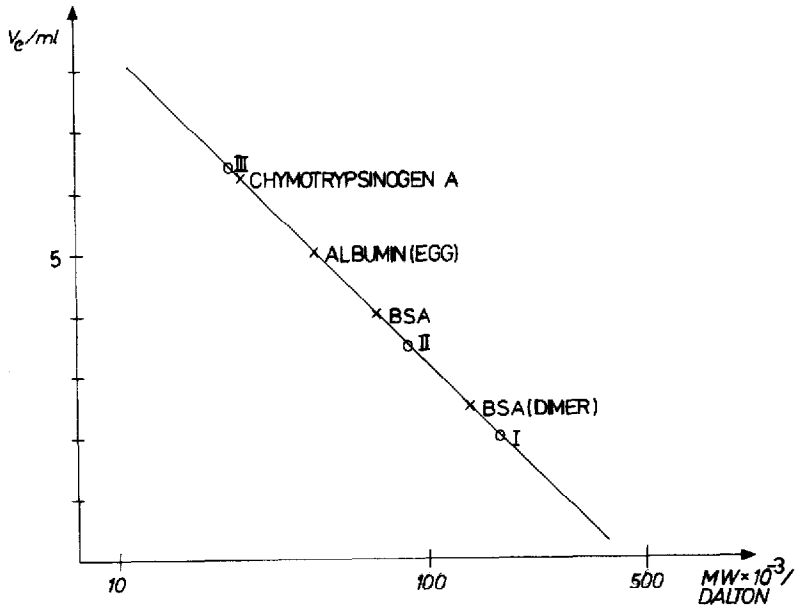

Fig. 2. Molecular-weight calibration of solubilized membrane protein HPLC fractions I–III.

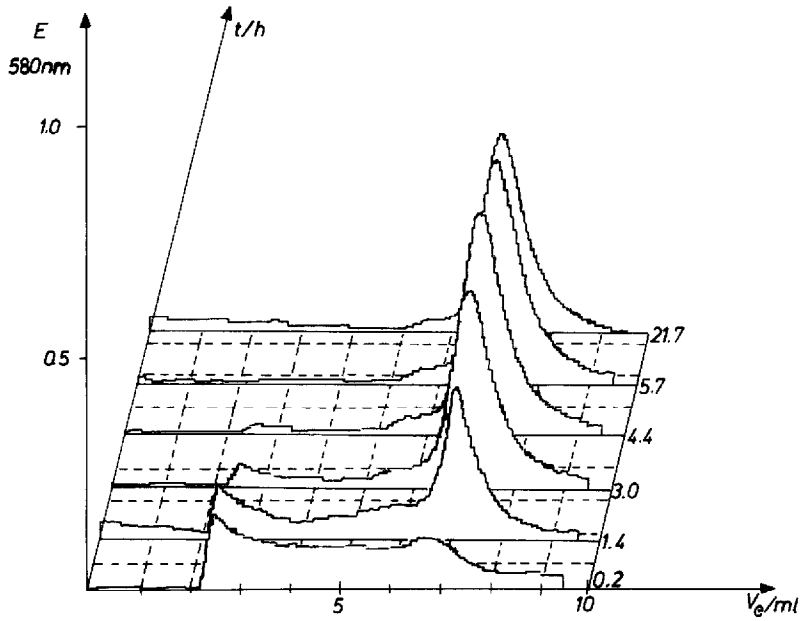

Fig. 3. Three-dimensional computerized plot of the time-dependent delipidation of BR at 2% (w/w) Triton X-100 and pH 7.0. Flux = 0.095 ml/min; BR concentration = 1.3 mg/ml.

to peaks I and III in Fig. 1. The first fraction comprises larger PM fragments. In the course of time, peak I disappears whereas peak III increases, indicating the formation of monomeric BR molecules. The fraction II was not detectable, suggesting that the intermediate state of BR trimers is not obtained. The HPLC fractions were collected and characterized spectroscopically.

The UV absorption spectra of the three HPLC fractions (I–III) are compared with that of native PM in Fig. 4. Upon addition of detergent, the absorption maximum of the chromophore undergoes a blue shift from 560 to 540 nm[13,23,24]. This suggests that either the semi-crystalline structure of the PM is dissociated during the delipidation process or that the Triton X-100 interacts directly with the chromophore[23]. The blue shift occurs immediately after the addition of Triton X-100[25], and there is no further change in the spectra during the subsequent monomerization process.

The effect of the pH on the delipidation of BR was investigated in 2% Triton solutions. The elution profiles at pH 8.0 resemble those at pH 7.0 except that peak II is somewhat more pronounced. At pH 9.0, however, the shape of the HPLC profiles is completely changed (see Fig. 5). In comparison to the experiments performed at pH 7.0 or 8.0, the elution volumes are larger, indicating a stronger adsorption to the detergent-saturated column. The first HPLC elution peak in Fig. 5 is comparable to peak I in Fig. 1 (larger PM fragments). The shoulder appearing after about 23 h probably indicates the presence of trimeric BR molecules corresponding to peak II in Fig. 1. Monomeric BR molecules were not detected. HPLC analysis of BR delipidation products at pH 5.0 and 6.0 also shows increased retention volumes in comparison to experiments at pH 7.0. In delipidation experiments carried out in acidic

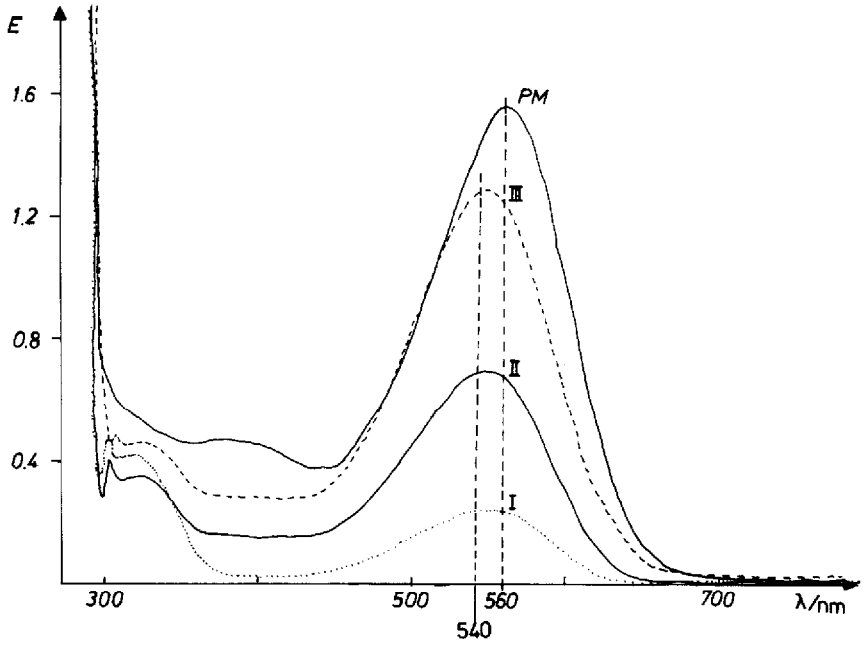

Fig. 4. UV absorption spectra of native PM fragments and detergent-treated HPLC fractions I–III.

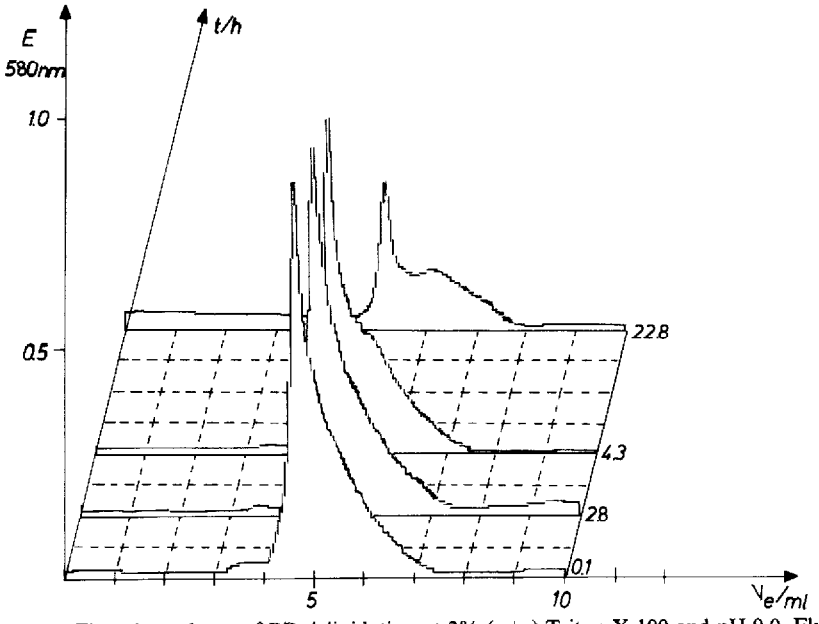

Fig. 5. Time dependence of BR delipidation at 2% (w/w) Triton X-100 and pH 9.0. Flux = 0.1 ml/min; BR concentration = 1.0 mg/ml.

detergent solution a broadening of the elution peaks was observed. The overall process of delipidation in these cases shows monomerization only of about 40–50% instead of more than 90% at pH 7.0, which is in agreement with the results of Dencher and Heyn[25]. We therefore conclude that the optimal conditions for the monomerization of BR are 2% (w/w) Triton X-100 at pH 7.0. Under these conditions the recovery of monomeric BR after HPLC is almost quantitative (80–90%). Although an analytical HPLC column was used in our research, the amount of BR separated in a single HPLC separation was about 380 μg, which is in the micro-preparative range.

The spectrophotometric analysis of HPLC eluents was carried out at 280 nm and 580 nm alternatively. Monitoring of the HPLC eluents at 580 nm yielded the amount of chromoprotein, whereas monitoring at 280 nm yielded the relative amount of protein due to the fact that the strong absorption of Triton X-100 at this wavelength was eliminated by using identical detergent concentrations in the incubation mixture and in the HPLC buffer. The absorption at 280 nm was always exactly accompanied by a corresponding absorption at 580 nm. As indicated by the coincidence of the absorption of the HPLC eluents at 580 and 280 nm and by the nearly quantitative yield of BR monomers (80–90%) as evidenced by spectroscopic determination of BR concentrations, no significant loss of BR chromophore occurs on the HPLC column.

The biological activity of monomeric BR molecules after HPLC separation was examined by measuring the proton pumping activity in reconstituted proteoliposomes according to Huang *et al.*[13].

DISCUSSION

A size-exclusion HPLC system has been applied to the kinetic analysis of the delipidation of the native chromoprotein BR from *Halobacterium halobium*. The separation of monomeric BR under the optimal conditions (2%, w/w Triton X-100, pH 7.0) was almost quantitative, and the monomerization process was completed after about 6 h. The described HPLC system is suitable for the rapid analytical as well as for micro-preparative separation of BR monomers. With detergent concentrations below 0.8% (w/w) the HPLC system yields an unstable baseline. We assume that this is caused by adsorption and desorption of detergent micelles on the column material. Saturation of the Polyol Si 200 HPLC material with detergent is achieved by application of Triton X-100 at concentrations of more than four times the cmc. Only the detergent-coated column is suitable for size-exclusion HPLC with native membrane proteins.

The MWs of the larger BR aggregates and the trimeric and monomeric BR molecules were determined by calibration of the HPLC system with marker proteins. The calculated molecular weight for BR monomers, MW = 23,000, is smaller than the value reported by Ovchinnikov *et al.*[5], but it is in good agreement with the results of Konishi[26] obtained by HPLC methods. The discrepancy between the generally accepted MW of about 26,000 daltons and our results can be explained by specific adsorption of detergent micelles by proteins. Because the detergent takes up up to 50% of the particle mass of some protein detergent aggregates[27–29] the apparent MWs obtained by size-exclusion chromatography are larger in the presence of detergents.

Thus the measured molecular weight is influenced by the nature of the protein surface in a specific manner for the individual proteins. However, the MW for trimeric BR as estimated by HPLC is larger than the value calculated on the basis of the MW of the monomer. This may be due to the larger micelle size of the Triton molecules surrounding the protein aggregate, because in its trimeric form the BR is still embedded in a lipid matrix.

At the optimal conditions for BR delipidation, the yield of BR monomers was 80–90%. Higher detergent concentrations do not result in a more complete BR monomerization. The variation of pH results either in a broadening of the elution peaks at pH 5.0 and pH 6.0 or in a slower delipidation process (pH 8.0). At pH 9.0 no significant monomerization of BR occurs; under these conditions the delipidation process yields BR trimers only.

The UV spectra of detergent-treated PM (Fig. 4) exhibit a 20-nm shift compared with untreated PM. The same shift is obtained for larger aggregates of (monomeric) BR molecules. Due to the similarity of the UV spectra of detergent-treated PM (Fig. 4) and monomerized BR it is not possible to distinguish spectrophotometrically between the different states of BR monomerization. Large BR aggregates, trimeric and monomeric BR molecules, can be distinguished by rapid HPLC analysis of BR monomerization products. The separation can be performed on a micro-preparative scale. Size-exclusion HPLC can be carried out in the absence of organic solvents and the native conformation of BR is thus preserved; highly purified, native BR protein molecules suitable for reconstitution experiments are obtained. The separation can be accomplished within about 90 min and the eluted protein shows full proton-pumping activity as revealed by reconstitution experiments.

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