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Using micellar mole fractions to assess membrane protein stability in mixed micelles

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Abstract

The increased focus on the structural and physical properties of membrane proteins has made it critical to develop methods that provide a reliable estimate of membrane protein stability. A simple approach is to monitor the protein's conformational changes in mixed detergent systems, typically consisting of an anionic (denaturing) and non-ionic (non-denaturing) component. Linear correlations between, e.g., the melting temperature and the bulk mole fraction of the anionic component have been observed. However, a potential complication is that the bulk mole fraction is not identical to the mole fraction in the mixed micelle, which is the local environment experienced by the membrane protein. Here, we present an extensive analysis of the thermal stability of the membrane-integrated domain of the outer membrane protein AIDA in the presence of different mixed micelles. In the micelle system SDS-octyl-polyoxyethylene, the melting temperature in the absence of SDS extrapolates to 113 °C using bulk mole fractions. However, for mixed micelles involving short-chain detergents or phospholipids, the melting temperature calculated using bulk mole fractions reaches values up to several hundred degrees higher than 113 °C and can only be obtained by extrapolation over a narrow mole fraction interval. Furthermore, there is a non-linear relationship between the melting temperature and bulk mole fractions for mixed micelle systems involving cationic detergents (also denaturing). We show that if we instead use the micellar mole fraction as a parameter for denaturing detergent strength, we obtain linear correlations which extrapolate to more or less the same value of the melting temperature. There remains some scatter in the extrapolated values of the melting temperature in different binary systems, which suggest that additional micellar interactions may play a role. Nevertheless, in general terms, the mixed micellar composition is a good parameter to describe the membrane protein's microenvironment. Note, however, that for the mixed micelle system involving SDS and dodecyl maltoside, which has been used by several research groups to determine membrane protein stability, the estimate provided by bulk mole fraction leads to similar values as that of micellar mole fractions.

Keywords: Outer membrane protein; Detergent; Mixed micelle; Thermal stability; Unfolding kinetics; Micellar composition

1. Introduction

Membrane proteins play a central role in many cellular functions such as nutrient uptake and cell signaling by virtue of their position in the cell membrane. They are adapted to their membrane environment through hydrophobic side chains, which interact with the acyl chains of the phospholipid bilayer, as well as aromatic, polar and charged residues which interact favorably with the interfacial region of the membrane [1]. The membrane is complex and consists of many different kinds of phospholipids and other hydrophobic molecules, such as cholesterol, besides a host of different integral and peripheral membrane proteins. To obtain more knowledge about the forces which stabilize membrane proteins, it is advantageous to

Abbreviations: α_i, bulk mole fraction of ionic detergent; α_n, bulk mole fraction of non-ionic detergent; AIDA, residues 951–1286 of Adhesin Involved in Diffuse Adherence; DCPC, 1,2-dicapryl-*sn*-glycero-3-phosphocholine; DecM, n-decyl-β-D-maltoside; DHPC, 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DM, n-dodecyl-β-D-maltoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; LTAC, lauroyl trimethyl ammonium chloride; NM, n-nonyl-β-D-maltoside; NPN, N-phenyl-1-napthylamine; OG, n-octyl-β-D-glucoside; OM, n-octyl-β-D-maltoside; oPOE, octyl-polyoxyethylene; SDeS, sodium decyl sulfate; SDS, sodium dodecyl sulfate; SHS, sodium hexadecyl sulfate; STS, sodium tetradecyl sulfate; T_m, melting temperature; UM, n-undecyl-β-D-maltoside; X_i, micellar mole fraction of ionic detergent; X_n, micellar mole fraction of non-ionic detergent

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study them in a simple and well-defined amphiphilic environment, which is accessible to spectroscopic measurements and also allow the experimenter to manipulate transitions between different conformational states. Such transitions can be used to measure energy differences between different states and thus provide a means of quantifying membrane protein stability. A simple example of such an environment is a binary mixture of a denaturing (typically anionic) and non-denaturing (non-ionic) detergent, typically sodium dodecyl sulfate (SDS) and dodecyl maltoside (DM), respectively. By combining these detergents, mixed micelles are formed. Pioneering work by Lau and Bowie showed that the trimeric α-helical membrane protein diacylglycerol kinase could be reversibly unfolded under equilibrium conditions in such a system by increasing the mole fraction of SDS, and demonstrated that the unfolding transition was consistent with a simple model in which the free energy of unfolding was proportional to the mole fraction of SDS [2]. This is analogous to the analysis of the stability of globular proteins by chemical denaturation in urea or guanidinium chloride, which is based on linear relationships between free energies of unfolding (or activation energies) and denaturant concentrations [3]. The binary detergent-mixture approach has subsequently been used to compare the stabilities of different mutants of bacteriorhodopsin [4,5]. The SDS-DM system has also been exploited to build up a kinetic model for the unfolding of DsbB [6], and a similar approach has been used to analyse the folding of the β-barrel protein OmpA in the SDS-octyl glucoside (OG) binary system [7,8].

An aspect which has hitherto not been studied so thoroughly in this approach is the fact that mixed detergent systems do not behave like solutions of the pure components but have unique properties [9]. The mole fractions of the two detergents in micelles (X_i and $X_n = 1 - X_i$, where "i" and "n" refer to the ionic and non-ionic component, respectively) are not identical to the bulk composition of detergents α_i and α_n, but rather vary with α in a rather complex and non-linear fashion. This arises from the simple phenomenon that the two detergents will invariably have different propensities to form micelles, and therefore one type will generally be preferred over the other for incorporation into micelles. Since the micelle is the local environment that the membrane protein experiences, the micellar composition would appear to be the best parameter to use when interpreting the behaviour of a membrane protein. Fortunately, it is straightforward to calculate this by measuring the critical micelle concentrations (cmc) of detergent mixtures of fixed bulk composition [10].

In this report, we investigate the consequences of this non-linear relationship between X and α using the membrane-integrated β-domain of the outer membrane protein AIDA as model system. AIDA is an autotransporter from *Escherichia coli*. The protein is transported to the outer membrane by an as yet not fully elucidated mechanism, leaving the C-terminal β-domain embedded in the outer membrane and the N-terminal passenger domain exposed on the bacterial surface, where it facilitates adherence to mucosal cell linings in the host [11–13]. In this study, we work with the trypsin-resistant core of the membrane-integrated β-domain, the β_2-domain, consisting of residues 951–1286 (in the following referred to as AIDA for simplicity). AIDA is typical of β-barrel proteins in that it does not unfold in SDS at room temperature, but needs to be exposed to elevated temperatures for this to occur [14]. We have previously shown that there is an empirical linear relationship between the midpoint temperature of denaturation T_m and the bulk mole fraction α_i of SDS in the SDS-octyl-polyoxyethylene (oPOE) binary system [14]. Here, we measure the thermal transition of AIDA in different binary systems and correlate them with the calculated micellar composition X_i to evaluate the importance of the direct micellar environment experienced by the membrane protein.

2. Materials and methods

2.1. Materials and protein preparation

n-octyl-β-D-maltoside (OM), n-nonyl-β-D-maltoside (NM), n-decyl-β-D-maltoside (DecM), n-undecyl-β-D-maltoside (UM), and n-octyl-β-D-glucoside (OG) were from Anatrace (Maumee, OH); n-dodecyl-β-D-maltoside (DM) was from Calbiochem (Canada); n-octyl-polyoxyethylene (OPOE) was from Bachem AG (Bubendorf, Switzerland); sodium dodecyl sulfate (SDS) and N-phenyl-1-napthylamine (NPN) were from Sigma-Aldrich (St. Louis, MO); sodium decyl sulfate (SDeS), sodium tetradecyl sulfate (STS), sodium hexadecyl sulfate (SHS) and lauroyl trimethyl ammonium chloride (LTAC) were from Lancaster (Eastgate, England). All phospholipids were from Avanti (Alabaster, AL). The purity of all chemicals was ≥99%. $AIDA^{951-1286}$, referred to as AIDA throughout this article, was expressed as inclusion bodies in *E. coli* and purified by folding on a Ni-NTA column as described [14].

2.2. Far-UV CD measurements

All measurements were performed in 10 mM Tris, pH 8 at 25 °C apart from thermal scans. Far-UV wavelength and thermal scans were recorded on a Jasco J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Hachioji City, Japan) as described [14]. A 1-mm quartz cuvette was used at a band width of 1 nm, using steps of 0.2 nm at a scan speed of 50 nm/min. To investigate possible two-state unfolding of AIDA in selected mixed detergent systems, wavelength CD scans were recorded in the range of 25 °C to 105 °C in steps of 5 °C at a protein concentration of 5 μM. At each temperature, five accumulations were averaged to yield the final spectrum. For thermal scans, 5 μM of refolded AIDA in different non-ionic detergents was mixed with ionic detergents to total concentrations of 15–62 mM (above the cmc, the total concentration of detergent did not make any difference to the thermal profile, which was only affected by the relative ratios of the two detergents). Thermal unfolding was monitored at 208 nm with a scan rate of 60 °C/h. The unfolding curves were fitted to the following equation, assuming a linear dependence of the pre- and post-transition baselines on temperature, to obtain T_m, the midpoint of denaturation [15]:

$$\theta_{208} = \frac{\alpha_N + \beta_N(T-298) + (\alpha_D + \beta_D(T-298))e^{\left(\frac{-\Delta H_{vH}}{R}\left(\frac{1}{T}-\frac{1}{T_m}\right)\right)}}{1+e^{\left(\frac{-\Delta H_{vH}}{R}\left(\frac{1}{T}-\frac{1}{T_m}\right)\right)}} \quad (1)$$

where θ_{208} is the observed ellipticity at a given temperature, α_N and α_D are the ellipticities of the native and denatured state, respectively, at 298 K, β_N and β_D are the slopes of the native and denatured state baselines, respectively, T is the temperature, T_m is the midpoint denaturation temperature, ΔH_{vH} is the Van't Hoff enthalpy change of unfolding, and R is the gas constant. Plots of T_m versus α_i (where α_i = [ionic detergent]/([ionic detergent]+[non-ionic detergent])) were fitted to a linear equation, except for the plots involving LTAC, where the following simple binding isotherm was found empirically to fit the data best:

$$T_m = \frac{K T_m^{\alpha_{LTAC}=0} + (1-\alpha_{LTAC}) T_m^{\alpha_{LTAC}=1}}{K + (1-\alpha_{LTAC})} \quad (2)$$

where $T_m^{\alpha_{LTAC}=0}$ and $T_m^{\alpha_{LTAC}=1}$ are the T_m-values at $\alpha_{LTAC}=0$ and 1, respectively, and K is an apparent dissociation constant for the hypothetical binding equilibrium between AIDA and LTAC (whose concentration is given as α_{LTAC}).

2.3. Kinetics of unfolding

AIDA and detergent were mixed in a preheated cuvette (path length 4 mm) to a final concentration of 2 μM protein and different mole fractions of ionic and non-ionic detergent at a total concentration of 15–62 mM detergent. The CD signal at an appropriate wavelength (200, 203, 206 or 209 nm) was followed until the signal reached a plateau at a band width of 1 nm. Data were recorded every 5 s and fitted to a single exponential equation to obtain the rate constant for unfolding k_u.

2.4. Fluorescence measurements

Steady-state fluorescence measurements were carried out on an LS-55 Spectrofluorometer (Perkin-Elmer Ltd., Wellesley, MA) with 5 μM N-phenyl-1-napthylamine (NPN) as a fluorescence probe, using an excitation wavelength of 350 nm and emission at the maximum of fluorescence intensity in the range of 420 to 440 nm. The incorporation of NPN into the micelles results in an increase in the fluorescence intensity of NPN. As NPN is present at less than 0.1% of total detergent concentration, we consider it reasonable to ignore its possible impact on cmc values. Fluorescence intensity was plotted against detergent concentrations, and the cmc was estimated as the break point of the curve. All cmc-values were measured at 25 °C.

2.5. Determination of the micellar composition of mixed micelles

Ideal cmc-values of the mixed detergent system (cmc*) are predicted by the Clint equation [16]:

$$\frac{1}{\mathrm{cmc}^*} = \frac{\alpha_i}{\mathrm{cmc}_i} + \frac{\alpha_i}{\mathrm{cmc}_n} \tag{3}$$

where α_i and α_n ($=1-\alpha_i$) are the mole fractions of detergent i (ionic) and n (non-ionic) in the total mixed solute, respectively, and cmc_i and cmc_n are the critical micellar concentrations of detergent i and n, respectively.

To analyse the synergistic behavior of all binary mixtures used in this work, we calculated their interaction parameter β and mixed micellar composition X_i using the regular solution approximation [10]. X_i can be iteratively computed from the following equation:

$$\frac{X_i^2 \ln\left(\frac{\mathrm{cmc}\alpha_i}{\mathrm{cmc}_i X_i}\right)}{(1-X_i)^2 \ln\left(\frac{\mathrm{cmc}(1-\alpha_i)}{\mathrm{cmc}_n(1-X_i)}\right)} = 1 \tag{4}$$

where cmc is the measured cmc of the mixed detergent system.

From the X_i-values, β can be computed as follows:

$$\beta = \frac{\ln\left(\frac{\mathrm{cmc}\alpha_i}{\mathrm{cmc}_i X_i}\right)}{(1-X_i)^2} \tag{5}$$

The β-value reveals the type of interaction between the two detergents which leads to a deviation from ideal behavior. Negative values indicate attractive interactions, and positive values indicate repulsion [16,17].

3. Results

Our results section is divided into three sections: Firstly, we establish the optimal conditions for evaluating AIDA stability in detergent micelles. Secondly, we present the results of these measurements in a large number of different binary detergent mixtures. Thirdly, we evaluate how these data correlate with the micellar composition of the detergent mixtures.

3.1. Conditions for evaluating AIDA stability: thermal scanning

In order to evaluate the stability of AIDA in binary detergent mixtures, we have to determine the best conditions for gathering our data. A simple approach is to measure the kinetics of unfolding at a given temperature, monitored in the region 203–210 nm where the change in ellipticity upon unfolding, as the temperature increases, is greatest (Fig. 1A). However, there are two disadvantages to the kinetic approach, firstly its complexity and secondly the relatively narrow mole fraction range over which unfolding kinetics can be measured. As shown in Fig. 1B (unfolding at a mole fraction $\alpha_{SDS}=0.7$ at 80 °C), the profile of ellipticity versus time requires two exponential decays with two half times t_1 and t_2 to obtain a satisfactory fit, suggesting that unfolding at a given temperature proceeds through one partially unfolded intermediate (though other explanations are also possible, such as parallel unfolding pathways). log t_1 and t_2 both depend linearly on the SDS mole fraction (Fig. 1C). However, the great sensitivity of the kinetics to the SDS mole fraction means that there is only a narrow window of experimental accessibility for kinetic studies of unfolding of AIDA at each temperature in a given binary detergent mixture. For example, even at 80 °C, it is only possible to measure unfolding rates between ca. 0.8 and 0.6 mole fractions SDS in our present experimental set-up, and even over this temperature interval the time required for equilibrium to be reached varies from around 20 h to 10 min.

In contrast, thermal scans in the same system, in which AIDA in a given binary micelle mixture is heated from 25 °C to 105 °C, allow us to measure over a broader range (between 0.975 and 0.4 mole fractions in the SDS-DM system, see below), simply because a thermal scan makes a broader "sweep" of the different energy levels available to the native and denatured state. Furthermore, unfolding under these conditions can be modelled according to a simple one-step transition, without requiring the introduction of an intermediate. This is supported by several independent observations. Firstly, our previously published analysis of AIDA thermal unfolding in the binary mixture SDS-oPOE [14] could be fitted to a two-state unfolding model (Eq. (2)). We observe the same correspondence for all other detergent mixtures analysed in the present work (see below).

Secondly, we have recorded far-UV CD wavelength scans of AIDA at different temperatures as it unfolds in different detergent mixtures. Provided there are only two states present during the whole unfolding process, there will be an isodichroic point corresponding to the wavelength where the ellipticities of the two absorbing species are identical. Fig. 1A shows the spectra of AIDA in the SDS-DecM detergent mixture over the temperature range 25–105 °C. We have obtained similar spectra for AIDA in other detergent mixtures such as SDS-NM, -UM and -DM (data not shown). The spectra of the natively folded and thermally unfolded states do

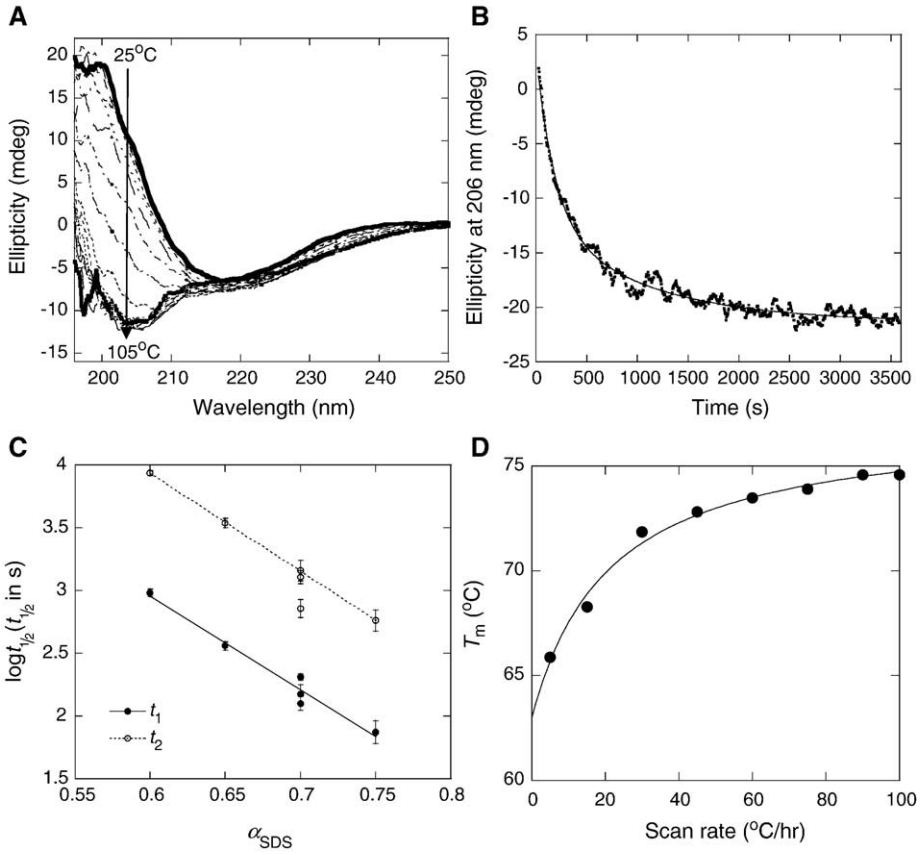

Fig. 1. AIDA Unfolds in a Single Step. (A) Far-UV CD scans of AIDA in the binary mixture SDS-DecM (at $\alpha_{SDS}=0.85$) over the temperature interval 25–105 °C. There is an approximate isodichroic point around 216 nm. (B) Kinetics of unfolding of AIDA in the binary mixture SDS-DM (at $\alpha_{SDS}=0.7$) at 80 °C measured at 206 nm. The solid line represents the best fit to a double exponential function to obtain the two half times of unfolding t_1 and t_2. (C) Plots of log t_1 (filled circles) and log t_2 (empty circles) versus α_{SDS} in the SDS-DM system at 80 °C. The lines indicate the best linear fits to the data points. All measurements are at 203 nm, except for $\alpha_{SDS}=0.7$, where measurements at 206 and 209 nm are also included. There is no systematic variation of half times with wavelength. (D) Dependence of the measured melting temperature T_m on the scan rate. The data are fitted to a simple hyperbolic function which predicts a T_m of 63±1 °C at a scan rate of 0 °C/h and an upper limit of a T_m of 77.2±0.7 °C. A scan rate of 60 °C/h is used in all subsequent experiments in this paper.

not intersect, but have the same value around 216 nm, and the ellipticity at this wavelength stays reasonably constant throughout the thermal scan. This indicates that only two states are significantly populated during the process. Therefore, we shall restrict our analysis to simple thermal scans of AIDA.

The two-state unfolding behaviour of AIDA makes analysis simple, since we obtain a single relevant parameter, namely T_m. Although in principle the enthalpy of unfolding ΔH_{unf} can provide further insight in the process, we find that generally there is no systematic correlation between ΔH_{unf} and T_m, and therefore do not include this in the present analysis. However, it should also be noted that unfolding is irreversible [14]. AIDA cannot be refolded from the urea-denatured state unless it is immobilized on a solid medium such as a Ni-NTA column, and we do not see any reversion to the native state spectrum when the thermally denatured state is allowed to cool (data not shown). This means that the native and denatured states are not in equilibrium during the thermal denaturation, and therefore the thermal midpoint of denaturation will depend on the thermal scan rate. As shown in Fig. 1D, T_m varies between 66 and 74 °C as the scan rate changes from 5 to 100 °C/h, although the scan-rate sensitivity decreases with increasing scan speed. To allow direct comparison between different binary detergent mixtures, all our experiments are carried out at the same scan rate (60 °C/h). We stress that the T_m-value is an apparent T_m-value, rather than a thermodynamically well-defined parameter, and is only used for comparative purposes in the following analysis.

3.2. Unfolding of AIDA is sensitive to the composition of the mixed micelles

In order to investigate the impact of the amphiphilic environment on AIDA's stability, we have carried out a comprehensive series of thermal scans of AIDA in different micelle mixtures (cf. Fig. 2A). The anionic detergent is an alkyl sulfate of chain-length 10 (SDeS), 12 (SDS), 14 (STS) or 16 (SHS), while the non-ionic detergent is an alkyl maltoside of chain-length 8 (OM), 9 (NM), 10 (DecM), 11 (UM) or 12 (DM). In addition, we have used the non-ionic detergents OG and oPOE as well as the zwitterionic phosphatidylcholine lipids with saturated alkyls of chain length 7 (DHPC), 10 (DCPC), 12 (DLPC), 14 (DMPC) as well as the unsaturated 18:1 lipid DOPC. Of these, only DHPC forms conventional micelles, while lipids with chain

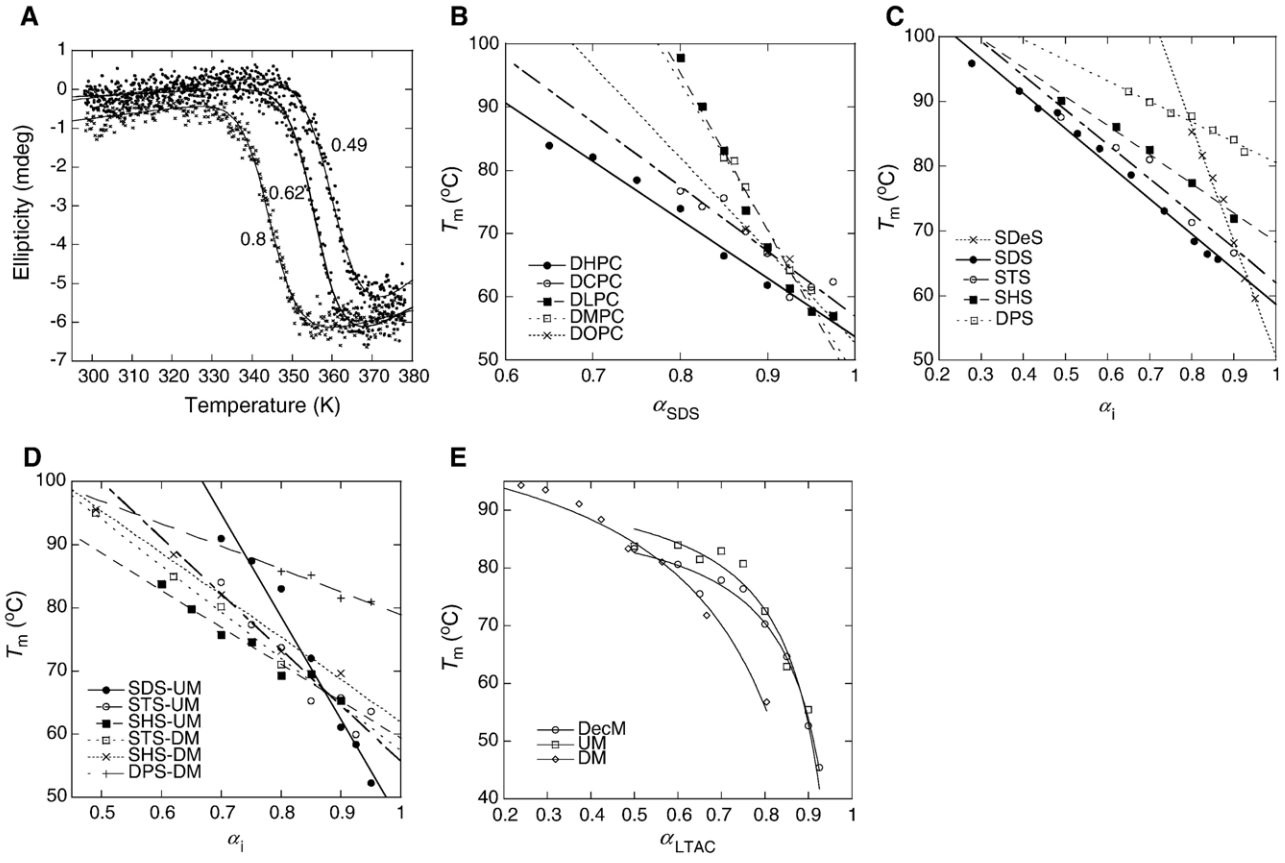

Fig. 2. Thermal Stability of AIDA using Bulk Mole Fractions. (A) Thermal unfolding of AIDA in the binary system STS-DM at an α_{STS} of 0.49, 0.62 and 0.8. (B)–(E) Plots of T_m of AIDA in different binary micelle systems versus α_i. Lines in panels B–D represent best linear fits, whereas the data in panel E are fitted to a hyperbolic binding curve. Data are summarized in Tables 1, 2.

lengths of 10 and above form membrane vesicles on their own. However, they easily incorporate into micelles when detergent molecules are present in excess [18]. We have also used the zwitterionic detergent DPS and the cationic detergent LTAC, both of which are destabilizing. For each micelle mixture, the mole fraction of the anionic surfactant (or the detergent DPS or LTAC) has been varied to give experimentally accessible T_m-values (typically between 55 and 95 °C). In all cases, we plot the T_m versus detergent bulk mole fraction, and obtain linear correlations which allow us to extrapolate to the T_m in the absence of anionic or denaturing detergent (T_m at $\alpha_i=0$) as well as T_m in micelles only containing anionic or denaturing detergent (T_m at $\alpha_i=1$). The data are summarized in Tables 1, 2, and representative data are shown in Figs. 2B–E.

We note two things in particular: Firstly, there is a great deal of variation in the values of T_m at $\alpha_i=0$ predicted for different binary detergent systems; they span from 431 °C (SDeS-UM)

Table 1
Extrapolated T_m-values for AIDA in binary ionic and non-ionic detergent systems for which the micellar composition has been determined

Non-ionic detergent (α_n)	Ionic detergent (α_i)	Experimental range (α_i)	T_m at $\alpha_i=0$	T_m at $\alpha_i=1$	T_m at $X_i=0$	T_m at $X_i=1$
OG	SDS	0.2–0.9	89.3±5.6	64.5±2.7	105±11	61.9±3.4
OM	SDS	0.5–0.9	101.3±4.6	61.7±2.0	130±11	54.6±3.7
NM	SDS	0.3–0.9	107.7±2.2	50.6±1.4	135.5±5.6	24.6±3.5
DecM	SDeS	0.875–0.975	410±39	44.2±3.5	119.5±5.8	33.5±4.7
DecM	SDS	0.6–0.9	175±13	47.4±4.9	157.0±5.8	7.2±5.2
DecM	STS	0.3–0.95	106.2±1.5	55.7±0.8	141.8±4.1	42.0±1.4
DecM	SHS	0.3–0.9	100.8±1.3	59.3±0.8	147.5±5.2	56.7±1.5
UM	SDeS	0.875–0.975	431±75	46.8±7.9	113.1±2.4	26.8±3.0
DM	SDeS	0.8–0.95	283.2±8.2	45.3±1.3	119±14	−9.6±25.9
DM	SDS	0.4–0.9	130.8±2.2	55.8±1.4	167±9.2	−105.3±20.9
DecM	LTAC	0.5–0.925	92.0±3.1[a]	–[a]	98.8±1.1	21.9±1.6
UM	LTAC	0.5–0.925	97.1±4.6[a]	–[a]	105.1±2.1	4.6±3.9
DM	LTAC	0.2–0.8	112.9±5.4[a]	–[a]	125.9±4.5	−171.9±26.7
DHPC	SDS	0.65–0.9	145.9±6.7	53.7±2.1	141.5±5.7	6.9±5.7

[a] Data are highly curved and are therefore fitted to a binding curve rather than a linear fit. This leads to a very unreliable estimate of T_m at $\alpha_i=1$.

Table 2
Extrapolated T_m-values for AIDA in binary ionic and non-ionic detergent systems for which the micellar composition has not been determined

Non-ionic detergent (α_n)	Ionic detergent (α_i)	Experimental range (α_i)	T_m at $\alpha_i=0$	T_m at $\alpha_i=1$
UM	SDS	0.7–0.95	208.2±10.9	46.1±2.4
UM	STS	0.7–0.95	144.3±9.9	55.7±2.1
UM	SHS	0.6–0.9	118.1±3.6	59.3±1.3
DM	STS	0.5–0.9	130.9±1.9	57.4±0.9
DM	SHS	0.5–0.9	128.8±3.5	61.8±1.6
DM	DPS	0.8–0.95	115±7.4	78.9±1.2
oPOE	SDeS	0.8–0.95	229.3±7.9	50.5±1.2
oPOE	SDS	0.28–0.86	112.9±1.2	58.7±0.9
oPOE	STS	0.5–0.9	115.2±5.0	62.0±2.3
oPOE	SHS	0.5–0.9	113.1±2.2	68.3±1.0
oPOE	DPS	0.65–0.925	112.2±1.7	80.6±0.5
DCPC	SDS	0.8–0.975	158.9±14.6	57.0±2.1
DLPC	SDS	0.8–0.975	293.2±17.1	45.7±2.4
DMPC	SDS	0.85–0.95	279.3±23.7	47.6±3.0
DOPC	SDS	0.875–0.975	198.7±23.3	52.7±2.1

to 89.3 °C (SDS-OG). Secondly, while most of the plots of T_m versus α_i are linear, the three alkyl maltoside-LTAC systems provide a notable exception (Fig. 2E). They all show an initial steep rise in T_m as α_{LTAC} decreases, which subsequently levels off to a plateau level that can be fitted to a simple binding isotherm. However, there is no basis to interpret these hypothetical isotherms as real binding events. As we shall see below, the introduction of micellar composition parameters leads to a significant convergence of the extrapolated values for T_m at $\alpha_i=0$ and also to a removal of the curvature in the alkyl-maltoside-LTAC systems.

3.3. Micellar mole fractions provide a better estimate of AIDA's stability than bulk mole fractions

The next step is to convert the bulk mole fractions to micellar mole fractions. By determining the cmc-values of each binary detergent system at different bulk mole fractions (cf. Fig. 3A), we have calculated the micellar mole fraction of ionic detergent X_i (see Table 3). Since all the binary mixtures in the present study are combinations of ionic and non-ionic detergents, a non-ideal behavior is expected due to significant differences between their head groups and hydrophobic tail. If the mixed cmc is lower than the ideal cmc*, the mixed micelle is stabilized by synergism between unlike monomers. These interactions results from the incorporation of non-ionic

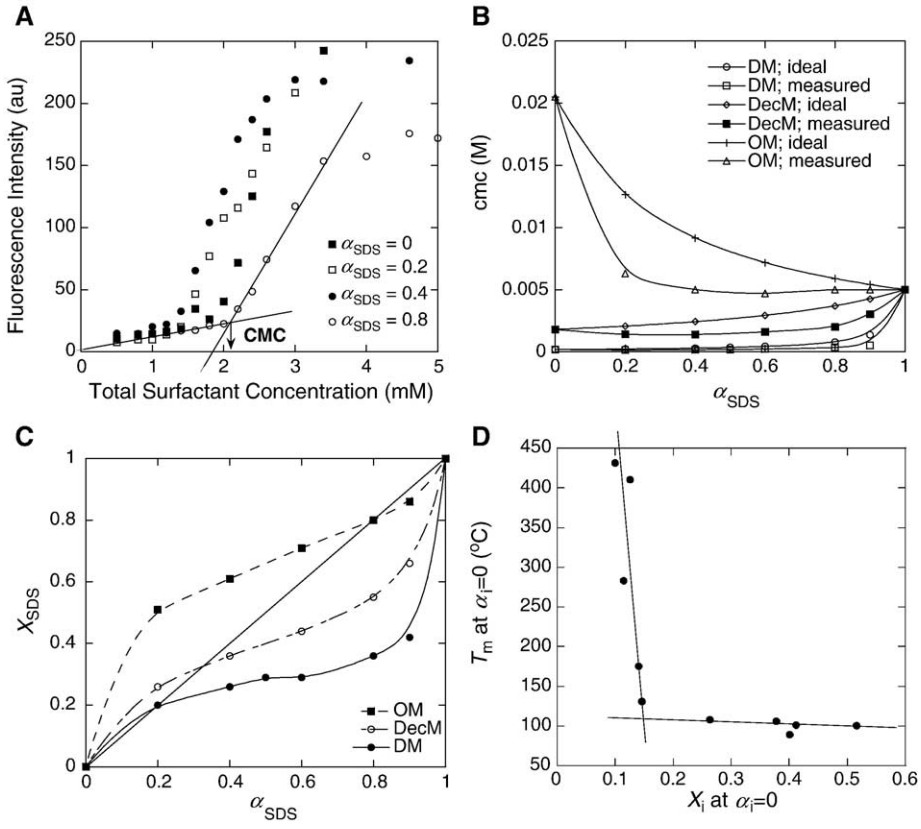

Fig. 3. Determining and using Micellar Mole Fractions. (A) Determination of cmc at different mole fractions of SDS in the mixed micelle system SDS-DecM using the fluorescent probe NPN. The cmc is determined as the break-point indicated by the arrow. (B) Ideal and measured cmc-values versus α_{SDS} in three different binary detergent systems. The measured cmc-values are for all measured detergent systems significantly below the ideal cmc-values predicted by the Clint equation (Eq. (3)). (C) Plots of micellar mole fraction X_i versus bulk mole fraction α_i reveal a complex relationship between the two parameters. (D) T_m in the absence of ionic detergent (extrapolated from plots of T_m versus α_i) versus the hypothetical micellar composition of mixed micelles (extrapolated to 0 mole fraction ionic detergent based on plots of X_i versus α_i in the bulk mole fraction range 0.2–0.8 (see (C) for an example)). This reveals that when the ionic component is predicted to be present at less than 0.1 mole fraction, the extrapolated T_m is dramatically exaggerated.

Table 3
Ideal critical micellar concentration (cmc*), experimental critical micellar concentration (cmc), micellar composition of ionic detergent (X_i), and the interaction parameter β for different ionic and non-ionic mixed systems

Mixed system	α_i	Ideal cmc* (mM)	Experimental cmc (mM)	X_i	β
SDeS-DecM	0.00	1.80	1.80	0.00	
	0.20	2.21	1.60	0.19	−4.04
	0.40	2.86	2.00	0.23	−3.25
	0.60	4.04	2.50	0.39	−3.04
	0.80	6.92	4.00	0.37	−2.57
	0.90	10.75	9.70	0.42	−0.42
	1.00	24.00	24.00	1.00	
					Average −2.66
SDS-DecM	0.00	1.80	1.80	0.00	
	0.20	2.06	1.40	0.26	−2.8
	0.40	2.42	1.38	0.36	−2.89
	0.60	2.92	1.70	0.44	−2.64
	0.80	3.69	2.00	0.55	−2.67
	0.90	4.25	3.00	0.66	−1.74
	1.00	5.00	5.00	1.00	
					Average −2.54
STS-DecM	0.00	1.80	1.80	0.00	
	0.20	1.33	0.62	0.47	−3.2
	0.40	1.05	0.52	0.57	−3.12
	0.60	0.87	0.50	0.65	−2.79
	0.80	0.75	0.50	0.75	−3.16
	0.90	0.69	0.50	0.8	−3.61
	1.00	0.65	0.65	1.00	
					Average −3.17
SHS-DecM	0.00	1.80	1.80	0.00	
	0.20	0.34	0.10	0.61	−5.86
	0.40	0.19	0.08	0.69	−5.67
	0.60	0.13	0.09	0.8	−4.24
	0.80	0.10	0.09	0.89	−3.8
	0.90	0.09	0.08	0.92	−3.43
	1.00	0.08	0.08	1.00	
					Average −4.6
SDeS-UM	0.00	0.55	0.55	0.00	
	0.20	0.68	0.57	0.12	−4.17
	0.40	0.90	0.75	0.14	−3.27
	0.60	1.33	1.10	0.16	−2.49
	0.80	2.52	1.30	0.30	−4.13
	0.90	4.56	3.20	0.31	−1.99
	1.00	24.00	24.00	1.00	
					Average −3.21
SDeS-DM	0.00	0.18	0.18	0.00	
	0.20	0.22	0.16	0.16	−6.78
	0.40	0.30	0.19	0.2	−6.47
	0.60	0.45	0.25	0.24	−6.31
	0.80	0.87	0.38	0.29	−6.21
	0.90	1.69	0.90	0.31	−4.26
	1.00	24.00	24.00	1.00	
					Average −6.00
SDS-DM	0.00	0.18	0.18	0.00	
	0.20	0.22	0.15	0.20	−5.47
	0.40	0.29	0.16	0.26	−5.49
	0.50	0.35	0.18	0.29	−5.29
	0.60	0.43	0.22	0.29	−4.75
	0.70	0.55	0.26	0.32	−4.78
	0.80	0.79	0.34	0.36	−4.61
	0.90	1.36	0.50	0.42	−4.57
	1.00	5.00	5.00	1.00	
					Average −4.99
SDS-NM	0.00	5.50	5.50	0	
	0.20	5.39	3.10	0.37	−2.75
	0.40	5.29	3.00	0.47	−2.39
	0.60	5.19	3.10	0.57	−2.31
	0.80	5.09	3.20	0.67	−2.47
	0.90	5.05	3.40	0.74	−2.8
	1.00	5.00	5.00	1	
					Average −2.54
SDS-OM	0.00	20.50	20.50	0.00	
	0.20	12.65	6.30	0.51	−2.94
	0.40	9.15	5.00	0.61	−2.77
	0.60	7.17	4.70	0.71	−2.48
	0.80	5.89	5.00	0.80	−2.38
	0.90	5.41	5.00	0.86	−2.54
	1.00	5.00	5.00	1.00	
					Average −2.62
SDS-OG	0.00	20.00	20.00	0.00	
	0.20	12.65	6.50	0.51	−2.61
	0.40	9.15	5.00	0.61	−2.77
	0.60	7.17	5.00	0.72	−2.32
	0.80	5.89	4.90	0.83	−1.97
	0.90	5.41	4.70	0.88	−2.74
	1.00	5.00	5.00	1.00	
					Average −2.48
LTAC-DecM	0.00	1.80	1.80	0.00	
	0.20	2.19	1.80	0.16	−2.78
	0.40	2.79	2.20	0.21	−2.14
	0.60	3.85	3.40	0.22	−0.89
	0.80	6.21	5.00	0.37	−0.98
	0.90	8.94	6.60	0.51	−1.32
	1.00	16.00	16.00	1.00	
					Average −1.62
LTAC-UM	0.00	0.55	0.55	0.00	
	0.20	0.68	0.50	0.17	−4.79
	0.40	0.90	0.55	0.23	−4.75
	0.60	1.31	0.95	0.23	−2.99
	0.80	2.42	1.50	0.3	−2.82
	0.90	4.20	2.80	0.36	−2.01
	1.00	16.00	16.00	1.00	
					Average −3.47
LTAC-DM	0.00	0.18	0.18	0.00	
	0.20	0.22	0.20	0.10	−4.49
	0.40	0.30	0.24	0.14	−4.25
	0.60	0.44	0.32	0.19	−4.20
	0.80	0.86	1.00	_[a]	_[a]
	0.90	1.63	1.20	0.25	−2.32
	1.00	16.00	16.00	1.00	
					Average −3.82
DHPC-SDS	0.00	0.90	0.90	0.00	
	0.65	1.93	0.63	0.43	−5.10
	0.70	2.11	0.60	0.45	−5.94
	0.85	2.97	1.00	0.51	−4.57
	0.90	3.44	1.20	0.54	−4.33
	0.95	4.07	1.46	0.60	−4.82
	1.00	5.00	5.00	1.00	
					Average −4.95

All data measured in 10 mM Tris, pH 8 at 25 °C.
[a] The programme used to calculate X_i and β did not converge to any meaningful value.

detergent monomers into the micelles, leading to a decrease in the electrostatic repulsion between the ionic head groups.

From the mixed cmc, it is possible to evaluate the interaction parameter β and micellar composition X_i using

Eqs. (4) and (5). Both sets of values are presented in Table 3. Negative and positive β-values indicate attractive and repulsive interactions between two detergents in a mixed micelle, respectively, while the β-value should be zero for ideal mixing. Over the whole mixing range, the β-values are negative, indicating favorable (attractive) interactions between non-ionic and ionic components in the mixed micelles. This is also seen by the experimentally determined cmc-values in the mixed micelle systems, which are all lower than the ideal mixed cmc (Fig. 3B, Table 3). Representative plots of X_i versus α_i are shown in Fig. 3C. Note that all measurements of X_i are carried out at 25 °C. For simplicity, we ignore the effect of temperature on X_i. We justify this by the fact that all T_m-values are used for comparative purposes only.

Now that we know the micellar composition of 14 different binary detergent systems, we can evaluate the effect on our analysis of the thermal stability of AIDA in different mixed micelle systems. Initially, we can perform a simple correlation between T_m and detergent miscibility. The latter may be determined as follows: In the range of α_i corresponding to 0.2–0.8, there is a good linear correlation between X_i and α_i (Fig. 3C), and a hypothetical value of X_i at $\alpha_i=0$ can be extrapolated from this region. A plot of T_m at $\alpha_i=0$ versus X_i at $\alpha_i=0$ reveals a remarkable hyperbolic relationship (Fig. 3D), in which X_i-values below ca. 0.15 lead to a dramatic increase in T_m at $\alpha_i=0$. This rather crude analysis reveals that micelles with a low general affinity for anionic detergents (low miscibility) have a dramatically reduced ability to destabilize AIDA. A similar plot using average β-values (from Table 3) instead of T_m at $\alpha_i=0$ also shows a steep drop in the value of β for X_i-values below ca. 0.15 (data not shown); that is, for detergent systems with strong synergy between the two components (more negative β-values), a significantly smaller fraction of the ionic detergent (compared to bulk) needs to be taken up into micelles for optimal interactions. A more thorough analysis is to plot the measured T_m-values versus X_i and extrapolate to 0 and 1 mole fraction denaturing detergent, as summarized in Table 1 and shown in Figs. 4A–D. It is clear that by converting to micellar compositions we obtain a much smaller spread in extrapolated T_m-values, indicating that this method of analysis is superior to simple bulk mole fractions. Remarkably, the curvature seen for the alkyl maltoside-LTAC systems in Fig. 2E is now completely abolished; instead, there is a simple linear relationship between T_m and X_{LTAC}. There remains a certain variation among the values for T_m at $X_i=0$ in different micellar systems. For example, for SDS in combination with alkyl maltosides, T_m at $X_i=0$ increases from around 130 °C in OM to 167 °C in DM, suggesting that longer chain lengths of the non-ionic detergent are stabilizing AIDA. In contrast, there is no clear trend for DecM in combination with alkyl sulfates of different chain lengths, where T_m at $X_i=0$ varies between ca. 120 and 160 °C. This suggests that there are some interactions

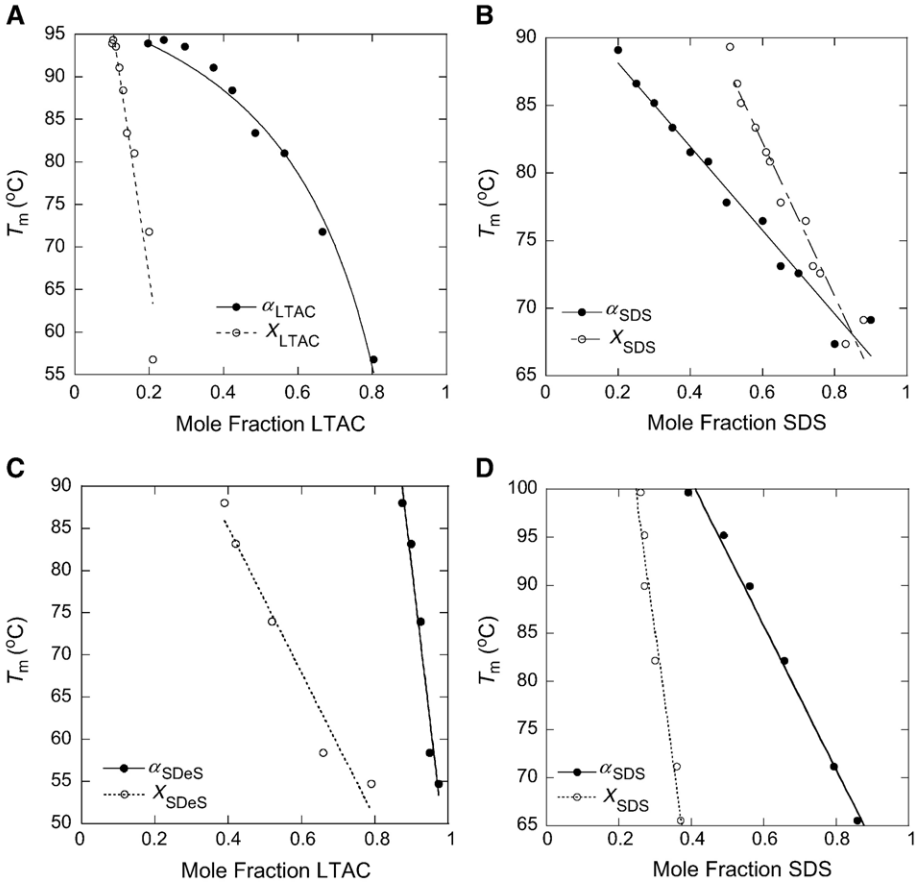

Fig. 4. Plots that reveal the difference between using bulk mole fractions and micelle mole fractions to calculate the thermal stability of AIDA in different mixed micelle systems. (A) LTAC-DM. (B) SDS-OG. (C) SDeS-DecM. (D) SDS-DM.

between micelles, which cannot fully be accounted for simply by using X_i-values rather than α_i-values.

It should also be noted that the values of T_m at $X_i=1$ become more scattered because there is now in several cases a long extrapolation to $X_i=1$. This leads to values as low as −105 °C (the SDS-DM system), and in many cases values are well below room temperature. This highlights two shortcomings of the use of X_i-values: Firstly, the sensitivity to small errors in determinations of cmc and consequent scatter in X_i; secondly, the steep rise in X_i for some mixed micelle systems as the bulk mole fraction of the ionic detergent approaches 1 (cf. Fig. 3C), which may restrict X_i to a range of low values.

In Table 2, we have included stability data for a number of binary systems for which mixed micelle compositions have not been determined. This is not experimentally feasible for the phospholipids of chain-lengths 10–18 carbon atoms, which in the absence of SDS form vesicles rather than micelles. It is clear that these lipids (in particular DLPC and DMPC) give rise to much exaggerated estimates of T_m, due most likely to a discrepancy between the bulk lipid mole fraction and micellar lipid mole fraction. This in turn suggests that the lipids are preferentially taken up into the micelles at the expense of SDS, in testimony to their excellent self-assembling properties. For oPOE in combination with a number of different anionic detergents, the values of T_m at $\alpha_i=0$ are in excellent agreement except for the short chain length detergent SDeS. This is similar to the observations in Table 1, where the combination SDeS-DecM gives a very high T_m-value (410 °C). Upon correction using X_i-values, SDeS-DecM gives the much more reasonable value of 120 °C. Further, UM gives a very high value of T_m at $\alpha_i=0$ with SDS. Thus, when one of the detergent components has a short chain length, there is likely to be a pronounced overestimate of the actual T_m-value (using α_i-values) due to deviations between α_i and X_i.

4. Discussion

We have shown that by converting detergent bulk mole fractions α_i to micellar mole fractions X_i, we are able to obtain simple linear relationships between melting temperatures and mole fractions that lead to consistent results between different systems. Thus, in general X_i appears to be a more appropriate parameter to use than α_i when describing the effect of changing mole fractions and using them to obtain T_m-values (and, for reversibly unfolding systems, free energies of unfolding) in the absence of ionic detergent (with the caveat that extrapolation to zero mole fraction non-ionic detergent may be less reliable). However, for some detergent-systems such as the SDS-DM combination, the linearity and predicted T_m at zero bulk mole fraction SDS gives a value (130 °C) that is in good agreement with the micellar composition data. Thus, previously published work on this system using the proteins diacylglycerol kinase, bacteriorhodopsin and DsbB [2,4,6] is in no way discredited. Nevertheless, we find that when the same SDS-DM system is used together with the buffer system required for kinetic studies on DsbB (25 mM sodium phosphate pH 8.0 and 100 mM NaCl), the parameters obtained from this analysis are dependent on whether X_i or α_i-values are used, although the originally proposed folding mechanism is not affected (Otzen, D.E., data not shown). This reflects the fact that micellar properties are sensitive to salt or polar solutes [19].

It seems reasonable that the micellar mole fraction is the appropriate parameter to describe the interaction between membrane proteins and the detergent environment, given that this is the local environment that the protein experiences. However, it should also be borne in mind that the mixed micelle system is not homogeneous in composition. There will be a Poisson-type distribution of populations with different mole fractions of the two components, and the measured X_i-value is merely an average [20]. In addition, micelles are not static and monodisperse, but deform, split and fuse, and may change in shape and size as detergent concentration increases [21]. Micelles are not simple spheres; in fact, their hydrophobic tails pack in a disorganized but compact fashion in the centre, forming a rough outer surface [22], so they are unlikely to be well ordered and efficiently packed in a spherical fashion around a protein. Given this complexity, it may be imagined that the protein can slightly skew the composition of the detergent micelles surrounding the hydrophobic region of the protein, depending on the relative binding affinity of different detergent types for the membrane protein surface, and the effect of binding on the optimal packing within the micelles. Different detergents will bind differently to proteins, with significant consequences for structure and function [23]. However, to our knowledge, there is no detailed structural or energetic information on the interactions between membrane proteins and mixed detergent systems; most structural studies focus on single detergent systems (cf. [24]). In the absence of other information, the experimentally determined micellar composition parameter remains the best approximation for the solvent.

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