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PREPARATIVE PURIFICATION OF FUNCTIONAL BACTERIORHODOPSIN BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

High-performance size-exclusion chromatography (HPSEC) was used to produce stable bacteriorhodopsin essentially free of native lipids. The purified bacteriorhodopsin was shown to be highly functional when reconstituted into phospholipid vesicles. Purple membrane was washed in the detergent 3-[(3-cholamidopropyl)dimethylammonio]-2,2-hydroxy-1-propanesulfonate (CHAPSO) to remove a large fraction (65%) of the membrane lipids, solubilized in Triton X-100 and purified on a Bio-Sil TSK G3000SW column using a CHAPSO mobile phase. Pooled column fractions of bacteriorhodopsin from 25-mg sample loads show a 280/548 nm absorbance ratio of 1.5–1.6 and contain less than 4% endogenous lipids. This HPSEC method requires much less expensive synthetic detergent and is much faster than open column methods [*cf.* L. J. W. Miercke, P. E. Ross, R. M. Stroud and E. A. Dratz, *J. Biol. Chem.*, 264 (1989) 7531–7535].

INTRODUCTION

Bacteriorhodopsin (BR) is an integral membrane protein which functions as a light-driven transmembrane proton pump[1]. The structure and mechanism of BR are widely studied and many of the approaches require detergent-solubilized protein in a stable, highly purified state free of native lipid. In a search for conditions to prepare pure, stable, delipidated BR monomers in a dialysable and exchangeable detergent, we modified the low-pressure column procedure of Huang *et al.*[2] since BR is not very stable in the deoxycholate detergent they used[3]. The method of Miercke *et al.*[3] employs a large, low-pressure size-exclusion column and the synthetic bile-like detergent 3 - [(3 - cholamidopropyl)dimethylammonio] - 2,2 - hydroxy - 1 - propanesulfonate (CHAPSO) at pH 5. CHAPSO allows purification to be run at the optimum pH for BR stability in detergent, yet retains the ability to remove native lipids from BR. In order to increase the speed of purification and decrease the amount of detergent required, we have extended the low-pressure purification technique to high-performance size-

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exclusion chromatography (HPSEC). In addition, the functional properties of the purified BR have been characterized.

Since the introduction of suitable HPSEC supports over the past several years, excellent resolution and recovery of soluble proteins[4] and membrane proteins[5,6] have been reported. HPSEC has also been applied to BR. Konishi[7] used HPSEC in the denaturing detergent sodium dodecyl sulfate (SDS) to follow the biosynthesis of membrane proteins in the plasma membrane of *Halobacterium halobium*; the resolution was apparently equal to SDS polyacrylamide gel electrophoresis (PAGE), but the HPSEC method was said to be superior in quantitative peak recovery. Pabst *et al.*[8] used analytical HPSEC in Triton X-100 mobile phase to follow the extent of purple membrane (PM) solubilization by Triton X-100. They obtained 80–90% recovery with maximum loads of 380 μg BR; a 280/445 nm absorbance ratio was not available due to the high 280-nm extinction of Triton X-100. Later, Pabst and Dose[9] used a larger HPSEC column (500 mm × 10 mm I.D.), larger loads (3–5 mg BR) and deoxycholate, taurodeoxycholate and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanosulfonate (CHAPS) as mobile phase detergents to delipidate Triton X-100-solubilized BR. Partial resolution, good delipidation and a BR 280/548 nm absorbance ratio <2 of was obtained; their best lipid and BR separation was with CHAPS. Mucio and De Lucas[10] used an octyl-β-D-glucopyranoside (OG) mobile phase and HPSEC to separate BR from BO (bacteriorhodopsin without the retinal chromophore). With maximum loads of 0.5 mg, improvement of the 280/548 nm absorbance ratio was marginal compared to BR initially solubilized in OG (2.10 to 2.01). These results indicate substantial BR denaturation reducing the 548-nm absorbance or protein impurities elevating the 280-nm absorbance since the 280/548 nm absorbance ratio of the purest BR was found to be 1.5–1.6[3].

Important factors for purifying membrane proteins by size exclusion are: a solubilization detergent that has high solubilization power, but is mild and non-denaturing; the mobile phase detergent should have a small micelle molecular weight and a relatively large critical micelle concentration; detergents, pH and other conditions must be consistent with high protein stability. These conditions allow solubilization to be performed at high protein concentrations, allow separation of solubilization detergent and detergent–lipid micelles from protein–detergent micelles and produce a stable protein where the detergent can be exchanged, if necessary, for lipids or other detergents.

In this paper, we report a large-scale (20–30 mg) HPSEC purification for the integral membrane protein BR where the above conditions have been optimized. The PM is washed in CHAPSO to remove the majority of lipids, solubilized in Triton X-100 and delipidated by HPSEC using a CHAPSO mobile phase at pH 5. This preparative method takes 4 h, uses 4 g of CHAPSO and produces 25 mg of delipidated BR (d-BR). The purified BR was shown to be in a highly stable and functional state by membrane reconstitution and assays of transmembrane proton pumping.

EXPERIMENTAL

BR purification

PM was produced and isolated as described by Miercke *et al.*[3]. BR monomers in Triton X-100 (BR–TX-100) were prepared by suspending PM (8 mg BR per ml) in 2%

(v/v) Triton X-100 (Pierce), 0.4 m*M* sodium azide, 20 m*M* sodium acetate, pH 5.0, and stirring in the dark for 25 h at room temperature in glass vials. Partial removal of lipids from PM without BR solubilization was performed by the method of Szundi and Stoeckenius[11] with the following changes: PM was incubated with CHAPSO (Calbiochem) at 4°C and the detergent/protein molar ratio was increased by 2. BR solubilized in TX-100 was centrifuged (200 000 *g* for 90 min at 15°C), filtered through a 0.2-μm membrane (Gelman Acrodisc) and concentrated using pressure ultrafiltration (10 ml Amicon and YM-10 filter).

A Spectra-Physics SP8700 high-performance liquid chromatographic (HPLC) system was used with a Bio-Sil TSK G3000SW (600 mm × 21.5 mm I.D.) column (Bio-Rad), 2-ml loading loop and a mobile phase containing 16 m*M* CHAPSO (Calbiochem), 0.4 m*M* sodium azide 100 m*M* sodium chloride and 20 m*M* sodium acetate, pH 5.0, at room temperature. Sample volume was 1.5 ml containing 20–30 mg BR. The eluate was continuously monitored at 280 nm using a Kratos Spectroflow 783 detector with a flow-rate of 1.5 ml/min.

Absorpton spectra, light adaption, protein concentration, BR stability, lipid analysis, SDS-PAGE and isoelectric focusing (IEF) were obtained as previously described[3], except a Shimadzu UV-160 spectrophotometer was used to characterize the absorption spectra of d-BR.

Assaying BR function

A modification of Huang *et al.*[2] was used to assay light-induced proton pumping of d-BR reconstituted into soybean phospholipid (Asolectin: 95% purified soy phosphatides; associated Concentrates) vesicles. All operations were carried out at 4°C unless otherwise stated. Vesicles were prepared by adding 0.2 mg d-BR in CHAPSO (d-BR–CHAPSO) to acetone-washed and dried phospholipid that had been solubilized in 0.4 ml of 200 m*M* CHAPSO at room temperature; the volume was brought to 1 ml with water and final sodium and potassium chloride concentrations of 0.15 *M*. The samples were stirred for 30 min and filtered through a 0.2-μm filter. Protein–detergent–lipid mixtures were dialysed in the dark against 1 l reconstitution buffer [0.15 *M* sodium chlorides, 0.15 *M* potassium chloride, 1 m*M* N-2-acetamidoiminodiacetic acid (ADA), pH 6.5, 0.025% sodium azide] for 60 h with five buffer changes followed by two days of dialysis against fresh buffer. A stirred 1-ml vesicle sample (diluted in reconstitution buffer), thermostated at 30°C and equilibrated for 30 min, was irradiated either with light from a 150-W halogen light source or a 30-W halogen lamp combined with the 150-W lamp using light pipes (Cole-Palmer) equipped with heat and yellow filters (Schott KG-4 and OG-530, respectively). A Minolta Auto Meter III light meter was used to measure light intensity.

Proton pumping was recorded with a gel-filled combination pH electrode (Sensorex No. SG900C) coupled with a PHM85 Radiometer pH meter; data were collected (0.8 s per time point) and analysed using Lotus Measure and Lotus 1-2-3 (Lotus Development). Initial proton pumping rates were determined over 10-s intervals. Changes in pH were calibrated using 1 μl of 10 m*M* hydrochloric acid (Fisher). Valinomycin (0.2 μ*M*) (Sigma; 1.5 m*M* stock in ethanol) was used to maintain the inhibitory light-induced membrane potential near zero[12–14].

Vesicle BR concentration was determined spectroscopically using a molar extinction coefficient of 52 000[15]. Vesicle optical scatter was overcome by Triton

X-100 solubilization (0.18 ml of vesicle plus 0.1 ml of 10% Triton in water, diluted with 0.52 ml of 5m*M* sodium acetate, pH 5.0). Corrections for residual optical scattering were made on a Hewlett-Packard 8452A diode array spectrophotometer using a Series 3000 Chem Station to fit the scattering background to a Y^{-b} form where b was adjusted for a best fit.

The transmembrane vectorial orientation of BR in lipid vesicles was determined by quantitating (Bio-Rad video densitometer) the chymotrypsin C-1 BR proteolysis fragment[16] separated on SDS-PAGE (15% total acrylamide; 0.75 mm thick) after silver staining and drying. Chymotrypsin (Sigma) digestion conditons were 0.1 mg BR per ml, 10 m*M* potassium chloride, 50 m*M* Tris, pH 8.0, 1:10 mol ratio of BR/chymotrypsin, dark and 37°C. Digestion at various time points was terminated by addition of SDS-PAGE application buffer and boiling for 3 min. Before chymotrypsin digestion, the vesicles were concentrated by centrifugation (90 000 *g* for 30 min at 4°C) to keep the ionic strength at a minimum.

Vesicle homogeneity was characterized by centrifugation[17] for 20 h at 4°C on continuous (2–20%) sucrose density gradients over a 40% cushion and size-exclusion chromatography[12] (70 cm × 1.5 cm CL-4B column run in reconstitution buffer at 4°C and 1 ml/min). Vesicle morphology was determined by electron microscopy using uranyl acetate-negative stain.

RESULTS

BR purification

The elution profile of BR–TX-100 prepared from native PM and purified by HPSEC in CHAPSO is shown in Fig. 1A. The 280-nm absorbance trace shows well

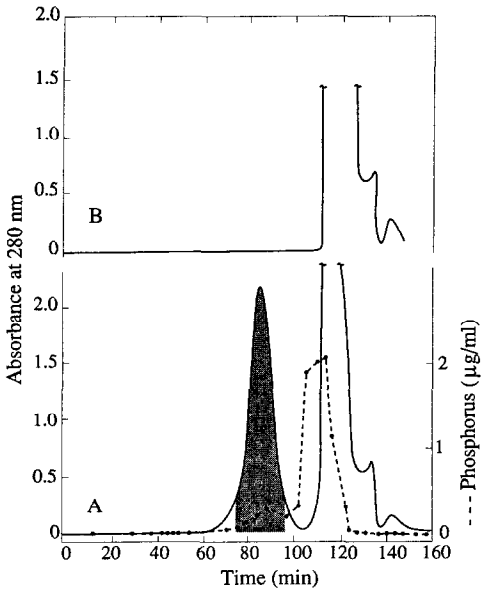

Fig. 1. HPSEC column elution profile of BR on a 60 cm × 2.15 cm TSK G3000SW column using he detergent CHAPSO in the mobile phase. The shaded region was pooled for recovery of purified d-BR. (A) 1.5-ml sample containing 22 mg BR and *ca.* 6% Triton X-100; (B) 1-ml sample of 7% Triton X-100.

separated BR and Triton X-100 peaks (compare in Fig. 1A and B where TX-100 alone was run). When the pooled d-BR sample containing 9% phospholipid (shaded region in Fig. 1A) was concentrated to 1.5 ml and reinjected into the HPSEC column, some slight additional lipid removal was accomplished with 7% of the initial phospholipid remaining in the rechromatographed BR.

In an attempt to remove all phospholipids by one HPSEC column run, PM was first washed in CHAPSO to remove a large fraction of the phospholipids from the membrane before solubilization by Triton X-100. Of lipid–phosphorus 65% was removed by three CHAPSO washes (58, 5 and 2% was removed by the 1st, 2nd and 3rd wash, respectively). When this sample was solubilized in Triton X-100 and applied to HPSEC in CHAPSO, a chromatogram identical to that in Fig. 1A was obtained. However, the total endogenous phospholipid removal on the pooled d-BR peak was 96% relative to the starting PM. BR purification by HPSEC in CHAPSO mobile phase is summarized in Table I.

TABLE I
SUMMARY OF BR PURIFICATION BY HPSEC

Sample	*mol P/mol BR*	*Phospholipid removal (%)*	*BR yield[a] (%)*
PM	12.0	–	–
BR monomers in CHAPSO			
1st column pass	1.1	91	90
2nd column pass	0.7	94	78
PM washed with CHAPSO	4.2	65	–
BR/CHAPSO from PM–CHAPSO[b]			
1 column pass	0.5	96	85

[a] Percentage yield relative to BR solubilized in TX-100 before concentration.

[b] HPSEC-purified BR monomers in CHAPSO prepared from PM washed in CHAPSO to deplete native lipid content and solubilized in TX-100 for chromatography.

For much smaller BR–TX-100 loads (0.6 mg or less), the narrowest analytical TSK G3000SW column (60 cm × 0.75 cm) worked equally well. Identical samples and mobile phases were used but the flow-rate was reduced to 0.6 ml/min.

The absorption spectra (Fig. 2) of the pooled d-BR CHAPSO band (shaded region in Fig. 1A) shows a dark-adapted (DA) absorption maximum at 548 nm and a 280/548 nm absorbance ratio of 1.5–1.6. Upon light adaption (LA), the maximum absorbance shifts to 556 nm with a 6–8% increase in extinction. The 280/248 nm absorbance ratio of BR is directly related to the ratio of total protein/native BR and is a convenient indicator of BR stability and integrity. Identical absorbance ratios are obtained using the conventional low solid-angle collection optics in the Shimadzu UV-160 spectrophotometer or the Shimadzu UV-3000 wide solid-angle scatter-transmission compartment for detergent-solubilized BR or purified d-BR solubilized in detergent. Due to high light scattering of native PM suspensions at 280 nm, poorer apparent absorbance ratios are obtained for PM with the Shimadzu UV-160 (2.1 *vs.* 1.8) compared to the Shimadzu UV-3000 scattered transmission spectrophotometer.

Other properties of d-BR–CHAPSO (stability, SDS-PAGE and IEF pattern) purified by HPSEC are identical to BR purified by our slower, less efficient, large open-column purification procedure[3].

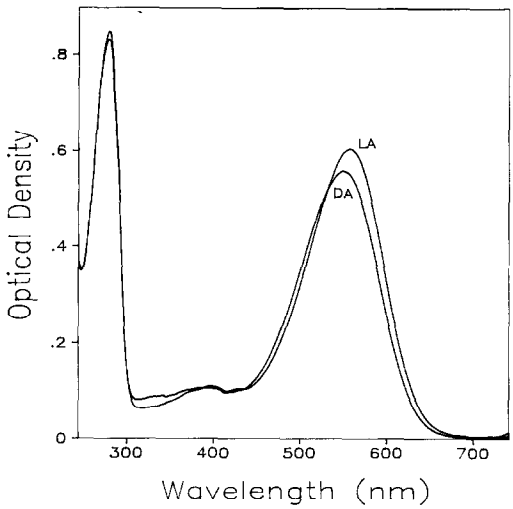

Fig. 2. Dark-adapted (DA) and light-adapted (LA) UV–VIS absorption spectra of pooled d-BR purified by HPSEC in 16 m*M* CHAPSO, pH 5. Light adaption was carried out as in ref. 3.

Assaying BR function

d–BR–CHAPSO reconstituted into soybean lecithin vesicles pump protons efficiently in response to light (Fig. 3). Greatly increased initial-rate (Fig. 3A) and steady-state (Fig. 3B) proton pumping activity was obtained when vesicles were diluted in high ionic strength buffer (2 *M* sodium chloride, 0.15 *M* potassium chloride, 1 m*M* ADA, pH 6.5). Proton pumping was reproducible and linear from 150 to 600 n*M* BR; however, longer times were required to reach steady state as BR concentration is increased; lower BR concentrations gave much larger standard deviations due to lower signal-to-noise ratio. Proton pumping activity was strongly pH-dependent with maximum activity being attained at pH 7.0 (Fig. 4).

Phospholipid vesicles with incorporated d-BR for functional assays showed one major and one minor (approximately 5% of total load) purple band on isopycnic sucrose density gradients at 6.0 and 10.5% density, respectively, and displayed a Gaussian-shaped peak (half width/elution volume = 0.06) with small tail on Sepharose CL-4B size-exclusion chromatography. The lipid vesicles are predominantly unilamellar and range in size from 1.4 to 2.2 μm as shown by electron microscopy.

Densitometry of BR's C-1 chymotrypsin proteolysis fragment (Fig. 5) showed that 69 $\pm$ 4% of d-BR reconstituted into vesicles was oriented inside out. Chymotrypsin digestion was completed within 20 h, and the C-1 fragment is clearly not further degraded by chymotrypsin (Fig. 5).

The pumping measurements were reproducible to within at least 15% since pumping measurements from five independent vesicles incorporated with d-BR had initial-rate and steady-state standard deviations of 15 and 13%, respectively, at low ionic strength and 13 and 9%, respectively, at high ionic strength (standard deviations generated using three light intensities and three data points per light intensity per vesicles). When pumping measurements were extended to thirteen independent liposome samples, reproducibility was within 20%.

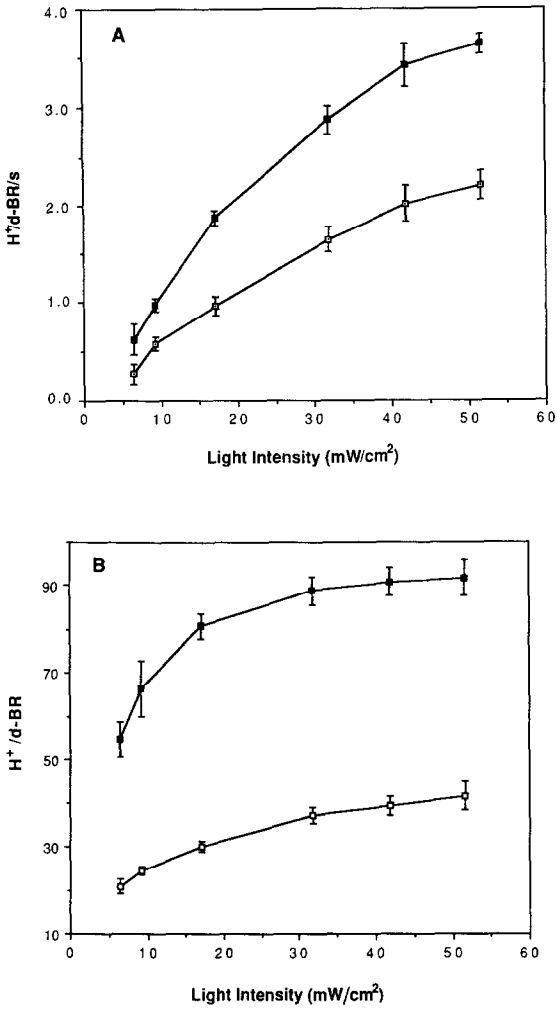

Fig. 3. Light-induced proton pumping of HPSEC–CHAPSO-purified d-BR reconstituted into soybean phospholipid vesicles *vs.* light intensity. BR concentration was 350 n*M* for all assays. (A) Initial proton pumping rate (proton per d-BR per s). (B) Steady-state proton pumping (proton per d-BR). The lower traces in (A) and (B) were obtained in low ionic strength (reconstitution buffer) while the upper traces were carried out at high-ionic-strength conditions (reconstitution buffer containing 2 *M* sodium chloride). High-ionic-strength samples were incubated for 2 h at room temperature in the dark prior to the 30-min equilibration time at 30°C. Values at each light intensity were averaged from six measurements (error bars represent standard deviations) generated from three separate assays and two data points per light intensity per sample. Measurements for each sample were carried out sequentially from lowest to highest light intensities. It was not necessary to correct the initial pumping rates for light-induced electrode response (-0.09 ± 0.13 proton per d-BR per s at maximum light intensity) but the steady-state measurements were corrected for light-induced electrode response using -0.9 ± 0.2, -2.0 ± 0, -2.7 ± 0.2, -4.2 ± 0.2, -4.6 ± 0.2 and -4.9 ± 0.3 proton per d-BR for low to highest light intensities, respectively.

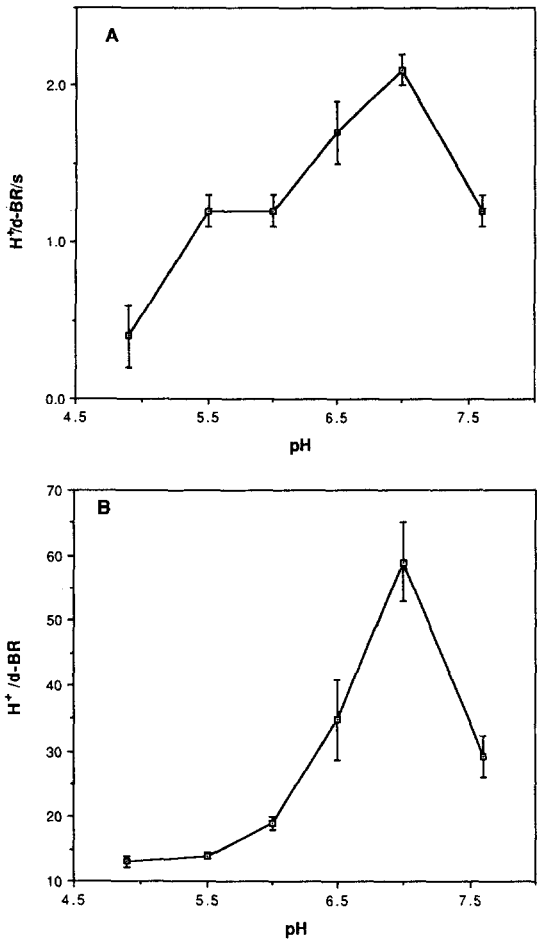

Fig. 4. Light-induced proton pumping as a function of pH. (A) Initial proton pumping rate. (B) Steady-state proton pumping. Vesicles were prepared in reconstitution buffer at pH 6.5 and diluted in same buffer which was adjusted to the defined pH with hydrochloric acid or sodium hydroxide. Values were averaged from three measurements at a light intensity of 32 mW/cm^2; data at pH 6.5 and 7.0 were averaged from nine measurements.

DISCUSSION

BR purification

When PM was washed with CHAPSO prior to Triton solubilization and HPSEC, less than 0.4 mol phospholipid per mol BR were present in the purified delipidated protein after one HPSEC run. It has not yet been possible to determine if this residual phosphate is bound by covalent or non-covalent linkages although rechromatography does not further lower the residual phosphate. Since there is less than a stoichiometric amount of phosphate present, any covalent linkages can only be present in a subset of the d-BR molecules.

The chromatographic profile of BR–TX-100 on HPSEC, percentage yield and

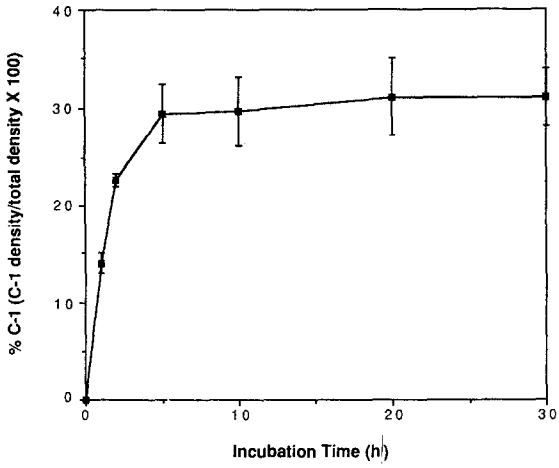

Fig. 5. Orientation of d-BR in the reconstituted vesicles assessed by production of the chymotrypsin C-1 fragment. The amount of BR's C-1 fragment generated *versus* chymotrypsin digestion time was determined by densitometry after SDS-PAGE and silver staining. Values were averaged from three separate digestions using two independent vesicle samples.

percentage phospholipid removal from CHAPSO-washed PM is very similar to our large open-column procedure. If a CHAPSO wash is omitted, HPSEC is less effective at lipid removal than the open column; this decrease in lipid removal is probable due to the considerably shorter HPSEC column length (60 cm *vs.* 180 cm). If CHAPSO washed PM is used, the HPSEC method produces 25 mg of d-BR in one fourth of the time of the open-column procedure and uses one half of the amount of expensive synthetic detergent.

When higher d-BR–CHAPSO concentrations are desired, pressure filtration with an Amicon YM-10 filter is effective; however, the final CHAPSO concentration is then unknown. Amicon-concentrated d-BR in CHAPSO is completely separated from excess concentrated CHAPSO micelles (d-BR and CHAPSO retention time are 14.0 and 22.9 ml, respectively) on the smaller (60 cm × 0.75 cm) TSK column. Therefore, an additional TSK step can be used after the concentration step to restore a known CHAPSO concentration if needed; optimum loads for removal of excess detergent is 2.5 mg d-BR per 0.4 ml using a 0.5-ml loading loop.

Assaying BR function

d-BR–CHAPSO prepared by HPSEC and reconstituted into soybean phospholipid vesicles pump protons with activity similar to d-BR prepared in the detergent deoxycholate[2,12]. The small standard deviations of initial transmembrane proton pumping rate and steady-state data indicates excellent reproducibility.

The initial and steady-state pumping rates are strongly dependent on the salt concentration in the assay mixture with higher salt concentrations giving much greater pumping (Fig. 3). A problem with using higher salt concentrations in the assays is that the pumping rates in high salt is rather strongly dependent on incubation time. For example, a peak pumping steady state is observed after 6 h incubation in 2 *M* sodium chloride but the steady state drops substantially as a function of incubation time over

at least 24 h (data not shown). Even though the light-induced proton pumping rates and steady states are lower, we have chosen to carry out routine assays at 0.3 *M* salt since proton pumping measurements are stable with variation in incubation time.

For precise light-induced proton pumping measurements, the transmembrane orientation of BR witin the vesicle bilayer should be quantitated. The percentage orientation of BR in vesicles was determined by monitoring over time the formation of the chymotrypsin fragment C-1 produced by cleavage of any BR molecules that have an exposed helix B-C loop, i.e., BR molecules that are oriented right-side out. When proton pumping data in the presence of 0.2 μM valinomycin and at pH 7.0 are corrected for BR vesicle orientation, d-BR, prepared by HPSEC–CHAPSO and incorporated into soybean phospholipids (2600 lipids per BR) by dialysis against 0.3 *M* ionic strength, has a proton pumping rate and steady state of 5.5 $\pm$ 0.6 protons per BR per sec and 155 $\pm$ 23 protons per BR at a light intensity of 32 mW/cm^2 and temperature of 30°C.

There is great interest in understanding the molecular mechanism of "uphill" transmembrane proton pumping by BR. Recent advances in protein engineering have provided mutant BR with a wide variety of altered amino acids[18]. The method presented has proved useful for isolation of BR mutants expressed in *Escheria coli* for functional and structural studies[19]. Pumping rates are very dependent on pH (as shown in Fig. 4). Clearly, one or more functional groups titrating in the p*K* range 6–6.5 must be deprotonated for effective pumping and group(s) titrating above p*K* 7.5 must be protonated. Pumping should be investigated as a function of pH in mutant BR proteins to attempt to identify essential groups.

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