

Use of amphipathic polymers to deliver a membrane protein to lipid bilayers

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Abstract Data are presented which suggest that a class of amphiphilic polymers known as ‘amphipols’ may serve as a vehicle for delivering complex integral membrane proteins into membranes. The integral membrane protein diacylglycerol kinase (DAGK) was maintained in soluble form by either of two different amphipols. Small aliquots of these solutions were added to pre-formed lipid vesicles and the appearance of DAGK catalytic activity was monitored as an indicator of the progress of productive protein insertion into the bilayers. For one of the two amphipols tested, DAGK was observed to productively transfer from its amphipol complex into vesicles with moderate efficiency. Results were not completely clear for the other amphipol. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Amphipol; Polymer; Membrane bilayer; Vesicle; Diacylglycerol kinase; Membrane protein

1. Introduction

Integral membrane proteins (IMPs) have traditionally been solubilized in aqueous solutions using detergent micelles (see review in [1]). This is not advantageous in all situations and for this reason Tribet, Audebert, and Popot introduced a new subclass of amphipathic polymers dubbed ‘amphipols’ which offer certain advantages as a vehicle for solubilizing membrane proteins [2,3]. Amphipols are distinct from other classes of amphipathic polymers [4–6] in that they are relatively low molecular weight linear polymers having alternating polar and non-polar side chains, which confer amphipathicity to the main chain. One attractive feature of amphipols is their ability to maintain the solubility of IMPs at very low bulk concentrations [2,3,7,8]. This is unlike classical detergents, where membrane protein solubility is maintained only as long as the detergent concentration exceeds the critical micelle concentration. In this Letter, we demonstrate that some amphipols can be used as a vehicle to facilitate the spontaneous insertion and folding of an IMP into pre-formed lipid vesicles. The membrane protein utilized for these studies is *Escherichia coli* diacylglycerol kinase (DAGK), a homotrimeric protein of 13 kDa subunits, each subunit having three transmembrane helices (see [9] and references therein).

2. Materials and methods

2.1. Purification of DAGK in amphipols

The amphipols employed in this work are illustrated in Fig. 1. OAPA-20 was prepared by refluxing polyacrylic acid (Aldrich, Milwaukee, WI, USA; average molecular weight: 5000 Da; polydispersity: 2.4) with octylamine (25 mol% relative to the free carboxylic acids of the polymer) in excess toluene for 36 h under conditions of azeotropic removal of the water produced in the condensation reaction between the amine and the carboxylic acid moieties. A Dean–Starke trap was used for this latter purpose. Following reaction, the toluene was removed by evaporation and the residue was suspended in water, neutralized with NaOH (resulting in polymer solubilization), dialyzed against water to remove unreacted amine, and then freeze-dried. The percent derivatization of OAPA-20 was determined to be $20 \pm 5\%$ by ^1H and ^{13}C NMR at 300 and 600 MHz (^1H frequency) field strengths. It is assumed that OAPA-20 prepared as described above is randomly amidated along the full length of the parent polyacrylic acid, but this was not directly evaluated.

PMAL-B (see Fig. 1) was prepared by a proprietary procedure, but is available from Anatrace, Inc. (Maumee, OH, USA) as catalog number P5002. The degree of amidation was determined to be 25% (on the average one amide for every two repeating units or a ratio of three free carboxyls for every ammonium amide) by ^{13}C NMR. The average molecular weight of this polymer was 10 000 Da based on the molecular weight of the precursor polymer (which had a polydispersity of 2) and the measured degree of derivatization.

N-terminal polyhistidine-tagged *E. coli* DAGK was overexpressed using a strain supplied by the lab of James Bowie of UCLA [10] and methods described elsewhere [11]. A cysteine-free DAGK mutant was employed in which the two native cysteines at positions 46 and 113 were replaced by alanine. This mutant is similar to wild-type in that it is fully active and stable, but has no cysteine residues which could form disulfide bonds and complicate these studies. Lysed cells were extracted by adding the detergent Empigen (Calbiochem, San Diego, CA, USA) to a final concentration of 3%, followed by gentle mixing for 30 min at 4°C. Ni(II)-NTA metal ion chelate resin (Qiagen, Chatsworth, CA, USA) was then added followed by incubation for 1 h at 4°C. Resin was transferred to a column and non-polyhistidine-tagged proteins were eluted with 40 mM imidazole, 1.5% Empigen in buffer A: 50 mM N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.3 M NaCl, pH 7.8. This left pure DAGK bound to the resin and bathed in an Empigen detergent solution. The DAGK/resin was then equilibrated with eight column volumes of 0.2% amphipol (OAPA-20 or PMAL-B) to exchange out detergent for amphipol. At this point there were two amphipol populations, one in complex with the resin-associated DAGK and the other free in solution. Two different procedures were used to effect the final elution of pure DAGK from the resin. In the first procedure, DAGK was eluted using a solution containing 0.2% amphipol in 0.3 M imidazole, pH 7.8. The DAGK pool generated by this procedure is referred to as ‘0.2% amphipol’ solution.

In a second procedure, all amphipol not tightly associated with the resin-bound DAGK was removed by re-equilibrating the column with eight column volumes of buffer A without any added amphipol or detergent. DAGK with tightly associated amphipol was then eluted

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with 0.3 M imidazole, pH 7.8. Under such conditions, the enzyme elutes in high yield in soluble form. The DAGK thus produced is referred to as being in a '0% amphipol' solution, meaning that there is no free amphipol population, only DAGK-complexed polymer.

DAGK was purified into 1% decyl maltoside (DM, Anatrace Inc., Maumee, OH) micelles according to a previously described procedure [1] also based upon metal ion chelate chromatography.

DAGK concentration was determined spectrophotometrically using an extinction coefficient of $\epsilon_{280, 0.1\%} = 1.8$. DAGK activity was determined using a coupled spectrophotometric mixed micellar assay described elsewhere [12]. 1 unit of DAGK activity equals 1 μ mol of DAG converted to PA per min.

2.2. Glutaraldehyde (GA) crosslinking of DAGK in amphipol solutions

In preparation for crosslinking, DAGK in 0% amphipol solutions was diluted to 14 μ M in buffer A. Protein in 0.2% amphipol solutions was diluted to 14 μ M in 0.2% amphipol in buffer A. GA was added to 32 mM from a fresh 25% stock solution in water. Samples were vigorously agitated for 16 h and products were visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.3. Insertion of DAGK into pre-formed lipid vesicles

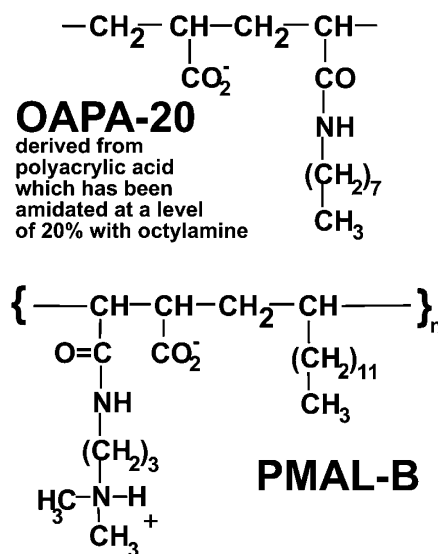
Multilamellar lipid vesicles were formed by dispersing 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC, Avanti, Alabaster, AL, USA) at a nominal concentration of 20 mM in assay buffer containing: 75 mM piperazine-*N,N'*-bis-2-ethanesulfonic acid (PIPES), 50 mM LiCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA); 0.1 mM ethylene-bis-(oxyethylenenitrilo)tetraacetic acid (EGTA), 1 mM DTT, 1 mM phosphoenolpyruvate (PEP), 0.25 mM reduced nicotinamide adenine dinucleotide (NADH), 3 mM ATP, 15 mM Mg(II), and 3 mM dibutylglycerol (DBG), pH 6.8. Unilamellar vesicles were formed by repeated extrusion through a 50 nm polycarbonate membrane. Vesicles were then diluted into the above assay mixture plus 37 units/ml PK and 52 units/ml LDH such that the nominal POPC concentration was 2 mM. To 1 ml of these vesicular mixtures were added small aliquots (2.5–5 μ l) of stock DAGK in 0% or 0.2% amphipol solutions, such that the final DAGK concentration was 40 nM while the vesicle concentration was ca. 80 nM. The appearance of DAGK activity as the enzyme inserts productively into vesicles was monitored spectrophotometrically at 340 nm through coupled NADH oxidation. Reactions were allowed to proceed until the activity was observed to plateau (typically 10 min) at 30°C. The resulting A_{340} vs. time data were smoothed using polynomial regression. Slope ($\Delta A_{340}/\Delta t$) vs. time curves were derived and converted into DAGK activity vs. time data using Axum 4.1 (MathSoft, Cambridge, MA, USA). From these latter data, final (plateau) activity and $t_{1/2}$ for maximal insertion could be measured.

3. Results

3.1. Purification of DAGK in amphipol solutions

Two different amphipols were employed in this work (Fig. 1). The first, OAPA-20, is closely related to the original amphipols developed by Tribet, Audebert, and Popot [1] and is polyanionic with a relatively short apolar side chain (octylamide) distributed among 20% of the side chain sites. The other amphipol (PMAL-B) has a longer (C_{12}) apolar side chain and is more highly ordered than OAPA-20 in the sense that all repeating units of the polymer have four backbone carbon positions, the first two of which have carbonyl-containing side chains (either two carboxyls or a carboxyl and an octylamide), followed by a site with no side chain, and finally a site with a dodecyl side chain. At neutral pH 50% of the repeating units of PMAL-B will be zwitterionic (containing both carboxylate and ammonium amide moieties), with the remainder being anionic (two carboxyls). Thus, PMAL-B has a considerable net negative charge at neutral pH, although not as dense as for OAPA-20.

DAGK was purified by metal ion chelate chromatography to yield pure protein in solutions containing either 0.2% am-



There is always 1 carboxyl and one dodecyl per unit, as shown; the other side chain is carboxyl in 50% of the units (not illustrated) and is ammoniumamide (as shown) in the other 50% of the units. The order between the free carboxyl and the amide within a unit is most likely random from unit to unit, as is the distribution of dicarboxylic units and monocarboxylate/ammoniumamide units along the polymer.

Fig. 1. Amphipols employed in this work. The average molecular weight of OAPA-20 is believed to be ca. 6.6 kDa, while that for PMAL-B is ca. 10 kDa.

phipol or in which the only amphipol present is that which is tightly associated with the enzyme ('0% amphipol'). For both amphipols under both 0% and 0.2% conditions, the yields of DAGK from equivalent batches and volumes of DAGK/Ni(II)–agarose resin were very similar, with the concentration of DAGK in each of the four eluted DAGK/amphipol pools falling with the range of 1.4 to 2.1 mg/ml. That DAGK was observed to remain highly soluble under 0% conditions points to a major difference between amphipols and traditional detergents. DAGK will not elute from detergent-exposed Ni(II)–agarose resin in solutions which do not contain detergent (unpublished), whereas amphipol-exposed DAGK will elute from the resin even after extensive washing with amphipol/detergent-free solution. This reflects the known high affinity of amphipols for membrane proteins [2,3,7] – their complexes persist even following prolonged exposure to a flow of amphipol-free solution.

3.2. Oligomeric state of DAGK in amphipol solutions

We examined whether DAGK present in the amphipol solutions retains its native homotrimeric oligomeric state. GA crosslinking results are illustrated in Fig. 2 for crosslinking of the enzyme in DM micelles or in lipid vesicles: conditions in which it is known that the homotrimer predominates [9]. The trimer band is observed to be the major crosslinked product, with some dimer and monomer also observed. In these cases, it is known that incomplete conversion to a crosslinked trimer indicates that the crosslinking reaction is not 100% efficient, rather than indicating multiple oligomeric states are present [9].

For DAGK in both 0.2% amphipol solutions, the trimer

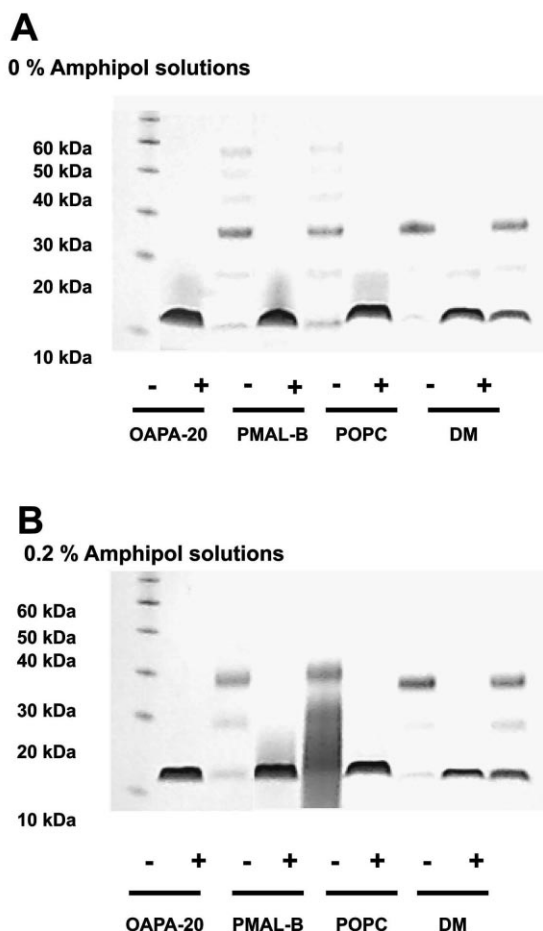


Fig. 2. Coomassie-stained SDS-PAGE gels of GA crosslinked (+) and non-crosslinked (–) DAGK in micelles, vesicles, and different amphipol solutions.

appears to be the major crosslinked product, with no higher oligomers being observed which would indicate aggregation (Fig. 2). The source of the smearing which obscures the monomer and dimer band in the cases of 0.2% PMAL-B is not known with certainty, but may reflect either some degree of GA-mediated crosslinking between the amphipol and DAGK or the steric trapping of DAGK-amphipol complexes by protein crosslinking.

SDS-PAGE of DAGK crosslinked in '0%' amphipol solutions also indicates that the trimeric oligomeric state predominates (Fig. 2). However, unlike the 0.2% amphipol cases, higher oligomers were detected, indicating that some of the DAGK present in both 0% amphipol solutions is partially aggregated. The absence of free amphipol evidently results in a moderately higher tendency for DAGK to aggregate than when 0.2% free amphipol is present. These results are consistent with the previous observation [7] that at low amphipol concentrations some IMPs exhibit a modest tendency to aggregate.

3.3. Transfer, insertion, and folding of DAGK from amphipol complexes into micelles and vesicles

The transfer of DAGK from amphipols into mixed micelles or into lipid vesicles was spectrophotometrically monitored using an assay system outlined in Fig. 3. Examples of the data which are generated by this assay are illustrated in Fig. 4. From such data it was in each case possible to determine $t_{1/2}$ for the process of DAGK insertion/assembly. By comparing the final (plateau) rate for a given set of conditions to a standard '100% active' rate for DAGK under the same final conditions it is also possible to determine the efficiency of insertion/folding. Sub-100% efficiency indicates that a certain fraction of DAGK never reaches the functional conformational state, perhaps because of misfolding to form kinetically trapped conformations.

An a priori assumption regarding interpretation of results from the above insertion assay is that dilution of DAGK/amphipol stock solutions into assay mixture which contain no micelles or vesicles will be inactive. This assumption was directly tested with results summarized in Table 1. For 0% OAPA-20, no DAGK activity was observed for a detergent/lipid-free control reaction, as expected. However, some DAGK activity was observed in detergent/lipid-free reactions initiated with 0.2% OAPA-20 or PMAL-B and for 0% PMAL-B stock DAGK solutions (Table 1). For 0.2% OAPA-20, the 'efficiency' was low (ca. 3%) and the $t_{1/2}$ was long (> 600 s). The long lag time of this reaction may suggest the assembly of DAGK or DAGK/amphipol aggregates which have some activity as opposed to monodisperse DAGK/amphipol complexes in which DAGK is partially active. In contrast, in the case of PMAL-B stocks the enzyme activity was observed to plateau rapidly ($t_{1/2} < 20$ s) with the

Table 1
Efficiencies and $t_{1/2}$ for transfer, insertion, and folding of DAGK from amphipol stock solutions to mixed micelles or pre-formed lipid vesicles

Initial state	Final state	$t_{1/2}$ (s)	Efficiency (%)
0% OAPA-20	no detergent or lipid ^a	no reaction	0
0.2% OAPA-20	no detergent or lipid ^a	720 ± 80	3.2 ± 1.9
0% PMAL-B	no detergent or lipid ^a	< 20	6.4 ± 3.4
0.2% PMAL-B	no detergent or lipid ^a	< 20	34 ± 8
0% OAPA-20	mixed micelles	< 10	75 ± 6
0.2% OAPA-20	mixed micelles	< 10	70 ± 10
0% PMAL-B	mixed micelles	< 10	57 ± 8
0.2% PMAL-B	mixed micelles	< 10	53 ± 5
0% OAPA-20	vesicles	130 ± 7	31 ± 4
0.2% OAPA-20	vesicles	72 ± 9	23 ± 3
0% PMAL-B	vesicles	272 ± 70	18 ± 2
0.2% PMAL-B	vesicles	11 ± 1	17 ± 4

Each reported value is the average of three trials.

^aAmphipol stocks were diluted by the usual factor into an insertion assay mix containing all usual components, but no detergent or lipid. The only amphiphiles which are present are the water soluble DBG which serves as the phosphoryl acceptor for the DAGK reaction and the amphipol from the stock aliquot of DAGK used to start the reaction.

'efficiencies' being substantial, higher (34%) for the 0.2% PMAL-B case compared to a 0% solution (6% efficiency). These remarkable observations will have to be followed up in later work, but suggest that PMAL-B itself can provide an membrane-mimetic environment capable of maintaining a significant level of DAGK activity. The fact that some DAGK activity was observed when three out of four DAGK/amphipol stock solutions were diluted into detergent/lipid-free assay mixtures indicates that caution must be exercised when interpreting results from insertion assays carried out in the presence of micelles or vesicles (see below), particularly in the case of PMAL-B.

When small aliquots of DAGK/amphipol stock solutions were added to mixed micelles, the rate of DAGK insertion into the micelles was in all cases quite rapid, with $t_{1/2}$ being shorter than the dead time following mixing (ca. 10 s). The apparent efficiency of insertion into mixed micelles varied from 50 to 75% (Table 1).

Rates and apparent efficiencies of DAGK transfer/insertion from amphipols into lipid vesicles were in all cases lower than into mixed micelles (Table 1). The apparent vesicular efficiencies were observed to be in the range of 17 to 31%, while $t_{1/2}$ ranged from 12 to 270 s. In the case of the OAPA-20 reactions, the rates and efficiencies were much higher than for the corresponding control reactions carried out in the absence of lipid and detergent, strongly supporting the notion that the vast majority of activity observed in the vesicular reactions

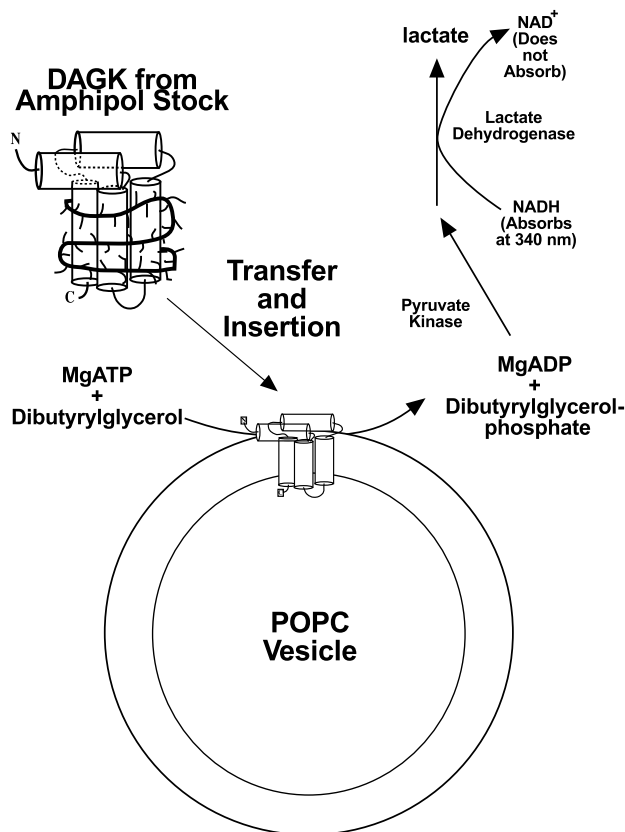


Fig. 3. Coupled spectrophotometric assay for monitoring the appearance of inserted and functional DAGK in lipid vesicles. For the sake of convenience, the assembled protein is depicted as a monomer, but is in fact a trimer [9] (PEP is not shown, in this scheme but is a necessary co-substrate for pyruvate kinase).

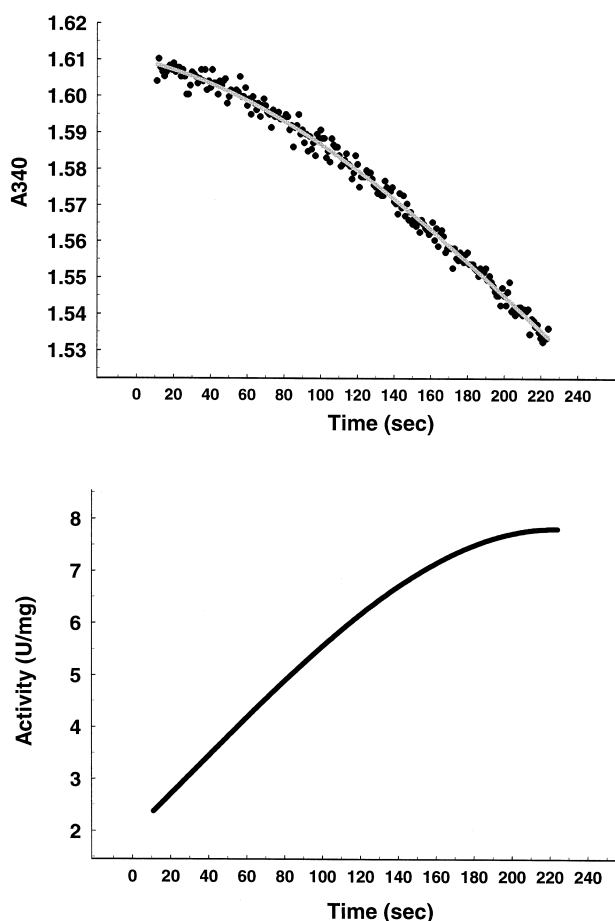


Fig. 4. Example of an A_{340} vs. time plot and the derived DAGK activity vs. time plot representing DAGK insertion into pre-formed POPC vesicles following dilution from a 0.2% OAPA-20 amphipol stock. Both the raw and smoothed (white line) data are illustrated for the A_{340} vs. time plot. This particular reaction was characterized by a $t_{1/2}$ of 66 s and reached an efficiency of ca. 24%. Fully active DAGK exhibits 32 U/mg under these particular vesicular conditions (which is different from the activity exhibited by the fully active enzyme under different assay conditions, see [9,12,14]).

initiated with OAPA-20 represent DAGK which has inserted into vesicles. This was supported by the observation that >90% of all recoverable DAGK activity was observed to pellet along with the lipid vesicles when the supernatant following low speed centrifugation of an insertion reaction mixture was subjected to high speed ultracentrifugation.

The fact that DAGK insertion from OAPA-20 solutions into mixed micelles occurred with much higher rates and efficiencies than insertion into vesicles suggests that the insertion into the lipid bilayer by DAGK represents a much more 'difficult' barrier to DAGK assembly than insertion into mixed micelles, such that the rates are lower and the potential for non-productive protein 'misfolding' or 'misinsertion' is higher for bilayers.

In the case of vesicular insertion reactions initiated with PMAL-B stock solutions, our results do not unambiguously establish the degree to which observed DAGK activity reflects enzyme which has inserted into vesicles relative to DAGK which remains PMAL-B solubilized and which is active in this form. In the 0% case, the final activity observed in the presence of vesicles was twice that observed in a model mem-

brane-free control and the $t_{1/2}$ was significantly shorter, suggesting a considerable degree of vesicular insertion. This is less clear for the 0.2% case, where $t_{1/2}$ were similar for vesicular and control reactions and where the apparent efficiency for the vesicular reaction is only 50% that observed for the detergent/lipid-free control. In support of the probability that considerable PMAL-B to vesicle transfer/insertion did indeed occur is the observation that following an insertion reaction >85% of all recoverable DAGK activity was observed to pellet along with the lipid vesicles when the supernatant from low speed centrifugation was subjected to high speed ultracentrifugation. However, this result is not completely definitive, since the possibility cannot be ruled out that active DAGK/amphipol complexes might sediment with vesicles if they were aggregated or somehow tethered to the vesicles. Based on the uncertainty regarding interpretation of the PMAL-B results, most of the remainder of this Letter will focus upon the more definitive results obtained with OAPA-20.

3.4. Attempts to solubilize lipid vesicles using amphipols

We tested the ability of the two amphipols examined in this study to solubilize lipid vesicles composed of POPC. 0.5% solutions of both PMAL-B and OAPA-20 at neutral pH were observed to be ineffective at solubilizing 0.033% POPC solutions under both low (ca. 0 mM) and high (300 mM) salt conditions, as judged by the persistence of light scattering even after several hours. This was also true when the mixtures were agitated by heating (to near 100°C) and by mild sonication. This result cannot be interpreted to indicate that amphipols do not interact with POPC vesicles; they almost certainly do (see [6,7]). However, this result is consistent with previous observations that amphipols are poor lipid solubilizing agents even under conditions where the polymer:lipid ratio is relatively high [3,7].

4. Discussion

The most significant observation of this paper is that at least one membrane protein (DAGK) appears to spontaneously transfer from an OAPA-20 amphipol complex into pre-formed lipid vesicles to adopt a properly inserted and folded structural state. This transfer/folding process was observed to occur at significant rates and efficiencies for reactions initiated using both stock enzyme solutions which contained only protein-associated OAPA-20 and a solution which contained excess free amphipol. Whether these results would be observed for other membrane proteins is a matter for future investigation. Vesicular insertion results for a second amphipol (PMAL-B) were ambiguous because DAGK exhibits considerable activity in PMAL-B solutions containing no lipid or detergent, such that the assay used did not allow us to distinguish between inserted active enzyme and non-inserted active enzyme.

Given the poor ability of concentrated amphipol solutions to solubilize very dilute lipid vesicles, the OAPA-20-assisted process of DAGK insertion into vesicles most likely does not involve catastrophic disruption of the target vesicles. These results suggest that, in addition to other appealing features, amphipols are worthy of further examination and development as a distinct method for delivering IMPs to pre-existing lipid bilayers.

IMPs have traditionally been reconstituted into lipid bilayers using methods by which lipids and membrane proteins are co-solubilized in detergent micelles, followed by introduction of conditions where lipid bilayers will spontaneously assemble in a membrane protein-inclusive manner (review in [13]). Applications can be imagined where it would be desirable to add a purified membrane protein to lipid vesicles or biological membranes under conditions where the protein could spontaneously insert and adopt its functionally active state without lysing the target bilayer. Applications for which this might be useful include a variety of possible biochemical/biotechnological experiments or applications, cell biology research involving manipulation of living cells or in vitro extracts, and 'drug delivery'-type applications where the goal is to insert a membrane protein into the membranes of cells within a living organism.

While it is possible that classical detergent/protein micelles might serve in some membrane protein delivery applications (cf., [14]), amphipols may offer unique advantages. Because many detergents are excellent membrane solubilizing agents (unlike amphipols), the potential for bilayer lysis is relatively high for classical detergents compared to amphipols. Moreover, when detergent/membrane protein complexes are diluted to below the critical micelle concentration, any protein which does not rapidly associate with the lipid phase can be expected to precipitate as it is depleted of detergent. This is in contrast to amphipol/membrane protein complexes which are stable for days, even under conditions where there is no free amphipol population, as confirmed for DAGK (Sanders, unpublished) and other membrane proteins [2,3,7]. In this work it was observed that OAPA-20 could facilitate DAGK folding/insertion into membranes with efficiencies in the range of 20–30%. This may be sufficient for many putative applications of amphipols as protein delivery agents. For other applications, the results of this paper hopefully represent a useful first step towards the development of novel amphipols capable of delivery with near 100% efficiency. Such development may be facilitated by specific follow-up to the results of this study to elucidate the exact fate of the DAGK populations which failed to productively fold and insert into bilayers from amphipols and to elucidate the molecular determinants of the partitioning ratio between productive and non-productive pathways during amphipol → bilayer transfers.

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