

Complexation of integral membrane proteins by phosphorylcholine-based amphipols

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Received 17 April 2007; received in revised form 18 June 2007; accepted 5 July 2007

Available online 24 July 2007

Abstract

Amphiphilic macromolecules, known as amphipols, have emerged as promising candidates to replace conventional detergents for handling integral membrane proteins in water due to the enhanced stability of protein/amphipol complexes as compared to protein/detergent complexes. The limited portfolio of amphipols currently available prompted us to develop amphipols bearing phosphorylcholine-based units (PC). Unlike carboxylated polymers, PC-amphipols remain soluble in aqueous media under conditions of low pH, high salt concentration, or in the presence of divalent ions. The solubilizing properties of four PC-amphipols were assessed in the case of two membrane proteins, cytochrome *b₆ f* and bacteriorhodopsin. The protein/PC-amphipol complexes had a low dispersity in size, as determined by rate zonal ultracentrifugation. Short PC-amphipols ($\langle M \rangle \approx 22$ kDa) of low dispersity in length, containing ~ 30 mol% octyl side groups, ~ 35 mol% PC-groups, and ~ 35 mol% isopropyl side groups, appeared best suited to form stable complexes, preserving the native state of BR over periods of several days. BR/PC-amphipol complexes remained soluble in aqueous media at $\text{pH} \geq 5$, as well as in the presence of 1 M NaCl or 12 mM calcium ions. Results from isothermal titration calorimetry indicated that the energetics of the conversion of BR/detergent complexes into BR/amphipol complexes are similar for PC-amphipols and carboxylated amphipols.

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Keywords: Amphipols; Integral membrane proteins; Phosphorylcholine; ITC; Amphiphilic macromolecules

1. Introduction

The isolation of integral membrane proteins (IMPs) and their solubilization in aqueous media remain major stumbling blocks hampering the structural characterization of this important class of proteins and the resulting biomedical applications. A variety of detergents and polymers have been employed successfully to extract IMPs from biological membranes or to keep them soluble in water. Examples of such compounds include neutral detergents, such as surfactants of the *n*-alkyloside and *n*-alkyl (polyethylene glycol) families [1–3], and polymeric surfactants, such as the amphipols (amphiphilic polymers) [4–6] and the

lipopeptides (peptidic detergents) [7]. Each of them however presents some drawback. IMP-adsorbed low molecular weight amphiphiles are in rapid equilibrium with the solution, which results in protein aggregation upon dilution below the detergent critical micellar concentration (cmc), and may affect IMP stability upon changes in buffer and salinity conditions. A number of the shortcomings of low molar mass detergents are alleviated through the use of amphipols, since multiple non-polar groups of a single amphipol molecule are involved in IMP complexation, which results in slower spontaneous desorption and higher affinity as compared to classical detergents [4,8–10]. Amphipols associate with the transmembrane domain of IMPs [11], presumably in a way such that the hydrophobic groups of the polymer interact with the hydrophobic surface of the protein while the polar groups provide a hydrophilic corona and thereby maintain the colloidal stability of the IMP/amphipol complex in surfactant-free buffer solutions. As a consequence, IMP/

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amphipol complexes are vulnerable to conditions that will affect the solubility of the polymer. For instance, amphipols such as A8-35 [4], which carry weak acid groups, form soluble complexes with IMPs as long as the acid groups remain ionized. However, a decrease of solution pH below a threshold value, ~ 6.5 for carboxylate-based polymers, triggers the aggregation of amphipols [12] and IMP/amphipol complexes [10,13]. An increase in ionic strength above a value of ~ 300 mM or the presence of calcium ions also affect the colloidal stability of IMP/amphipol complexes ([14] and unpublished data), which limits the range of conditions accessible for IMP purification, functional assays, and structural determinations, e.g. by NMR or crystallographic techniques. This situation has prompted the development of amphipols with alternative chemical structures [14–18].

An attractive approach to enhance the solubility of amphiphilic polymers in buffers of high salinity and to expand the pH range in which they remain useful involves replacing the carboxylate units of the first-generation amphipols by units that possess covalently linked cationic and anionic groups (betaines) [19,20]. Polybetaines tend to be poorly soluble in pure water, due to the formation of intra- and interchain ionic contacts resulting in an ionically crosslinked network structure. However, they become soluble upon addition of salts. This dissolution process can be understood in terms of the electrolyte penetrating the ionic network, screening the net attractive electrostatic interactions between the polymer chains, and hence promoting solubility [21]. The addition of salt thus results in chain expansion, an effect opposite to the response of typical polyelectrolytes, which contract in salt solutions and become insoluble. Several types of polymeric betaines are known [19]. Among them, polymers bearing the phosphorylcholine (PC) group are ideally suited for biochemical and biomedical applications. A number of PC-containing synthetic polymers are currently available and employed successfully as coatings of biomedical devices as well as in pharmaceutical and cosmetics formulations [22]. The development of these materials was based on the concept of biomimicry, in which phosphorylcholine-based copolymers create a surface that mimics a biomembrane, thus reducing biological interactions [23]. Various groups have demonstrated that PC-copolymer coatings are non-thrombogenic and that they improve the biocompatibility of ocular devices and cardiovascular implants. The hypothesis that has driven the work described here is that amphipols bearing PC-groups would (i) provide an enlarged pH- and salt stability window, as a result of their betaine structure, and (ii) possibly increase the functional stability of solubilized IMPs, due to the similarity of the structure of their hydrophilic moieties to that of phosphatidylcholine. This design strategy, whereby the anionic and cationic groups are linked to the same unit of a macromolecule, differs from the methodology of Nagy et al., who reported an amphipol containing anionic and cationic groups attached to distinct units, such that in solutions of neutral pH the negative units were in 50% excess [20].

None of the PC-based copolymers currently available can be employed readily as amphipols, which, to be effective, need to have a controlled architecture and lipophilic/hydrophilic ba-

lance, as demonstrated by previous studies on carboxylate-based amphipols [4]. Miyazawa and Winnik [24] recently devised a versatile synthesis of PC-containing amphiphilic polymers by post-modification of a copolymer of *n*-alkylacrylamide, *N*-*t*-BOC-*N*-[(ethylenedioxybis(ethyl) acrylamide)] and *N*-isopropylacrylamide. Using the design rules established for amphipols of the A8-35 class, we prepared four PC-based amphipols, which are random copolymers of *n*-octylacrylamide, *N*-isopropylacrylamide, and *N*-phosphorylcholine-*N*-ethylenedioxybis(ethyl)acrylamide (Fig. 1 and Table 1) and tested their ability to keep soluble bacteriorhodopsin from *Halobacterium salinarium* (BR) and the cytochrome *b₆ f* complex from *Chlamydomonas reinhardtii*. We have compared PC-amphipols with A8-35 at the stage of IMP trapping, which involves the exchange of detergent for amphipol. We determined the amphipol/IMP ratio required to maintain IMPs soluble and assessed the size dispersity of the complexes by rate zonal sedimentation. We measured by isothermal titration calorimetry the enthalpy of mixing IMP/detergent complexes with excess amphipol. We have also assessed the stability of the IMP/PC-amphipols complexes upon storage in neutral aqueous buffer and in solutions of acidic pH (pH 5.0), in the absence of salt and in saline solutions. Our study points to the remarkable solubility of IMP/PC-amphipols complexes in acidic media and in solutions of high ionic strength, thus extending the range of conditions accessible to studies of amphipol-complexed IMPs.

2. Experimental procedures

2.1. Materials

Water was deionized with a Millipore Milli-Q water purification system. Anilino-naphthalene sulfonic acid (ANS), dodecyl maltoside (98%, DDM), and *n*-octylthioglucoside (98%, OTG, for ITC experiments) were purchased from Sigma Chemicals and used without further purification. An OTG sample (>99%) purchased from Anatrace was employed for protein solubilization. The five amphipols, A8-35 [12], C22-43, C60-44, C60-30, and C45-68 [17] (according to the amphipol nomenclature used in [4], A and C stand for anionic and cationic, respectively, the digit adjacent to the letters refers to the molar mass (in kg mol⁻¹), and the second digit is the molar percent of carboxylate or phosphorylcholine residues) were synthesized as described elsewhere [12,17]. Briefly, they were obtained by modification of copolymers *N*-*t*-BOC-*N*-[ethylenedioxybis(ethyl) acrylamide], *N*-isopropylacrylamide, and *N*-*n*-octylacrylamide obtained by free-radical polymerization of suitable mixtures of the three comonomers carried out in dioxane at 65–70 °C for 17 h using azobis(isobutyronitrile) as initiator. The precursor polymer used to prepare C22-43 was synthesized from the same monomers and initiators, but in the presence of the chain transfer agent *S*-1-isobutyl-*S'*-(α,α' -dimethyl)- α'' -*N*-isopropylacetamide) trithiocarbonate in order to control the molecular weight and polydispersity index of the polymer. In all cases, the precursor polymers were purified by two precipitations from THF into hexane and dried in vacuo. The polymers were characterized by gel permeation chromatography (eluent: DMF, 40 °C, TSK-gel α -M and a TSK-gel α -3000, Tosoh Biosep columns). The *N*-*t*-BOC protecting group was cleaved by treatment with trifluoroacetic acid. The deprotected polymers were purified by treatment with the ion exchange resin Dowex 2X8-400 in order to remove trifluoroacetate ions. Phosphorylcholine groups were linked to each precursor polymer by treatment with phosphorylcholine glyceraldehyde and sodium borohydride. The PC-containing polymers were purified by dialysis against water. They were isolated by freeze-drying and characterized by ¹H NMR spectroscopy. The molecular characteristics of the polymer are listed in Table 1. All other chemicals were of analytical grade.

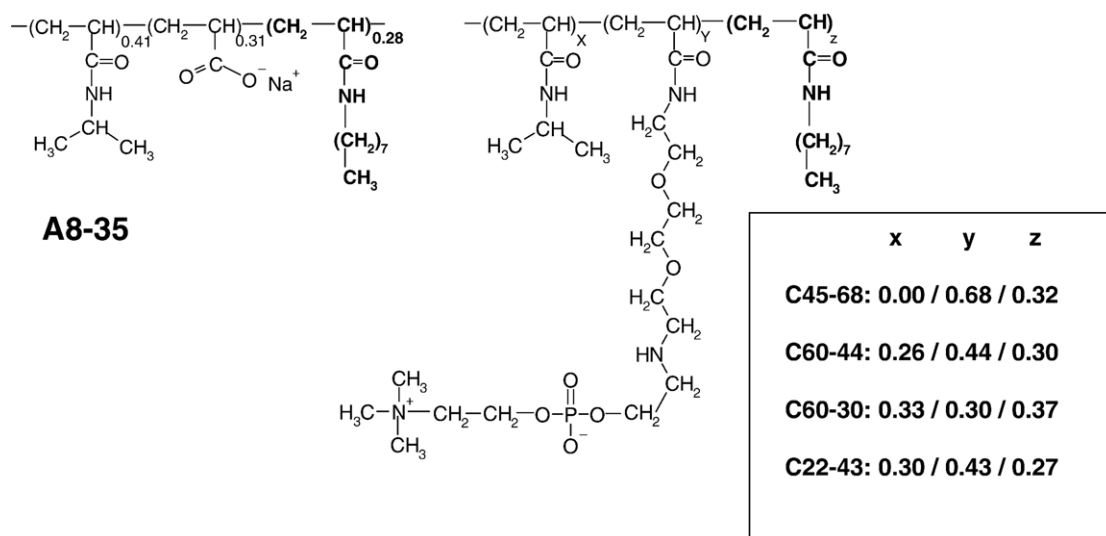


Fig. 1. Structure of A8-35 and of PC-amphipols.

2.2. Purification of bacteriorhodopsin (BR) and cytochrome b_6f

Purple membrane from *H. salinarium* (strain S9) was solubilized for 48 h in 100 mM OTG in 20 mM sodium phosphate buffer (pH 6.8) [25]. Soluble BR/OTG complexes were recovered in the supernatant after centrifugation for 30 min at 200,000×g in the TLA100 rotor of a Beckman TL100 centrifuge and diluted 3-fold in buffer to obtain a 33-mM OTG stock solution; the final BR concentration was ~1 g/L, determined from the absorbance at 554 nm ($\epsilon=1.60 \text{ L g}^{-1} \text{ cm}^{-1}$) [25] using a UV-visible Hewlett-Packard 8453 diode array spectrometer.

Cytochrome b_6f complex containing a six-histidine tag at the C-terminus end of cytochrome f was produced by a genetically modified strain of *C. reinhardtii* [26]. The protein was purified as described by Stroebel et al. [26] from thylakoid membranes solubilized with DDM, followed by anion-exchange (Source 30Q, Amersham) and immobilized nickel (HiTrap chelating, Amersham) chromatography. The final b_6f concentration in 0.2 mM DDM and 20 mM Tris-HCl pH 8.5 buffer was 0.4 g/L (~4 μM cytochrome f), as determined from the redox difference spectra at 554 nm and 563 nm [27]. After 6 days of storage at 4 °C, the preparation comprised a mixture of the inactive monomer and the native dimer forms of b_6f , as revealed by the two bands obtained by rate zonal sedimentation in DDM solution (Fig. 3A) [32].

2.3. Amphipol solution preparations

Stock polymer solutions (2 to 20 g/L) were prepared by dissolving freeze-dried samples in deionized water under gentle stirring for at least 2 h at room temperature. The polymer solution in water was supplemented with concentrated buffer (100 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 6.8) and NaCl in amounts such that the final concentrations of phosphate and NaCl were 20 mM and 100 mM, respectively. The solutions were filtered through a 0.22- μm syringe filter (Millex, Millipore USA) prior to measurements.

2.4. Fraction of BR maintained soluble by polymers

Aliquots of polymer stock solutions were added to a stock solution of BR/OTG in 33 mM OTG (20 μL) to reach final polymer/BR ratios of 1–12 g/g. The resulting solutions were incubated for 15 min at 4 °C in the dark. They were mixed with 160–180 μL of a solution of 100 mM NaCl, 20 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 6.8, to reach a final OTG concentration of 3.3 mM, i.e. below its cmc (~10 mM). After a second incubation for 10–15 min, samples were centrifuged in the A-110 rotor of a Beckman Airfuge centrifuge (20 psi, ~210,000×g, 10 min). The absorbance of the initial samples and supernatants at 554 nm was measured to determine the fraction of soluble complexes. In the case of polymer C60-44, some experiments were carried out with polymer solutions that had been centrifuged (10 min, 200,000×g) prior to mixing with the BR stock solution.

2.5. Trapping of IMPs in amphipols and rate zonal sedimentation

To obtain BR/amphipol complexes depleted of OTG, a BR/OTG stock solution (~1 g/L BR, 33 mM OTG, 20 mM phosphate buffer) was mixed with a solution of polymer in 33 mM OTG, 20 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 6.8, with or without 100 mM NaCl. The final polymer/BR ratio was 9:1 g/g. After incubation for 15 min in the dark at 4 °C, the solution was treated with Bio-Beads SM-2 (Bio-Rad Bio-Beads/OTG 10:1 wt/wt) and the mixture was stirred in the dark at 4 °C overnight. Bio-Beads are known to adsorb detergents but not amphipols [10]. The supernatant (100–200 μL) was layered onto 2 mL of a 5–20% (wt/wt) linear sucrose density gradient in the same buffer, except for the absence of detergent (20 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 6.8, with or without 100 mM NaCl). The tubes were centrifuged at 4 °C for 5 h at 55,000 rpm in a Beckman TLS 55 rotor (~250 000×g), or for 18 h at 37,000 rpm in an MLS50 one (~145,000×g). Typically 18–20 fractions (120 μL or 200 μL , respectively) were collected from the top of the tubes and analyzed by UV-visible absorbance spectroscopy. A control sedimentation experiment was performed simultaneously with the BR/OTG stock solution diluted in the phosphate buffer down to ~15 mM OTG (~0.5g/L BR), without removal of OTG. The BR/OTG samples were layered onto 5–20% sucrose gradients in 20 mM phosphate buffer, 10 mM OTG, and centrifuged in the same conditions as the BR/polymer samples.

The same procedure was adopted for the sedimentation of b_6f /amphipol complexes, starting with a mixture of b_6f /DDM complexes and the polymer in a 20 mM Tris/HCl buffer, pH 8, NaCl 100 mM, in which the DDM concentration exceeded the cmc of DDM, incubated overnight with Bio-Beads to remove DDM. The concentration of b_6f in the collected fractions was determined from their absorbance at 554 nm, 420 nm, and 280 nm.

Table 1
Characteristics of the polymers employed in the study

Polymer	Composition (mol%)				M_n (g/mol)	M_w/M_n
	NIPAM	AA	PC	C_8H_{17}		
A8-35 ^a	41	31	–	28	9,000	1.6
C22-43 ^b	30	–	43	27	22,000	1.2
C45-68 ^b	–	–	68	32	45,000	2.5
C60-44 ^b	26	–	44	30	60,000	2.0
C60-30	33	–	30	37	60,000	2.0

AA: acrylic acid; NIPAM: *N*-isopropylacrylamide; PC: *N*-phosphorylcholine - *N'*-[ethylenedioxybis(ethyl) acrylamide]; C_8H_{17} : *n*-octylacrylamide.

^a See Refs. [12,31] (batch HAPol-1).

^b See Ref. [17].

2.6. Rate zonal sedimentation of polymers in sucrose gradients

ANS (0.2 mM final concentration, from a 10 mM stock solution in water) was added to solutions of amphipols (1 g/L) in 20 mM NaH_2PO_4 – Na_2HPO_4 , pH 6.8. Aliquots (100 μL) of these solutions were layered onto a 2 ml 5–20% (w/w) linear sucrose density gradient in the same buffer, containing 35 μM ANS. The tubes were centrifuged at 20 °C (35,000–45,000 rpm, Beckman MLS 50 rotor, $\sim 145,000$ – $210,000\times g$). The position of the fluorescent band was noted for increasing centrifugation times (1–18 h).

2.7. Isothermal titration calorimetry

Measurements were carried out with a VP-ITC instrument from Microcal Inc (USA). Prior to each experiment the sample cell (1.43 mL) was filled with a polymer solution. BR solubilized in OTG (cf. Section 2.2) was purified by centrifugation on a 5–20% (wt/wt) sucrose gradient in 15 mM OTG, 20 mM NaH_2PO_4 – Na_2HPO_4 buffer pH 6.8 (Beckman MLS50 rotor, 18 h at 37,000 rpm, $\sim 145,000\times g$). The band of BR/OTG was collected and dialyzed for 48 h against the same buffer supplemented with 15 mM OTG and 100 mM NaCl, to remove the sucrose and to achieve a good control of the concentrations of detergent and salt in equilibrium with the protein. The heat exchanges generated by the interactions of BR/OTG complexes with the polymer were obtained from titrations of the BR/OTG solution (0.45 g/L BR) into aqueous polymer solutions (either 1.0 or 5.0 g/L) in the same buffer. During the titration the 300- μL syringe containing the BR/OTG solution was stirred at a speed of 300 rpm, allowing rapid mixing of the aliquots injected into the sample cell [28]. Aliquots (2 to 10 μL) were injected into the sample cell at 300-s intervals. All measurements were carried out at 25 °C. Data were analyzed using the Microcal ORIGIN software. The experimental enthalpy change h_i resulting from the i th BR/OTG solution injection was obtained by integration of the raw data signal. The integrated molar enthalpy change per injection (ΔH_i , in J/mol BR) was obtained by dividing h_i by the number of moles of OTG or BR added, n_i ; hence, $\Delta H_i = h_i/n_i$, resulting in enthalpograms, which are plots of ΔH_i as a function of free OTG or BR concentrations in the calorimeter sample cell.

2.8. Assessment of the stability of BR/amphipol solutions

Two fractions of purified BR/amphipol complexes were collected by sedimentation experiments (Section 2.5, fractions containing the maximum BR concentration). The absorbance spectrum of these fractions kept in the dark at 4 °C was measured regularly for 11 days. Alternatively, an aliquot of the fraction was supplemented with either 5 M NaCl (final NaCl 1 M), 250 mM citric acid–NaOH buffer, pH 5.0 (final citrate 25 mM), or CaCl_2 120 mM (final Ca^{2+} 12 mM). The UV-visible spectra of the samples were measured immediately after supplementation and after centrifugation (10 min at $\sim 200,000\times g$) at incubation times 15 min and 4 h. Similar measurements were carried out for b_6/f /C60-44 solutions in the same buffers containing either 1 M NaCl, or 25 mM citrate buffer to check the stability and absence of aggregation after incubation for 1 h.

3. Results

3.1. Design and properties of PC-amphipols

Four different PC-amphipols were used in this work (Fig. 1 and Table 1). Three samples, C45-68, C60-44, and C60-30, were prepared by conventional free radical polymerization and exhibit a broad distribution of chain sizes characterized by a polydispersity index above two. To illustrate the implications of this polydispersity index, estimates of the average molar masses of truncated populations of chains help in understanding the implications of a high polydispersity value; for example, in C60-44 having an average mass of 60,000 g/mol, as determined by size exclusion chromatography, the first 10% by weight of chains (i.e. the shortest macromolecules) have a molecular

weight of 24,000 g/mol whereas the last 10% by weight (longest macromolecules) in the total distribution have a mass of 285,000 g/mol. The chains that are present in significant amount thus cover a range of lengths of one decade. The dispersity of the C60-44 chains is similar to that of the amphipol A8-35 developed by Tribet, Audebert and Popot (Table 1). In contrast, the size distribution of C22-43, which was obtained by a controlled free-radical polymerization [29], is much narrower. Estimates of the average molar masses of the 10% shortest and 10% longest chains in C22-43 gave 13,000 g/mol and 34,000 g/mol, respectively, which are values close to the average value (22,000 g/mol) of the mass distribution. The PC-amphipols C22-43, C60-44 and C60-30 are random copolymers of the neutral monomer *N*-isopropylacrylamide, the oleophilic monomer *n*-octylacrylamide, and a hydrophilic unit bearing a phosphorylcholine group. The fractions of the three monomers in each sample are listed in Table 1. We also used a PC-amphipol (C45-68) consisting exclusively of PC units and *n*-octyl units. Note that all the PC-modified polymers are cationic under the conditions of our measurements as a consequence of the presence of a secondary amine in the fragment linking the PC group to the polymer main chain [17].

Previous dynamic light scattering studies indicated that, in aqueous media, PC-amphipols self-assemble into particles containing a small number of polymer chains. Thus, in the pH 6.8 phosphate buffer used in this study, C22-43 forms micelles having a Stokes radius of 4.4 ± 1.1 nm, without detectable amounts of larger particles [17]. C60-44 assembles into larger objects which have a hydrodynamic radius of ~ 15 nm and a broader size distribution, as compared to C22-43 particles. A small fraction of larger aggregates (~ 150 nm in size) could be detected in solutions of C60-44. These particles were effectively removed by centrifugation (10 min at $200,000\times g$).

3.2. Formation of bacteriorhodopsin/PC-amphipols complexes

The ability of PC-amphipols to form water-soluble complexes with IMPs was examined using purified preparations of BR ($M \approx 27,000$ g/mol) in octylthioglucoside (OTG). In a typical experiment, the protein/detergent solution was supplemented with PC-amphipols and diluted with buffer such that the concentration of OTG dropped below its cmc. The sample was then subjected to centrifugation and the extent to which the protein was kept soluble was determined from the absorbance of the supernatant [4]. In the absence of amphipols, this dilution-centrifugation procedure resulted in almost complete precipitation of the protein (Fig. 2A). In contrast, in the presence of all PC-amphipols tested, as well as with A8-35, a significant fraction of BR was detected in the supernatant as long as the polymer/BR weight ratio was higher than 3 g/g (Fig. 2A and B). The fact that BR remained in the supernatant suggests that the polymers bind to the protein, thereby preventing its aggregation and precipitation. With C45-68, C60-44 and C60-30, a plateau of $\sim 70\%$ retention of the protein in the supernatant was reached for polymer/protein weight ratios above 5/1. As noted earlier (Section 3.1), solutions of C60-44 in buffer contain a small amount of large particles,

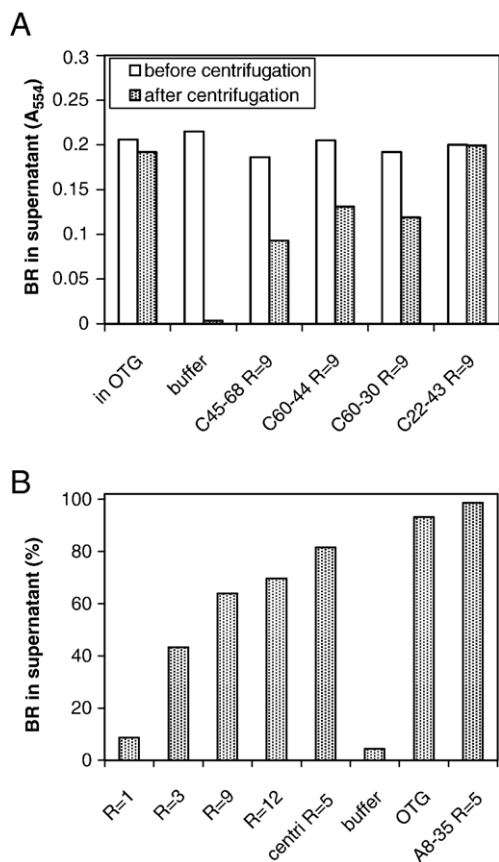


Fig. 2. Solubility of BR at submicellar concentrations of OTG in the presence of PC-amphipols or A8-35. Aliquots of a stock solution of polymer were added to the stock solution of BR (~ 1 g/L BR solubilized in 33 mM OTG, 20 mM phosphate buffer, pH 6.8) to obtain various polymer/BR ratios, R (in g/g). After incubation for 15 min at 4 °C, samples were diluted 10 \times in 100 mM NaCl, 20 mM phosphate buffer, pH 6.8, and centrifuged at 200,000 \times g, 10 min, 8 °C, in a Beckmann Airfuge. The fraction of BR kept soluble was determined from the absorbance at 554 nm of the supernatants. “OTG” and “Buffer” refer respectively to dilutions into the phosphate buffer, in the absence of polymer, with and without 33 mM OTG. “Centri $R=5$ ” corresponds to the use of a stock solution of polymer that was pre-centrifuged (200,000 \times g, 10 min) to remove traces of polymer aggregates prior to mixing with BR. (A) Comparison of polymers at $R=9$; (B) solubility of BR as a function of C60-44 concentration. A polymer/BR ratio of 5 g/g corresponds approximately to mol/mol ratios of 2.2:1 (C60-44) or 15:1 (A8-35).

which can be removed by centrifugation. The presence of such aggregates may have decreased the retention yield of the protein. Indeed, using a pre-centrifuged C60-44 solution to prepare BR/C60-44 complexes enhanced the solubility of BR to 80% (Fig. 2B). In all cases, the UV-visible spectrum of BR, which is highly sensitive to the functional state of the protein, did not change significantly upon association with PC-amphipols, indicating that these polymers can preserve the native state of the protein. In all cases, however, some loss of protein ($\sim 20\%$) took place, and this even at high polymer concentration. Two phenomena, protein denaturation and aggregation/precipitation, can concur to lower the overall yield. Denaturation is expected to increase at high ratios of APols or detergent, which may favor delipidation of the protein (the mere dilution of protein/OTG complexes into an excess of

OTG results in the loss of $\sim 10\%$ BR). Aggregation is favored at low APol/IMP ratios which are conditions driving the formation of protein/protein interactions at the expense of protein/amphipol interactions. Milder procedures of mixing and adjustments of handling conditions (temperature, ionic strength, etc.) are likely to enhance the current 80% recovery. In the present study, however, we did not attempt further optimization of the transfer from OTG.

Based on previous studies, we hypothesize that the solubility of BR in the presence of PC-amphipols is due to the formation of soluble protein/polymer complexes [4] and that the treatment with Bio-Beads (Section 2.5) removes most of the OTG from the complexes [10,13]. We set about next to assess by rate zonal centrifugation the size dispersity of the OTG-depleted BR/PC-amphipol complexes. In Fig. 3A, we show, as a control experiment, the absorbance at 554 nm of fractions collected after centrifuging BR/OTG complexes (i.e. the starting material for trapping experiments) in a gradient containing OTG. The sedimentation band of the OTG/BR complexes extends mainly over three consecutive fractions in the sedimentation gradient. The trailing of the band towards the bottom of the gradient indicates that, while BR is predominantly monomeric, some oligomers are also present (cf. [13]). When amphipol-trapped BR was deposited onto a surfactant-free gradient, a single sedimentation band migrating towards the middle of the sucrose gradient was observed for the complexes formed with C22-43, C60-44, C60-30, and A8-35. The presence of a single band confirms the absence of BR aggregation upon entrapment by any of the four polymers. In one case, however, namely that of BR/C45-68 complexes, the sedimentation band was much broader and aggregates were collected at the bottom of the sedimentation gradient (not shown). This behavior was noted only in the case of BR/C45-68 complexes. It may indicate that the chemical composition of this PC-amphipol, which comprises no *N*-isopropylacrylamide units, is not well suited to its function as an amphipol. Consequently, we did not carry out further measurements with this polymer.

Under identical conditions, BR/PC-amphipol complexes migrate deeper in the gradient than BR/OTG complexes: as shown in Fig. 3B in the case of BR/C60-44 complexes, the protein concentration is maximal in fractions 8 and 9, instead of 4–5 for OTG/BR, which means that the polymer/BR complexes migrated twice as far during the same centrifugation time (18 h). The penetration in the gradient of BR/PC-amphipol complexes is also about twice as deep as that of BR/A8-35 complexes (Fig. 3C). In all cases, the band containing the BR/polymer complexes featured a UV-visible spectrum characteristic of native BR (Fig. 4), devoid of an absorbance maximum at 382 nm, the wavelength at which free retinal absorbs when released from denatured BR.

The rate at which a particle sediments depends on its molecular weight, hydrodynamic radius, and density. It seems unlikely that the BR/PC-amphipol complexes have a hydrodynamic radius smaller than that of compact BR/OTG complexes. Consequently, their faster sedimentation rate points to a significantly higher density and/or larger molar mass of the particles, likely due to a higher density and/or mass of the BR-

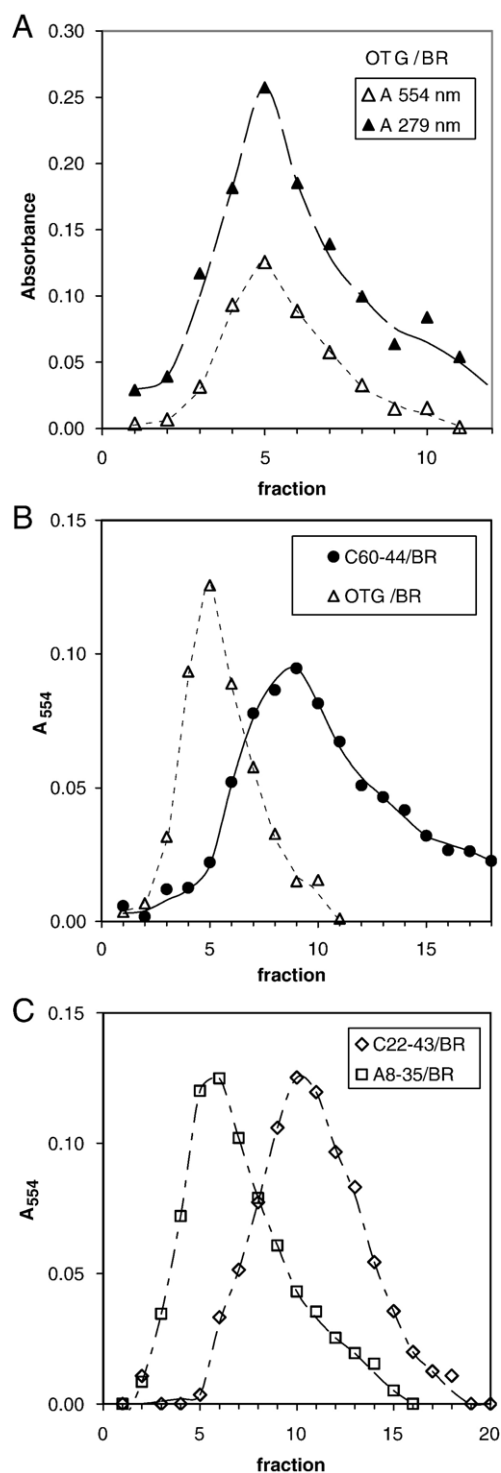


Fig. 3. Rate zonal sedimentation of BR/OTG and BR/polymer complexes in sucrose gradients. Samples containing ~ 0.5 g/L BR and either a polymer:BR ratio $R=9$ g/g, or, in the absence of polymer, ~ 15 mM OTG, were layered onto 5–20% (w/w) sucrose gradients in 20 mM phosphate buffer, pH 6.8, containing (A, B) 10 mM OTG (“BR/OTG complexes”), or (B, C) no surfactant (“BR/C60-44”, “BR/C22-43” and “BR/A8-35” complexes). OTG had been removed from polymer-containing samples by adsorption onto Bio-Beads prior to deposition onto the gradient (see Section 2). After centrifugation, 18–20 fractions were collected from the top of the tubes. The concentration of BR in the fractions was determined by absorbance measurements at (A–C) 554 nm and (A) 279 nm.

bound amphipol. In order to examine whether the polymers associated to BR have significantly different sedimentation properties, which could affect the sedimentation of the complexes, we compared the sedimentation rates of the free polymers. The sedimentation band of the polymers, in the absence of BR, was visualized with a fluorescence probe (ANS) known to partition in the hydrophobic core of surfactant micelles [30]. C22-43 and C60-44, in the absence of BR, were found to sediment about twice as far as A8-35 (Fig. 5A). The particles of A8-35 and their sedimentation properties have been extensively characterized [12,31]. PC-polymer particles have similar (C22-43) or larger (C60-44) hydrodynamic radii in solution [17]. Consequently, in the absence of protein, the faster sedimentation rate of PC-amphipols as compared to A8-35 must arise from an increase in the density and/or the mass of the PC-amphipols particles. The sedimentation of C22-43 produces a sharp band (Fig. 5B), whereas the band formed by C60-44 is much broader, betraying the polydispersity in size of the particles formed by this amphipol. It is important to note that it is most probably

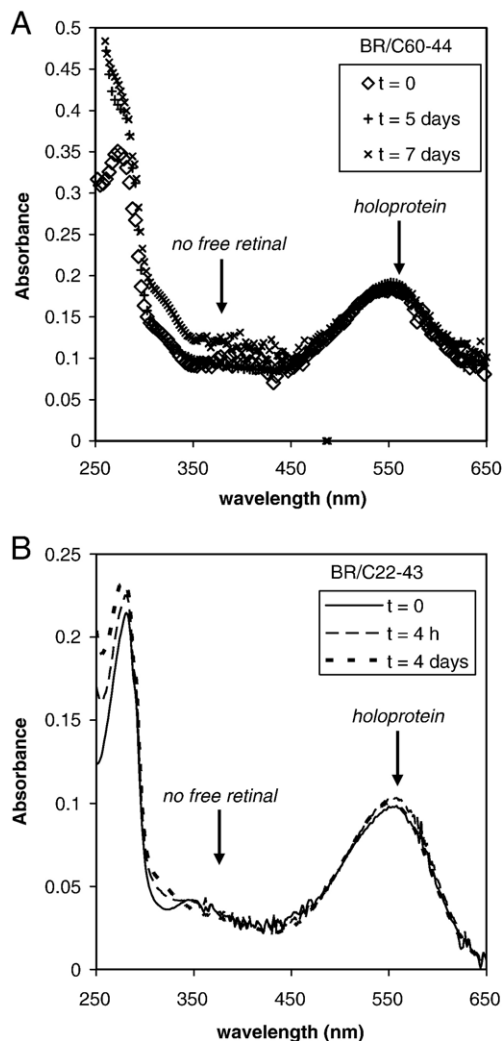


Fig. 4. UV-visible spectra of BR/PC amphipol complexes collected from sucrose gradients and incubated in the dark at 4 °C for the time indicated. (A) BR/C60-44 (B) BR/C22-43, with 1 M NaCl added at time zero. Arrows indicate peaks corresponding to native BR (~ 554 nm) and free retinal (~ 382 nm).

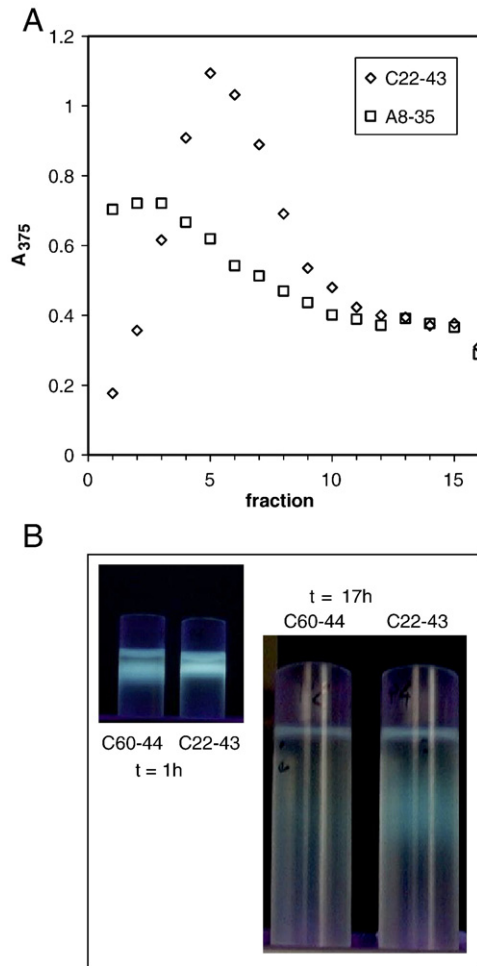


Fig. 5. Rate zonal sedimentation analysis of polymers A8-35, C60-44 and C22-43, labeled with 200 μ M ANS, in 5–20% (w/w) sucrose gradients. The gradients contained 35 μ M ANS. (A) Absorbance of ANS in fractions collected from top to bottom of the gradient after an 18 h centrifugation. (B) Photographs of the tubes exposed to UV light after 1 h and 17 h of centrifugation. The fluorescence of ANS exposed to UV (360–400 nm) reveals the presence of polymer particles. The same volume of samples was layered on the top of gradients (20 mM phosphate buffer, pH 6.8), which were centrifuged at 37,000 rpm (Beckman TLA centrifuge, MLS 50 rotor, $\sim 145,000\times g$).

because of the polydispersity in size of C60-44 particles that BR/C60-44 complexes form a significantly broader peak in the gradients than BR/OTG complexes (cf. Fig. 3B). In the case of C22-43, which, in the absence of BR, features a much reduced particle size polydispersity, complexes with BR indeed appear nearly monodisperse in size and do not form aggregates. Absence of aggregates and a sharp sedimentation band of BR/A8-35 complexes were essential criteria in the assessment of the efficiency of A8-35 as a suitable amphiphile for the handling of membrane proteins [4,13]. The only difference of behavior between the two types of BR/amphiphil complexes is a downshift by four fractions for C22-43 complexes, as compared to BR/A8-35 ones, under identical centrifugation conditions (Fig. 3C). As already noted for A8-35 (see e.g. refs. [10,13,14]), these observations point to the important impact of the properties of amphiphils in solution on the characteristics of their complexes with IMPs.

The contribution of the molecular properties of the polymer to the sedimentation properties of IMP/amphiphil complexes should be less pronounced when the protein is significantly more massive than the polymer. To test the effect of protein molar mass on the dispersity of IMP/PC-amphiphil complexes, we transferred to C60-44 or C60-30 a preparation of detergent-solubilized cytochrome b_6f . The protein samples used, which had been obtained by incubating for ~ 6 days at 4 $^{\circ}$ C a preparation of dimeric b_6f purified in DDM [26], contained both native b_6f dimers (16 protein subunits, $M \approx 215,000$ g/mol) and some inactive monomers (6 subunits, 87,000 g/mol) (see refs. [26,32]). The complexes were subjected to rate zonal sedimentation experiments similar to those carried out with BR, and fractions of the gradients were collected to measure their absorbance at 420 nm (Soret absorption band of the b_6f hemes). The mixture of monomer and dimer enables one to check on the resolution achieved (observation of two peaks in the gradients). In Fig. 6A, we present the results of a control experiment carried out with the same b_6f sample, kept in DDM and centrifuged in DDM-containing gradients. The presence of monomer and dimer was evidenced by peaks in fractions 6 and 9, respectively.

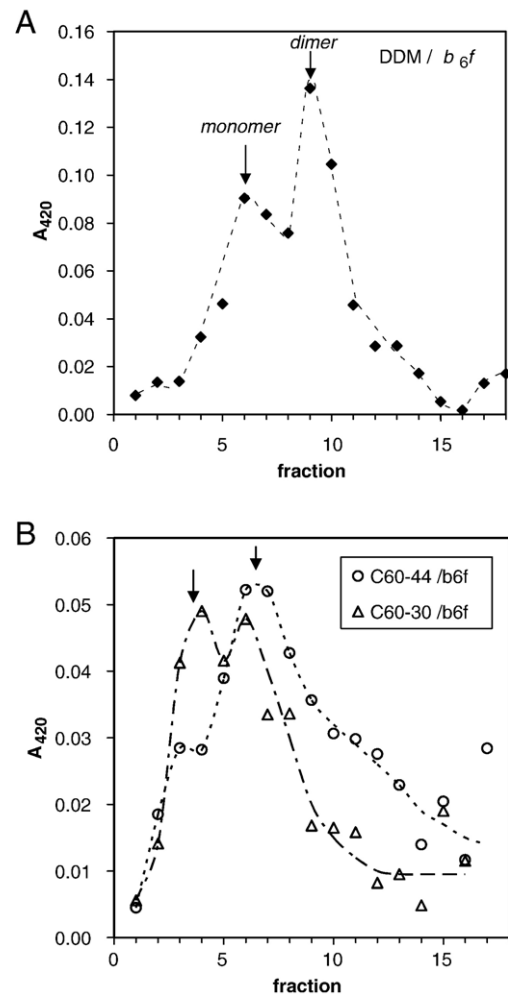


Fig. 6. Rate zonal sedimentation of b_6f /DDM (A) and b_6f /PC-amphiphil complexes (B) in sucrose gradients. The distribution of the protein was determined from heme absorbance at 420 nm.

For the protein trapped by polymers (Fig. 6B), the monomers and dimers were not as clearly distinguished as with $b_6 f$ /DDM complexes, presumably because of the broader size distribution of the complexes (arrows in the figure tentatively point to their expected positions). The $b_6 f$ /PC-amphipol complexes were recovered in fractions located at a similar depth in the gradient as $b_6 f$ /DDM complexes (namely between fractions 4 and 7, Fig. 6B), at variance with the different rates of migration observed for the complexes formed with BR. This result indicates that the sedimentation of proteins of high molar masses is much less sensitive to the properties of the polymers entrapping them.

3.3. Isothermal calorimetry titrations

The energetics of the formation of IMP/PC-amphipol complexes were assessed by isothermal calorimetric titrations monitoring the heat exchange that takes place upon titration of BR/OTG complexes into a solution of amphipol. Heat is released during the transfer of a protein from a detergent solution to an amphipol solution as a result of several processes: (i) dispersion of the OTG micelles into monomers; (ii) association of OTG with the polymer; and (iii) formation of BR/polymer complexes. BR/OTG complexes were prepared and purified by sedimentation through a gradient containing OTG micelles (13.5 mM OTG). The resulting OTG/BR solution was equilibrated by dialysis against a solution of 13.5 mM OTG in buffer (100 mM NaCl, 20 mM NaH₂PO₄–Na₂HPO₄, pH 6.8). The polymer present in the calorimetric cell, either C22-43 or A8-35, was dissolved in the same buffer but without OTG.

Control titrations including (i) the titration of OTG into either buffer or a polymer solution in the same buffer and (ii) the titration of a buffer solution (100 mM NaCl) into a buffer of slightly different salt concentration (95 mM NaCl) are presented as supporting information, together with the raw data corresponding to the enthalpograms presented in Fig. 7. Fig. 7A shows that dilution of 13.5 mM OTG into buffer is an exothermic process [17]. The demicellization enthalpy of OTG has been determined to be 5.4 kJ/mol [17]. Upon dilution of OTG micelles into a solution of either A8-35 or C22-43, concomitant re-association of surfactant molecules to the polymer takes place. The corresponding endothermic contribution has been analyzed in detail elsewhere [17]. The heat evolved upon dilution of BR/OTG complexes into a polymer solution was significantly higher than the heat evolved upon addition of micellar OTG containing no BR (Fig. 7A), implying that a large exothermic effect is associated with the overall interaction of BR/OTG with polymers. The magnitude of this effect can be established by subtracting from the signal measured upon dilution of BR/OTG complexes into polymer the contribution due to the dilution of the free OTG into the polymer (determined using the OTG solution against which the complexes had been dialysed). The enthalpy difference associated with the trapping of BR into polymers is presented in Fig. 7B as a function of protein concentration in the ITC cell containing either A8-35 or C22-

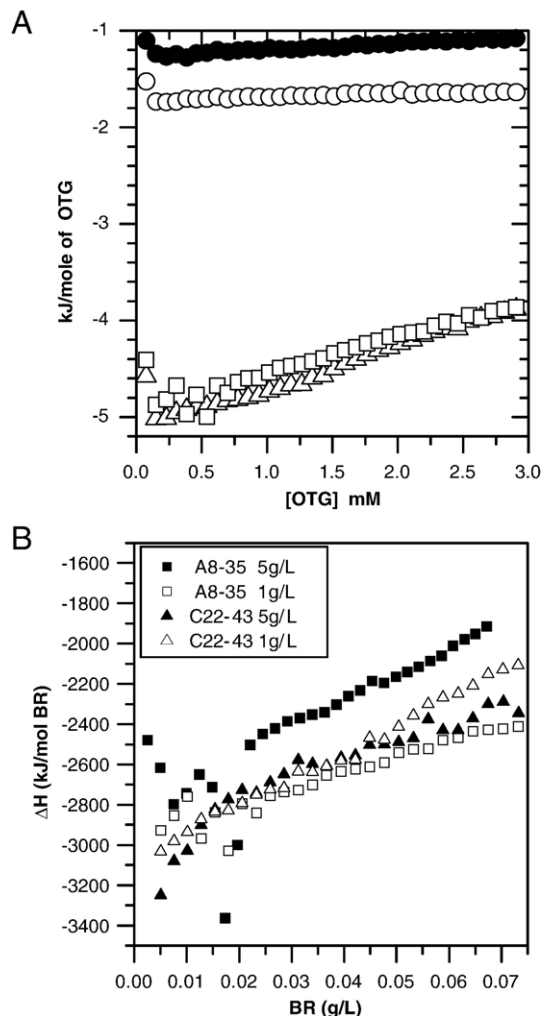


Fig. 7. Enthalpy of dilution (ΔH) of OTG or BR/OTG complexes into solutions of amphipols. All solutions were prepared in 100 mM NaCl, 20 mM NaH₂PO₄–Na₂HPO₄ buffer, pH 6.8. (A) Injection of OTG into solutions of 1 g/L C22-43 (○) or A8-35 (●), and corresponding injection of BR/OTG complexes (BR 0.45 g/L in 15 mM OTG) into 1 g/L C22-43 (△) or 1 g/L A8-35 (□). In order to facilitate the comparison of the enthalpies in the presence and absence of BR, the x-axis refers to the concentration of free OTG in the samples injected. (B) Difference between the enthalpy of transfer of BR/OTG and that of pure OTG in (△) 1 g/L C22-43, (□) 1 g/L A8-35, (▲) 5 g/L C22-43, and (■) 5 g/L A8-35. Temperature: 25 °C.

43. Remarkably, the enthalpy vs. [BR] traces are nearly identical in all cases, independently of the structure of the polymer and of its concentration in the cell (1 g/L or 5 g/L). The fact that the polymer concentration does not affect the enthalpy of association suggests that an excess of amphipol was present throughout the titration and that complete conversion to BR/polymer complexes was achieved. Invariance with polymer structure was not expected, though it points to some similarity in the origin (presumably hydrophobic) of BR trapping, irrespective of the exact structural details of the hydrophilic moieties in the polymer. The ITC measurements provide a strong indication of the similarity of the properties of A8-35 and C22-43 in terms of complexation energetics.

Table 2
Stability of IMP/amphipol complexes in presence of salts and at low pH

Conditions	NaCl			pH			Ca ²⁺		
	1 M	5	12 mM	1 M	5	12 mM	1 M	5	
Amphipol	A8-35			C22-43			C60-44		
Turbidity at $t=0$	+	+	+	–	–	+	–	–	
% BR in supernatant $t=15$ min	80	0	22	91	97	94	n.d.	n.d.	
% BR in supernatant $t=4$ h	79	0	6	93	94	92	93	94	
% b_6f in supernatant $t=1$ h							78	77	

^a A white precipitate forms (presumably calcium phosphate) upon addition of CaCl₂.

3.4. Stability of amphipol/BR complexes

Whenever sedimentation in sucrose gradients yielded a well-defined band of BR/polymer complexes, (C60-44, C60-30, C22-43, and A8-35), it was collected and its UV-visible spectrum was examined as a function of storage time. Judging from previous observations with IMP/anionic amphipol complexes [8], it is likely that detergent depletion was extensive. Thus the purified complexes are suitable objects for the assessment of the extent to which the polymer layer protects the protein from aggregation and inactivation. All BR/polymer complexes exhibited the absorption spectrum of native BR (Fig. 4) and were devoid of free retinal, as indicated by the absence of an absorbance peak at 382 nm. Upon storage at 4 °C in the dark, the spectrum of BR was invariant over a period of 13 days at pH 6.8.

At low pH or in the presence of high salt or calcium ions, A8-35 and IMP/A8-35 complexes tend to form aggregates (refs. 12–14 and unpublished data). We tested the ability of PC-amphipols to preserve IMPs from aggregation under such conditions. Aliquots of purified BR/C22-43 and BR/A8-35 solutions (the two most concentrated fractions after sedimentation in sucrose gradients) were supplemented with either a NaCl solution, a citric acid-NaOH buffer, or a CaCl₂ solution to reach final concentrations of 1 M NaCl, 25 mM citrate, pH 5, and 12 mM Ca²⁺, respectively (cf. Section 2). Immediately after mixing (t_0), UV-visible spectra of the samples were measured to determine whether their transparency was altered. All samples containing A8-35 became turbid. In the case of C22-43, the samples treated with NaCl and those brought to low pH remained clear, while that supplemented with Ca²⁺ became turbid (Table 2). After 15 min or 4 h of incubation at 20 °C, the samples were centrifuged (10 min at ~200,000×g) and UV-visible spectra of the supernatants were recorded to determine the amount of protein present (Table 2). The PC-amphipols were highly efficient at maintaining more than 90% of the BR soluble in all cases. The turbidity observed in the presence of calcium was presumably due to the formation of calcium phosphate, judging from the presence of a white precipitate at the bottom of the centrifugation tubes. In contrast, a purple pellet formed upon centrifugation of the BR/A8-35 samples that had been

treated with calcium ions or subjected to low pH conditions. A significant fraction of BR/A8-35 complexes, on the other hand, remained soluble in 1 M NaCl. Similar experiments carried out with b_6f /C60-44 complexes in the same buffers showed similar preservation of solubility in either 1 M NaCl or 25 mM citrate buffer (Table 2).

4. Discussion

While tens, probably hundreds of detergents have been tested for their usefulness in membrane biochemistry (see e.g. refs. [1,3], and references therein), only a handful of amphiphilic polymers have been hitherto applied to keeping IMPs soluble [4,14–16]. Developing a range of alternative structures is of interest on many counts. For one thing, comparison of the structures of inefficient vs. efficient polymers will help delineating general rules for their design. Thus, most current studies suggest that the dispersity in size of the self-associated structures formed by A8-35 tends to correlate with that of IMP/A8-35 complexes [10,13,14]. A similar observation is reported here for complexes of BR and PC-amphipols. This effect is seen – none too surprisingly – to depend on the size of the protein: monomers and dimers of cytochrome b_6f (~87 and ~215 kDa, respectively) were still relatively well resolved following complexation with the rather polydisperse C60-44 PC-amphipol, consistent with the view that the polymer represents a smaller fraction of the total bulk and mass of the particle than in the case of BR (~27 kDa) (cf. ref. [8]).

A number of similarities between IMP/A8-35 and IMP/PC-amphipol complexes, especially C22-43, were anticipated given that the two polymers share the same general structure. The fact that the energetics of BR transfer from OTG to amphipols appear nearly identical for A8-35 and C22-43 seemed surprising at first, however, given the very different nature of the hydrophilic groups of two polymers. This observation emphasizes the importance of hydrophobic interactions in the mechanism of complexation of IMPs by APols, consistent with the fact that these polymers seem to interact preferentially with the transmembrane surface of the protein [11]. ITC measurements provide new insights into the properties of self-association and complexation of amphipols, which may prove particularly relevant to understanding both IMP-associated amphipol layers and pure amphipol particles. Further ITC experiments are in progress and will be reported elsewhere.

Another incentive for the development of new amphipols is to provide the biochemist and biophysicist with a variety of molecules with complementary ranges of applications and conditions of uses. Examples of applications abound, where a diversification of amphipol structures and physical–chemical properties would be desirable. We may cite the following situations:

- (i) The best-characterized amphipol to date, A8-35, has repeatedly proven its efficiency at biochemically stabilizing the functional state of IMPs, e.g. BR or the sarcoplasmic calcium pump [4,14,33]. It cannot be used,

however, in acidic media nor in the presence of calcium ions, if the monodispersity of the particles is to be preserved [10,14].

- (ii) IMP/A8-35 complexes can be studied by NMR [11], but not at the slightly acidic pH that is optimal for most NMR studies involving amide protons.
- (iii) A8-35 provides an excellent medium for IMP renaturation [34] – an issue of great importance given the difficulty to overproduce IMPs in their native state – but its pH-sensitivity limits the range of refolding conditions that can be explored.

In all such cases, having access to pH-insensitive structures would broaden the range of applicability of amphipols. While organic chemistry suggests many possibilities, physical chemistry imposes rather severe constraints. Indeed, because a high density of grafting with hydrophobic chains is essential to the efficiency of amphipols, the polar moieties that keep the polymer soluble have to be highly hydrophilic. In addition to the original carboxylates [4], this had been achieved to date thanks to multiple hydroxyle groups [15], a combination of quaternary amines and carboxylates [16], or sulfonates [14,18]. While all of these amphipols have been shown to be able to maintain IMPs soluble and native, none of them have been thoroughly optimized nor characterized in great details yet.

5. Conclusion

The present work establishes the usefulness of a new family of amphipols, PC-amphipols, whose solubility is ensured by phosphorylcholine groups. As shown here, this type of polar group, which is highly soluble over a very wide range of conditions, including low pH or the presence of Ca^{2+} ions, makes it possible to prepare amphipols that have the potential to overcome many of the physical–chemical limitations of A8-35. In addition, the phosphorylcholine group is a good mimic of the polar moiety of phosphatidylcholine, which comprises a large proportion of membrane lipids in eukaryotic organisms. The PC moiety is likely to endow the complexes with particularly good biocompatibility.

Acknowledgments

Particular thanks are due to Tassadite Dahmane (UMR 7099, Paris) for a gift of purple membrane. CT thanks the Center for Self-Assembled Chemical Structures (Québec, Canada) for a visiting professorship. This work was supported in part by a grant of the Natural Sciences and Engineering Research Council of Canada to FMW and grant RG00223/2000-M from Human Frontier Science Program Organization to JLP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbame.2007.07.007.

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Supporting information for:

Complexation of integral membrane proteins by phosphorylcholine-based amphipols

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1. Raw data corresponding to the traces represented in Figure 7.

Note that the scale of the ordinates is the same in all plots to facilitate comparisons

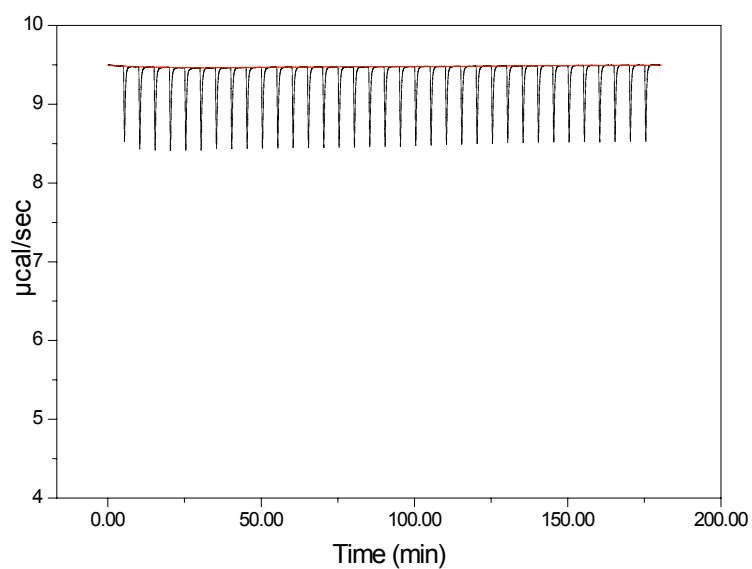


Figure 1. Calorimetric trace of the titration of an OTG solution (13.5 mM) into a solution of A8-35 (1 g/L).

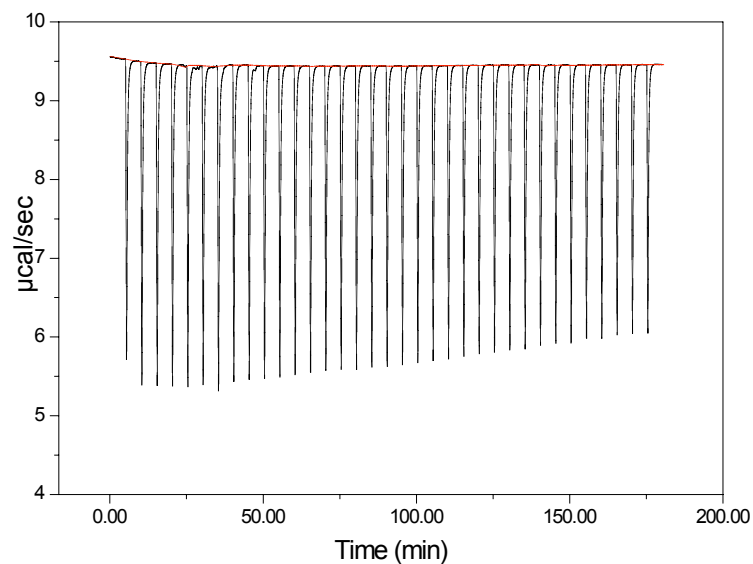


Figure 2. Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of A8-35 (1 g/L).

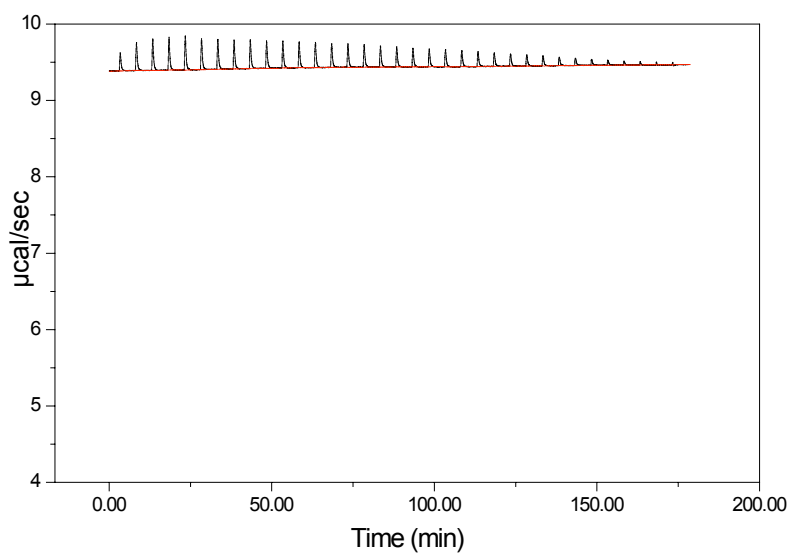


Figure 3. Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of A8-35 (5 g/L).

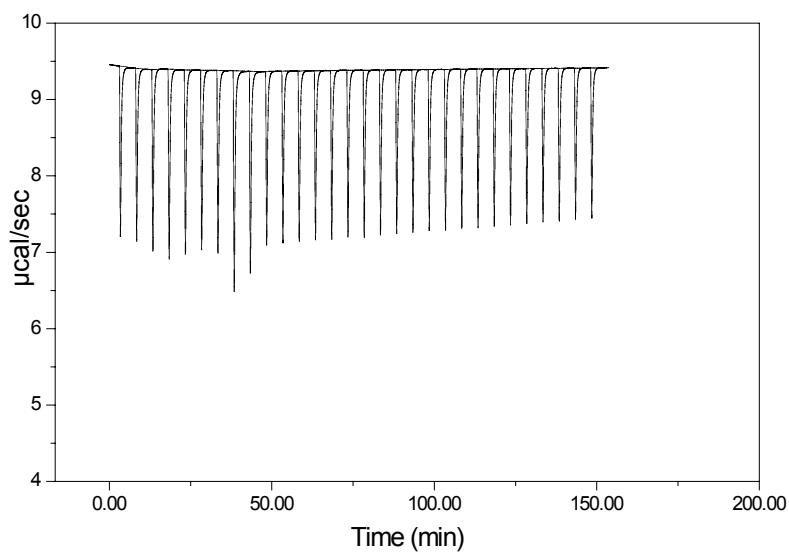


Figure 4. Calorimetric trace of the titration of a solution of BR/OTG complexes ($[\text{OTG}] = 13.5 \text{ mM}$) into a solution of A8-35 (5 g/L).

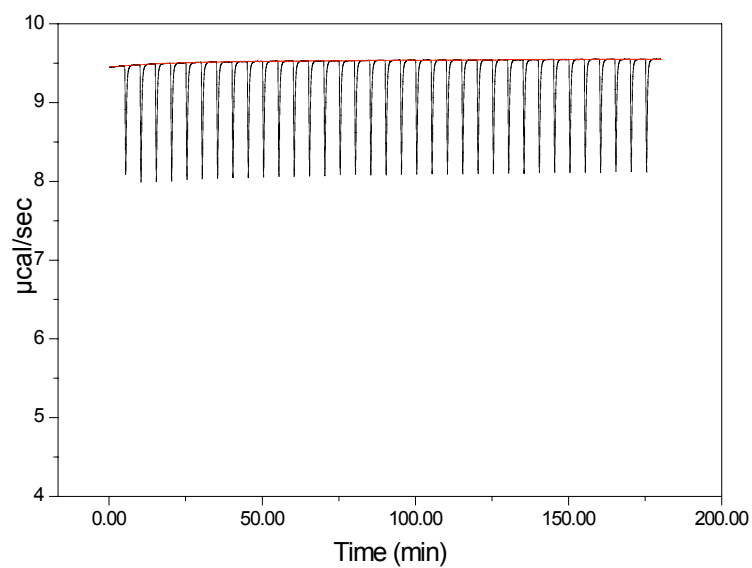


Figure 5. Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of C22-43 (1 g/L).

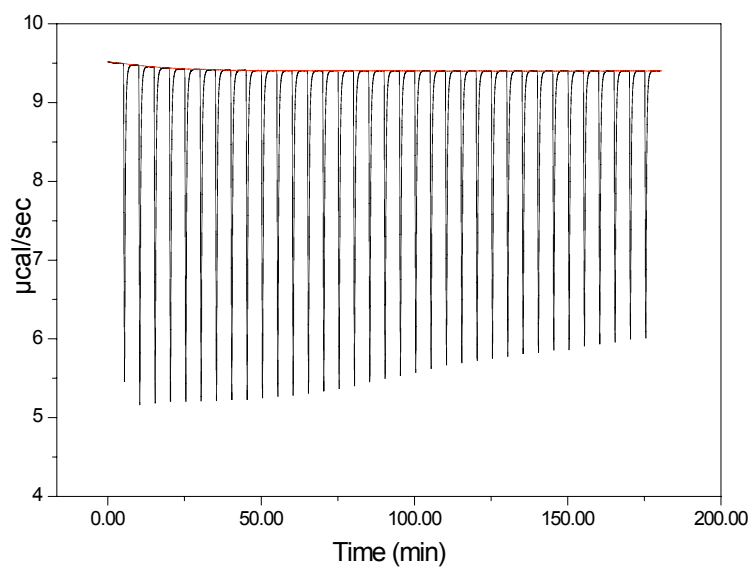


Figure 6. Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of C22-43 (1 g/L).

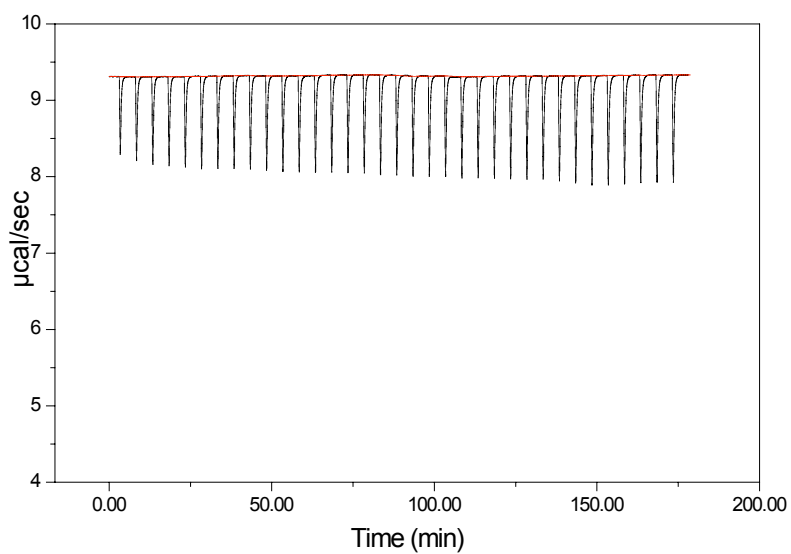


Figure 7. Calorimetric trace of the titration of a solution of OTG (13.5 mM) into a solution of C22-43 (5 g/L).

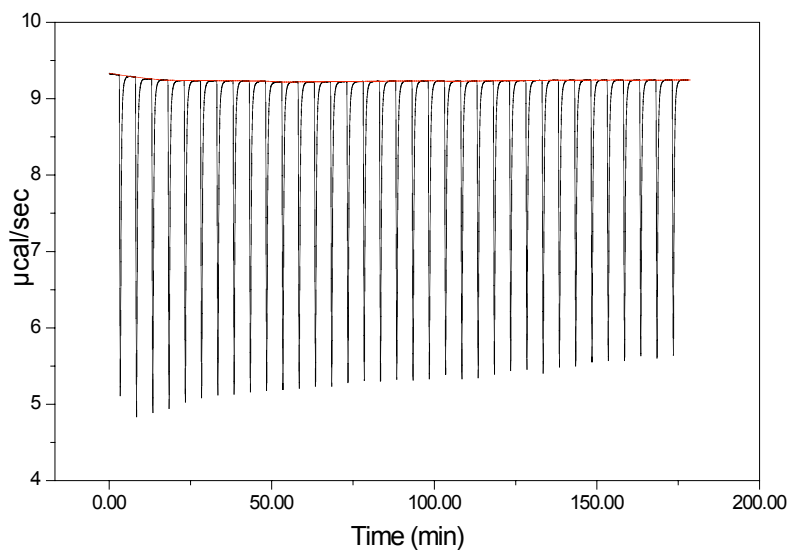


Figure 8. Calorimetric trace of the titration of a solution of BR/OTG complexes ([OTG] = 13.5 mM) into a solution of C22-43 (5 g/L)

2. Control experiments.

Two titrations were performed under experimental conditions identical to those described in the article to assess the effect of an imbalance of the buffer concentrations after dialysis on the results of the ITC experiments. Assuming that the error upon addition of NaCl to the solution after dialysis was 2% or less, we performed two control experiments: in one measurement, a buffer solution containing 100 mM NaCl was titrated into the identical buffer ; in another titration, a buffer solution containing 95 mM NaCl was titrated into a buffer containing 100 mM NaCl. The enthalpograms obtained in each case are presented below. It can be observed that the difference in enthalpy in the latter case is

~22 J/mol, a negligible value as compared to the enthalpies of interactions between amphipols and BR (Figure 7).

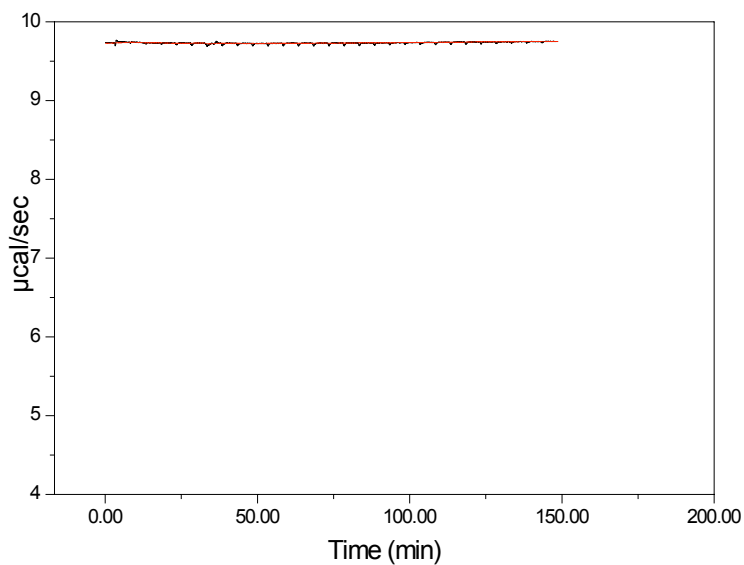


Figure 11. Calorimetric trace of the titration of a buffer solution containing 100 mM of NaCl into the same buffer.

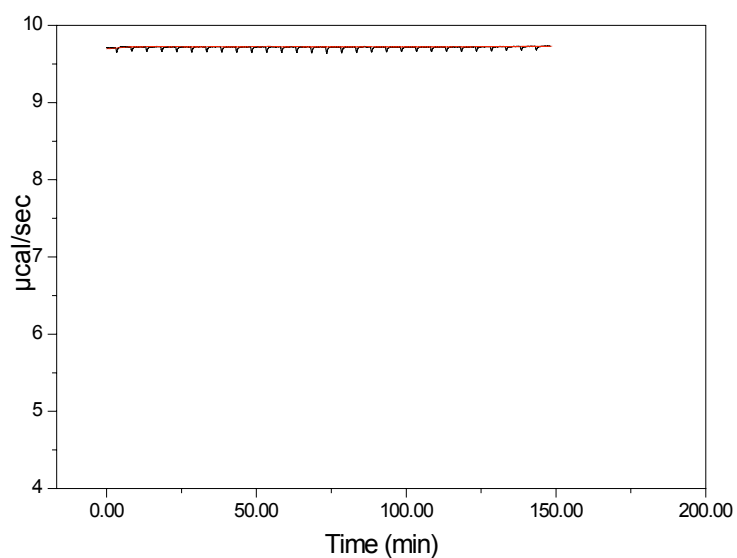


Figure 12. Calorimetric trace of the titration of a buffer solution containing 95 mM of NaCl into the same buffer containing 100 mM of NaCl.