Protective and Inhibitory Effects of Various Types of Amphipols on the Ca^{2+}-ATPase from Sarcoplasmic Reticulum: A Comparative Study†

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ABSTRACT: Amphipols are amphipathic polymers designed to replace or supplement detergents in membrane protein solution studies. Previous work has suggested both advantages and disadvantages to the use of a polyacrylate-based amphipol, A8−35, for studying the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1a). We investigated this issue further using a set of four amphipols with different chemical structures. Previous size exclusion chromatography experiments had shown that A8−35 and SERCA1a/A8−35 complexes aggregate under certain conditions. We show here that aggregation can be prevented by omitting calcium from buffers or by using a sulfonated version of A8−35. A8−35 had previously been shown to protect Ca^{2+}-ATPase from irreversible denaturation, while inhibiting its activity in a reversible manner. We show here that the other three amphipols tested also display these properties and that all four amphipols slow down backward calcium dissociation from the nonphosphorylated solubilized enzyme, a priori an unrelated step. As this calcium dissociation involves the opening up of the bundle of transmembrane ATPase segments, the slowing of this process may indicate that multipoint attachment of the polymers to the hydrophobic transmembrane surface damps protein dynamics ("Gulliver" effect). Damping might be the reason why amphipols also simultaneously protect membrane proteins against irreversible denaturation and may inhibit the activity of those of them that display large rearrangements of their transmembrane surface during their catalytic cycle.

Integral membrane proteins are the object of intensive studies, because they fulfill essential physiological functions and constitute important biomedical targets. However, in vitro studies of membrane proteins are hampered by aggregation in aqueous solutions, due to the high hydrophobicity of the surface of their transmembrane region. Detergents prevent this aggregation by adsorbing onto transmembrane surfaces, thereby providing an interface with the hydrophilic medium (*1*). However, detergents are dissociating surfactants, which very often destabilize membrane proteins. One of the major challenges in membrane protein biochemistry is therefore to achieve an acceptable compromise between solubility and biochemical stability (for discussions, see, e.g., refs *2* and *3*). This problem has prompted the design of milder surfactants, which may not necessarily extract proteins from biological membranes efficiently but can substitute for detergents after solubilization and are more efficient at keeping membrane proteins in solution without inactivation (for reviews, see, e.g., refs *4* and *5*).

"Amphipols" (APols)[1] are amphipathic polymers specially designed for this purpose (*6*). First-generation APols have a polyacrylate backbone onto which fatty amines are grafted (Scheme 1A) (*6*). APols and their uses in membrane biology have been the subject of two recent reviews (*5*, *7*). Polyacrylate-based APols, as well as some nonionic or zwitterionic APols, keep membrane proteins soluble in the absence of detergent (in most cases, after extraction from the membrane with detergents). Membrane proteins are generally more stable after trapping with APols than in detergent solution. Thus, APols are potentially useful substitutes for detergents for in vitro functional or structural studies of detergent-sensitive membrane proteins (see, e.g., refs *7* and

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[1] Abbreviations: APol, amphipol; SAPol, a sulfonated amphipol; SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; A8−35, a specific type of polyacrylate-based amphipol (see Scheme 1A); $C_{12}E_8$, octaethylene glycol monododecyl ether; HPLC, high-pressure liquid chromatography; K_d, distribution coefficient; R_S, Stokes radius; SEC, size exclusion chromatography; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; quin2, 2-[(2-amino-5-methylphenoxy)-methyl]-6-methoxy-8-aminoquinoline-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; CMC, critical micelle concentration.

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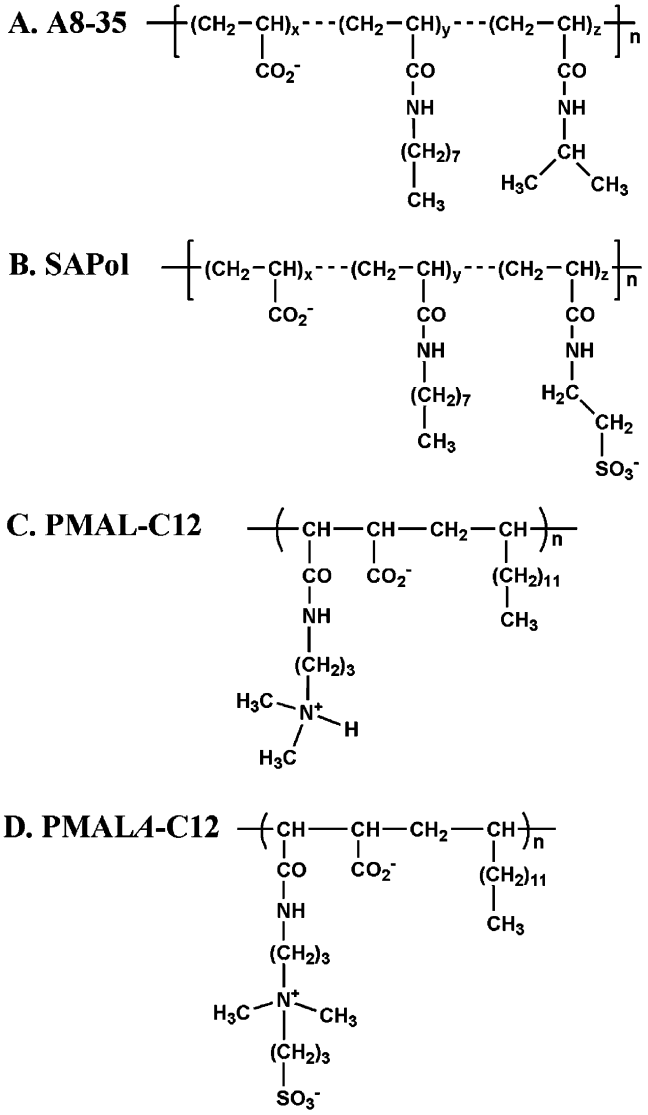
Scheme 1: Chemical Structure of the Four Amphipols Used[a]



[a] (A) A8−35, a polyacrylate-based APol, anionic at neutral and basic pH; $\langle x \rangle \approx 35\%$, $\langle y \rangle \approx 25\%$, $\langle z \rangle \approx 40\%$, $\langle n \rangle \approx 76$, average molecular mass 8−10 kDa (*6*, *17*); (B) SAPol, a sulfonated APol, anionic at any pH, $\langle x \rangle \approx 35\%$, $\langle y \rangle \approx 25\%$, $\langle z \rangle \approx 40\%$, $\langle n \rangle \approx 76$, average molecular mass ∼11 kDa (F. Giusti, unpublished data); (C) PMAL-C12, an APol designed to be zwitterionic at neutral and basic pH (*12*; Anatrace no. P5012, PMAL-B-100 in ref *12*), $\langle n \rangle \approx 30$, average molecular mass ∼12 kDa; (D) PMALA-C12, a sulfonated version of PMAL-C12 (Anatrace no. PA5012), $\langle n \rangle \approx 30$, average molecular mass ∼16 kDa.

8 and references cited therein). However, the behavior of these molecules and of membrane protein/APol complexes is far from being completely understood. Furthermore, there is an enormous variety of conceivable APol chemical structures (see, e.g., refs *9* and *10*). Optimizing APols for work with specific types of membrane protein, or for particular experimental approaches, requires a more detailed understanding of the solution behavior of APol and protein/APol complexes and of the stability and activity of APol-trapped proteins.

Early studies of polycarboxylate-based or nonionic APols established that both classes of polymers allowed membrane proteins to retain their native structure (*6*, *7*, *10*, *11*). Most data suggest little or no interference of APols with membrane protein function (*7*, *11*, *12*; see Supporting Information for a more detailed review). However, a different conclusion was reached in an earlier study of complexes between APol A8−35 and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1a): while A8−35 was found to strongly stabilize the calcium pump against irreversible inactivation, it also inhibited its ATPase activity, an inhibition that could be reversed by excess detergent (*13*). Thus, the prevalence and mechanism(s) of membrane protein functional inhibition by APols remain open questions; addressing them was the main purpose of the present work. Previous studies also found that, while the formation of complexes between membrane proteins and A8−35 generally yields monodisperse particles (see, e.g., refs *6*, *7*, and *14*−*17*, and Supporting Information), A8−35 and Ca^{2+}-ATPase/A8−35 complexes exhibited a strong tendency to aggregate (*13*). The secondary aim of this study, of practical relevance, was to identify the cause of this aggregation and to find ways of preventing it.

In the course of the present work, we used four different APols: A8−35 and a polysulfonated analogue thereof ("SAPol"; Scheme 1B), both synthesized by us, and two commercial APols, the zwitterionic PMAL-C12 (*12*) and its sulfonated (and thus anionic) analogue, PMALA-C12 (Scheme 1D). Their comparative study led to interesting insights into the mechanisms of membrane protein functional inhibition by APols and the causes of aggregation.

EXPERIMENTAL PROCEDURES

The structures of the four types of APol tested are shown in Scheme 1. A8−35, an anionic, polyacrylate-based APol (Scheme 1A), was synthesized either as described in ref *6* (batch GPPHR, hereafter referred to as "old") or with the modifications described in refs *16* and *17* (batches FGH15 and FGH20, hereafter referred to as "new"). The average molecular mass of A8−35 was between ∼9 and ∼10 kDa, depending on the size of the poly(acrylic acid) precursor; such variability has no detectable effect on solution properties (*17*). Some early A8−35 batches, including batch GPPHR, were found to contain an excessive amount of grafting by dicyclohexylurea (DCU), a derivative of the activating agent dicyclohexylcarbodiimide, which confers them a tendency to aggregate (see refs *16* and *17*). The synthesis of SAPol, a sulfonated analogue of A8−35 (Scheme 1B; batch FGHS8), will be described elsewhere (F. Giusti, in preparation). The level of DCU grafting, determined by NMR, was ∼10% (GPPHR), ∼2% (FGH15 and FGH20), and ∼0% (FGHS8) of the acrylate units. PMAL-C12 (Scheme 1C; referred to as PMAL-B-100 in ref *12*) and PMALA-C12 (Scheme 1D) were bought from Anatrace [references P5012 (batch 12PM08) and PA5012 (batch 12PA01), respectively]. Polymer stock solutions were made at a concentration of 50 mg/mL in Milli-Q deionized water. SR vesicles were prepared as previously described (*18*). $C_{12}E_8$ (CMC = 50 μg/mL, about 0.09 mM) was bought from Nikko and dodecyl maltoside (CMC = 90 μg/mL, about 0.18 mM) from Calbiochem.

Size exclusion HPLC chromatography (SEC) of APols alone was performed as previously described (*12*), using a Beckman Gold system with diode array detection (1 cm optical path length) and calibration (K_d vs R_S; see ref *19*) with Bio-Rad standards (catalog number 151-1901). We loaded 200 μL samples of 5 mg/mL APol onto an Amersham TSK 3000 SW silica gel column (7.5 mm × 30 cm, total

volume ~11.5 mL), run at 23 °C. Some experiments were carried out with a ProteinPak TSK 300 silica column loaded with 100 μL samples, with Waters HPLC chromatographic equipment, with similar results. Most experiments were carried out in 100 mM KCl and 20 mM TES−NaOH, pH 7 ("KCl/TES, pH 7, buffer"), with or without metal ions and/or chelators.

The turbidity and absorbance of APol or SR suspensions were assessed with a diode array HP 8453 spectrophotometer. Samples in the controlled temperature cuvette were stirred continuously.

ATPase activity was determined at 20 °C with a coupled enzyme assay in the HP 8453 spectrophotomer in a medium comprised of 100 mM KCl, 1 mM Mg^{2+}, 50 mM TES−Tris, 0.1 mM Ca^{2+}, and 0.05 mM EGTA (pH 7.5), supplemented with 5 mM MgATP, 0.1 mg/mL pyruvate kinase, 1 mM phosphoenolpyruvate, 0.1 mg/mL lactate dehydrogenase, and an initial concentration of about 0.3 mM NADH ("ATPase activity assay medium") (*20*). Where indicated, $C_{12}E_8$ was added at the desired concentration.

Calcium dissociation from Ca^{2+}-ATPase was monitored by a stopped-flow method with BioLogic SFM3 equipment and the Ca^{2+}-sensitive fluorophore quin2 (potassium salt; Calbiochem) as a Ca^{2+} chelator (*21*). The excitation wavelength was 312 nm.

RESULTS

Effect of Divalent Cations on the Aggregation of Various Amphipols. Earlier SEC studies of pure A8−35 particles and Ca^{2+}-ATPase/A8−35 complexes showed that, in our typical SERCA1a buffer (KCl/TES, pH 7, buffer plus 1 mM Mg^{2+} and 0.5 mM Ca^{2+}), both of them formed aggregates, some of which were eluted in the void volume of the column (*13*). Subsequent studies with other proteins, such as bacteriorhodopsin (*14*) or the transmembrane domain of OmpA (*15*), indicated that the aggregation behavior of the polymer largely determines that of membrane protein/APol complexes. We therefore reinvestigated buffer effects on the behavior of A8−35 particles. Chromatogram A in Figure 1 confirms that A8−35 alone (in the absence of detergent or protein), prepared as for the experiments in ref *13* ("old" batch), diluted to 5 mg/mL in SERCA1a buffer and subjected to SEC on a TSK column equilibrated with the same medium, elutes as extremely polydisperse particles. It has recently been shown that the mode of synthesis and purification of A8−35 can have a profound influence on its solution behavior: in particular, excessive artifactual grafting of dicyclohexylurea (DCU) may occur under certain conditions, as was the case for the old batch used here (see Experimental Procedures), which predisposes the resulting polymer to aggregation (*16*−*17*). We therefore repeated the SEC experiments using a recent batch of A8−35 ("new" batch) with a low level (~2%) of artifactual grafting. Under the same SEC conditions, this new batch also appeared polydisperse but with far fewer very large particles (Figure 1B).

Polydispersity was reduced even more markedly if EDTA was added to chelate all divalent cations (dotted line in Figure 1C): the elution profile revealed a main population of particles with a Stokes radius of ~3 nm (by reference to protein standards). This value is identical to that previously obtained, in the absence of divalent cations, by both SEC and small-angle neutron scattering (3.15 ± 0.15 nm; see refs *14* and *17*). A small fraction of particles eluting slightly earlier than the main fraction was still present (Figure 1C), possibly due to slow aggregation of A8−35 at pH 7 (*17*). The effect of divalent cations on the aggregation of A8−35 was confirmed using the old batch of A8−35 and a different type of column (ProteinPak) (see Figure S1 in Supporting Information). Further experiments were performed to sort out the extent to which Ca^{2+} and Mg^{2+} contribute to the aggregation of A8−35. The presence in the elution buffer of 1 mM Mg^{2+} (but no Ca^{2+}) modified the elution pattern of A8−35 only slightly from that obtained in the total absence of divalent cations (Figure 1C, solid vs dotted lines), whereas the addition of this concentration of Mg^{2+} plus 0.5 or 1 mM Ca^{2+} induced aggregation much more strongly

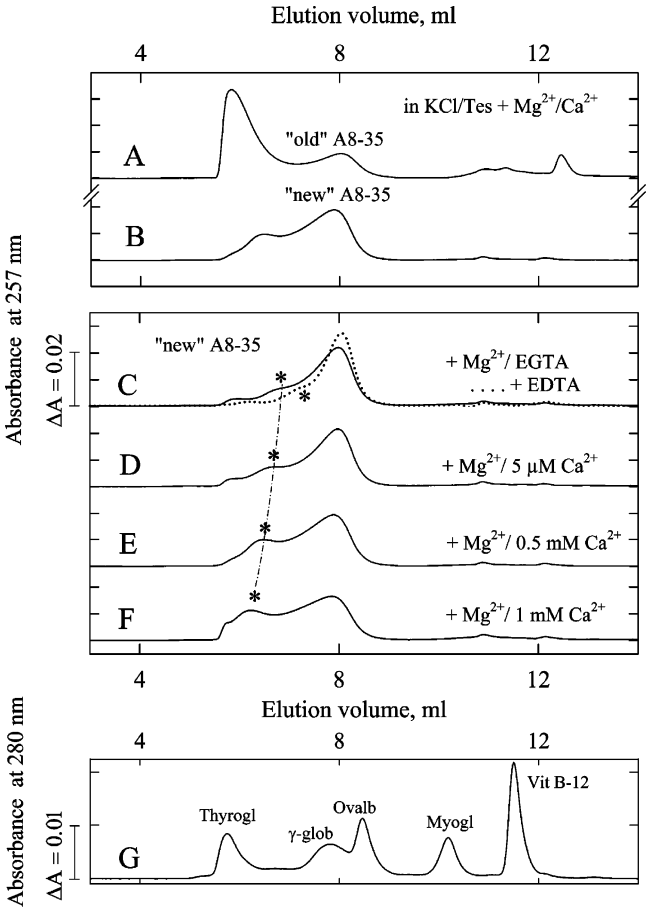


FIGURE 1: Size exclusion chromatography analysis of two different batches of amphipol A8−35 and effect of divalent cations on A8−35 aggregation. For Figures 1 and 2, 200 μL samples of A8−35 at 5 mg/mL were loaded onto a TSK 3000 SW column equilibrated at room temperature (23 °C). For chromatograms A and B, the medium consisted of KCl/TES, pH 7, buffer supplemented with 1 mM Mg^{2+} and 0.5 mM Ca^{2+}: A, A8−35 from batch GPPHR (old); B, A8−35 from batch FGH15 (new). For chromatograms C−F, A8−35 from batch FGH15 (new) was used, and the medium consisted of KCl/TES, pH 7, buffer supplemented with either 0.5 mM EDTA (C, dotted line) or 1 mM Mg^{2+} plus 0.5 mM EGTA (C, solid line), 0.5 mM EGTA and 0.45 mM Ca^{2+} (free $Ca^{2+} \approx 5$ μM) (D), 0.5 mM Ca^{2+} [E; reproduced from (B) above], or 1 mM Ca^{2+} (F). G: R_S standards, separated in the presence of 1 mM Ca^{2+}: thyroglobulin (670 kDa, $R_S = 8.6$ nm), γ-globulin (158 kDa, $R_S = 5.2$ nm), ovalbumin (44 kDa, $R_S = 2.8$ nm), myoglobin (17 kDa, $R_S = 1.9$ nm), and vitamin B_{12} (1.3 kDa, $R_S = 0.85$ nm). Absorbance was measured at 257 nm for A8−35 and at 280 nm for standards. For clarity, the various traces have been shifted vertically with respect to each other.

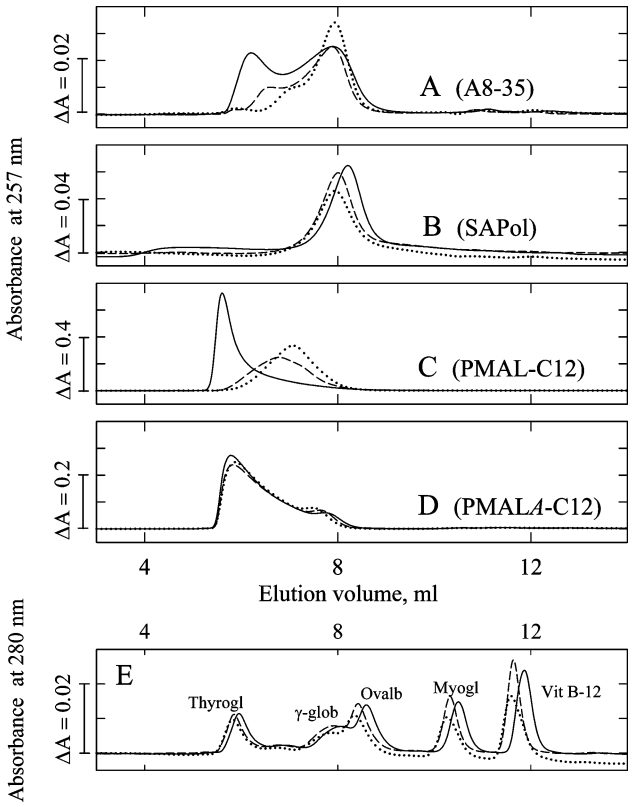


FIGURE 2: Effect of divalent cations on the aggregation of four different amphipols. Various APols [A, A8−35 (batch FGH15); B, SAPol; C, PMAL-C12; D, PMAL*A*-C12] and R_S standards (E) were subjected to SEC in KCl/TES, pH 7, buffer supplemented with 0.5 mM EDTA (dotted lines), 1 mM Mg^{2+} (dashed lines), or 1 mM Mg^{2+} plus 0.5 mM Ca^{2+} (solid lines).

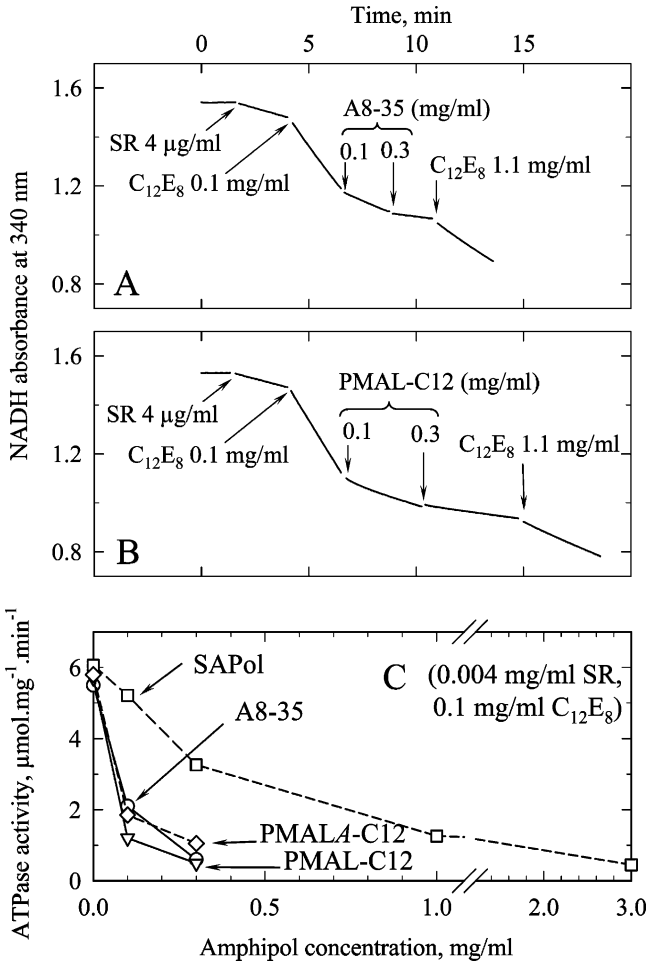


FIGURE 3: Reversible inhibition by amphipols of the ATPase activity of detergent-solubilized SR membranes. SR vesicles were diluted to 0.004 mg/mL in a detergent-free assay medium, and ATPase activity was monitored continuously with a coupled enzyme system. Samples were successively supplemented with (1) 0.1 mg/mL (i.e., twice the CMC) $C_{12}E_8$, which solubilizes the SR membranes, thereby relieving inhibition due to the accumulation of Ca^{2+} within SR vesicles, (2) APols [either A8−35 (batch FGH15), panel A, or PMAL-C12, panel B] at final concentrations first of 0.1 mg/mL and then of 0.3 mg/mL, and (3) a larger concentration of $C_{12}E_8$, partly relieving inhibition by APols (1 mg/mL was added to the 0.1 mg/mL already present). Panel C: ATPase activities as a function of APol concentration for the four APols tested.

(Figure 1E,F). The presence of ~5 μM free Ca^{2+} (Figure 1D) only slightly increased the effect of Mg^{2+} alone. In our previous work (*13*), divalent cations were added to the HPLC buffers because they are known to slow the irreversible denaturation of solubilized Ca^{2+}-ATPase (e.g., refs *22*−*24*). This was most likely the reason for the observed tendency of both A8−35 alone and Ca^{2+}-ATPase/A8−35 complexes to aggregate.

The aggregative effect of calcium on A8−35 can constitute a serious hindrance when designing experiments requiring both the presence of calcium and monodispersity of membrane protein/APol particles. We therefore examined whether aggregation could be avoided by using APols with different chemical structures. No aggregation was observed with a sulfonated analogue of A8−35, SAPol, which, unlike A8−35, remained monodisperse in the presence of 1 mM Mg^{2+} and 0.5 mM Ca^{2+} (compare panels A and B of Figure 2). In contrast, PMAL-C12 (PMAL-B-100 in ref *12*), a zwitterionic APol that also contains carboxylic groups, experienced even stronger aggregation than A8−35 in the presence of Ca^{2+} (Figure 2C). PMAL*A*-C12, a sulfonated analogue of PMAL-C12, gave fairly large particles both in the presence and in the absence of divalent cations (Figure 2D). The addition of very high concentrations (e.g., 30 mM) of Ca^{2+} to A8−35 solutions resulted in the development of visible turbidity, at a rate strongly dependent on the concentrations of APol and Ca^{2+} concentrations. On the other hand, even in these conditions, no aggregation was observed with SAPol (see Figure S2 in Supporting Information for further details).

Functional Consequences of the Complexation of Detergent-Solubilized Ca^{2+}-ATPase by APols. The complexation of detergent-solubilized SERCA1a by A8−35 has been shown to decrease ATPase activity, while inhibition is reversed by excess detergent (*13*). This observation was confirmed here with a new batch of A8−35 (Figure 3A). Similar observations were made with PMAL-C12 (Figure 3B), SAPol (not shown), and PMAL*A*-C12 (not shown). Comparison of the effects of the four APols on ATPase activity showed that higher doses of SAPol are required for similar levels of inhibition (Figure 3C).

Following its dilution in a detergent-free solution, the solubilized Ca^{2+}-ATPase is immediately and irreversibly inactivated in the absence of APols (e.g., ref *24*; see top right trace in Figure 4A). We previously showed that adding APol A8−35 to the protein/detergent solution before dilution protects the enzyme against this inactivation (*13*). This protective effect was confirmed here with the new batch of

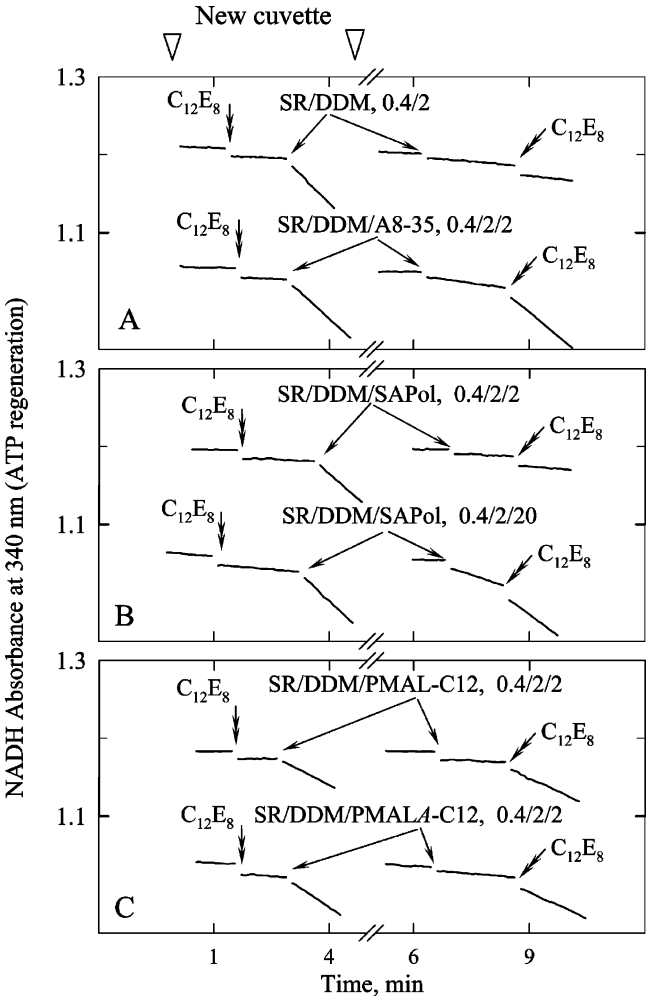


FIGURE 4: Protection conferred by various amphipols against irreversible inactivation of detergent-solubilized ATPase upon dilution into a detergent-free medium. Solubilized SR membranes (0.4 mg/mL SR protein and 2 mg/mL dodecyl maltoside) were incubated in the presence or absence of various APols in a medium containing 50 mM KCl, 25 mM TES−Tris, 0.5 mM Mg^{2+}, and 0.05 mM total Ca^{2+} at pH 7.5 and 23 °C. After 15−60 min, 10 μL aliquots were diluted (single arrows) in 2 mL of ATPase activity assay medium, either after (left) or before (right) addition of 1 mg/mL $C_{12}E_8$ (double arrows) to the assay medium. NADH oxidation was followed as a reporter of ATPase activity. Panel A: top traces, no polymer; bottom traces, preincubation with A8−35 (batch FGH15) at 2 mg/mL. Panel B: Preincubation with either 2 (top) or 20 mg/mL (bottom) SAPol. Panel C: Preincubation with 2 mg/mL of either PMAL-C12 (top) or PMALA-C12 (bottom).

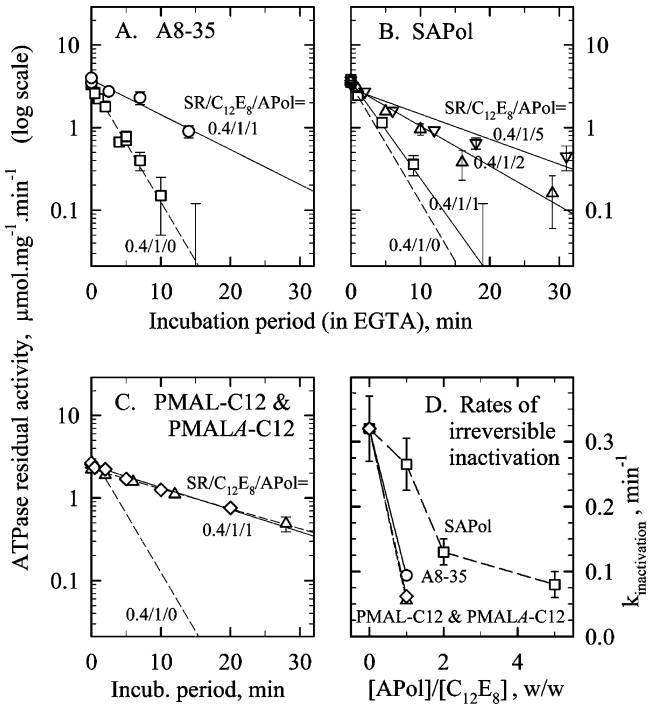


FIGURE 5: Protection conferred by various amphipols against irreversible inactivation of detergent-solubilized ATPase upon calcium depletion. Panels A−C: Detergent-solubilized SR membranes (0.4 mg/mL SR protein, 1 mg/mL $C_{12}E_8$) were incubated in the presence or absence of various APols in a medium containing 100 mM KCl, 50 mM MOPS−Tris, 0.02 mM total Ca^{2+}, and 1 mM EGTA (hence with a very low free Ca^{2+}concentration), at pH 7.0 and 23 °C. After various periods, 20 μL aliquots were diluted in 2 mL of ATPase activity assay medium, supplemented with 1 mg/mL $C_{12}E_8$, and ATPase activity was measured. Panel A: No polymer (□) or A8−35 at 1 mg/mL in the incubation medium (○). Panel B: SAPol at 1 (□), 2 (△), or 5 mg/mL (▽). Panel C: 1 mg/mL PMAL-C12 (△) or PMALA-C12 (◇). In panels B and C, the dotted line represents the control curve in the absence of polymer, reproduced from panel A. In all cases, control incubation in EGTA-free medium (i.e., in the presence of 20 μM Ca^{2+} alone) showed no significant inactivation over 30 min (not shown). Panel D: Rate constants for irreversible inactivation as a function of APol concentration (○, A8−35; □, SAPol; △, PMAL-C12; ◇, PMALA-C12).

A8−35 (bottom right trace in Figure 4A), as well as with the other three APols (Figure 4B,C, right traces); as for inhibition, higher concentrations of SAPol than of the other APols were required to achieve the same degree of protection (Figure 4B, right traces).

A8−35 has also been shown to protect against irreversible inactivation under different conditions: incubation of detergent-solubilized ATPase in detergent-containing but calcium-free solutions (*13*). This effect also was reproduced here with all four APols tested (Figure 5); again, a higher concentration of SAPol (Figure 5B) than of the other APols (Figure 5D) was required for maximal protection.

It would be conceivable to try to identify the step in the ATPase catalytic cycle that is the main target of the inhibitory effect of APols [as attempted in studies of the modulatory effects of detergents (*25*), for example]. However, this would not necessarily lead to identifying the molecular mechanism responsible for the effect. Instead, we explored possible reasons for the apparent correlation between inhibition and protection by measuring the effects of APols on an a priori unrelated step: calcium dissociation from the transmembrane Ca^{2+}-binding sites of nonphosphorylated ATPase toward the cytosolic side of the membrane ($Ca_2E1 \rightarrow E2$, top left of Figure 6). This step is clearly not rate-limiting for overall ATPase activity, as its direction in the enzyme cycle (top left in Figure 6) is opposite to that of Ca^{2+}-dependent ATP hydrolysis.

The rate of calcium dissociation from unphosphorylated ATPase was measured by mixing in a stopped-flow instrument (see, e.g., ref *21*) preformed ATPase/Ca^{2+} complexes with an excess of quin2, a chelator that binds Ca^{2+} (released from the ATPase) with an immediate change in fluorescence (*26*−*28*). In control experiments with SR membranes (top trace in Figure S3 in Supporting Information), Ca^{2+} rapidly dissociated from its binding sites, as expected, with a k_{obs} of about 16 s^{-1}. The rate constant of the quin2 fluorescence

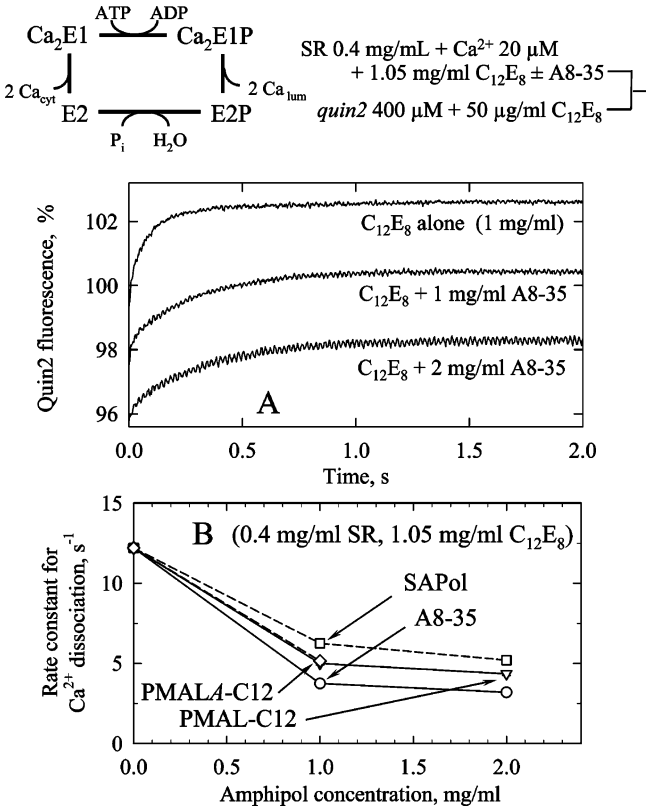


FIGURE 6: Amphipols slow Ca^{2+} dissociation from detergent-solubilized Ca^{2+}-ATPase. Top: Catalytic cycle of Ca^{2+}-ATPase and experimental protocol. Panel A: Stopped-flow traces. For the control experiment (top trace), $C_{12}E_8$-solubilized SR membranes (0.4 mg of protein/mL plus 1 mg/mL $C_{12}E_8$ in the ATPase syringe), equilibrated with 20 μM Ca^{2+}, were mixed volume for volume with quin2 (at a concentration of 400 μM in the quin2 syringe), and quin2 fluorescence was monitored with an excitation wavelength of 312 nm and a green filter (MTO DA531) in the emission pathway. For both syringes, the medium otherwise contained 100 mM KCl, 50 mM MOPS−Tris, and 50 μg/mL $C_{12}E_8$ (i.e., close to the CMC) at pH 7 and 20 °C. For experiments in the presence of APols (middle and bottom traces), SR in the ATPase syringe was first solubilized with 1 mg/mL $C_{12}E_8$ before APol (here, A8−35) was added at concentrations of either 1 or 2 mg/mL; the quin2 syringe contained 0.05 mg/mL $C_{12}E_8$. Quin2 fluorescence, reflecting the kinetics of dissociation of Ca^{2+} from the ATPase, is expressed as a percentage of its value at the end of the mixing time. For clarity, the two bottom traces have been shifted vertically by about −2% and −4%, respectively. Panel B: Summary of the rate constants found in parallel experiments with the four APols.

signal did not change significantly ($k_{obs} \approx 14\ s^{-1}$) after the addition of detergent in sufficient amounts (2.5 g/g) to solubilize SR membranes (*29*), but the amplitude of the signal was halved (see bottom trace in Figure S3 in Supporting Information). This suggests that solubilization speeds up the dissociation of the first Ca^{2+} ion (bound at site II), which now occurs within the dead time of the stopped-flow instrument, while having no significant effect on the rate of dissociation of the second Ca^{2+} ion (bound at site I). Under these conditions, the addition of A8−35 to $C_{12}E_8$-solubilized ATPase did not affect the overall amplitude of the change in quin2 fluorescence but decreased its rate by a factor of up to 4 (Figure 6A). This effect was also observed with the other three APols (Figure 6B). SAPol again was the least efficient of the four APols at slowing down backward Ca^{2+} dissociation (Figure 6B), just as it was the least efficient at slowing down forward ATP hydrolysis (Figure 3) or at protecting the ATPase from irreversible inhibition (Figures 4 and 5); yet, ionic conditions and protein/APol/detergent ratios are fairly different in these four experiments.

DISCUSSION

Earlier work on complexes of A8−35 with Ca^{2+}-ATPase (*13*) identified a couple of difficulties that had not been encountered with other membrane proteins. We tried here to establish the origin of such difficulties and to determine whether they are intrinsic to the use of APols or can be circumvented. We also further explored whether the fact that APols both stabilize and reversibly inhibit the ATPase is coincidental or is underlain by a common molecular mechanism.

Divalent Cations Induce the Aggregation of Polyacrylate-Based Amphipols: A Caveat, plus Solutions. One problem encountered earlier (*13*) was the strong tendency of A8−35 to form aggregates under certain conditions. In the present study, size exclusion chromatography and turbidity measurements showed that Ca^{2+} (and, to a much lesser extent, Mg^{2+}) induces A8−35 aggregation. A broadening of the HPLC profile is already detectable in the presence of 0.5 mM Ca^{2+}, and massive time-dependent aggregation occurs at [Ca^{2+}] higher than a few millimolar. Thus, in conjunction with the fact that the batches of A8−35 used for our earlier experiments did not yield as monodisperse particles in divalent cation-free buffers as the new batches used in the present work, the presence of Ca^{2+} and Mg^{2+} appears to have been primarily responsible for the polydispersity of Ca^{2+}-ATPase/APol particles observed previously (*13*). Possible physical−chemical mechanisms accounting for this effect are briefly discussed in Supporting Information (see also refs *30*−*33*).

We investigated the sensitivity to calcium ions of three other APols with different chemical structures, as potential alternatives to A8−35. PMAL-C12, a zwitterionic APol at neutral pH, was found to aggregate even more strongly than A8−35 in the presence of calcium. On the contrary, a sulfonated version of A8−35 (SAPol) displayed very low, if any, sensitivity to calcium. Thus, the problem of calcium-induced aggregation can be circumvented by using different APols. Note, however, that most membrane proteins can be handled in the absence of calcium. Our size exclusion chromatography data also indicate that the combination of a millimolar concentration of Mg^{2+} and a micromolar concentration of Ca^{2+} does not result in the formation of large A8−35 aggregates. Thus, A8−35 can probably be used for most experiments with membrane proteins requiring only a cytosol-like medium.

Reversible Inhibition of ATPase Activity and Protection against Denaturation: A Case of "Gulliver and the Lilliputians"? All four APols tested inhibited the catalytic activity of detergent-solubilized Ca^{2+}-ATPase. As ternary complexes are formed in membrane protein solutions containing both APols and detergent (above or below the CMC) (*13*, *34*−*35*), this inhibitory effect is not particularly surprising in itself. However, more remarkably, the various APols tested proved effective for protecting the ATPase from irreversible inhibition with similar relative efficiencies: PMAL-C12 and PMAL*A*-C12 had effects similar to those of A8−35, whereas higher concentrations of SAPol were required for equivalent levels of inhibition or protection. Recent experiments have

led to the suggestion that part of the mechanism by which A8−35 supports the renaturation of membrane proteins is the creation of repulsive interactions between complexes: these would protect the refolding proteins from interacting one with another, thereby preventing the formation of unproductive aggregates (*53*). Such an isolating mechanism could well contribute to protecting the Ca^{2+}-ATPase against irreversible denaturation. However, it does not explain the observed correlation with inhibition. A previous survey of many different detergents found no obvious correlation between their effect on steady-state ATPase activities and their effect on the rate of irreversible denaturation (*24*). Thus, the observation that SAPol was simultaneously the least inhibitory and the least protective of the four APols tested, if not a simple coincidence, suggests that there may be a common mechanism underlying inhibition and protection by APols.

Independently, we found that all four APols slowed the dissociation from solubilized ATPase of at least one Ca^{2+} ion (such dissociation runs in the opposite direction to normal ATPase turnover), SAPol again being the least efficient of the molecules tested (Figure 6). As control activity measurements revealed no evidence for any increase of the apparent affinity of the solubilized ATPase for Ca^{2+} in the presence of A8−35 (data not shown), the slowing of the rate of Ca^{2+} dissociation may well reflect a true kinetic effect, i.e., an increase in the activation energy for Ca^{2+} escape from its binding site (destabilization of the "transition state" rather than a shift in the equilibrium between initial and final states). In known SERCA1a structures, the high-affinity binding site for Ca^{2+} ("site I") is deeply embedded within the bundle formed by the protein transmembrane segments, with no open pathway leading to the cytosol (*36*). Ca^{2+} dissociation, which is accompanied by considerable conformational rearrangements of the transmembrane region (*37*−*39*), must therefore involve some sort of "breathing" of the protein, providing a transient escape route from the inside of the bundle to the aqueous phase. Thus, the results of our Ca^{2+} dissociation experiments may indicate that, in the presence of detergent, this process of breathing is slowed by APols.

At the molecular level, a possible mechanism for this effect could be offered by what we previously dubbed the "Gulliver effect" (*7*), so named by analogy to Swift's character tied to the ground by the tiny strings of the Lilliputians (*40*). Although most individual points of contact between APol alkyl chains and the hydrophobic surface of the protein are likely to be transient on the millisecond time scale of enzymatic reactions (for a discussion, see ref *7*), the large number and close spatial proximity (within nanometers) of these contacts may damp collective movements. Specifically, multipoint attachment of APol molecules to the ATPase may reduce the frequency and/or amplitude of movements tending to open up the ATPase transmembrane bundle: APols thereby may slow Ca^{2+} dissociation from site I. This would not necessarily interfere in a detectable manner with Ca^{2+} dissociation from site II, as the latter mainly depends on reorientation of the side chain of the more peripheral residue E309 (a reorientation which may be specifically accelerated by the detergent-dependent increase in *local* microviscosity): Gulliver can still move his head or feet, but large-scale movements are hampered (more on this in Supporting Information).

We cannot exclude other mechanisms for the slowing of Ca^{2+} dissociation by APols. For example, APols might also interfere with the large conformational changes in the *cytosolic* domains of the ATPase, which are crucial for long-range coupling to movements of the transmembrane segments. However, the Gulliver mechanism, suggesting that APol-dependent restriction of the movements of the ATPase transmembrane section accounts for the reduced rate of Ca^{2+} dissociation, has the advantage of simultaneously providing a tentative explanation for ATPase protection, by the same APols, against irreversible denaturation. Indeed, it has been suggested that the irreversible denaturation of detergent-solubilized monomeric ATPase exposed to high temperatures is dominated by the behavior of the transmembrane segments, with the Ca^{2+}-binding domain being unfolded first (*41*). The fact that ATPase inactivation in the presence of detergent is faster in the absence of Ca^{2+} than in its presence may also be attributed in part to "cross-linking" of the transmembrane segments by the Ca^{2+} ions (bound to the negatively charged ligand residues on M4, M5, M6, and M8; see ref *36*): denaturation of the ATPase presumably starts with a limited, transient, reversible unfolding of its transmembrane region, which would become irreversible upon further unfolding and/or formation of intermolecular contacts. By damping the dynamics of large-amplitude transmembrane movements, APols may thus also slow denaturation.

As breathing of the transmembrane segments is also required for the forward cycle of ATP hydrolysis, the Gulliver mechanism may also provide a tentative molecular mechanism for the fact that ATPase activity is reversibly inhibited by APols. But, interestingly, it also provides a simple tentative explanation for the fact that slowing down by APols can be observed for one membrane protein (e.g., the Ca^{2+}-ATPase), but not for others [e.g., the nicotinic acetylcholine receptor (*11*) or bacteriorhodopsin (*53*)], since it ties inhibition to the amplitude of the rearrangements of the transmembrane surface that occur during the functional cycle. A related hypothesis has been put forward concerning the mechanism of inhibition of ion channels by general anesthetics: the most sensitive channels would be those whose function entails the largest transient changes in transmembrane cross-sectional area (*42*). Indeed, another member of the P-type ion-transporting pump family to which Ca^{2+}-ATPase belongs, Na^+,K^+-ATPase, has been shown to present unusually large activation volumes for the forward and backward transitions corresponding to occlusion and deocclusion of potassium ions (*43*). The activity of the Ca^{2+}-ATPase itself is known to depend on hydrostatic pressure (e.g., ref *44*), again suggesting fairly large activation volumes during ATP hydrolysis. In keeping with these observations, X-ray studies have revealed that transitions between functional states during the enzymatic cycle involve extensive changes of the Ca^{2+}-ATPase/membrane interface (*37*−*39*, *45*−*48*). Such does not seem to be the case for membrane proteins whose transconformations are not inhibited by A8−35, such as the nicotinic acetylcholine receptor or bacteriorhodopsin (*49*−*52*). The inhibition of Ca^{2+}-ATPase by APols may thus be related to the large amplitude of its transmembrane conformational transitions.

CONCLUSION

This work represents a first step toward determining which of the physical and biochemical properties of membrane protein/APol complexes are general and which depend on the specific chemical structure of the APol or on the membrane protein studied. Our observations confirm that tailoring the chemical structure of APols can be used to overcome some of the problems encountered with first-generation APols, such as calcium-dependent aggregation. They also suggest that APols may help to stabilize membrane proteins by slowing down movements of the polypeptide chain that involve substantial rearrangements of the transmembrane surface, such as transmembrane helix bundle breathing in an ion-transporting pump. The APol layer surrounding the transmembrane region of the protein may therefore act not only as a "diving suit" (*5*) but also as a "strait jacket". Thus, for membrane proteins such as the Ca^{2+}-ATPase, the catalytic activity of which requires large-amplitude transmembrane rearrangements (*42*), functional (and reversible) inhibition by APols might be the price to be paid for biochemical stabilization. This hypothesis, although speculative, provides a framework for the planning or interpretation of functional or structural investigations of other membrane proteins using APols. The dynamics of APol-trapped membrane proteins and relationships to functional effects form a fascinating subject for further studies, which may prove informative for both polymer adsorption studies and membrane protein biophysics.

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SUPPORTING INFORMATION AVAILABLE

Previously documented effects of APols on membrane protein function, previously documented properties of A8−35 in solution, Figure S1 showing that the presence or absence of millimolar divalent cations has a critical effect on the aggregation state of amphipol A8−35 in the absence of detergent, Figure S2 showing the effect of calcium on the turbidity of A8−35 and SAPol solutions, possible physical−chemical mechanisms for the aggregating effect of divalent cations, Figure S3 showing Ca^{2+} dissociation from detergent-solubilized Ca^{2+}-ATPase, as measured with a quin2 stopped-flow assay, and possible implications for the "flickering gate" mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. le Maire, M., Champeil, P., and Møller, J. V. (2000) Interaction of membrane proteins and lipids with solubilizing detergents, *Biochim. Biophys. Acta 1508*, 86−111.
2. Garavito, R. M., and Ferguson-Miller, S. (2001) Detergents as tools in membrane biochemistry, *J. Biol. Chem. 276*, 32403−32406.
3. Bowie, J. U. (2001) Stabilizing membrane proteins, *Curr. Opin. Struct. Biol. 11*, 397−402.
4. Gohon, Y., and Popot, J.-L. (2003) Membrane protein-surfactants complexes, *Curr. Opin. Colloid Interface Sci. 8*, 15−22.
5. Sanders, C. R., Hoffmann, A. K., Gray, D. N., Keyes, M. H., and Ellis, C. D. (2004) French swimwear for membrane proteins, *ChemBioChem 5*, 423−426.
6. Tribet, C., Audebert, R., and Popot, J.-L. (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions, *Proc. Natl. Acad. Sci. U.S.A. 93*, 15047−15050.
7. Popot, J.-L., Berry, E. A., Charvolin, D., Creuzenet, C., Ebel, C., Engelman, D. M., Flötenmeyer, M., Giusti, F., Gohon, Y., Hong, Q., Lakey, J. H., Leonard, K., Shuman, H. A., Timmins, P., Warschawski, D. E., Zito, F., Zoonens, M., Pucci, B., and Tribet, C. (2003) Amphipols: polymeric surfactants for membrane biology research, *Cell. Mol. Life Sci. 60*, 1559−1574.
8. Zoonens, M., Catoire, L. J., Giusti, F., and Popot, J.-L. (2005) NMR study of a membrane protein in detergent-free aqueous solution, *Proc. Natl. Acad. Sci. U.S.A. 102*, 8893−8898.
9. Nagy, J. K., Kuhn Hoffmann, A., Keyes, M. H., Gray, D. N., Oxenoid, K., and Sanders, C. R. (2001) Use of amphipathic polymers to deliver a membrane protein to lipid bilayers, *FEBS Lett. 501*, 115−120.
10. Prata, C., Giusti, F., Gohon, Y., Pucci, B., Popot, J.-L., and Tribet, C. (2001) Nonionic amphiphilic polymers derived from tris-(hydroxymethyl)-acrylamidomethane keep membrane proteins soluble and native in the absence of detergent, *Biopolymers 56*, 77−84.
11. Martinez, K. L., Gohon, Y., Corringer, P.-J., Tribet, C., Mérola, F., Changeux, J.-P., and Popot, J.-L. (2002) Allosteric transitions of *Torpedo* acetylcholine receptor in lipids, detergent and amphipols: molecular interactions vs physical constraints, *FEBS Lett. 528*, 251−256.
12. Gorzelle, B. M., Hoffman, A. K., Keyes, M. H., Gray, D. N., Ray, D. G., and Sanders, C. R. (2002) Amphipols can support the activity of a membrane enzyme, *J. Am. Chem. Soc. 124*, 11594−11595.
13. Champeil, P., Menguy, T., Tribet, C., Popot, J.-L., and le Maire, M. (2000) Interaction of amphipols with sarcoplasmic reticulum Ca^{2+}-ATPase, *J. Biol. Chem. 275*, 18623−18637.
14. Gohon, Y. (2002) Etude structurale et fonctionnelle de deux protéines membranaires, la bactériorhodopsine et le récepteur nicotinique de l'acétylcholine, maintenues en solution aqueuse non détergente par des polymères amphiphiles, Thèse de Doctorat d'Université, Université Paris-6.
15. Zoonens, M. (2004) Caractérisation des complexes formés entre le domaine transmembranaire de la protéine OmpA et des polymères amphiphiles, les amphipols. Application à l'étude structurale des protéines membranaires par RMN haute résolution, Thèse de Doctorat d'Université, Université Paris-6.
16. Gohon, Y., Pavlov, G., Timmins, P., Tribet, C., Popot, J.-L., and Ebel, C. (2004) Partial specific volume and solvent interactions of amphipol A8−35, *Anal. Biochem. 334*, 318−334.
17. Gohon, Y., Giusti, F., Prata, C., Charvolin, D., Timmins, P., Ebel, C., Tribet, C., and Popot, J.-L. (2006) Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8−35, *Langmuir 22*, 1281−1290.
18. Champeil, P., Guillain, F., Vénien, C., and Gingold, M. P. (1985) Interaction of magnesium and inorganic phosphate with calcium-deprived sarcoplasmic reticulum adenosinetriphosphatase as reflected by organic solvent-induced perturbation, *Biochemistry 24*, 69−81.
19. le Maire, M., Ghazi, A., Møller, J. V., and Aggerbeck, L. P. (1987) The use of gel chromatography for the determination of sizes and relative molecular masses of proteins. Interpretation of calibration curves in terms of gel-pore-size distribution, *Biochem. J. 243*, 399−404.
20. Møller, J. V., Lind, K. E., and Andersen, J. P. (1980) Enzyme kinetics and substrate stabilization of detergent-solubilized and membraneous (Ca^{2+}+Mg^{2+})-activated ATPase from sarcoplasmic reticulum. Effect of protein−protein interactions, *J. Biol. Chem. 255*, 1912−1920.
21. Champeil, P., Henao, F., and de Foresta, B. (1997) Dissociation of Ca^{2+} from sarcoplasmic reticulum Ca^{2+}-ATPase and changes in fluorescence of optically selected Trp residues. Effects of KCl and NaCl and implications for substeps in Ca^{2+} dissociation, *Biochemistry 36*, 12383−12393.
22. Andersen, J. P., Møller, J. V., and Jørgensen, P. L. (1982) The functional unit of sarcoplasmic reticulum Ca^{2+}-ATPase. Active site titration and fluorescence measurements, *J. Biol. Chem. 257*, 8300−8307.
23. McIntosh, D. B., and Ross, D. C. (1985) Role of phospholipid and protein−protein associations in activation and stabilization

of soluble Ca^{2+}-ATPase of sarcoplasmic reticulum, *Biochemistry 24*, 1244−1251.
24. Lund, S., Orlowski, S., de Foresta, B., Champeil, P., le Maire, M., and Møller, J. V. (1989) Detergent structure and associated lipid as determinants in the stabilization of solubilized Ca^{2+}-ATPase from sarcoplasmic reticulum, *J. Biol. Chem. 264*, 4907−4915.
25. Champeil, P., le Maire, M., Andersen, J. P., Guillain, F., Gingold, M., Lund, S., and Møller, J. V. (1986) Kinetic characterization of the normal and detergent-perturbed reaction cycles of the sarcoplasmic reticulum calcium pump. Rate-limiting step(s) under different conditions, *J. Biol. Chem. 261*, 16372−16384.
26. Bayley, P., Ahlström, P., Martin, S. R., and Forsen, S. (1984) The kinetics of calcium binding to calmodulin: Quin 2 and ANS stopped-flow fluorescence studies, *Biochem. Biophys. Res. Commun. 120*, 185−181.
27. Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S., and Claret, M. (1989) Fast kinetics of calcium release induced by myo-inositol trisphosphate in permeabilized rat hepatocytes, *J. Biol. Chem. 264*, 17665−17673.
28. Wakabayashi, S., and Shigekawa, M. (1990) Mechanism for activation of the 4-nitrobenzo-2-oxa-1,3-diazole-labeled sarcoplasmic reticulum ATPase by Ca^{2+} and its modulation by nucleotides, *Biochemistry 29*, 7309−7318.
29. de Foresta, B., le Maire, M., Orlowski, S., Champeil, P., Lund, S., Møller, J. V., Michelangeli, F., and Lee, A. G. (1989) Membrane solubilization by detergent: use of brominated phospholipids to evaluate the detergent-induced changes in Ca^{2+}-ATPase/lipid interaction, *Biochemistry 28*, 2558−2567.
30. Magny, B., Iliopoulos, I., and Audebert, R. (1994) Aggregation of hydrophobically modified polyelectrolytes in dilute solution: ionic strength effects, in *Macromolecular Complexes in Chemistry and Biology* (Dubin, Bock, Davis, Schultz, and Thies, Eds.) pp 51−62, Springer-Verlag, Berlin and Heidelberg.
31. Wang, T. K., Iliopoulos, I., and Audebert, R. (1991) Aqueous solution behavior of hydrophobically modified poly(acrylic acid), in *Water-Soluble Polymers: synthesis, solution properties, and applications* (Shalaby, S. W., McCormick, C. L., and Butler, G. B., Eds.) pp 218−231, ACS Symposium Series, Washington, DC.
32. Magny, B., Iliopoulos, I., Zana, R., and Audebert, R. (1994) Mixed micelles formed by cationic surfactants and anionic hydrophobically modified polyelectrolytes, *Langmuir 10*, 3180−3187.
33. Petit, F., Audebert, R., and Iliopoulos, I. (1995) Interactions of hydrophobically modified poly(sodium acrylate) with globular proteins, *Colloid Polym. Sci. 273*, 777−781.
34. Tribet, C., Audebert, R., and Popot, J.-L. (1997) Stabilization of hydrophobic colloidal dispersions in water with amphiphilic polymers: application to integral membrane proteins, *Langmuir 13*, 5570−5576.
35. Nowaczyk, M., Oworah-Nkruma, R., Zoonens, M., Rögner, M., and Popot, J.-L. (2004) Amphipols: strategies for an improved PS2 environment in aqueous solution, in *Biohydrogen III* (Miyake, J., Ed.) pp 151−159, Elsevier, Dordrecht, The Netherlands.
36. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution, *Nature 405*, 633−634.
37. Toyoshima, C., and Nomura, H. (2002) Structural changes in the calcium pump accompanying the dissociation of calcium, *Nature 418*, 605−611.
38. Toyoshima, C., and Inesi, G. (2004) Structural basis of ion pumping by Ca^{2+}-ATPase of the sarcoplasmic reticulum, *Annu. Rev. Biochem. 73*, 269−292.
39. Obara, K., Miyashita, N., Xu, C., Toyoshima, I., Sugita, Y., Inesi, G., and Toyoshima, C. (2005) Structural role of countertransport revealed in Ca^{2+} pump crystal structure in the absence of Ca^{2+}, *Proc. Natl. Acad. Sci. U.S.A. 102*, 14489−14496.
40. Swift, J. (1726) *A Voyage to Lilliput*, Chapter 1.
41. Merino, J. M., Møller, J. V., and Gutiérrez-Merino, C. (1994) Thermal unfolding of monomeric Ca^{2+},Mg^{2+}-ATPase from sarcoplasmic reticulum of rabbit skeletal muscle, *FEBS Lett. 343*, 155−159.
42. Cantor, R. S. (1997) The lateral pressure profile in membranes: a physical mechanism of general anesthesia, *Biochemistry 36*, 2339−2344.
43. Bugnon, P., Doludda, M., Grell, E., and Merlbach, A. E. (1997) High-pressure stopped-flow spectrometer for kinetic studies of fast bioorganic reactions by absorbance and fluorecence detection, in *High-Pressure Research in the Biosciences and Biotechnology* (Heremans, K., Ed.) pp 143−146, Leuven University Press, Belgium.
44. Hasselbach, W. (1988) Pressure effects on the interactions of the sarcoplasmic reticulum calcium transport enzyme with calcium and dinitrophenyl phosphate, *Z. Naturforsch. C 43*, 929−937.
45. Toyoshima, C., and Mizutani, T. (2004) Crystal structure of the calcium pump with a bound ATP analogue, *Nature 430*, 529−535.
46. Toyoshima, C., Nomura, H., and Tsuda, T. (2004) Lumenal gating mechanism revealed in calcium pump crystal structures with phosphate analogues, *Nature 442*, 361−368.
47. Sørensen, T. L.-M., Møller, J. V., and Nissen, P. (2004) Phosphoryl transfer and calcium ion occlusion in the calcium pump, *Science 304*, 1672−1675.
48. Olesen, C., Sørensen, T. L.-M., Nielsen, R. C., Møller, J. V., and Nissen, P. (2005) Dephosphorylation of the calcium pump coupled to counterion occlusion, *Science 306*, 2251−2255.
49. Unwin, N. (1995) Acetylcholine receptor channel imaged in the open state, *Nature 373*, 37−43.
50. Unwin, N. (2003) Structure and action of the nicotinic acetylcholine receptor explored by electron microscopy, *FEBS Lett. 555*, 91−95.
51. Miyazawa, A., Fujiyoshi, Y., and Unwin, N. (2003) Structure and gating mechanism of the acetylcholine receptor pore, *Nature 423*, 949−955.
52. Neutze, R., Pebay-Peyroula, E., Edman, K., Royant, A., Navarro, J., and Landau, E. M. (2002) Bacteriorhodopsin: a high-resolution structural view of vectorial proton transport, *Biochim. Biophys. Acta 1565*, 144−167.
53. Pocanschi, C. L., Dahmane, T., Gohon, Y., Apell, H.-J., Kleinschmidt, J. H., and Popot, J.-L. (2006) Amphipathic polymers: tools to fold integral membrane proteins to their active form (submitted for publication).

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