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Folding of diphtheria toxin T-domain in the presence of amphipols and fluorinated surfactants: Toward thermodynamic measurements of membrane protein folding☆,☆☆

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ABSTRACT

Solubilizing membrane proteins for functional, structural and thermodynamic studies is usually achieved with the help of detergents, which, however, tend to destabilize them. Several classes of non-detergent surfactants have been designed as milder substitutes for detergents, most prominently amphipathic polymers called 'amphipols' and fluorinated surfactants. Here we test the potential usefulness of these compounds for thermodynamic studies by examining their effect on conformational transitions of the diphtheria toxin T-domain. The advantage of the T-domain as a model system is that it exists as a soluble globular protein at neutral pH yet is converted into a membrane-competent form by acidification and inserts into the lipid bilayer as part of its physiological action. We have examined the effects of various surfactants on two conformational transitions of the T-domain, thermal unfolding and pH-induced transition to a membrane-competent form. All tested detergent and non-detergent surfactants lowered the cooperativity of the thermal unfolding of the T-domain. The dependence of enthalpy of unfolding on surfactant concentration was found to be least for fluorinated surfactants, thus making them useful candidates for thermodynamic studies. Circular dichroism measurements demonstrate that non-ionic homopolymeric amphipols (NAhPols), unlike any other surfactants, can actively cause a conformational change of the T-domain. NAhPol-induced structural rearrangements are different from those observed during thermal denaturation and are suggested to be related to the formation of the membrane-competent form of the T-domain. Measurements of leakage of vesicle content indicate that interaction with NAhPols not only does not prevent the T-domain from inserting into the bilayer, but it can make bilayer permeabilization even more efficient, whereas the pH-dependence of membrane permeabilization becomes more cooperative. This article is part of a Special Issue entitled: Protein Folding in Membranes.

Abbreviations: APols (amphipols), a class of amphipathic polymers designed for handling membrane proteins in aqueous solutions; NAhPols, Non-ionic homopolymeric amphipols (Fig. 1); A8-35, an anionic, polyacrylate-based APol (Fig. 1); F_6TAC (or FTAC) (6 refers to the number of fluorinated carbons), fluorinated surfactants 4-thia-7,7,8,8,9,9,10,10,11,11,12,12,12-tridecafluorododecanamyl-*poly-Tris*-(hydroxymethyl)acrylamidomethane (the same compound was called FTAC-C6 in some of our previous publications [1, 2]); F_6-Diglu (6 refers to the number of fluorinated carbons), fluorinated surfactant *N*-1,1-di[(β-D-glucopyranosyl)oxymethyl]hydroxyethyl-4-thia-7,7,8,8,9,9,10,10,11,11,12,12,12-tridecafluorododecanamide; OG, *n*-octyl-α-D-glucopyranoside; DDM, *n*-dodecyl-α-D-maltopyranoside; CMC, critical micelle concentration; DTT (or T-domain), diphtheria toxin T-domain; ANTS, 8-aminonapthalene-1,3,6 trisulfonic acid; DPX, *p*-xylene-*bis*-pyridinium bromide; LUV, extruded large unilamellar vesicles of 100 nm diameter; POPC, palmitoyloleoylphosphatidylcholine; POPG, palmitoyloleoylphosphatidylglycerol; CD, circular dichroism

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1. Introduction

The thermodynamic characterization of membrane proteins (MPs) lags substantially behind that of their soluble counterparts. One of the principal difficulties that severely limits the application of such classical tools as thermal unfolding is the insolubility in water of both their native and denatured states, as MPs aggregate and precipitate outside of their native membrane environment. Detergent solubilization, which is a general way of handling MPs *in vitro*, very often makes them unstable. Several approaches have been suggested to try to circumvent this problem, (*e.g.,* see Refs. [1–5], among which is the use of new, milder non-detergent surfactants such as amphipols [6–9] or fluorinated non-ionic surfactants [10–16].

Amphipols (APols) are "amphipathic polymers that are able to keep individual MPs soluble under the form of small complexes" [5]. Applications of APols include MP folding and cell-free synthesis, structural studies by NMR, electron microscopy and X-ray diffraction, APol-mediated immobilization of MPs onto solid supports, proteomics, delivery of MPs to preexisting membranes, and vaccine formulation (for a review see [9]). Over the years, several types of APols have been designed, synthesized, and tested [9]. In the present study, we used either a polyacrylate-based APol called A8–35 [6] or a recently developed non-ionic glucose-based amphipathic homopolymer, NAhPol [17,18] (Fig. 1A). NAhPols are the most advanced members of a series of non-ionic APols [19–21]. For the sake of the present studies, they present the advantage of remaining soluble at acidic pH [20], while A8–35 does not [22].

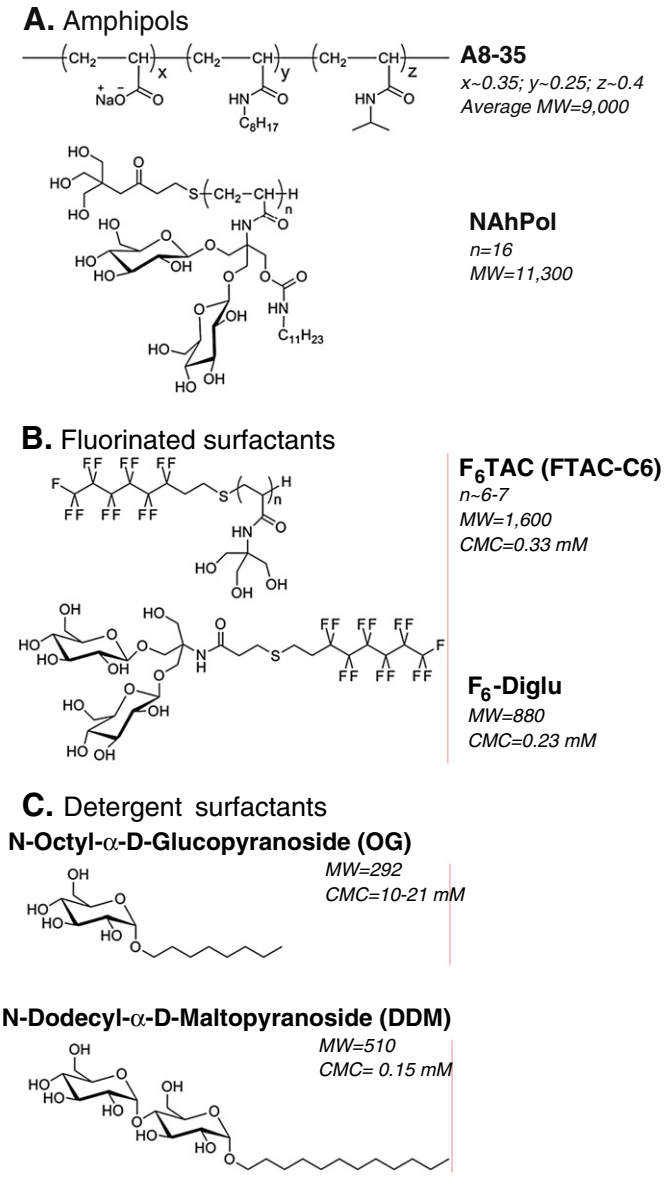

Fig. 1. Molecular structures of amphipols (A), fluorinated surfactants (B) and regular detergents (C) used in this study.

Fluorinated surfactants are small compounds composed of a polar head and a hydrophobic moiety that contains fluorinated atoms (Fig. 1B). Due to poor packing of fluorinated and hydrocarbon chains, these surfactants, while they look like detergents, possess an unusual and useful combination of properties: being at the same time good solvents for proteins and poor solvents for lipids. Previously, we have demonstrated the usefulness of fluorinated surfactants in chaperoning the insertion of a diphtheria toxin (DT) T-domain into preformed lipid bilayers, by reducing non-productive aggregation in the aqueous phase without compromising membrane insertion [10,11,15]. The DT T-domain (DTT) is a particularly useful model system, as it can be easily converted from a properly folded, water-soluble monomeric globular state with a known structure [23] to a membrane-competent conformation, by acidification. The physiological function of the T-domain is associated with its membrane insertion and the translocation of the catalytic domain attached to its *N*-terminus. This translocation is believed to proceed via a transmembrane pore of unknown structure formed by the T-domain itself [24,25]. The insertion pathway, however, is rather complex and contains multiple intermediate steps [26–31]. Here we explore the interactions of the T-domain with APols and fluorinated surfactants, as a first step towards their future applications in thermodynamic studies of MPs.

2. Materials and methods

2.1. Materials

POPC and POPG were purchased from Avanti Polar Lipids (Alabaster, AL). OG and DDM detergents were from Anatrace (Maumee, OH). 50 mM sodium phosphate buffer with pH values ranging from 6.0 to 8.0 was used in this study. Rapid acidification during the leakage experiment was achieved by addition of a small amount of concentrated sodium acetate. Diphtheria toxin T-domain (amino acids 202–378) was cloned into NdeI–EcoRI-treated pET15b vector containing an N-terminal 6xHis-tag and a thrombin cleavage site and isolated as described in Ref. [32]. The concentration of T-domain was 5 μM for samples used in CD experiments and 2 μM for samples used in fluorescence experiments, unless otherwise specified.

2.2. LUV preparation for permeabilization measurements

Large unilamellar vesicles (LUV) of diameter 0.1 μm were prepared by extrusion [33,34] from 3:1 molar mixtures of POPC and POPG. The vesicles were preloaded with 1 mM ANTS and 10 mM DPX and leakage was measured as described in [35].

2.3. Syntheses of APols and fluorinated surfactants

APols and fluorinated surfactants were prepared according to previously described procedures as follows: A8–35 [6,36], F_6TAC [37], F_6-Diglu [38]. The NAhPol series is a novel series of NAPols [17,18], which is prepared by homopolymerization of an amphiphilic diglucosylated monomer in the presence of a transfer agent.

2.4. Fluorescence measurements

Fluorescence was measured either using a SPEX Fluorolog FL3-22 steady-state fluorescence spectrometer (Jobin Yvon, Edison, NJ)

equipped with double-grating excitation and emission monochromators as described previously [10]. The measurements were made in 4×10 mm cuvettes, oriented perpendicular to the excitation beam and maintained at 25 °C using a Peltier device from Quantum Northwest (Spokane, WA).

2.5. CD measurements and analysis of thermal unfolding

CD measurements were performed using an upgraded Jasco-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo). Normally, 50 scans were recorded between 190 and 260 nm with a 1 nm step at +20 °C, using a 1 mm optical path cuvette. All spectra were corrected for background. Temperature-dependencies of unfolding were measured at 222 nm with a 1°/min scan rate and analyzed as described previously [10]. The thermal unfolding was analyzed using thermodynamic equations for a reversible two-state, $N \leftrightarrow U$ unfolding transition, where N and U are the native and the unfolded states of the protein, respectively. In order to obtain the transition temperature (T_m) and the enthalpy changes (ΔH), raw data were fitted applying nonlinear least-square analysis with six fitting parameters, Y_N, m_N, Y_U, m_U, ΔH and T_m with the following equations [39]:

$$Y = (Y_N + m_N \cdot T) \cdot X_N + (Y_U + m_U \cdot T) \cdot (1 - X_N) \quad (1)$$

$$X_N = 1/[1 + \exp(-\Delta H(1 - T/T_m)/RT] \quad (2)$$

where Y is the experimentally observed CD signal at a given temperature, Y_N and Y_U, represent the signals of the pure N and U states at zero temperature, and m_N and m_U are the temperature-dependencies of these CD signals for the N and U states, respectively.

3. Results

Thermal unfolding of the T-domain in the presence of various surfactants and polymers was monitored by CD spectroscopy. Typical examples of temperature scans of the ellipticity signal at 222 nm, which is sensitive to the unfolding of helical segments, are presented in Fig. 2. Whereas the presence of NAhPol (open symbols) does not affect the signal from either the folded or unfolded state, it clearly lowers both the melting temperature and the cooperativity of the transition. The quantitative analysis of thermal denaturation traces was performed by fitting the data to Eqs. ((1)–(2)) (solid lines), which allowed the determination of melting temperature T_m and transition enthalpy ΔH.

A summary of the quantitative analysis of the T-domain unfolding in the presence of APols is presented in Fig. 3. The measurements

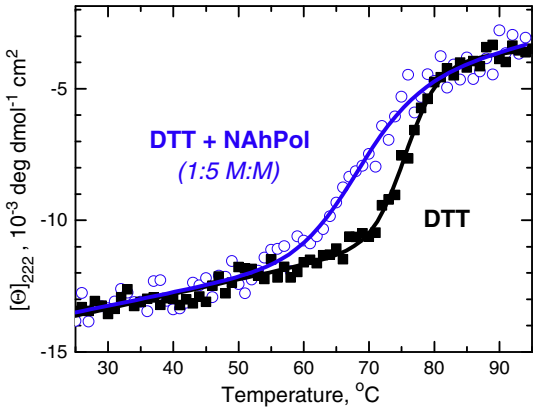

Fig. 2. Thermal unfolding of the T-domain monitored by changes in molar ellipticity at 222 nm in the absence (solid symbols) and presence of NAhPol (open symbols). Solid lines correspond to the least-square analysis with Eqs. (1–2) to determine transition temperature T_m and enthalpy ΔH.

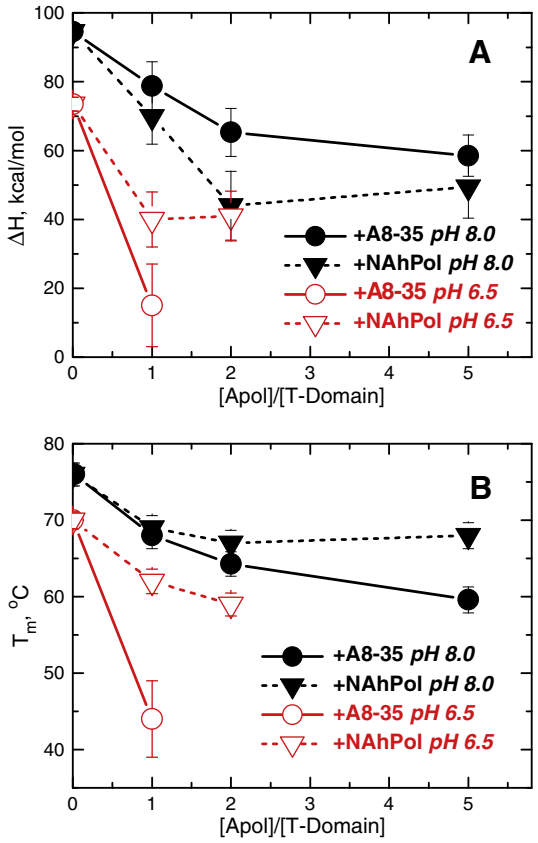

Fig. 3. Influence of A8-35 (circles) and NAhPols (triangles) of the enthalpy (A) and transition temperature (B) of thermal unfolding of the T-domain measured at pH 8 (solid symbols) and pH 6.5 (open symbols).

were performed either at pH 8, when the T-domain is in a tightly folded soluble state, or at pH 6.5, at the beginning of the transition leading to the formation of the membrane-competent state [27]. At neutral pH, both APols lead to similar decreases in ΔH (top panel) and T_m (bottom panel). At pH 6.5, only NAhPol provides useful data, as the T-domain/A8-35 sample shows a dramatic decrease in melting cooperativity, possibly related to the loss of solubility of A8–35 upon acidification [22].

The changes in thermodynamic parameters appear to be saturating at higher polymer-to-protein ratios, consistent with the formation of individual complexes of these molecules. This is different from the effect of detergents and fluorinated surfactants, which show no such saturation ([10] and data in Fig. 4). To appropriately compare the effects of various surfactants on the unfolding of the T-domain, we plotted their effects on transition enthalpy as a function of the surfactant concentration in terms of weight units rather than molar units (Fig. 4). At either pH, the slope observed with DDM was the steepest, indicating the strongest perturbation. The slope observed with F_6TAC was generally the lowest, although at pH 6.5 it coincided with that of another fluorinated surfactant, F_6-Diglu.

CD spectra of the T-domain free in solution and in complex with NAhPol are presented in Fig. 5. Remarkably, exposure of the T-domain/NAhPol complexes to even minor acidification leads to a substantial alteration of the CD spectrum, with loss of ellipticity and change of the shape of the spectrum. (It is not immediately clear whether the shape of the spectrum is affected by absorbance flattening [40,41], although this would be rather unusual for non-membrane system.) The spectral change, however, is quite different from that induced by thermal denaturation: The spectrum that develops during the latter is consistent with the presence of random coil structure, even in the presence of NAhPol (dotted line). This difference suggests

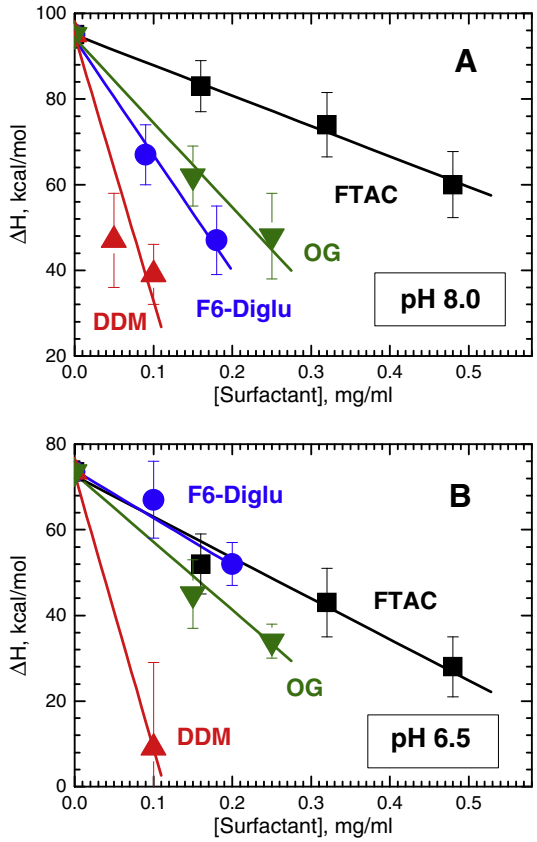

Fig. 4. Dependence of the enthalpy of thermal unfolding of the T-domain (ΔH) on the concentration of the following surfactants in the sample: F_6TAC (squares); F_6-Diglu (circles); OG (down triangles); DM (up triangles). All concentrations are below corresponding CMCs.

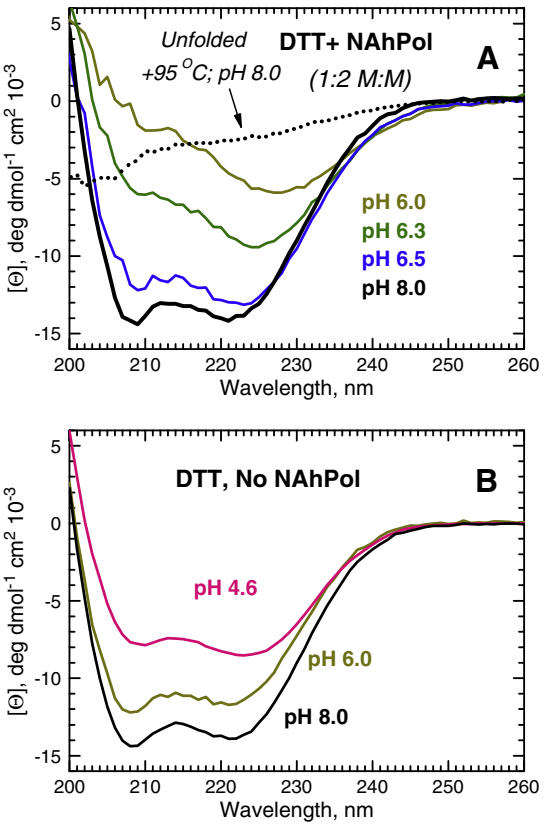

Fig. 5. CD spectra of the T-domain measured in the presence of NAhPol (A) or in the absence of any surfactant (B). Solid lines correspond to the spectra measured at pH indicated on the plots (top to bottom) measured at 25 °C, while dotted lines correspond to the spectrum of denatured T-domain measured at 95 °C.

that in the 6–7 pH range, the T-domain undergoes a specific polymer-induced conformational change, rather than simple unfolding. The conformational change of the aqueous T-domain observed in a more acidic environment has a similar spectral appearance (*e.g.,* at pH 4.5, as shown in Fig. 5B), although the magnitude of the change in this case is lower (Fig. 6). None of the detergents or fluorinated surfactants caused such conformational changes (not shown).

To check on the ability of the T-domain to maintain its membrane activity in the presence of NAhPol, we performed a vesicle permeabilization assay. Lipid vesicles were loaded with the ANTS/DPX dye/quencher pair and their release was monitored by the resulting increase in fluorescence. While APols can cause membrane permeabilization by themselves (see Refs. [7,9]), we found that under the conditions of our experiments the background leakage was rather mild, especially for NAhPol (Supplemental Fig. S2). At neutral pH, the leakage caused by T-domain/NAhPol complexes was quite low, while acidification to pH 4.5 caused massive release of the markers (Fig. 7A). When leakage was initiated at pH 6.5, i.e., after the T-domain had changed its conformation in the presence and with the assistance of the polymer, the leakage was strongly reduced. Subsequent lowering of the pH to 4.5, however, restored the activity (Fig. 7B), which indicates that the T-domain can be released from the polymer and then insert into the bilayer.

4. Discussion

The APols used in the present study, namely A8–35 and NAhPol (Fig. 1A), have been developed to keep MPs soluble outside the native environment of the lipid bilayer under less denaturing conditions than is achievable in detergent solutions. The idea is that flexible polymer carrying hydrophobic and hydrophilic groups will self-organize into a solubility cage around MPs, with nonpolar groups facing the transmembrane surface of the MP and polar groups the water phase. NMR and electron microscopy studies have found, indeed, that APols specifically adsorb onto the transmembrane surface of the MPs they keep soluble [42–44]. APols appear much less inactivating than detergents, which has prompted the development of many APol applications in the MP field (reviewed in [9]). Here, for the first time, we apply APols for thermodynamic studies of thermal denaturation of a model protein, diphtheria toxin T-domain.

In our previous studies, we have tested the ability of various fluorinated surfactants to chaperone the insertion of the diphtheria toxin

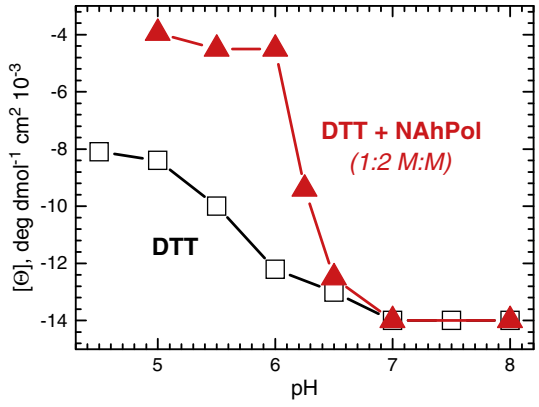

Fig. 6. pH-dependent changes in ellipticity of the T-domain measured at 222 nm in the absence (open symbols) and presence of NAhPol in 1:2 molar ratio (solid symbols).

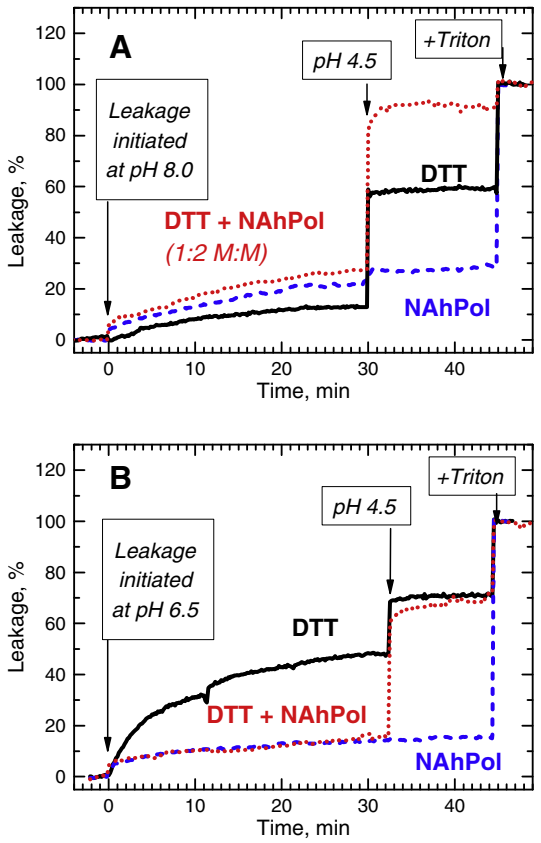

Fig. 7. Effect of NAhPol on the permeabilization of lipid vesicles by the T-domain. Stock solutions of T-domain (solid lines), NAhPol (dashed lines) or their complex (dotted lines) were mixed with LUV preloaded with ANTS/DPX markers at zero time. Release of markers was followed by changes in fluorescence and normalized to 100% after complete solubilization of LUV in 1% Triton. The final mixture contained 0.2 mM lipid and 0.4 μM T-domain and 0.8 μM NAhPol, when present. Stock solutions containing 5 μM T-domain and 10 μM NAhPol were preincubated overnight either at pH 8.0 (A) or pH 6.5 (B).

T-domain into lipid bilayers [10,15]. The advantage of the T-domain as a model system is that it can be easily converted from a soluble to a membrane-competent form by simple acidification. The first generation of APols, comprising A8–35 and its congeners, is insoluble at pH<7 [22], which is not compatible with studying the effect of pH on the T-domain. The recent introduction of non-ionic APols [20,21], in the present case NAhPols [17,18] (Fig. 1), has gone around this limitation.

The main idea of applying APols to thermodynamic studies of MPs, specifically to thermal denaturation, is to ensure the solubility of the folded or unfolded hydrophobic chain. It is not clear, however, how MP/APol interactions in both the native and the denatured states will affect the thermodynamic parameters of unfolding. As a first step towards developing this approach for regular MPs, we have tested it on the T-domain and used T_m and ΔH obtained in the absence of APols as references. At pH 8, when the T-domain is in the folded water-soluble form, addition of APols results in both lowering the transition temperature and decreasing the transition enthalpy (Fig. 3). From these data, and the value for heat capacity ΔC_p, it is possible to estimate the difference in free energy of the folded and unfolded state ΔG. Assuming that $\Delta C_p = 1.4$ kcal/mole [Kyrychenko, Rodnin, Makhatadze, Ladokhin unpublished], we estimate that interaction with NAhPol reduces ΔG for thermal denaturation of the T-domain by ~5 kcal/mol at pH 8 and ~3 kcal/mol at pH 6.5. We suggest that the stabilization of the unfolded state inside the polymer hydrophobic cage is responsible for a substantial part of this free energy difference. With regular MPs, the situation will be substantially different, because the protein has to be surrounded by a surfactant (lipid, detergent, APol, fluorinated surfactant…) in both the native and the unfolded states.

Thermodynamic stabilization or destabilization depends on the balance of the interactions of the surfactant with the two forms. APols have been shown to strongly stabilize most MPs as compared to detergents (reviewed in Refs. [5,7,9]). The effect, however, is not purely thermodynamic. It has been proposed to involve three types of mechanisms: *i)* a poorer competition of APols, as compared to detergents, with the protein/protein and protein/lipid interactions that stabilize the native 3D structure of the protein; *ii)* diminishing the volume of the "hydrophobic sink" (detergent micelles, free APol particles), whose presence favors the dissociation of lipids, subunits, and other stabilizing factors; and *iii)* a dampening effect on large conformational excursions, *i.e.*, a kinetic effect (for a discussion, see ref. [9]). Factor *i* is obviously favorable for thermodynamic studies, since it provides the MP under study with a more neutral and less aggressive environment. Factor *ii* could be eliminated by working with lipid- and cofactor-free monomeric MPs, as is the case with the DT T-domain. Eliminating factor *iii* implies that measurements be carried out under carefully monitored conditions of reversibility. Unfortunately, the membrane-competent T-domain is not suitable for testing this approach experimentally, as the acid-induced conformational change is accompanied by the loss of cooperative unfolding.

Perhaps the most surprising result of this study is the ability of NAhPol to efficiently change the conformation of the T-domain (presumably to that of the membrane-competent state) even at the very mildly acidic pH of ~6–6.5 (Figs. 5, 6). Normally, the T-domain undergoes conformational changes leading to ultimate membrane insertion at a lower pH. It seems likely that binding of APol to exposed hydrophobic regions facilitates the transition. This is consistent with the small, but measurable shift of intrinsic fluorescence of the T-domain observed at pH 6.0 upon addition of NAhPol (Supplement Fig. S1). It is remarkable, however, that no such facilitating effect is seen with detergents. It is difficult to study the membrane-competent form of the T-domain in solution, as it tends to aggregate, which affects low-resolution structural measurements (e.g., CD, fluorescence) and makes high resolution structural studies completely impossible. MP/NAhPol complexes yield NMR spectra of a quality comparable to those of MP/A8–35 [45]. Thus, it would be interesting to examine whether T-domain/NAhPol complexes will be useful for structural studies of the membrane-competent conformation using NMR.

The complex of the T-domain with NAhPol appears to be quite stable at pH 6.5, as can be seen by the lack of membrane permeabilizing activity (Fig. 7B, dotted line). Lowering the pH to 4.5, however, causes efficient release of the T-domain into the membrane, even after a prolonged incubation (in our case we incubated T-domain/ NAhPol complexes overnight at pH 6.5 prior to collecting the data in Fig. 7). The released T-domain is membrane-competent and causes efficient permeabilization of the vesicles. Similar behavior is also observed when T-domain/NAhPol complexes are incubated at pH 8 before adding to the vesicles (Fig. 7A). Moreover, in this case the final activity of the complex at pH 4.5 is even higher than that of the T-domain alone (compare solid and dotted lines in Fig. 7A). This indicates that NAhPol, similarly to fluorinated surfactants [10,15], is capable of chaperoning membrane insertion of the T-domain, presumably by preventing non-productive aggregation.

Fluorinated surfactants (e.g., F_6TAC and F_6-DiGlu, Fig. 1B) act similarly to regular detergents. Their important advantage is poor miscibility with membranes, which makes them particularly useful for thermodynamic studies of membrane insertion under equilibrium conditions [11]. At least in the case of the diphtheria toxin T-domain, fluorinated surfactants can prevent aggregation of the membrane-competent form at concentrations lower than their CMC [10]. Here we compared the effects of sub-CMC concentrations of several of the fluorinated and nonfluorinated surfactants on the thermal

denaturation of the T-domain (Fig. 4). (Note that even at these concentrations the reduction in enthalpy values are so pronounced that increasing detergent concentration will lead to a complete elimination of cooperative melting transition before CMC is reached) The idea behind the experiment is to identify compounds that would exhibit lowest perturbation, *i.e.,* will have the shallowest concentration dependence of unfolding enthalpy ΔH. Clearly, fluorinated surfactants, especially F_6TAC, have an edge over regular detergents. At pH 6.5, when the T-domain has more exposed hydrophobic area, F_6-DiGlu showed an equally good result, suggesting that it also may be a good potential candidate for thermodynamic studies of MPs. Its potential for equilibrium thermodynamic measurements of insertion, however, is somewhat compromised by its interaction with lipid bilayers. As our data indicate, F_6-DiGlu can cause leakage of vesicle contents (Supplement Fig. S2), a shortcoming shared by regular detergents, but not by F-TACs [10], which stand out as the best candidates for various thermodynamic studies of MPs.

The results presented here suggest that, while the application of APols to thermodynamic studies of MPs is potentially useful, they should be approached with caution, as APols may affect conformational equilibria. It is clear that we are in the early stages of understanding all the physical/chemical properties of these extremely useful molecules, and that further studies will reveal other potential applications. One intriguing possibility is suggested by the fact that the T-domain, when in complex with NhAPols, is not membrane-active at pH 6.5 but regains full activity upon further acidification. This property is potentially important for use in targeted delivery of therapies to cancerous cells, which usually have a more acidic environment [46]. Membrane action of the T-domain in complex with NAhPol has a sharper (more cooperative) pH-sensitive conformational switching than the protein alone and therefore may potentially provide more selective targeting to pathogenic cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamem.2011.09.012.

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