Journal of Chromatography, 350 (1985) 427–434
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 149

RAPID DELIPIDATION OF THE MEMBRANE PROTEIN BACTERIORHODOPSIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received August 30th, 1985)

SUMMARY

Bacteriorhodopsin from *Halobacterium halobium* was monomerized in Triton X-100 solution. The protein was subsequently delipidated by a detergent exchange procedure employing a size-exclusion high-performance liquid chromatographic (HPLC) procedure. The applicability of four detergents (DOC, TDOC, CHAPS and octyl-β-D-glucopyranoside) to the delipidation process was investigated. The HPLC method shows advantages over other delipidation procedures. The delipidated protein was fully active in light-dependent proton translocation after reconstitution into lipid vesicles.

INTRODUCTION

Investigations of the function and properties of integral membrane proteins (*e.g.*, bacteriorhodopsin or ATP synthase) in their natural environment is often problematical owing to the complex structure of biomembranes and isolation and purification of the enzymes is therefore generally required. The purification procedures usually involve delipidation of the membrane protein in the presence of a detergents[1,2]. For precise chemical and biochemical studies, reconstitution of the delipidated proteins into artificial membranes such as liposomes or planar lipid bilayers is necessary[3–5].

For bacteriorhodopsin (BR), the light-driven proton pump of *Halobacteria*[6,7], a number of studies on the solubilization of the purple membrane and the exchange or removal of the endogenous phospholipids have been reported[8–14]. A highly efficient delipidation and reconstitution procedure, however, has been reported only by Huang *et al.*[14]. As a first step, they described the solubilization of the purple membrane by Triton X-100. This is the detergent of choice for this purpose, as also demonstrated by Dencher and Heyn[15]. In addition, we have recently described the kinetic analysis of the monomerization of the native chromoprotein by Triton X-100 with a size-exclusion high-performance liquid chromatographic (HPLC) system[16].

The disadvantages of Triton X-100 are, however, its strong absorbance at 280 nm, its chemical impurity and, as far as the reconstitution of proteoliposomes is concerned, its low critical micellar concentration. Because lipid model membranes

0021-9673/85/$03.30

become leaky and unstable in the presence of traces of Triton X-100, a detergent exchange procedure must be performed prior to reconstitution of BR into model lipid membranes. This is usually achieved by low-pressure liquid chromatography on ion-exchange or gel permeation media[14,17]. In a previous paper[18] we showed that detergent exchange of Triton X-100 against deoxycholate (DOC) by HPLC can be achieved.

Here we compare three different bile salt detergents and octyl-β-D-glucopyranoside with respect to their applicability to detergent exchange against Triton X-100 and demonstrate a successful delipidation of active monomeric bacteriorhodopsin (MBR) by HPLC without denaturation.

EXPERIMENTAL

Bacteriorhodopsin-containing purple membranes were isolated from *Halobacterium halobium* S9 according to a standard procedure[19]. Monomerization of BR was performed in 0.1 *M* Tris–acetate buffer (pH 7.5) at 4% (w/w) Triton X-100 as described previously[18]. Samples for HPLC were passed through 0.4-μm Nucleopore filters (Nucleopore, Pleasanton, U.S.A.). Buffer solutions were passed through 0.45-μm Millipore filters (Millipore, Eschborn, F.R.G.) and degassed for at least 1 h *in vacuo* before use. The protein concentrations were calculated by absorbance measurements assuming molar absorption coefficients of $\varepsilon = 63\,000$ l mol^{-1} cm^{-1} for the purple membranes[20] and 58 000 l mol^{-1} cm^{-1} for solubilized BR[14].

HPLC measurements were carried out on an Si 200 Polyol 0.005 mm column (500 × 10 mm I.D.; Serva, Heidelberg, F.R.G.) connected to a Biotronik BT 3020 HPLC pump (Biotronik, Maintal, F.R.G.). The flow-rate of 0.5 ml/min yielded a pressure of 30–40 bar in the HPLC system. Prior to each delipidation experiment the column was equilibrated for at least 20 h with detergent buffer. The HPLC elution buffer contained 5 m*M* Tris–acetate (pH 7.5), 75 m*M* sodium chloride, 0.012% (w/w) sodium azide and alternatively 6 m*M* deoxycholate (DOC), 10 m*M* taurodeoxycholate (TDOC), 16 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-propanesulphonate (CHAPS) (Fluka, Buchs, Switzerland) or 44 m*M* octyl-β-D-glucopyranoside (Riedel de Haen, Hannover, F.R.G.). Protein samples of 1 ml containing 3–5 mg of BR were routinely injected. The eluents were analysed optically at 580 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. The eluted fractions (1 ml) were collected with a ISCO Model 328 fraction collector and scanned for determination of the A_{280}/A_{538} absorbance ratio on a Perkin-Elmer 554 spectrophotometer (Perkin-Elmer, Überlingen, F.R.G.).

Molecular weights were determined by calibration of the HPLC system with marker proteins.

HPLC fractions were examined for detergent and lipid content by thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, F.R.G.) using chloroform–methanol–ammonia (14:6:1, v/v) as the solvent. Detection of Triton X-100 and lipids was achieved by staining with iodine vapour or copper(II)–phosphoric acid spray reagent[21]. Quantitative determination of phospholipids was performed according to Fiske and Subbarow[22] after removing the detergent by dialysis.

Circular dichroism (CD) spectra were recorded on a Jasco J-500 A spectro-

polarimeter (Japan Spectroscopics, Tokyo, Japan) using a quartz cell with a 1-cm path length.

RESULTS

Fig. 1 shows the elution profile of a Triton X-100–DOC HPLC exchange run scanned at 280 nm. The dashed line represents the absorbance at 580 nm and the dotted line the absorbance ratio (A_{280}/A_{538}) of the eluent. As described by Bayley *et al.*[1], fractions containing delipidated BR show $A_{280}/A_{538} \leq 2.0$. At an elution volume of about 33 ml (fraction No. 33) the absorbance ratio rises abruptly. This is due to the strong absorption of Triton X-100 at 280 nm, indicating the almost complete separation of Triton X-100 from the BR, as was confirmed also by TLC (see below).

The HPLC profile scanned at 280 nm generally shows four absorption peaks (I–IV in Fig. 1). The first peak represents the void volume (V_0) of the column. Here non-solubilized purple membrane fragments also appear. The second peak contains the monomeric, delipidated BR, as could be ascertained both by its coincidence with the BR absorption peak scanned at 580 nm and by obtaining an A_{280}/A_{538} ratio of <2.0. Here the BR however, is not completely separated (see Fig. 1) from the Triton X-100 and halobacterial lipids. The third elution peak is relatively broad because MBR, lipids and Triton X-100 appear together, possibly because of the presence of mixed micelles of protein, lipids and detergent. Within the fourth and last elution fraction the major amount of Triton X-100 is detected.

Absorption spectra of the HPLC fractions containing BR reveal a blue shift of the absorption maximum of the retinal chromophore from 560 to 538 nm compared with intact purple membranes[14,23]. This effect could be due to reduced cooperativity of the chromophores in solubilized MBR samples in comparison with the

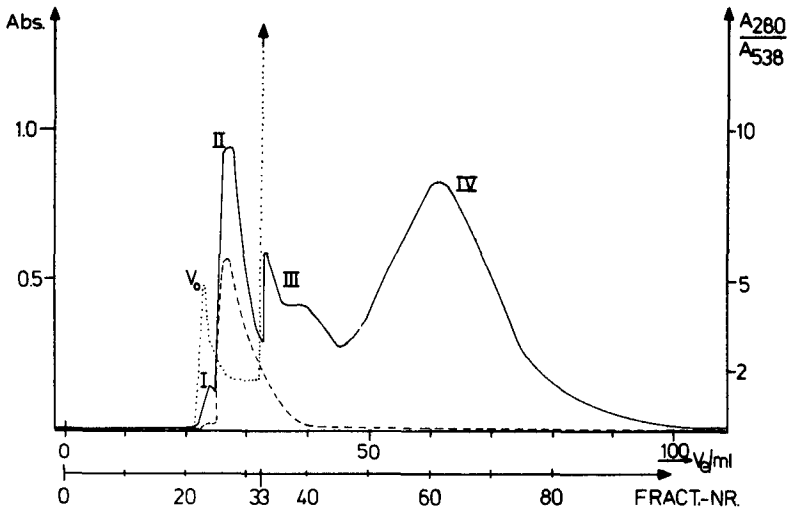

Fig. 1. HPLC elution profiles of a Triton X-100–DOC exchange run scanned at (———) 280 nm and (– – –) 580 nm. Dotted line, A_{280}/A_{538} absorbance ratio. V_0 = Void volume; flow-rate = 0.5 ml min^{-1}; p = 30 bar; c_{BR} = 3.9 mg ml^{-1}.

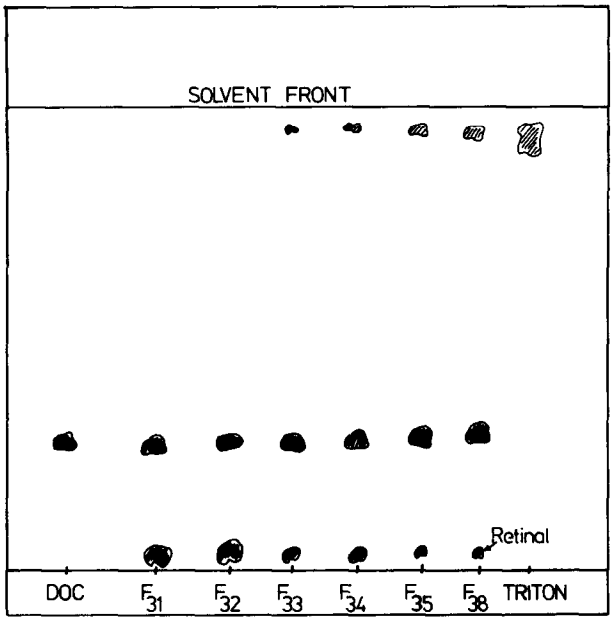

Fig. 2. Thin-layer chromatogram of HPLC fractions from Fig. 1 obtained on silica gel 60 plates. Solvent: chloroform–methanol–ammonia (14:6:1, v/v). Detection: iodine vapour.

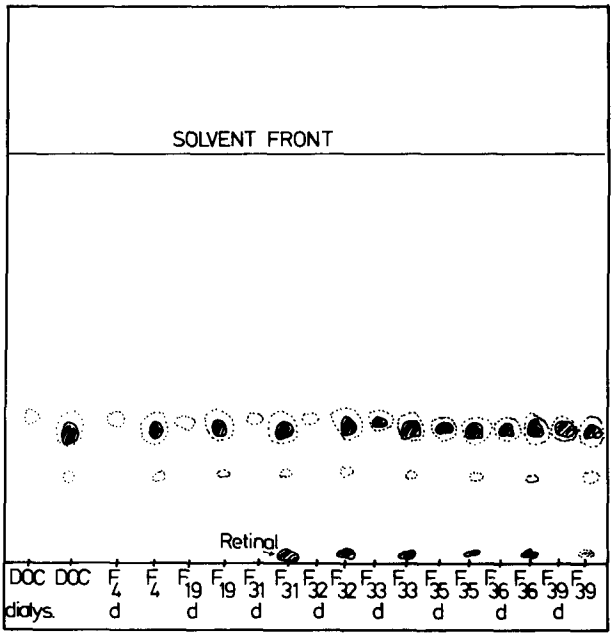

Fig. 3. Thin-layer chromatogram of dialysed (d) and non-dialysed HPLC fractions from Fig. 1. Solvent as in Fig. 2. Detection: copper(II)–phosphoric acid spray reagent.

semi-crystalline arrangement of the protein in the purple membrane, thereby confirming the dissociation of the purple membrane into monomeric BR[23].

The molecular weights (MW) of the HPLC fractions were determined by calibration of the chromatographic system with marker proteins. The protein–detergent micelles (peak II in Fig. 1) show an apparent MW of about 45 000 daltons, which, with respect to the calculated MW of BR (26 000 daltons[24]), rules out the existence of dimers or higher aggregates.

The analysis of the eluted HPLC fractions by TLC is shown in Figs. 2 and 3. Fig. 2 demonstrates that the extreme rise in the A_{280}/A_{538} ratio appearing after fraction 33 (see Fig. 1) is due to the co-elution of Triton X-100 and MBR. Halobacterial lipids are not detected by this method, but we assume that they would coincide with DOC. In order to test this assumption, we analysed a number of HPLC fractions (as listed in Fig. 3). Half of the individual samples were used as reference and the remainder was dialysed for 8 days against doubly distilled water to remove the detergent. Subsequently, the dialysed and non-dialysed samples were analysed by TLC. Staining was performed with copper(II)–phosphoric acid spray reagent, which allows the detection of polar lipids and of DOC but not of Triton X-100. The results are shown in Fig. 3.

In contrast to the references, the dialysed samples (fractions 4, 19, 31 and 32) were not stained. Beginning with fraction 33, however, even the dialysed samples showed a reaction with the copper(II)–phosphoric acid spray reagent. This result supports the inference that the halobacterial lipids are eluted together with Triton X-100. The fact that only one lipid component was detected can be explained by the composition of the purple membrane. It consists of about 70 mol.-% of phosphatidylglycerophosphate, about 20 mol.-% of a glycolipid sulphate and about 10 mol.-%

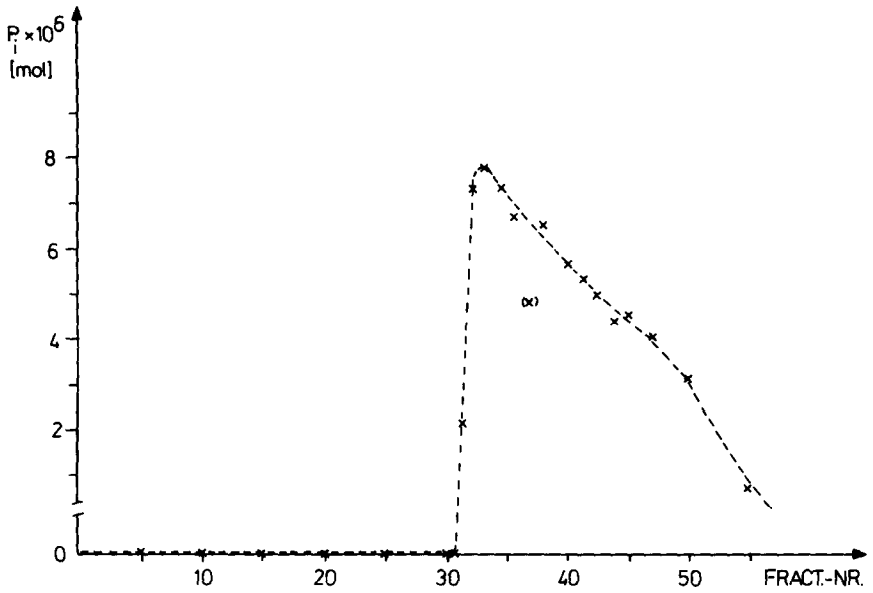

Fig. 4. Determination of inorganic phosphate in HPLC samples from Fig. 1 by the method of Fiske and Subbarow[22].

of two other lipid components (phosphatidylglycerol and phosphatidylglycerosulphate)[25]. With the copper(II)–phosphoric acid spray reagent, however, no glycolipids can be stained and therefore only the major lipid component was detected. Quantitative determinations of the phospholipids were performed by the method of Fiske and Subbarow[22] and the results are shown in Fig. 4. Halobacterial phospholipids are present in the elution volumes that coincide with peak III in Fig. 1. Hence the TLC findings are confirmed (see above).

In Fig. 5, detergent exchange procedures using bile salt detergents are compared by a three-dimensional plot of the elution profiles for Triton X-100 exchange against the detergents DOC, TDOC and CHAPS. The fourth detergent tested (octyl-β-D-glucopyranoside) did not lead to effective delipidation of the MBR samples with our HPLC procedure (data not shown). A similar result for octyl-β-D-glucopyranoside was obtained by Huang *et al.*[14] using low-pressure liquid column chromatography on a 0.5-m Bio-Gel A column. The three bile salt detergents, however, proved to be useful in our HPLC procedure, although they also revealed some differences regarding their applicability. For a comparison of the elution profiles, the profile obtained with DOC will serve as a reference. The shapes of the elution profiles obtained with TDOC and DOC for smaller elution volumes are similar as far as the recovery of MBR is concerned. The final elution peak, however, which contains the major amount of Triton X-100, shows a lower retention with TDOC. Hence the use of TDOC is advantageous, as the complete HPLC separation can be terminated about 30% earlier than with the use of DOC. For the detergent CHAPS the elution profile is different, the retention volume for MBR being higher. Because the subsequent elution of the lipid–detergent micelles (peak III in Fig. 1) is also retarded, a more complete separation of the MBR and the lipid–detergent micelles is achieved. This leads to a higher yield of monomeric, delipidated protein. The overall time

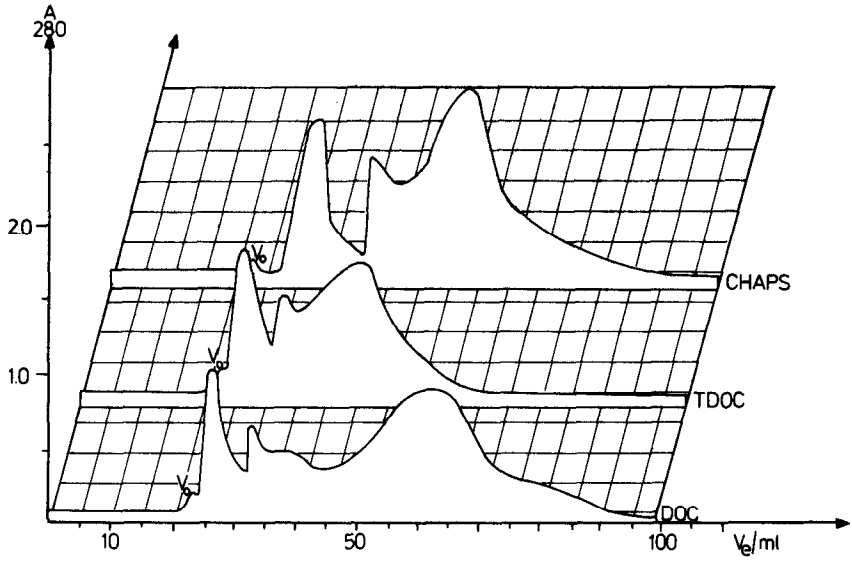

Fig. 5. Comparison of detergent exchange procedures by HPLC with DOC-, TDOC- and CHAPS-containing elution buffers. The elution profiles were scanned at 280 nm. c_{BR} = 3.9–4.3 mg ml^{-1}; p = 30–35 bar; flow-rate 0.5 ml min^{-1}.

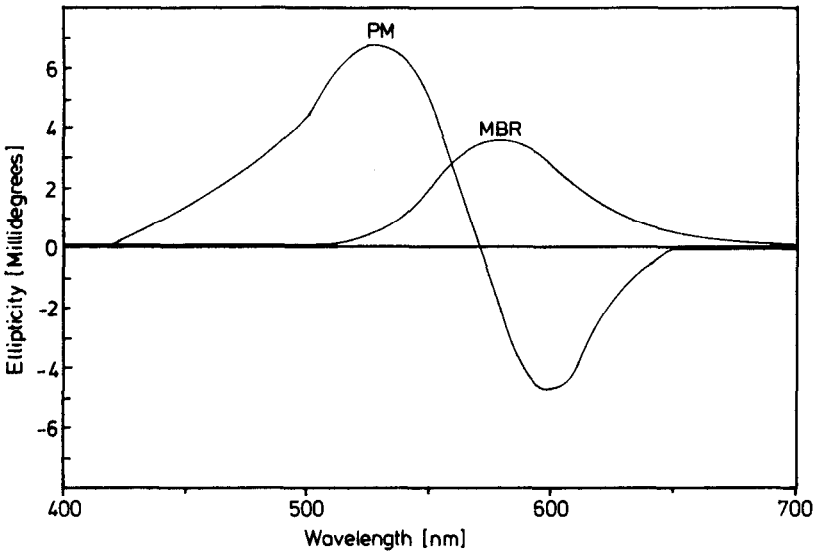

Fig. 6. CD spectra of a purple membrane suspension in 0.1 *M* KCl and of MBR in CHAPS–HPLC buffer. c_{PM} = 0.8 mg ml^{-1} of BR; c_{MBR} = 0.65 mg ml^{-1} of BR.

required for an HPLC run is about the same as that with DOC.

The optimal amounts of BR applied to the HPLC column were between 3 and 4.5 mg. The recovery of active MBR increased from about 85% for TDOC or DOC to more than 95% for CHAPS. In agreement with the observations of Huang *et al.*[14], we obtained lower recoveries when a freshly packed column was used. The recoveries improved, however, and reached the values reported after two or three runs.

Fig. 6 shows the CD spectra of a purple membrane suspension in 0.1 *M* potassium chloride solution and of MBR in CHAPS–HPLC buffer are shown. As reported by Heyn and co-workers[23,26], the CD spectra of BR in the aggregated (purple membrane patches) and solubilized (Monomeric) forms are significantly different. The CD spectrum of MBR shown in Fig. 6 suggests that the protein is obtained in the solubilized and delipidated form by application of the above detergent exchange procedure.

As shown recently[18], the BR samples prepared show full biological activity.

DISCUSSION

The application of a size-exclusion HPLC system to the detergent exchange of MBR–Triton X-100 solutions shows some advantages over other detergent exchange procedures[7–13]. The most effective method up to now was that described by Huang *et al.*[14]. Their low-pressure column liquid chromatographic method applied to the delipidation of BR, however, shows some disadvantages with respect to the elution time and the recovery of MBR. Moreover, the elution procedure must be performed at 4°C, which sometimes leads to gelatinization of the detergent buffer. With our HPLC method, the delipidation procedure can be performed at room temperature.

Owing to the high velocity of the detergent exchange process using HPLC, no loss of retinal chromophore occurred under the elution conditions described. The active MBR is obtained about seven times faster than by the method of Huang *et al.* and also the maximal recovery of protein is higher. Using low-pressure liquid chromatography, the reported yield was about 75%, whereas by our HPLC method yields of 85–95%, depending on the detergent employed, were routinely obtained. On comparing the bile salt detergents used in our investigation, it is obvious that the bile salt derivative CHAPS has advantages over DOC and TDOC with respect to the total amount of active MBR obtained. With TDOC, however, the detergent exchange process is faster than with the other detergents tested.

The HPLC elution process under our elution conditions renders the MBR fully biologically active. The MBR so prepared is superior to purple membrane patches when it is to be used in co-reconstitution experiments together with bacterial ATP synthase, as will be shown in a forthcoming paper[27].

ACKNOWLEDGEMENTS

This work is part of the thesis of R. Pabst for a Dr. rer. nat. degree at the Johannes Gutenberg Universität, Mainz. It was supported by grants from the Deutsche Forschungsgemeinschaft (Do 192/5-1) and from the Bundesministerium für Forschung und Technologie No. 03 B 21 C 02.

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