

Amphipol-assisted folding of bacteriorhodopsin in the presence or absence of lipids: functional consequences

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Abstract Amphipols are short amphipathic polymers designed to stabilize membrane proteins in aqueous solutions in the absence of detergent. Bacteriorhodopsin (BR), a light-driven proton pump, has been denatured, either by direct solubilization of the purple membrane in sodium dodecylsulfate (SDS) solution or by a procedure that involves delipidation with organic solvent followed by transfer to SDS, and renatured in amphipol A8-35. The effect of different renaturation procedures and of the presence or absence of lipids and the cofactor retinal have been investigated. The resulting samples have been characterized by absorbance spectroscopy, size-exclusion chromatography, thermostability measurements, and determination of photocycle kinetics. Transfer to A8-35 can be achieved by SDS precipitation, dilution, or dialysis, the first route resulting in the highest yield of refolding. Functional BR can be refolded whether in the presence or absence of lipids, higher yields being achieved in their presence. Retinal is not

required for the protein to refold, but it stabilizes the refolded form and, thereby, improves folding yields. Lipids are not required for BR to perform its complete photocycle, but their presence speeds up the return to the ground state. Taken together, these data indicate that a membrane or membrane-mimetic environment is not required for correct decoding of the chemical information contained in the sequence of BR; functional folding is possible even in the highly foreign environment of lipid-free amphipols. BR interactions with lipids, however, contribute to an effective photocycle.

Keywords Amphipol A8-35 · Membrane protein · Folding · Photocycle · Anfinsen's principle

Introduction

The principle that self-organization applies to biological objects as complex as proteins was established 50 years ago by Anfinsen's experiments on the renaturation of ribonuclease (Anfinsen 1973; Anfinsen et al. 1961). It posits that the native structure of a protein corresponds to the state of lowest free energy of the system comprising the protein and its environment. Whether this principle applies to integral membrane proteins (MPs) remained an open question until Khorana and coworkers, 20 years later, demonstrated that bacteriorhodopsin, a polytopic MP that folds into a bundle of seven transmembrane α -helices around its cofactor, retinal (Henderson et al. 1990; Henderson and Unwin 1975), could be brought back to its native three-dimensional (3D) structure after extensive denaturation in organic solvents (Huang et al. 1981). Three decades later, this demonstration has been extended to only a dozen more α -helical MPs (Banères et al. 2011; Booth

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et al. 2001; Kiefer 2003) and approximately as many β -barrel MPs (Buchanan 1999). In some respects, this process remains as amazing today as it was 30 years ago, if not more so, because of the progress made in deciphering the mechanisms that lead to the insertion and folding of MPs in vivo. MPs, indeed, insert vectorially into preformed membranes, with the help of chaperones and, in most cases, a sophisticated insertion-assisting device, the translocon (Park and Rapoport 2012). The membrane into which they insert is asymmetrical and is the site of strong gradients of electrical potential, salt concentration, redox potential, etc. For MPs folded into transmembrane bundles of helices, specific features of the peptide sequence, namely the hydrophobicity of amino acid side chains and the distribution of charges in the vicinity of hydrophobic segments, are decoded by the translocon and determine whether a given segment will insert, remain in the cytosol, or be translocated, and, if it inserts, in which orientation (Park and Rapoport 2012; White and von Heijne 2008). In view of this complexity, it may seem unlikely that Anfinsen's principle applies to MPs, and it is truly surprising that at least some denatured MPs, placed in a mixture of detergent and lipids, manage to find their way to a functional 3D structure.

Bacteriorhodopsin (BR) is a particularly convenient model for MP folding studies. It is produced in large amounts by overproducing strains of the archaebacterium *Halobium salinarum*, in the plasma membrane of which it accumulates in so-called purple membrane (PM) patches, which are easy to purify. PM patches contain BR and BR-bound lipids in a 3:1 mass ratio, the protein and lipids being organized in a highly regular two-dimensional lattice. The purple color results from the presence of retinal, which is bound through a Schiff base to lysine-216, a residue located in the seventh and last helix, close to the mid-plane of the membrane. Free retinal is yellow, because its visible absorption peak is near 382 nm, but it undergoes a strong bathochromic shift on protonation of the Schiff base, giving BR in its ground state its characteristic purple color. Upon illumination, the retinal isomerizes from the all-*trans* to the 13-*cis* form, initiating in the protein a complex cascade of transconformations. The major steps of BR's photocycle (below, Fig. 8a) are:

1. deprotonation of the Schiff base, the proton being taken up by an aspartate residue on the extracellular side of the membrane;
2. reisomerization of the retinal to the all-*trans* form;
3. reprotonation of the Schiff base from the intracellular side; and
4. relaxation to the ground state.

In this process, each photon absorbed drives the extrusion of a proton from the cytosol, building up the proton

gradient that the bacterium uses to drive its physiological processes. Because the absorption spectrum of the retinal is exquisitely sensitive to its environment, most of the many transconformational steps in the photocycle are accompanied by detectable spectral changes (below, Fig. 8b), the most spectacular one being a hypsochromic shift to ~ 412 nm when the Schiff base deprotonates, yielding state M (reviewed by Haupts et al. 1999).

Upon denaturation by dissolution of PM in a solution of sodium dodecylsulfate (SDS), BR unfolds and retinal is released, turning yellow. The resulting mixture of denatured bacterio-opsin (BO), lipids, and retinal has been used as a starting material in innumerable refolding studies, the most popular approach being to dilute the BO/SDS mixture with an excess of detergent/lipid mixed micelles, in which refolding occurs (Huang et al. 1981). The degree to which the refolded protein is characterized ranges from the mere recovery of the purple color (which indicates that the retinal has rebound to a protonated Schiff base) and of a native-like circular dichroism (CD) spectrum (Huang et al. 1981), to that of the light-induced 7–11 nm red shift that accompanies the illumination of the dark-adapted BR (due to the conversion of a fraction of 13-*cis* retinal to the all-*trans* form, indicative that the photocycle is functional) (London and Khorana 1982), to detailed spectroscopic analyses of the photocycle (Sonar et al. 1993), to reformation and crystallographic analysis of two-dimensional crystals (Popot et al. 1986, 1987), and to the recovery of proton-pumping activity after transfer to lipid vesicles (Huang et al. 1981). CD analysis indicates that SDS-denatured BO is ~ 50 % α -helical in structure compared with ~ 70 % for native and refolded BR (Huang et al. 1981; London and Khorana 1982; Riley et al. 1997; Swords and Wallace 1993). This could indicate that the protein has not relaxed to full equilibration with the SDS solution and has retained some structural information acquired during biogenesis. Khorana and coworkers therefore developed an extensive denaturation scheme that involves solubilizing lyophilized PM in formic acid, which achieves complete unfolding of BO and enables its separation from retinal and PM lipids, followed by transfer to SDS solutions, where some α -helicity reappears and from which BR could be renatured in a variety of lipid/detergent mixtures (Huang et al. 1981). This confirmed that BR indeed obeys Anfinsen's principle. The refolding medium most often contained lipids. In some rare cases, it comprised pure detergent (London and Khorana 1982), the general structure of which could, however, be regarded as somewhat similar to that of lipids. This has left uncertain the extent to which a membrane-mimicking environment is required for the protein to acquire its native structure.

Amphipols (APols) are amphipathic polymers developed for handling MPs in aqueous solutions under less

destabilizing conditions than those of detergent solutions (Popot 2010; Popot et al. 2011; Tribet et al. 1996). They have proved efficient tools for folding MPs to their native state starting from a denatured state in SDS (Banères et al. 2011; Bazzacco et al. 2012; Catoire et al. 2010a; Dahmane et al. 2009; Pocanschi et al. 2006) or urea (Dahmane et al. 2011; Pocanschi et al. 2006). APol-assisted MP folding is of interest for two reasons. From a practical perspective, it provides experimental access to MPs, for example G protein-coupled receptors (GPCRs), that can be produced in substantial amounts in a misfolded state only (Banères et al. 2011; Bazzacco et al. 2012; Catoire et al. 2010a; Dahmane et al. 2009). From a fundamental point of view, the use of APols also opens new vistas. Beyond its amphipathic nature, the chemical structure of A8-35, the APol used in the present study, has very little in common with that of lipids. Its hydrophobic and hydrophilic moieties are both very small by lipid standards (Fig. 1), and, rather than occupying the opposite ends of elongated molecules, they are interspersed along the main chain of the polymer. In aqueous solutions, A8-35 does not form bilayers, but forms globular particles that resemble detergent micelles (Gohon et al. 2006; Perlmutter et al. 2011). Upon associating with MPs, it covers their transmembrane surface (Althoff et al. 2011; Catoire et al. 2010b; Zoonens et al. 2005) with an amphipathic layer that is unlikely to reproduce, beyond screening the protein surface from the water phase, any of the physical constraints provided by a lipid bilayer in terms of thickness, fluidity, electrostatic field, packing and lateral pressure, effective dielectric constant, hydration gradients, etc. With regard to molecular interactions, it seems a priori unlikely that A8-35 can recognize specific lipid binding sites. Indeed, indirect evidence suggests that APols compete for these sites less efficiently than detergents and that transferring a membrane protein from a detergent/lipid solution to A8-35 favors lipid rebinding (Gohon et al. 2008; Martinez et al. 2002). APols, therefore, offer a fascinating opportunity to re-investigate the extent to which the folding of MPs depends on information provided by their environment.

In this work we have examined the extent to which different methods for transferring BO from a denaturing environment to APol A8-35 result in functional refolding, and the involvement of lipids and retinal in the process. We

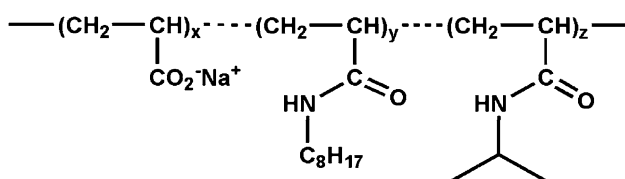


Fig. 1 Chemical structure of amphipol A8-35 ($x \approx 0.4$, $y \approx 0.25$, $z \approx 0.35$) (Tribet et al. 1996)

show that BR can be efficiently folded in pure, lipid-free A8-35, where it performs its entire photocycle. In addition to their fundamental implications regarding MP folding, these experiments therefore provide insight into the involvement of lipids in the photocycle of BR, a question that, until now, has been studied mostly with partially delipidated PM sheets (Fitter et al. 1998; Fukuda et al. 1990; Hartsel and Cassim 1988; Jang and el-Sayed 1988) and detergent-solubilized BR (Hendler and Bose 2003; Hendler and Dracheva 2001; Joshi et al. 1998; Milder et al. 1991; Mukhopadhyay et al. 1996; Váró and Lanyi 1991).

Materials and methods

Chemicals

SDS was from Bio-Rad, OTG (*n*-octyl- β -D-thioglucopyranoside) from Calbiochem, DDM (*n*-dodecyl- β -D-maltoside) from Affymetrix, formic acid (88 %, HPLC-grade) from Fluka, ethanol (HPLC-grade) from Carlo Erba Réactifs/Solvants, Sephadex LH-60 and Superose 12/HR from Pharmacia, all-*trans* retinal and Triton X-100 from Sigma, and bacteriological peptone from Oxoid. Trisodium citrate, potassium chloride, and magnesium sulfate were from Normapur; all other salts were from Sigma. All aqueous solutions were prepared in water purified by use of a Milli-Q system (Millipore). A8-35 (batch FGH20) was synthesized and purified by F. Giusti (UMR 7099) as described elsewhere (Gohon et al. 2004, 2006).

Buffers

SDS-buffer:	5 % (w/v) SDS, 0.025 % (w/v) NaN_3 , 50 mM sodium phosphate, pH 7.0
SDS ₁ -buffer:	2.5 % (w/v) SDS, 0.025 % (w/v) NaN_3 , 50 mM sodium phosphate, pH 7.0
SDS ₂ -buffer:	0.25 % (w/v) SDS, 50 mM sodium phosphate, pH 7.0
K-buffer:	150 mM KCl, 0.025 % NaN_3 , 30 mM sodium phosphate, pH 7.0
NaPi ₁ buffer:	25 mM sodium phosphate, pH 7.0
NaPi ₂ buffer:	100 mM NaCl, 25 mM sodium phosphate, pH 7.0
NaPi ₃ buffer:	50 mM sodium phosphate, pH 7.0
NaPi ₄ buffer:	100 mM NaCl, 50 mM sodium phosphate, pH 7.0

Purification of purple membrane, solubilization of BR, and transfer of native BR to A8-35

Halobium salinarum (strain S9, a gift from G. Zaccai, IBS, Grenoble) was grown on bacteriological peptone (12 g L⁻¹),

and purple membrane (PM) was purified as described elsewhere (Gohon et al. 2008; Oesterhelt and Stoerkenius 1974). Purple membrane stocks were stored at -80°C . For solubilization, PM samples, containing $4\text{--}6\text{ g L}^{-1}$ BR, were incubated for 40 h with constant magnetic stirring with 100 mM OTG (final concentration) in NaPi_2 buffer. The solubilized PM was ultracentrifuged ($200,000\times g$ for 20 min) to remove unsolubilized material. Solubilized BR was diluted in NaPi_2 buffer to a final concentration of OTG of 18 mM. The concentration of BR in the supernatant was estimated by use of $\epsilon_{554} = 47\text{ mM}^{-1}\text{ cm}^{-1}$ and $\epsilon_{280} = 81\text{ mM}^{-1}\text{ cm}^{-1}$ (Gohon et al. 2008; London and Khorana 1982). A8-35, from a stock solution at 10 % w/v in water, was added to BR solubilized in OTG to a final protein/A8-35 ratio of 1:5 w/w. After incubation for 15 min the detergent was removed by adding Bio-Beads for 2 h (Bio-Beads SM2, Bio-Rad, 1:10 detergent/beads mass ratio).

Preparation of denatured bacterio-opsin in SDS

Preparation of SDS-solubilized purple membrane (BO/SDS). BR was denatured by incubating PM (1 g L^{-1}) in SDS-buffer for 2 h at room temperature in the dark, as described elsewhere (Pocanschi et al. 2006; Popot et al. 1987).

Preparation of SDS-solubilized delipidated bacterio-opsin (dBO/SDS). Delipidation of PM was performed as described elsewhere (Huang et al. 1981; Pocanschi et al. 2006; Popot et al. 1987). Briefly, lyophilized PM was solubilized in formic acid (88 %) at room temperature. After 5 min, ethanol was added to furnish a 30:70 (v/v) formic acid/ethanol mixture. The final protein concentration was typically 4 g L^{-1} . The separation of dBO from lipids and retinal was performed by size-exclusion chromatography on a 200-mL Sephadex LH-60 column equilibrated with formic acid/ethanol (Huang et al. 1981; Popot et al. 1987). The absence of lipids in dBO fractions ($<2\%$ of the lipid/BR ratio in PM) was established by thin-layer chromatography (Gohon et al. 2008). Transfer of dBO from formic acid/ethanol to SDS was achieved by dialysis as described elsewhere (Popot et al. 1987). The final concentration of dBO in SDS/buffer was $\sim 0.25\text{ g L}^{-1}$, taking ϵ_{280} for BO to be $65\text{ mM}^{-1}\text{ cm}^{-1}$ (Liao et al. 1984). When specified, dBO solutions in SDS-buffer were supplemented with retinal before renaturation in 1.3:1 (molar ratio) retinal/dBO, taking ϵ_{382} of retinal to be $42.8\text{ mM}^{-1}\text{ cm}^{-1}$ (Rehorek and Heyn 1979).

Renaturation of bacteriorhodopsin from SDS

Renaturation by precipitation of dodecylsulfate using KCl. BR renaturation was performed by precipitating dodecylsulfate as its potassium salt (PDS), as described elsewhere

(Pocanschi et al. 2006; Popot et al. 1987). A8-35 was added, at ratios of 2, 5, or 10 g A8-35 g^{-1} BO, either to SDS-solubilized PM (BO/SDS) or to dBO in SDS-buffer supplemented or not with retinal. After incubation for 15 min at room temperature, SDS was precipitated by addition of KCl. After 30 min, the PDS precipitate was removed by centrifugation and the supernatant was dialyzed twice against 100 volumes of K-buffer. The final concentration of renatured BR was in the range $0.2\text{--}0.4\text{ g L}^{-1}$.

Renaturation by dialysis of SDS. BR was denatured by incubating PM (1 g L^{-1}) in SDS_1 -buffer for 2 h at room temperature in the dark. A solution of A8-35 was added to the denatured BO (at final mass ratios 5 or 10 g A8-35 g^{-1} BO) in such a manner that the initial sample of BO/SDS was diluted twice with water. After incubation for 15 min, the samples were dialyzed twice against 100 volumes of NaPi_1 or NaPi_2 buffer for a period of 48 h, using a membrane with a molecular weight cut-off of 10 kDa.

Renaturation by dilution of SDS. BR was denatured by incubating PM (1 g L^{-1}) in SDS_2 -buffer for 30 min at 45°C in the dark, as described by London and Khorana (1982). BO/SDS was diluted $5\times$ in SDS-free buffer containing either detergent (DDM or Triton X-100) at the specified concentration or A8-35 at a ratio of 10 or 25 g A8-35 g^{-1} BO. Renaturation was followed by monitoring the UV-visible spectrum in a 1-mL cuvette with constant stirring.

Renaturation from organic solvent

Delipidated dBO in formic acid/ethanol was dried under vacuum and resuspended in trifluoroethanol (TFE) to a final concentration of dBO of $\sim 1.8\text{ g L}^{-1}$. Retinal was added at a protein/retinal molar ratio of 1:1.5 and A8-35 at a protein/A8-35 mass ratio of 1:10. Typically, $80\text{-}\mu\text{L}$ samples were dialyzed for 2 days against 1 L 20 mM phosphate buffer, pH 7.0, the dialysis buffer being changed once.

Characterization of renatured BR samples

Percentage renaturation was estimated by comparing the A_{280}/A_{554} ratio of dark-adapted samples with that of native BR in OTG. Spectra were recorded in $100\text{-}\mu\text{L}$ quartz cuvettes (Hellma, France) on a Hewlett–Packard HP-8453 spectrophotometer. Thermal denaturation was performed by using the temperature controller of the spectrophotometer. Samples were placed in a $100\text{-}\mu\text{L}$ quartz cuvette covered with a plastic film (Parafilm) to prevent evaporation. UV spectra were measured between 20 and 70°C . Each spectrum was recorded 20 min after the temperature was reached.

Size-exclusion chromatography

The homogeneity of the renatured samples was examined by size-exclusion chromatography (SEC) on a Superose 12/HR column equilibrated in K-buffer, connected to an Äkta system (Pharmacia). Elution profiles were typically followed at 280, 382, and 550–554 nm.

Time-resolved absorption spectroscopy

The procedure used was the same as that described by Gohon et al. (2008). Native BR trapped in A8-35 at a 1:5 BR/A8-35 mass ratio (nBR/5A8-35) and samples renatured either from BO/SDS (rBR/5A8-35) or from dBO/SDS (rdBR/5A8-35), generally at a concentration of 0.2–0.4 g L⁻¹, were pre-illuminated for 4 min at 4 °C to induce the light adapted state. Absorption changes associated with the photocycle of BR were recorded on a home-built instrument (Béal et al. 1999). The photocycle was initiated by means of a 640-nm pulse of 5-ns duration from a frequency-doubled Nd:Yag laser. Absorption changes for a photoactivated sample and one in the initial state were recorded (Fig. 8c) in the spectral range 370–500 nm. These changes were fitted by a sum of five exponentials (Gohon et al. 2008; Milder et al. 1991), each one characterized by a half-time $t_{1/2}$ and a wavelength-dependent amplitude (further details are given by Gohon et al. 2008). The so-called “decay-associated spectra” (DAS) representing these amplitudes as a function of wavelength are plotted in Fig. 8e.

Results

Dark-adapted BR in its native PM environment has an absorption peak in the visible at ~560 nm, which reflects the presence of a mixture of all-*trans* and 13-*cis* retinal bound to the folded protein by a protonated Schiff base. Upon solubilization by use of non-denaturing detergents, e.g. octylthioglucoside (OTG), this peak shifts to 554 nm; this has been attributed to a shortening of the distance between the Schiff base and the negatively charged proton acceptor Asp-85 (Milder et al. 1991). This feature is characteristic of the soluble state and is maintained after transfer of detergent-solubilized BR to A8-35 (Gohon et al. 2008) or to non-ionic APols (Bazzacco et al. 2012). Dissolution of PM in SDS causes denaturation of the BR and release of free retinal, with an absorption peak at ~382 nm. Addition of A8-35 and removal of SDS results, under appropriate conditions, in the refolding of BO to its native state, rebinding of retinal, and recovery of the purple color (Pocanschi et al. 2006) (Fig. 2a). In a previous publication we described a standard procedure, derived from that developed for refolding of BR in lipids (Popot et al.

1987), which leads to nearly quantitative refolding (Pocanschi et al. 2006). It relies on precipitating dodecylsulfate as its potassium salt (PDS), removing the PDS crystals by centrifugation, and removing residual dodecylsulfate by dialysis against buffer containing 150 mM KCl (to prevent redissolution of residual PDS crystals) and 30 mM potassium phosphate, pH 7.0 (K-buffer). Under these conditions, refolding yields $\geq 90\%$ are routinely achieved (Table 1, line 1a). The same procedure has been used to fold several GPCRs, with yields ranging from 40 to 70 %, using either A8-35 (Banères et al. 2011; Dahmane et al. 2009) or non-ionic APols (Bazzacco et al. 2012) as the accepting medium. In the following discussion, we first examine the consequences of various modifications of the standard procedure, with the objective of identifying alternative approaches that could be better suited either to the folding of other MPs or to obtaining other types of samples. We then characterize the resulting samples in respect of the structure and function of refolded BR.

Is there more than one way to skin a cat? Effects of departing from the standard refolding protocol

The conventional starting material for refolding experiments is SDS-solubilized PM (hereafter called BO/SDS), which contains lipids and retinal (Huang et al. 1981). Alternatively, delipidated BO (dBO) can be produced by solubilizing lyophilized PM in formic acid, supplementing the solution with ethanol, and separating BO from lipids and retinal by hydrophobic size-exclusion chromatography (SEC) (Huang et al. 1981). dBO can then be transferred either to other organic solvents or to an SDS solution (the latter preparation being denoted dBO/SDS), and supplemented or not, before renaturation, with retinal and/or lipids, which introduces much flexibility to investigation of the factors that affect refolding.

Refolding in the absence of lipids

Renaturation of BO/SDS and dBO/SDS in the presence of A8-35 are compared in Fig. 2. In the case of BO (Fig. 2a), PM lipids and retinal are present in the original mixture; in that of dBO (Fig. 2b), retinal was added back after its elimination during hydrophobic SEC, but no lipids were present. Two points are worth noting:

1. refolding yields are almost, although not totally, as good when refolding is carried out in the absence as in the presence of lipids (typically 70–75 % vs. ~90 %, respectively; *cf.* Table 1, part 1, line 1b vs. 1a); and
2. more A8-35 is needed to achieve a maximum yield from renaturation in the presence (A) than in the absence (B) of lipids (Table 1, part 1).

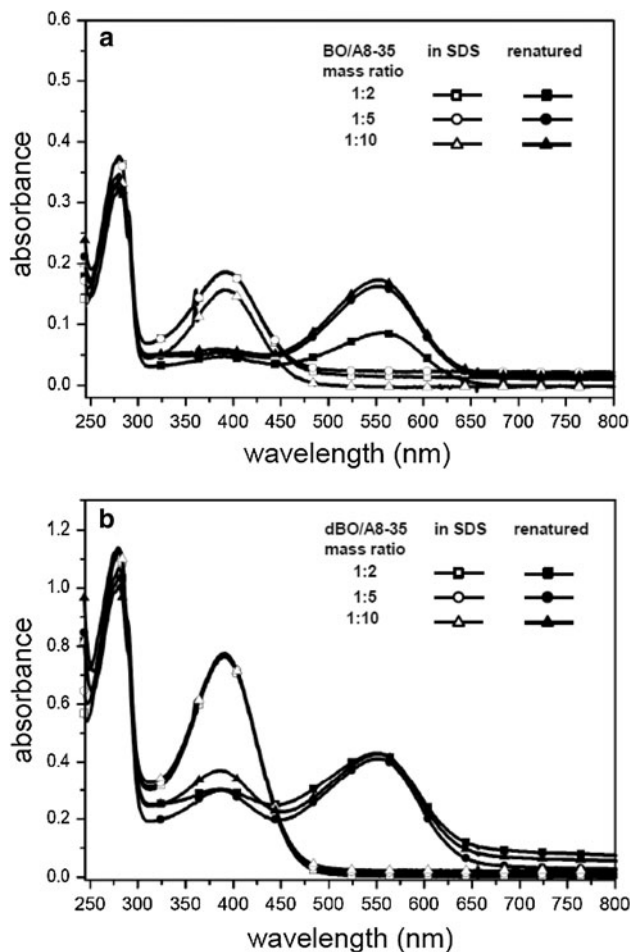


Fig. 2 Renaturation by dodecylsulfate precipitation. A8-35 was added to either SDS-solubilized bacterio-opsin (BO; data from Pocanschi et al. 2006) (a) or delipidated bacterio-opsin (dBO) (b) at ratios of 2, 5 or 10 g g⁻¹ protein. Dodecylsulfate was precipitated with KCl, PDS crystals eliminated by centrifugation, and the samples dialyzed against K-buffer for 48 h. UV-visible absorption spectra were recorded before (*open symbols*) and after (*solid symbols*) renaturation. Refolding yields are given in Table 1, part 1

This suggests that lipids, although they slightly improve the maximum yield of renaturation, may, at low concentrations of APols, facilitate the aggregation of refolding molecules. SEC analysis of samples refolded at different BO/A8-35 ratios (Pocanschi et al. 2006) indeed suggests that aggregation is the likely cause for the lower yield observed at 1:2 BO/A8-35 ratio (Fig. 2a). Finally, the visible absorbance peak of lipid-free renatured samples appeared slightly blue-shifted compared with that of lipid-containing samples (550–552 vs. ~554 nm).

Renaturation from organic solvent

Attempts were made to refold dBO by transferring it from formic acid/ethanol to TFE, supplementing it with retinal and A8-35, and exchanging TFE for aqueous buffer by

dialysis. Results were erratic, one experiment achieving ~40 % chromophore regeneration and the others failing. Despite numerous attempts to vary the conditions of transfer to TFE and the exchange conditions, the source of variability was not identified. It may be affected by the state of the protein when redissolved in TFE, which may depend on the exact manner in which the transfer is performed. These attempts, although mainly negative, are nevertheless worth mentioning, because they show that this approach can be made to work.

Renaturation by dialysis

BR can be refolded by supplementing BO/SDS with A8-35 and removing the SDS by dialysis (Fig. 3a; Table 1, part 2). The renaturation yields observed after dialysis against phosphate buffer (NaPi₁; 25 mM sodium phosphate, pH 7.0, no salt) are similar to, although somewhat lower than, those obtained by precipitation, if A8-35 is present at a protein/A8-35 mass ratio of 1:10 (70–80 %; Table 1, lines 2a,b). At a mass ratio of 1:5, renaturation yields tend to be lower (50–60 %; *ibid.*). Renaturation yields can be improved by increasing the ionic strength of the dialysis buffer, e.g. by supplementing it with 100 mM NaCl (NaPi₂ buffer; Fig. 3a; Table 1, lines 2c,d). As an example, for a mass ratio of 1:5, the renaturation yield for BO in 5 % SDS increases from ~52 % in NaPi₁ (Table 1, line 2a) to ~76 % in NaPi₂ (Table 1, line 2c).

To further examine this issue, renaturation was carried out by dialysis of BO/SDS against solutions of detergents such as 5 mM DDM or 0.5 % Triton X-100. At low ionic strength, this approach leads to good renaturation yields (≥80 %; Fig. 3b). In contrast with the behavior observed with A8-35, however, the refolding yield decreased when the dialysis buffer contained 100 mM NaCl (Fig. 3b).

The contrasting effects of ionic strength, depending on whether refolding is conducted in A8-35 or detergent solutions, can possibly be related to the dependence on salt concentration of the critical micellar concentration (cmc) of SDS, which, at 20 °C, drops from ~8 mM (~0.23 %) in pure water to ~1.4 mM (~0.04 %) in buffer-free 100 mM NaCl (Mukerjee and Mysels 1971). This increases the partitioning of SDS in mixed micelles and decreases the rate of SDS elimination by dialysis. Why is it, then, that upon refolding in the presence of A8-35 the opposite effect is observed—addition of salt having a positive effect on the renaturation yield? Two phenomena may be involved. First, it seems that APols carrying a net charge are more stabilizing at high than at low ionic strength. This has been observed for sulfonated APols, whose charge density is higher than that of A8-35 (Dahmane et al. 2011). Although this effect is not obvious for BR/A8-35 complexes (*ibid.*) it may be important during the delicate process of refolding.

Table 1 Yields of A8-35-assisted renaturation of BO/SDS or dBO/SDS by use of different procedures

	Denatured sample	Method of transfer to A8-35	Folding yield at protein/A8-35 mass ratios	
			1:5	1:10
<i>1</i>		<i>Precipitation</i>		
1a	BO in SDS 5 %	Add A8-35, precipitate dodecylsulfate with KCl, dialyze against K-buffer	87 ± 8 % (<i>n</i> = 4)	92 ± 8 % (<i>n</i> = 4)
1b	dBO in SDS 5 %	Same as 1a	70 ± 9 % (<i>n</i> = 4)	76 ± 10 % (<i>n</i> = 4)
<i>2</i>		<i>Dialysis</i>		
2a	BO in SDS 5 %	Add A8-35, remove SDS by dialysis against NaPi ₁ buffer (no NaCl)	52 ± 12 % (<i>n</i> = 3)	72 ± 10 % (<i>n</i> = 3)
2b	BO in SDS 2.5 %	Same as 2a	62 ± 13 % (<i>n</i> = 3)	80 ± 10 % (<i>n</i> = 3)
2c	BO in SDS 5 %	Add A8-35, remove SDS by dialysis against NaPi ₂ buffer (100 mM NaCl)	76 ± 2 % (<i>n</i> = 3)	76 ± 5 % (<i>n</i> = 3)
2d	BO in SDS 2.5 %	Same as 2c	74 ± 8 % (<i>n</i> = 3)	80 ± 12 % (<i>n</i> = 3)
<i>3</i>		<i>Dilution</i>		
3a	BO in SDS 0.25 % (denaturation was completed by incubating the sample at 45 °C for 30 min)	Dilute 5× with SDS-free NaPi ₃ buffer containing A8-35 (no NaCl)	~0 %	~40–50 %
3b	Same as 3a	Dilute 5× with SDS-free NaPi ₄ buffer containing A8-35 (100 mM NaCl)	Not available	~70–80 %

The second mechanism may be specific to BR, or, at least, to MPs whose stability depends on the binding of cofactors, lipids, or subunits. Retinal is a highly hydrophobic molecule, expected to partition into A8-35 particles. At a 1:10 BO/A8-35 mass ratio, the number of those exceeds that of retinal molecules by a factor of ~7, meaning that most newly refolded BO molecules will find themselves surrounded by a retinal-free layer of A8-35. Uptake of retinal, which is required to stabilize the refolded protein (see below), depends on the rate of collision of retinal-free BO/A8-35 complexes with retinal-containing A8-35 particles, a process that is highly dependent on ionic strength (Zoonens et al. 2007). By accelerating the uptake of retinal by freshly refolded BO, raising the ionic strength can prevent its denaturation and, thereby, increase the renaturation yield. Similar phenomena may be expected to be involved whenever the stability of a freshly refolded MP depends on its binding components that are dispersed among APol particles. Such a mechanism may explain, for instance, why the folding of the leukotriene BLT1 receptor goes through a maximum, in the absence of lipids, at a protein/A8-35 mass ratio of 1:5 (Dahmane et al. 2009). Although BLT1/A8-35 mass ratios of 1:10 or 1:20 might be expected to further decrease the probability of counterproductive intermolecular association during refolding, they will also decrease the rate of dimerization, a process that is likely to stabilize the refolded receptor.

Renaturation by dilution of SDS

BR can be renatured by diluting a BO/SDS/A8-35 mixture with SDS-free buffer (Fig. 4). The rate and final yield of

refolding in A8-35 were markedly improved in the presence of more APols, namely for a 1:25 rather than 1:10 mass ratio (Fig. 4a). Two mechanisms may contribute to this effect. First, adding more A8-35 reduces the concentration of SDS in the environment of the refolding molecules. Second, the probability that two refolding molecules will come into contact and establish intermolecular associations is reduced by diluting them with more APol. The same effect of salt using this mode of renaturation was observed as upon renaturation by dialysis. Figure 4b shows the evolution of the absorbance at 554 nm after addition of A8-35 and dilution with SDS-free buffer, NaPi₃ (50 mM sodium phosphate, pH 7.0, no salt), or NaPi₄ (same plus 100 mM NaCl); improvement of the final renaturation yield by a factor of ~2× was observed under the second condition. As previously observed with the dialysis procedure, the presence of 100 mM NaCl in the dilution buffer, in contrast, reduced renaturation yields for BO/SDS in Triton X-100 (Fig. 4c), presumably because, as a result of the lower cmc of SDS under these conditions, the mixed SDS/Triton particles in which refolding occurred contained a higher proportion of SDS.

The effect of ionic strength on the refolding yield observed by use of the dialysis procedure led to the hypothesis that rapid rebinding of retinal to the freshly refolded BO was likely to be an important factor in improving the folding yield. This hypothesis was further checked by use of the dilution procedure. Being obtained by solubilizing PM in SDS, BO/SDS solutions contain retinal in a strict 1:1 molar ratio to BO. At a 1:10 BO/APol ratio, this corresponds to one molecule of retinal per

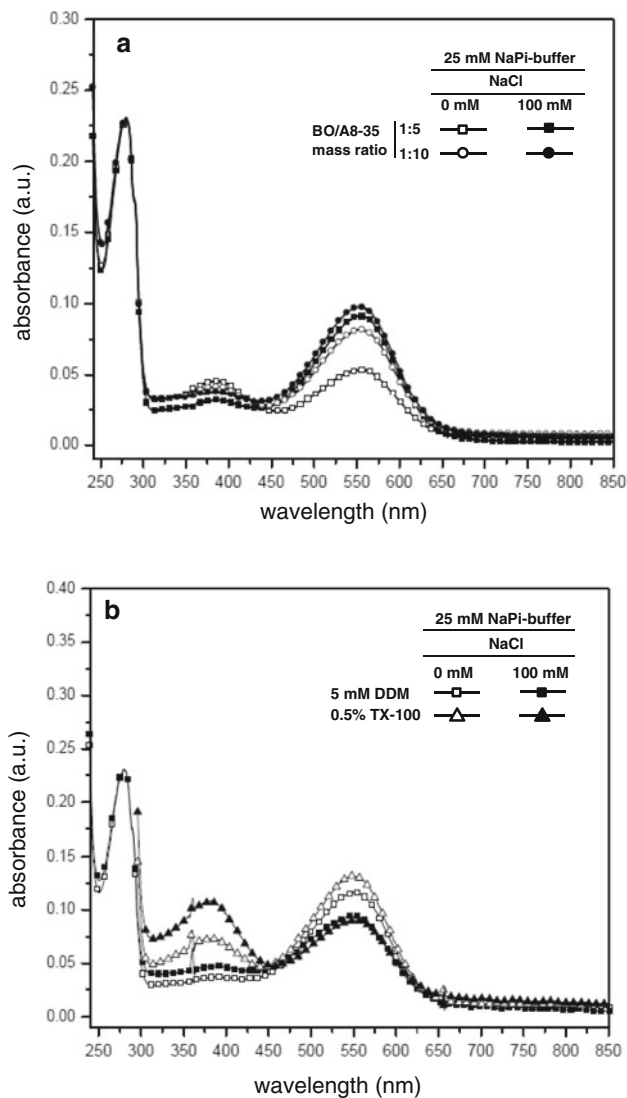


Fig. 3 Renaturation by dialysis. PM was denatured in 2.5 % SDS (SDS₁/buffer). BO/SDS samples (1 g L⁻¹ BO) were supplemented with either A8-35 at the indicated protein/APol ratio (a) or detergent (5 mM DDM or 0.5 % Triton X-100) (b), diluted twice (with the same concentration of detergent, if applicable), and the samples dialyzed against SDS-free buffer containing either no NaCl (NaPi₁) or 100 mM NaCl (NaPi₂), supplemented, if applicable, with the same concentration of detergent. UV–visible spectra were recorded after the samples had been dialyzed for 48 h. The spectra in Triton X-100 were cut below 295 nm because of the absorbance of the detergent. Refolding yields in A8-35 are given in Table 1, part 2

270 kDa A8-35. BR has been shown to bind ~2 g of A8-35 g⁻¹ BR, i.e. ~54 kDa (Gohon et al. 2008). Assuming BO binds ca. the same amount of APol as BR, the chances that a molecule of retinal will be present in the APol layer bound to a refolding molecule of BO are about 1 in 5. Molecules that fold in a retinal-free environment are at risk of being inactivated before the cofactor can be delivered to them by collision with a retinal-containing A8-35 particle.

Providing extra retinal to the preparation, which should accelerate its uptake by refolded BO, could, therefore, improve the yield. This turned out to be the case, as shown in Fig. 4d: supplementing the preparation with extra retinal to reach a retinal/BO molar ratio of 4:1, which, taking into account the dilution into APol particles, amounts to a probability of ~1:1.25 of a retinal molecule being present in the environment of a refolding BO, increased the refolding yield by a factor of ~2, from ~40 to ~70 % (in the absence of salt in the renaturing buffer) (Fig. 4d). As expected, this was accompanied by an increase in the rate of reformation of the holoprotein, as detected by the re-appearance of absorbance at 554 nm, consistent with the view that most of the refolding BO molecules now found a cofactor readily available in the protein-bound APol layer (Fig. 4d).

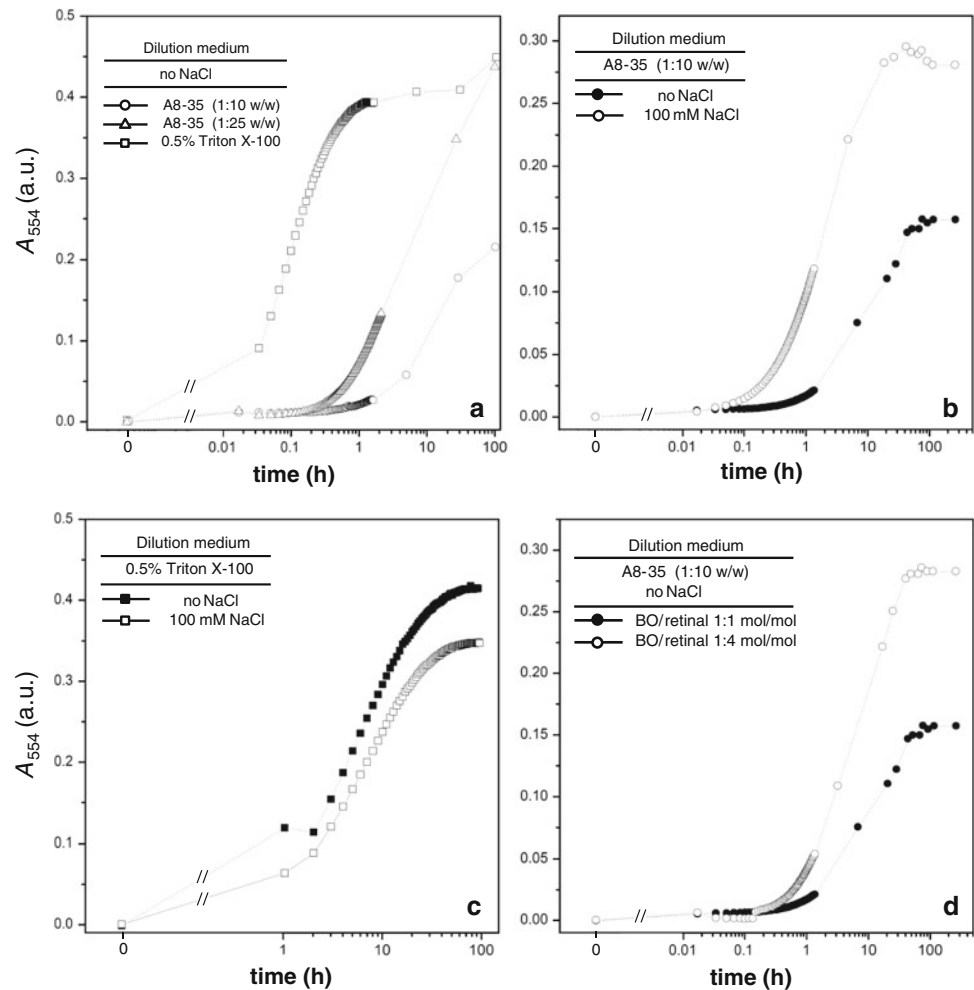
Renaturation in the absence of retinal

In the experiments described in Fig. 2b, refolding of BR from dBO/SDS by precipitation was carried out after adding retinal to the denatured preparation. To check the hypothesis that the relative instability of refolded BO was a factor in determining the yield of refolding, dBO/SDS preparations were refolded in the absence of retinal and retinal was added in a second stage (Fig. 5). Aliquots from an ethanolic solution of retinal were added in a 1.3:1 retinal/BO molar ratio, either immediately after the precipitation and removal of dodecylsulfate, i.e. ~1 h after the transfer from one to the other surfactant, or after dialysis for 3 days. When retinal was added shortly after the transfer from SDS to A8-35, the refolding yield was found to be as high as that observed when retinal is present during the transfer (~80 %, which is typical for lipid-free preparations; Fig. 5). If, on the other hand, retinal was first provided 3 days after the transfer, the percentage of holoprotein in the renatured preparation dropped to <30 % (Fig. 5). The most straightforward interpretation of this observation is that, in the 3-day interval, ~2/3 of the BO that had initially refolded had denatured again. This observation confirms:

1. that BO folds efficiently in the absence of retinal (renatured BO indeed features the same secondary structure as BR refolded from dBO/SDS in the presence of retinal; see below); and
2. that early enough rebinding of retinal is critical in stabilizing the refolded state and, thereby, in the final yield of renaturation.

An obvious extrapolation is that fragile MPs might advantageously be folded in the presence of one of their ligands.

Fig. 4 Renaturation of BO/SDS by dilution. PM was denatured by incubation at 45 °C in 0.25 % SDS (SDS₂-buffer). BO/SDS (1 g L⁻¹ BO, BO/SDS mass ratio 1:2.5) was diluted 5× with SDS-free buffer containing either A8-35 (final concentration 2 or 5 g L⁻¹, BO/A8-35 mass ratio 1:10 or 1:25) or Triton X-100 (final concentration 0.5 % w/v, mass ratio of BO/micellar Triton ~ 1:24). Absorbance at 554 nm was monitored as a function of time. **a** Comparison of the rate of refolding in Triton X-100 versus A8-35 in NaPi₃ buffer. **b, c** Effect of ionic strength on the rate and final yield of refolding in A8-35 (**b**) and Triton X-100 (**c**). **d** Effect of the concentration of retinal on the rate and final yield of refolding in A8-35. Final refolding yields in A8-35 are given in Table 1, part 3



Characterization of native, denatured and refolded samples

Native, denatured and refolded samples were characterized by their UV–visible absorbance spectrum, their size distribution as observed by SEC, their resistance to thermal denaturation, and the photocycle of BR as observed by time-resolved absorbance spectroscopy after an actinic flash (this work), and by synchrotron-radiation circular dichroism (Popot et al. 2011). In the following discussion samples are designated by the following codes: PM, native purple membrane; nBR, native BR solubilized from PM with OTG; BO, PM solubilized in SDS, still containing lipids and retinal; rBR, BR refolded from BO; dBO, delipidated BO, which has been separated from lipids and retinal by SEC in organic solvents; rdBO, delipidated BO refolded in the absence of lipids and retinal; and rdBR, BR refolded from rdBO after addition of retinal. Unless otherwise indicated, refolding was performed by use of the precipitation procedure. After a slash is indicated the surfactant associated with the protein, with, if appropriate, an

indication of the protein/APol ratio at which refolding was performed. Thus, rdBR/5A8-35 indicates a sample that was folded using delipidated BO supplemented with retinal and A8-35, the latter at a 1:5 BO/APol mass ratio. For easy reference, the nature, mode of preparation, and composition of the main native and refolded samples described below are summarized in Table 2.

Size-exclusion chromatography

Refolded samples obtained by the dodecylsulfate precipitation procedure were analyzed by SEC in aqueous buffer. Whether the samples had been obtained in the presence (rBR/5A8-35) or absence (rdBR/5A8-35) of lipids, the complexes were essentially monodisperse (Fig. 6). Their elution profiles were similar to that of native BR trapped in A8-35 after solubilization in OTG (nBR/5A8-35), a preparation that has been shown to retain all of the lipids initially present in PM (~ 1 g per 3 g BR) and to bind ~ 1.8 g A8-35 g⁻¹ protein (Gohon et al. 2008).

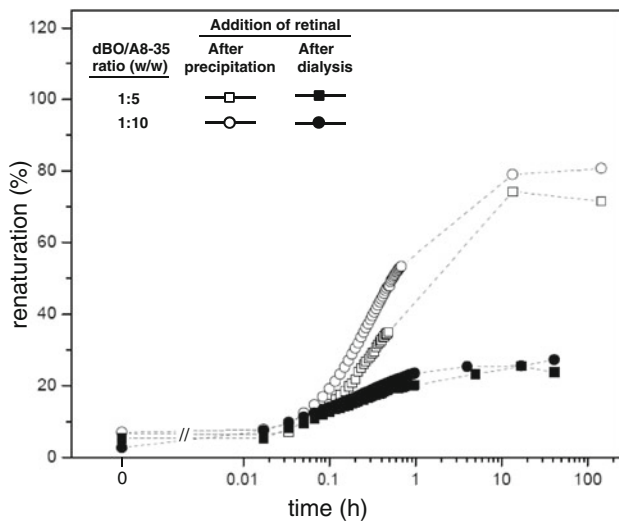


Fig. 5 Renaturation of dBO/SDS in A8-35 in the absence of retinal. dBO was transferred from SDS to A8-35 by KCl precipitation, in the absence of retinal, at two different dBO/A8-35 mass ratios, 1:5 (*squares*) and 1:10 (*circles*). Retinal, at a 1:1.3 BO/retinal molar ratio, was added to the resulting dBO/A8-35 samples at two different times—either immediately after precipitation and elimination of dodecylsulfate (*open symbols*) or after the samples had been centrifuged and dialyzed for 3 days (*solid symbols*). The percentage of renaturation was estimated from the relative absorbance of the samples at 554 and 280 nm

Thermostability

The thermostability of samples refolded in A8-35 in the presence or absence of lipids (rBR/A8-35 and rdBR/A8-35, respectively) was examined by monitoring the evolution of

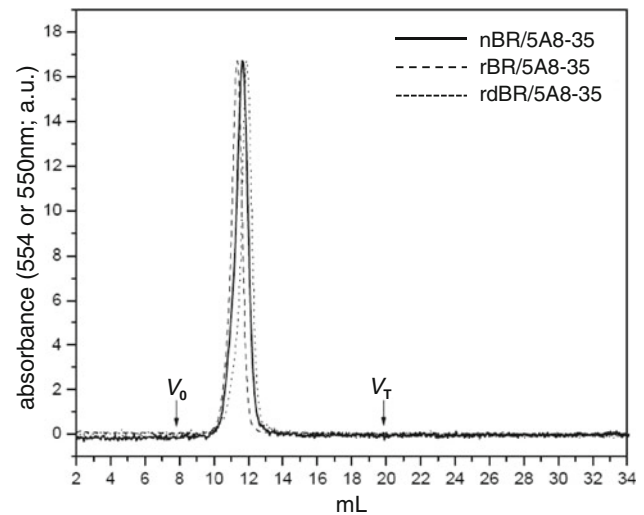


Fig. 6 Size-exclusion chromatography of native, amphipol-trapped BR (nBR/5A8-35) and BR renatured in A8-35 either in the presence (rBR/5A8-35) or absence (rdBR/5A8-35) of lipids. SEC was performed on a Superose 12/HR column equilibrated in K-buffer. V_0 and V_T : void and total volumes, respectively

their UV–visible spectra upon heating from 20 to 70 °C with continuous stirring, and compared with that of native BR in PM, in OTG, and after trapping with A8-35 (Fig. 7). The temperature was increased in 10° steps, starting from 20 °C, and the samples were incubated for 20 min before spectra were recorded. PM, as has long been observed (Jackson and Sturtevant 1978), does not denature in this range of temperature, its UV–visible spectrum remaining essentially unchanged from the beginning to the end of the

Table 2 Native and refolded BR and BO samples characterized in the present work

Sample short name	Nature	Mode of preparation	Content
PM	Native purple membrane	Purified from <i>H. salinarum</i>	BR, PM lipids
nBR/OTG	Native BR in OTG	PM solubilized in 100 mM OTG. After solubilization, diluted to 18 mM OTG	Contains BR, OTG, and most endogenous lipids (Gohon et al. 2008)
nBR/5A8-35	Native BR transferred to A8-35 from nBR/OTG	A8-35 added to nBR/OTG (mass ratio A8-35/BR 5:1). OTG eliminated by use of BioBeads	Contains BR, A8-35, and most endogenous lipids (Gohon et al. 2008)
rBR/5A8-35	BR renatured in A8-35 from BO/SDS	PM was solubilized in 5 % SDS. A8-35 was added to an A8-35/BR mass ratio of 5:1. SDS eliminated by precipitation with KCl	Contains renatured BR, endogenous lipids, A8-35
rdBR/5A8-35	BR renatured in A8-35 from dBO/SDS + retinal	PM solubilized in organic solvents, BO separated from lipids and retinal by SEC in organic solvents. Transfer to an SDS solution. Addition of retinal (molar ratio dBO/retinal 1:1.3). A8-35 added to a BR/A8-35 mass ratio of 5:1. SDS eliminated by precipitation with KCl	Contains renatured BR, less than 2 % of the endogenous lipids (Huang et al. 1981; Gohon et al. 2008), A8-35
rdBO/5A8-35	BO renatured in A8-35 from dBO/SDS in the absence of retinal	Same as above but without addition of retinal	Contains renatured BO, less than 2 % of the endogenous lipids (Huang et al. 1981; Gohon et al. 2008), A8-35

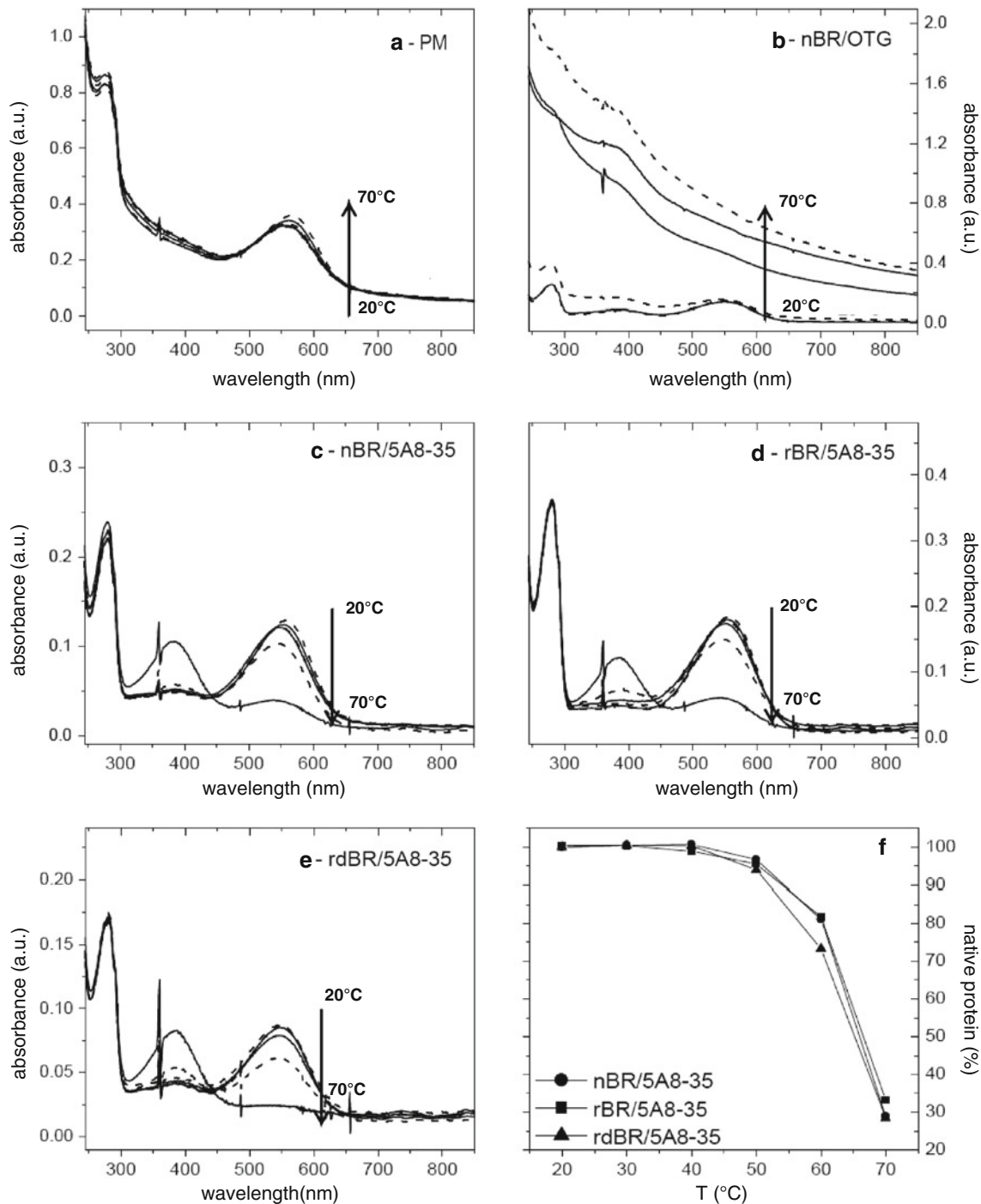


Fig. 7 Thermal denaturation of BR in different environments. **a** In PM (in water). **b** Solubilized in OTG (in NaPi₂ buffer). **c** Transferred from OTG to A8-35 (in NaPi₂ buffer). **d**, **e** Renatured by transfer from SDS to A8-35 in the presence (**d**) or absence (**e**) of lipids, using the KCl precipitation procedure (in K-buffer). For all BR/A8-35 samples, the mass ratio of protein to polymer was 1:5. UV-visible spectra were recorded after incubation for 20 min at the temperature indicated.

experiment. OTG-solubilized BR is poorly stable: even at 40 °C, the intensity of the visible absorption peak of the holoprotein starts to decrease, and light scattering indicates

the onset of aggregation. At higher temperature, the protein is totally denatured, the absorption band of retinal having moved to ~380 nm, and aggregation is massive. Native

They are represented by, alternately, *solid and dashed lines* at 10 ° intervals from 20 °C (*dashed line*) to 70 °C (*solid line*). **f** Comparison of the thermal denaturation of native, A8-35-trapped BR (*filled circles*) versus BR renatured in A8-35 in the presence (*filled squares*) or absence (*filled triangles*) of lipids. Data are expressed as a percentage of native BR at 20 °C

the onset of aggregation. At higher temperature, the protein is totally denatured, the absorption band of retinal having moved to ~380 nm, and aggregation is massive. Native

BR transferred to A8-35 (nBR/5A8-35), starts showing signs of denaturation at approximately 50 °C and is ~70 % denatured at 70 °C (Fig. 7c, f). Very similar behavior is observed for the sample refolded in A8-35 from BO/SDS (rBR/A8-35), which is expected to have rebound PM lipids upon renaturation (Fig. 7d, f). Finally, the thermostability of the sample renatured from dBO/SDS in the absence of lipids (rdBR/A8-35) is also relatively high (Fig. 7e), but release of retinal starts somewhat earlier than for the nBR/5A8-35 or rBR/5A8-35 (Fig. 7f). This observation suggests that, in rBR/A8-35, PM lipids indeed have rebound to the refolded protein, increasing its stability up to the level of native, lipid-associated BR.

Synchrotron-radiation circular dichroism

UV-CD spectra recorded by F. Wien on the synchrotron beamline Bessy (Helmholtz-Zentrum, Berlin) have been published by Popot et al. (2011). They show, in brief, that there is no difference between the secondary structure of nBR/5A8-35 and rBR/5A8-35, or between that of rdBO/5A8-35 and rdBR/5A8-35. As previously observed (Huang et al. 1981; Riley et al. 1997), denaturation of BR in SDS induces a drop of ~20 % in α -helical content, which is integrally recovered after renaturation in A8-35 in the presence of lipids. In the absence of lipids, recovery is lower by a few percent, whether or not retinal is present (F. Wien, personal communication), which may result either from the presence of more misfolded protein and/or from a minor difference in the conformation of BR.

Functional state of refolded samples: time-resolved absorption spectroscopy

Based on a comparison with native, APol-trapped BR, refolded samples, whether or not they contain lipids, seem to have returned to the native state as far as can be deduced from the size and solution properties of the complexes and the absorption spectrum and secondary structure of the protein. The thermostability of samples refolded in the presence of PM lipids is also identical with that of APol-trapped native BR, but that of BR refolded in the absence of lipids is slightly inferior, a clear indication that lipids, in the same way as retinal, must rebind to the refolded protein. Because it is difficult to refold BR efficiently in the absence of lipids, the photocycle of BR has always been studied in their presence, be it on native PM (reviewed by Haupts et al. 1999), on detergent-solubilized native BR (Milder et al. 1991), or on BR that had been denatured and refolded in the presence of lipids (Sonar et al. 1993). In the following discussion we compare the photocycle of native, APol-trapped BR with that of BR refolded in A8-35 in the presence or absence of lipids, in order to:

1. examine whether there are functional differences between the native BR and that refolded in the presence of lipids; and
2. determine which effects the absence of lipids has on the function of BR.

Proton pumping by BR is the outcome of a complex photocycle in the course of which the protein passes through a series of conformational states, each defined by their spectral properties and the kinetics of their appearance and disappearance (Fig. 8a, b). Deprotonation of the Schiff base causes the appearance of state M, which is characterized by a strongly blue-shifted absorption peak at 412 nm. The spectral state M comprises two successive conformational states, M_1 and M_2 , so that the kinetics of appearance and disappearance of M involves a spectrally neutral step of equilibration between M_1 and M_2 . After an actinic flash, the absorption changes in the “short-term” (until ~200 μ s after the flash) reveal the appearance of the M state, and therefore feature an increase of absorption at 412 nm, whereas the “long-term” spectra reveal the disappearance of the M state and the return to the ground state (Fig. 8c). In the time range studied (from 5 ns to 500 ms), the time-resolved spectral changes can be fitted with five exponentials (Gohon et al. 2008; Milder et al. 1991), each representing a transition from a state X to a state Y and characterized by a difference spectrum (“decay-associated spectrum”, or DAS; Fig. 8d) and a half-time $t_{1/2}$ (Fig. 8e).

In a previous study, the photocycles of native BR in PM, in OTG, and after trapping with A8-35 were compared (Gohon et al. 2008). The main conclusions were:

1. in all three environments, BR undergoes its entire photocycle;
2. in the two solubilized states, the rise of state M is faster than in PM, which, for the detergent-solubilized state, has been attributed to a conformational relaxation that brings Asp-85, the proton acceptor, closer to the Schiff base (Milder et al. 1991); and
3. in OTG, M undergoes a polyphasic decay, with, compared with PM, a major phase that is strongly accelerated and a minor one that is slowed.

Deconvolution of multiwavelength spectral data reveals that this is because of a transient population of state N that lasts longer in OTG solution than in PM (Gohon et al. 2008). Upon transfer to A8-35, the kinetics of M decay return to nearly, although not fully, those observed in PM (Gohon et al. 2008; Pocschi et al. 2006).

As shown in Fig. 8, for all the samples analyzed here, i.e. nBR/A8-35, rBR/A8-35, and rdBR/A8-35, the characteristic absorption changes attesting to the formation of the M state and its consecutive decay were observed. The DAS of the fourth component (t_4), with a positive band in the

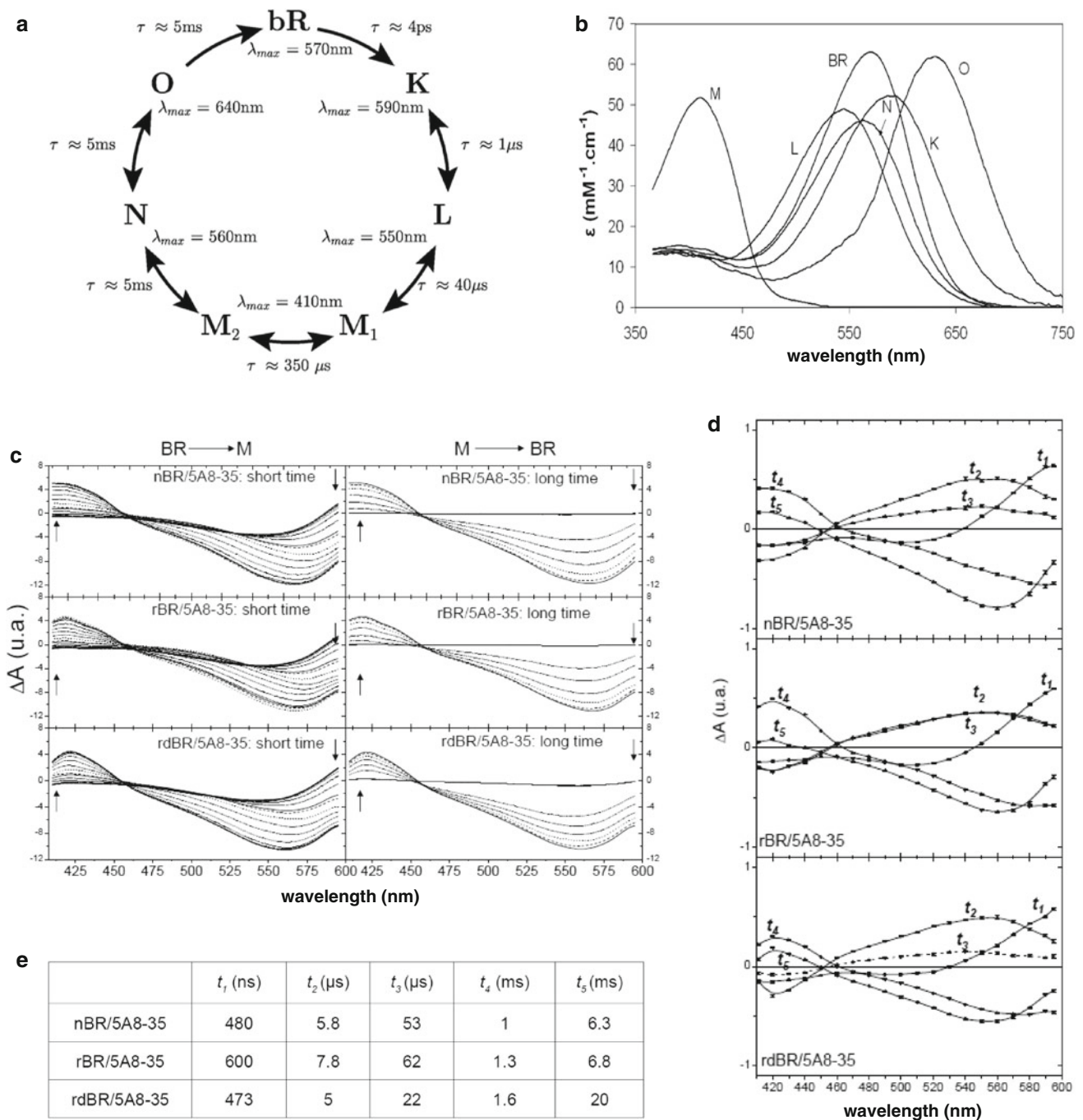


Fig. 8 Time-resolved absorption spectroscopy of native BR trapped in A8-35 (nBR/A8-35) and of BO/SDS and dBO/SDS renatured in A8-35 (rBR/A8-35 and rdBR/A8-35, respectively). **a** BR photocycle (from Neutze et al. 2002). **b** UV–visible spectra of BR intermediates (from Váró 2000). **c** Absorbance changes plotted as a function of

wavelength for nBR/A8-35, rBR/A8-35, and rdBR/A8-35. **d, e** Decomposition of spectral changes into five exponentials, characterized by decay associated spectra (**d**) and half-times (**e**). Further experimental details are given by Gohon et al. (2008)

420 nm range and a trough above 590 nm, is likely to be indicative of conversion of the M state into the O state, indicating that all samples went through the entire photocycle. From a kinetic perspective, there are no significant differences between the nBR/A8-35 and rBR/A8-35 samples, which had similar time constants for the five

components (Fig. 8e). There were, however, differences between the relative amplitudes of the second and third components, which correspond to the conversion of the L state into the M state. A somewhat faster L to M transition (22 μs compared with 50–60 μs for the samples with lipids) was observed for the sample renatured in A8-35 in the

absence of lipids (rdBR/A8-35). Most strikingly, the M state was longer-lived in the absence of lipids (20 ms instead of 6–7 ms). These two features are reminiscent of the findings reported by Gohon et al. (2008), who observed a faster L → M transition and a more stable M state for BR solubilized in OTG than for nBR/A8-35. This similarity between nBR/OTG and rdBR/5A8-35 suggests that these two features arise from the absence or disturbance of the lipids surrounding BR.

Discussion

The data presented here are of interest for the following reasons:

1. They extend the range of procedures that can be resorted to when attempting to fold MPs using APols.
2. They provide novel insight into the possible function of lipids in facilitating conformational transitions during the photocycle of BR.
3. More generally, they contribute to a better understanding of the functional effects of APols on MPs.
4. They shed further light on the nature of the constraints that MPs require from their environment to acquire a functional 3D structure.

Presumably because their dissociating power is weak, APols have proved to be an excellent environment in which to fold denatured MPs to their native state. This is of great practical import, because most MPs are difficult to produce in biochemically relevant amounts in a functional state. Producing large quantities of misfolded MPs is possible, e.g. by directing them to inclusion bodies. However, identifying efficient conditions to fold them, typically from the denatured state recovered after solubilizing inclusion bodies in SDS, tends to be difficult, time-consuming, and highly protein-specific, and the yields achieved are often low (reviewed by Banères et al. 2011; Booth et al. 2001; Kiefer 2003). Exploring the limits of any novel folding methodology is therefore of great interest. APol-assisted folding was first demonstrated by using, as models, BR and two β -barrel MPs (Pocanschi et al. 2006). It has since been extended to seven G protein-coupled receptors (GPCRs), some of which had never been folded with acceptable yield by use of conventional approaches (Banères et al. 2011; Bazzacco et al. 2012; Catoire et al. 2010a; Dahmane et al. 2009). Most folding procedures to-date have resorted to A8-35 (Banères et al. 2011; Catoire et al. 2010a; Dahmane et al. 2009; Pocanschi et al. 2006). More recently, folding of a β -barrel MP in sulfonated APols and of a GPCR in non-ionic APols have been reported (Dahmane et al. 2011; Bazzacco et al. 2012). For β -barrel MPs, folding was initiated by dilution of a protein solution in concentrated urea

into a urea-free buffer containing the APol (Dahmane et al. 2011; Pocanschi et al. 2006). For MPs whose transmembrane domain folds into an α -helical bundle, the procedure, until now, has always been to precipitate the potassium salt of dodecylsulfate from a solution of the denatured protein in concentrated SDS after supplementing it with APols (Bazzacco et al. 2012; Catoire et al. 2010a; Dahmane et al. 2009; Pocanschi et al. 2006). In this work we have compared this and alternative procedures, using BR as a model. Transfer from SDS to A8-35, by removing the dodecylsulfate by dialysis or by diluting the mixture into a large volume of SDS-free buffer, also gave high yields of folded BR (typically 70–80 % under the best conditions), although not as high as those yielded by the precipitation method (≥ 90 %). These two approaches offer interesting alternatives when, for one reason or another, the PDS precipitation method is not appropriate, for example when folding kinetics are to be observed spectroscopically on a short time scale (dodecylsulfate precipitation results in extremely turbid samples, from which PDS crystals must be removed by centrifugation before any spectroscopic observation is possible). The precipitation method also has rather strict requirements about the concentrations of salt to be used (Popot et al. 1987), which may not suit all proteins.

A further observation made in the course of this work is that BO refolded in A8-35 in the absence of retinal is, as might be expected, not as stable as BR. A8-35-trapped native BR is stable for months at 4 °C (Gohon et al. 2008). We show here that its thermostability and that of BR refolded in A8-35 in the presence of lipids are comparable. After refolding in the absence of lipids, BR is only marginally less stable. Refolded dBO, in contrast, is ~ 70 % inactivated after 3 days at 4 °C. As a result, conditions that accelerate the uptake of retinal by freshly refolded BO improve the final folding yield. The presence of lipids also increases the yield, as it does for GPCRs (Dahmane et al. 2009). APol-trapped BO is most probably more stable in the presence of lipids than in their absence, which may account, at least in part, for this effect. Another possible mechanism is that, in the course of folding, lipids bind to reforming binding sites at the transmembrane surface of the partially folded protein, thus steering the folding process toward the native conformation. BR, nevertheless, refolds quite efficiently (up to 70–80 %) in the total absence of lipids, which has interesting applications and implications (see below).

A procedure that did not yield consistent results is direct transfer of denatured BO to A8-35 from trifluoroethanol (TFE), an organic solvent that, similar to SDS, tends to promote the formation of α -helices. A previous attempt to transfer a synthetic transmembrane peptide from TFE to A8-35 also gave unsatisfactory results, although in that case the very nature of the peptide may have prevented it

from folding correctly in A8-35 (Duarte et al. 2008). Whereas our observations indicate that refolding of BR by direct transfer from TFE to A8-35 can work, establishing whether or not such an approach can be made reliable will require further investigations using, perhaps, simpler proteins or peptides.

Our results afford novel insights into the effect lipids may have on the function of BR. Lipid-free BR refolded in A8-35 performs its entire photocycle. Several differences with lipid-containing native or refolded BR/A8-35 complexes are noticeable, however. First, lipid-free, APol-trapped BR is slightly more sensitive (by <10 °C) to thermal denaturation than the other two preparations. Second, the peak of absorbance in the visible region is slightly blue-shifted (550–552 vs. \sim 554 nm). Third, the CD spectrum reveals a slight deficit of α -helical structure, identical for BR and BO, which may, at least in part, be a result of the presence of more misfolded protein. And, fourth, the photocycle is slightly different: whereas the kinetics of appearance of the M states are similar to those observed with native BR or with BR refolded in the presence of lipids, the return to the ground state is somewhat slower (by a factor of \sim 3). This may suggest either that interactions with lipids contribute to guiding the transconformations that bring BR back to the ground state, or that pure A8-35 slows this process. It is worth noting, regarding the second hypothesis, that:

1. the interior of an A8-35 particle is, according to molecular dynamics calculations, less fluid than that of a detergent micelle or a lipid bilayer (Perlmutter et al. 2011); and
2. APols slow the enzymatic cycle of the sarcoplasmic Ca^{2+} -ATPase (Champeil et al. 2000; Picard et al. 2006).

The latter effect has been tentatively attributed to damping by the polymer of large-scale transmembrane helix movements (discussed by Popot et al. 2011). It is possible that the difference seen here between lipid-associated and lipid-free APol-trapped BR is a weak reflection of the stronger effect seen with the Ca^{2+} -ATPase.

As far as we are aware, the photocycle of fully delipidated BR has not been studied previously. Very few refolding experiments using delipidated BO have been performed in the absence of lipids, and the photocycle of refolded BR has been studied only in their presence (Sonar et al. 1993). Delipidation of PM by non-solubilizing detergents, for example bile salts or Tween 20 (Fitter et al. 1998; Fukuda et al. 1990; Hartsel and Cassim 1988; Jang and el-Sayed 1988), is only partial (up to \sim 80 %) and likely to create lattice constraints, which complicates the interpretation of perturbations of the photocycle. In delipidated PM, both the rise of M and its decay are slowed,

although the decay phase is the most strongly affected. The first effect is absent with lipid-free A8-35-trapped BR (there is, actually, a slight acceleration). The slowing of M decay, which is more pronounced for delipidated PM (ca. one order of magnitude) than the relatively weak effect seen for A8-35-trapped, fully delipidated BR (\sim 3 \times), has been attributed to “rigidification” of the lipid environment (Fukuda et al. 1990) or, otherwise stated, to the loss of the “lubricant” provided by hydrated lipids (Fitter et al. 1998). This may be reminiscent of the “damping” by pure A8-35 postulated above. Whether the same kind of mechanism operates in environments that are so different remains, however, an open question. Alternatively, the slowing of the last part of the photocycle in lipid-free APol could be related to the loss of interactions with specific lipids, e.g. squalenes, as proposed for Triton X-100-solubilized BR (Hendler and Bose 2003; Joshi et al. 1998). The methodology developed in this work should make it possible to determine whether kinetic differences result from a general, physical effect of pure A8-35 versus the lipid/A8-35 environment, or whether they involve specific protein/lipid molecular interactions (cf. Hendler and Bose 2003; Hendler and Dracheva 2001; Joshi et al. 1998; Milder et al. 1991; Mukhopadhyay et al. 1996; Váró and Lanyi 1991).

Finally, these experiments further support the rather surprising conclusion that neither a lipid bilayer nor even lipids are required by MPs—or, at least, some MPs—to reach their functional state starting from an unfolded state. We have previously shown that several GPCRs purified from inclusion bodies can be folded with very substantial yields (30–55 %) in pure A8-35 (Dahmane et al. 2009). More recently, it has been found that BR can be expressed in vitro, in a cell-free expression system, in the presence of non-ionic APols, under which conditions it correctly folds and binds retinal (Bazzacco et al. 2012; Popot et al. 2011). In neither of these two cases is it expected that more than traces of lipids are present. In these experiments, delipidated BO has been shown to contain less than 2 % of the lipids initially present in the PM (Gohon et al. 2008; London and Khorana 1982), i.e. less than one lipid per five BR molecules (Corcelli et al. 2002). APols, whether ionic or non-ionic, are structurally very different from lipids, and do not organize into anything resembling a lipid bilayer, but into micelle-like particles (Gohon et al. 2006; Perlmutter et al. 2011; Sharma et al. 2012). The inescapable conclusion is that at least some MPs can fold to their native state without any specific information from a membrane environment. Not only is all the information needed contained in their sequence, but this information does not need to be decoded either by the proteins that catalyze, in vivo, their insertion into target membranes, or by specific constraints imposed by a membrane, or even by specific interactions with lipids. The application of Anfinsen’s

principle requires only that their hydrophobic transmembrane surface be screened from exposure to water by a surfactant mild enough not to interfere with the interactions the polypeptide establishes with itself. This is consistent with the view that, despite its complexity, the translocon acts as a catalyst that lets “membrane proteins come into thermodynamic equilibrium with the lipid membrane, where physicochemical interactions determine the final three-dimensional structure” (White and von Heijne 2008). Folding in amphipols emphasizes the fact that guidance by specific interactions with the membrane is, at least for some MPs, dispensable.

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