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Review

Protein–surfactant interactions: A tale of many states

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ABSTRACT

The scientific study of protein surfactant interactions goes back more than a century, and has been put to practical uses in everything from the estimation of protein molecular weights to efficient washing powder enzymes and products for personal hygiene. After a burst of activity in the late 1960s and early 1970s that established the general principles of how charged surfactants bind to and denature proteins, the field has kept a relatively low profile until the last decade. Within this period there has been a maturation of techniques for more accurate and sophisticated analyses of protein–surfactant complexes such as calorimetry and small angle scattering techniques. In this review I provide an overview of different useful approaches to study these complexes and identify eight different issues which define central concepts in the field. (1) Are proteins denatured by monomeric surfactant molecules, micelles or both? (2) How does unfolding of proteins in surfactant compare with "proper" unfolding in chemical denaturants? Recent work has highlighted the role of shared micelles, rather than monomers, below the critical micelle concentration (cmc) in promoting both protein denaturation and formation of higher order structures. Kinetic studies have extended the experimentally accessible range of surfactant concentrations to far above the cmc, revealing numerous different modes of denaturation by ionic surfactants below and above the cmc which reflect micellar properties as much as protein unfolding pathways. Uncharged surfactants follow a completely different denaturation strategy involving synergy between monomers and micelles. The high affinity of charged surfactants for proteins means that unfolding pathways are generally different in surfactants versus chemical denaturants, although there are common traits. Other issues are as follows: (3) Are there non-denaturing roles for SDS? (4) How reversible is unfolding in SDS? (5) How do solvent conditions affect the way in which surfactants denature proteins? The last three issues compare SDS with "proper" membranes. (6) Do anionic surfactants such as SDS mimic biological membranes? (7) How do mixed micelles interact with globular proteins? (8) How can mixed micelles be used to measure the stability of membrane proteins? The growing efforts to understand the unique features of membrane proteins have encouraged the development of mixed micelles to study the equilibria and kinetics of this class of proteins, and traits which unite globular and membrane proteins have also emerged. These issues emphasise the amazing power of surfactants to both extend the protein conformational landscape and at the same time provide convenient and reversible short-cuts between the native and denatured state for otherwise obdurate membrane proteins.

1. A historical introduction

1.1. Ancient surfactants

If cleanliness is next to godliness, then there must be a strong moral dimension to the study of protein–surfactant molecules. The attention to personal hygiene using man-made soaps can be considered one of the defining traits of modern man—and not least woman. Man-made protein–surfactant interactions probably started before the dawn of history with the use of soap to remove (amongst others) proteinacious types of dirt. An inscription in ancient Babylon dating to 2200 BC describes the preparation of soap from water, alkali (from bone ashes) and oil of cassia (the "bastard cinnamon" tree) [1]. The ancient Egyptians, fond of bathing, made soaps from vegetable and animal fats combined with alkali salts [2]. Pliny the Elder mentions that tallow (Latin *sebum*, whence soap) may be combined with ashes to form soap (which he disapprovingly mentions is used by Germans and Gauls as a hair pomade by men, rather than women) [3]. Galen, the doyen of Roman medical sciences, mentions the use of lye or sodium hydroxide to make soaps [4].

1.2. Modern surfactants and the detergent industry

The industrial production of soaps is a late child of the chemical revolution of the 19th century, which only gradually evolved to the fully-fledged petrochemically-derived detergent industry of today.

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Soaps were made by adding sodium or potassium hydroxide to animal fats right up to World War I, when shortages forced Imperial Germany to develop synthetic soaps in the form of branched chain alkyl benzene sulfonates and short chain alkyl naphthalene sulfonates, which only showed moderate detergency. However, the first synthetic detergent with phosphate builder (Tide, marketed by Procter & Gamble) was not introduced until 1946. In modern washing technology terms, a *detergent* is a product formulated to promote detergency or ability to disperse soil (dirt or grease) from different surfaces. It comprises surface active agents or *surfactants* (i.e. substances preferentially absorbed at interfaces) and subsidiary constituents such as builders and boosters (to enhance surfactant performance by *e.g.* softening the water), alkali and auxiliaries such as zeolites, antideposition and anticorrosion agents [5]. The main role of surfactants is to disperse otherwise insoluble oily droplets. The most common anionic surfactants are linear alkyl benzene sulfonates and alkyl sulfates (though carboxylates and phosphates are also used), while ethoxylates are the most common nonionic surfactants [5]. The alkyl chains are usually manufactured using petroleum as primary raw material.

1.3. The modern detergent industry

Protein–surfactant interactions have been relevant in the detergent industry for a long time [6]. It was realised at an early stage that enzymes could improve detergent performance by reducing the energetically costly high temperatures and agitation that also shorten clothing life. As early as 1913, the industrial magnate and chemist Otto Rohm filed a patent (GP283923) for detergent including pancreatin, i.e. the enzyme extract from pancreas where trypsin is a major component. However, little happened in practice before 1963, when Novo Terapeutisk Laboratorium introduced Alcalase, a derivative of the bacterial protease subtilisin Carlsberg from *Bacillus licheniformis*, into detergents. Alcalase was the first industrial enzyme produced by microbial fermentation and is sufficiently SDS-resistant to be used to probe the degree of unfolding of proteins in the presence of surfactant [7]. This compatibility with detergent matrices spurred the development of enzymes in detergents. Protein–surfactant complexes help solubilise the soil components [8]. Some of the first examples of protein engineering were stimulated by the detergent industry. Subtilisin and its derivatives turned out to be inactivated by chemical oxidants which modify a Met residue near the catalytic Ser, so this had to be replaced by nonoxidisable residues [9]. In practice, the oxidising environment and metal chelators effectively bar thiol proteases and metalloproteases from playing a role in detergents. Nowadays more than half of all detergents contain enzymes, and the detergent industry is the largest single market for industrial enzymes (25–30% of all sales) [10]. In powder detergents, enzymes are usually prepared as dust-free granulates covered by protective sugars salts and coated by waxy materials and hydrophilic builders to protect them against damage by detergent components. Nevertheless, upon release they must still retain enzymatic activity in the presence of both anionic and nonionic detergents (as well as the other chemical components of detergents). The stability challenge is even greater for enzymes in liquid formulations with shelf lives of many months. In 2006, the US laundry marked had annual sales of $3 billion, with liquid detergents leading by a 2:1 margin [11]. Given that detergents contain both ionic and non-ionic surfactants which will form mixed micelles, it is also relevant to understand how these more complex surfactants affect protein structure and stability. So there is a strong practical aspect to the understanding of what drives protein inactivation or unfolding in the presence of surfactants. Companies such as Novozymes and Genencor (now part of Danisco) have built up an enormous empirical data base of mutations that affect enzyme stability and performance in surfactant solutions, though little of that is published, and few published protein engineering studies address these aspects.

1.4. Early research on surfactants

In addition to these mundane purposes, surfactants have long been used in science as practical tools. Bile salts were used to extract the photosensitive pigment protein of the eye in 1879 [12]. Amphiphiles were used 18 years later to detoxify snake venom and tetanus toxin [13,14]. Tobacco mosaic virus particles were shown in 1938 to be dissociated by SDS into constituent proteins and nucleic acid components [15]. Possibly the earliest focused report on the effect of surfactants on protein conformation by Anson in 1939 duly noted the low amounts of surfactants needed to induce denaturation of methemoglobin (followed by colour changes) and the rapidity of the reaction [16]. In 1941, Smith dissociated the chlorophyll–protein complex with sodium dodecyl sulfate [17]. In 1943 Lundgren et al. used electrophoretic mobility analysis to demonstrate denaturation of albumin by alkylsulfonates at surfactant weight fractions above 0.3 [18]. Only 2 years later, SDS was shown to bind by strong electrostatic interactions, so that the binding stoichiometry is determined by the number of cationic protein groups, forming 1:2 and 1:1 SDS:cationic complexes at low SDS concentrations [19]. Thus by the end of World War II, many important concepts were already sketched out, such as the solubilising, dissociating and denaturing properties of surfactants, the forces driving their interaction with proteins and their use in electrophoretic mobility assays. Table 1 summarises several practical applications of protein–surfactant interactions.

2. The scope of this review

Protein–surfactant interactions are an enormous topic. Excellent reviews have summarised work on protein–surfactant interactions up to 1948 [20] and 1969 [21] and have treated surfactants' multi-step binding isotherms [22] before the advent of Tanford's classic monograph from 1980 [23]. More recent reviews provided by Jones [24] and Randolph and (another) Jones [25] focus particularly on the thermodynamics of adsorption. Protein–surfactant interactions have to be studied by many different techniques to approach the full view of the structural, stoichiometric and calorimetric changes accompanying different binding stages. In this review I will start by providing some general information about surfactants and techniques to study protein–surfactant interactions. Rather than plod through a tedious enumeration of the behavior of different proteins in surfactant contexts, I have identified what I consider to be eight key issues in protein–surfactant interactions which may be addressed by comparing different proteins. The present review is by no means an exhaustive survey, but the emphasis is on proteins which have been studied by as large as coterie of complementary techniques as possible, including both equilibrium and kinetic approaches. The reader must forgive a perhaps slightly excessive focus on work from my own laboratory, which draws its start from a 2-year post-doctoral period as research chemist in the enzyme detergent industry. The rather presumptuous excuse for this focus is that the concerted multidisciplinary efforts in my group and with highly inspiring collaborators over the last decade have allowed us to provide new angles on the

Table 1
Some examples of protein–surfactant interactions.

Emulsification of soil in detergent industry and oils in personal hygiene products.
Modification of food texture, viscosity and stability in food technology using esters of polyvalent alcohols and animal/plant fatty acids [273].
Assisted-protein refolding of inactivated aggregates in inclusion bodies [274].
Solubilisation and extraction of membrane proteins.
SDS–PAGE to estimate size and purity of protein samples [74].
Purification/extraction in aqueous two-phase systems which separate into surfactant-rich and surfactant-poor phases [275,276]. Also reverse micellar systems involving a nonpolar solvent, where proteins partition into water-filled core of micelles away from hydrophobic phase [277].

structures and dynamics of surfactant–protein complexes which make this review timely. It is my hope that this approach will provide the reader with an overview of key concepts which can be used to understand *post hoc* what "really" goes on when proteins encounter surfactants.

3. General properties of surfactants

3.1. Basic concepts

All surfactant molecules are amphiphilic, that is, they contain a polar moiety and a hydrophobic moiety, typically an alkyl chain. The polar head group helps solubilise the surfactant despite the presence of the alkyl chain; nevertheless, at high enough aqueous concentrations of surfactant (the critical micelle concentration or cmc), it becomes favourable for the surfactant molecules to associate via their hydrophobic chains to form micelles with a generally hydrophobic interior and a hydrophilic water-exposed exterior. Above the cmc, the monomer concentration does not increase further (Fig. 1). Although often pictured (mostly for convenience) as symmetrical spheres, micelles are a dynamic jostling blob of rather irregularly associated surfactant molecules, according to molecular simulations (Fig. 2), though the segregation into a hydrophobic interior and hydrophilic exterior is generally maintained [26]. The cmc is the single most important characteristic of a surfactant, and can be determined by many different techniques [27]. Cmc values are important because proteins interact very differently with monomeric and micellar surfactants, so the concentration ranges for these different interactions are closely associated with (but not always entirely dictated by) the cmc. The number of surfactant molecules per micelle (the aggregation number) trails as a distant second in importance, although straightforward to determine by simple quenching experiments [28].

3.2. The cmc as a variable parameter

Two aspects of the cmc are always important to be aware of whenever comparing different protein–surfactant interactions. Firstly, the cmc (particularly for ionic surfactants) is very sensitive to ionic strength, since the increase in ionic strength reduces the electrostatic repulsion between the ionic headgroups, Therefore the cmc should be determined for the given buffer conditions used for the experiment; for SDS it is 7–8 mM in water but around 0.8–1 mM in PBS buffer [29]. Secondly, the formation of free micelles will be displaced to higher concentrations in the presence of proteins, because they sequester surfactant molecules and reduce the concentration of free monomeric surfactant. This forms the basis for the quantification of the number of bound surfactant molecules (see below). However, proteins also induce the formation of micelle-like surfactant clusters well below the cmc, and these clusters can interact with proteins in a very different way than bulk micelles.

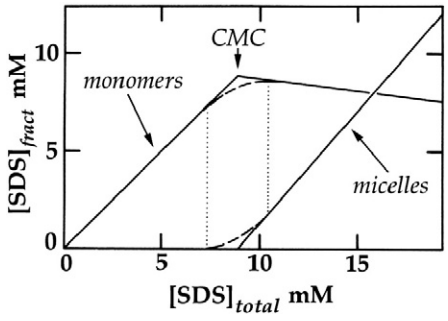

Fig. 1. Change in concentration ([SDS]fract) of monomer and micellar fractions versus the total detergent concentration with increasing SDS concentration. Micelles form at the critical micelle concentration, but this occurs in a relatively smooth transition rather than a sharp boundary, according to techniques such as pyrene fluorescence, surface tension, conductance, osmotic pressure, solubilisation ability or isothermal titration calorimetry.
Reprinted from Ref. [26] with permission.

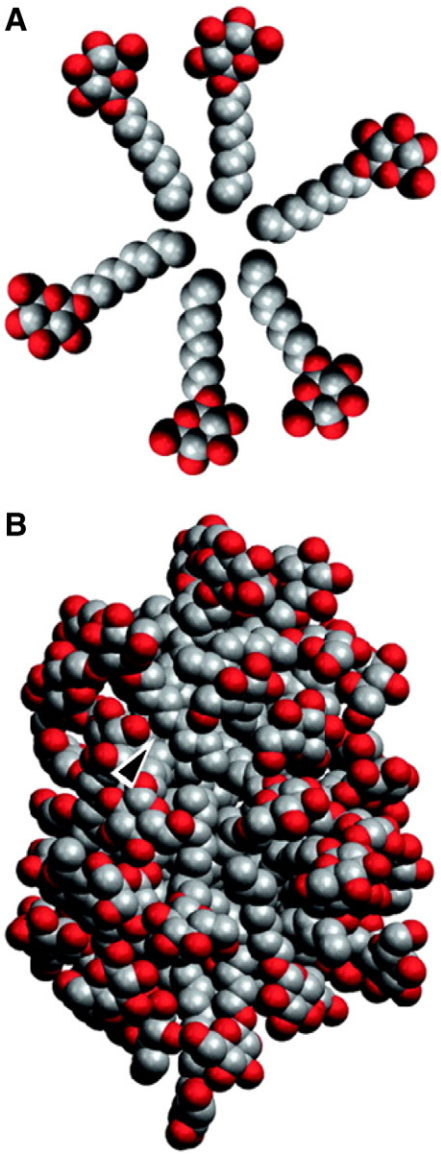

Fig. 2. Space filling models of β-D-octyl glucoside micelles. A, simple representation based on 6 monomers, showing the geometric impossibility of rigorous segregation of a hydrophilic exterior from a hydrophobic interior. B is a 50-monomer micelle derived from 40 ns molecular dynamics. Note the disorganised but compact packing, where a significant part of the hydrocarbon core is exposed to bulk solvent. The arrow head depicts alkyl chains lying along the micelle surface.
Reprinted from Ref. [26] with permission.

3.3. Surfactant classifications and denaturation potency

The most important classification of surfactants is whether their head groups are charged (and if so, anionic or cationic) or overall neutral (either because they are nonionic or zwitterionic). A more detailed description of these classes is provided in e.g. [30,31]. This classification decides whether or not the surfactants bind cooperatively to proteins and thus denature them. With few exceptions [32], neutral surfactants do not denature proteins, whereas ionic surfactants do so at very low concentrations, often well below their cmc (see below), which is typically a few mM. This makes anionic surfactants around 1000 times more efficient denaturants than traditional chemical denaturants such as urea and guanidinium chloride (GdmCl), and thus the most

potent protein denaturants known (throughout this review the term *denaturant* refers only to urea and GdmCl for simplificity). It is this enormously enhanced potency that makes the study of protein–surfactant interactions so fascinating. Clearly the surfactants must interact with proteins in a completely different way than chemical denaturants. They differ from all other ligands (except H^+) in binding to proteins in multiple equilibria to both native and denatured proteins, and unfolding is driven by the higher affinity for the denatured state(s). The formalism for this binding was developed by Reynolds and co-workers who combined the binding of SDS to the two states (measured by equilibrium dialysis, where the surfactant concentration was subsequently determined by complexing with methylene blue) with the explicit unfolding of the native state [33,34]. This leads to the well-known binding curve (Fig. 3) where early binding leads to a plateau as ionic interactions saturate [35], followed by more cooperative binding at concentrations closer to the cmc.

3.4. Oter surfactant properties

In addition to these non-covalent interactions, surfactants can also affect covalent bond formation. They catalyse the acid hydrolysis of amide and peptide bonds (mainly below pH 3.0 and above 37 °C) in a manner that is unrelated to their denaturing potency [36]. SDS has also been reported to alter the autooxidation of *G. paulistus* hemoglobin [37]. These properties are however of little interest in this context.

4. Techniques involved in the study of protein–surfactant interactions

4.1. The many stages of surfactant-binding

The strong binding affinity of ionic surfactants for proteins makes surfactants bind in several steps. All these steps are amenable to analysis by many complementary approaches that can be combined to provide a very accurate picture of the changes that occur both at the level of the protein and the complexed surfactant molecules. Anionic surfactants such as alkyl sulfates are spectroscopically silent, making them compatible with essentially all spectroscopic techniques; cationics with bromide counter ions show an often unacceptable large absorption in the far-UV spectrum, and chloride ions are

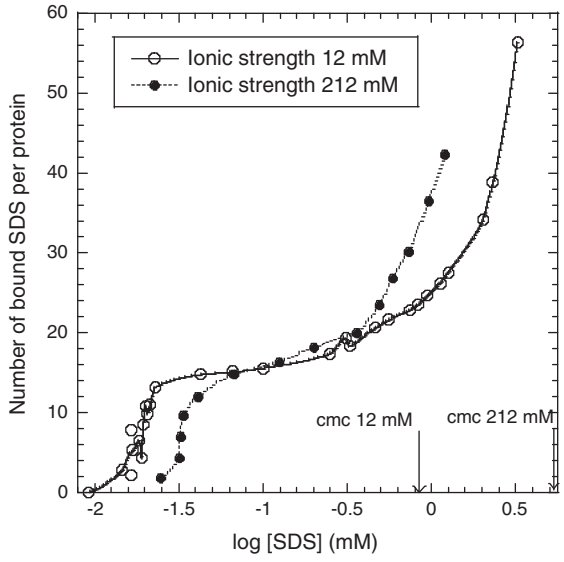

Fig. 3. Binding isotherms for binding of SDS to lysozyme at different ionic strengths at pH 3.2 (where the protein is natively folded). Note how the increase in ionic strength decreases the cmc and also reduces the affinity of the initial binding steps, effectively shrinking the accessible binding concentration range. Lines included to guide the eye. Adapted from Ref. [35].

preferable [38]. Rigorous thermodynamic treatment involving attempts to model multiple binding steps, typically based on equilibrium dialysis data, have been developed with great elegance and insight [24], but in addition to the extreme care and long equilibration times needed for these experiments, it is often difficult to extract simple conclusions from these approaches because of the complexity of binding. SDS molecules bind with different affinities at different sites, different clusters can be expected to have different levels of cooperativity and the protein unfolds in several steps with different degrees of denaturation. Instead it may be more productive to monitor the conformational changes associated with stepwise titration of surfactants into protein. This requires a combination of complementary techniques that provide information on the number, nature and mechanisms of protein conformational changes, the stoichiometry and association of bound surfactant molecules and the overall structure of the complex. The most common techniques to study protein–surfactant interactions are summarised in Table 2.

4.2. A multidisciplinary approach to surfactant–protein interactions

We have found the following "combinatorial" approach particularly powerful when analysing the mechanism of surfactant-induced denaturation of a given protein [32,39–42]. A good place to start is to measure changes in the protein's tryptophan fluorescence as a function of surfactant concentration. Due to the high sensitivity of Trp fluorescence to even small changes in its environment, this will typically report not only on changes in the protein's conformation, but also changes in polarity caused by the binding of surfactant molecules (even if this binding does not lead to conformational changes). Conformational changes can be distinguished from inconsequential surfactant binding by at least two different approaches: Firstly, far-UV and near-UV circular dichroism report on actual structural changes at the level of secondary and tertiary (aromatic) structure, respectively, but show much less sensitivity to polarity changes *per se* than Trp fluorescence does (Fig. 4A). Secondly, the kinetics of "proper" conformational changes will usually be much slower than bimolecular protein–surfactant binding reactions which occur within the 2–5 ms dead-time of typical stopped-flow machinery, so if a relaxation signal is picked up at the ms-s time scale, this is a good sign that a proper conformational change is occurring. Kinetics also provide information about the number of steps involved in denaturation (though it is generally not possible to use the same simple formalism employed for chemical denaturation, where the log of microscopic rate constants varies linearly with denaturant concentration [43–45]). Kinetics are also useful under conditions where solvent background effects need to be filtered out. This is particularly appropriate for membrane proteins where surfactant micelles need to be present at all times to maintain solubility and it is only the composition and thus the polarity of the micelles that can be varied [46–48]. In practice, early binding steps that are picked up by Trp fluorescence are found to be accompanied by conformational changes; any changes in local polarity that are caused by surfactants at this early stage usually require a cluster of surfactant molecules to form, and this is invariably tied to the process of denaturation (Fig. 4B).

4.3. Isothermal titration calorimetry to measure surfactant binding

Formation of surfactant clusters on the protein can be probed by fluorophores such as pyrene which change fluorescence upon transfer into a hydrophobic environment. This is a very important technique in view of the central role played by shared micelles in protein denaturation (Fig. 4C). However, no quantitative information is provided in this way about the number of surfactant molecules bound. Spectroscopically invisible binding of surfactant molecules at early stages of binding is seen for many proteins. We have observed it for ACBP [39], myoglobin [40], S6 [42] and α-lactalbumin [32]. This binding, as well as the other stages of surfactant binding, may be

Table 2
Techniques used to study protein–surfactant interactions.

Property	Technique	Comments	Protein concentration required	References
Protein secondary structure	Far-UV CD	Changes in backbone structure.	0.1–0.4 mg/ml in ~0.4 ml	[278] Chapter 3 [279]
Protein tertiary structure	Near-UV CD	Changes in degree of immobilisation of aromatic residues. Insensitive to changes in solvent polarity, *e.g.* caused by surfactant binding.	~1 mg/ml in 2–3 ml	[278] Chapter 4
	Trp fluorescence	Changes in Trp environment due to changes in conformation or binding of surfactant (distinguish by near-UV CD).	~0.01 mg/ml in 0.1–0.7 ml	[279,280]
	Natural ligands	Mainly for heme proteins, where Soret band at 410 nm and absorbance at 695 nm report on the local heme environment. Usually reports on same conformational changes as Trp. May be sensitive to polarity [105].	Usually 0.1–1 mg/ml	[281]
Comparison of conformational changes under different conditions	^{1}H-NMR combined with Principal Component analysis	NMR spectra collected under different experimental conditions are analysed together to provide protein folding state maps.	Very broad: from 0.1 to 10 mg/ml in ~0.6 ml	[150]
Protein flexibility	Trp anisotropy	Measures degree of Trp mobility. Proteins increase Trp mobility (and thus decrease anisotropy) upon unfolding with a small SDS cluster; subsequent binding to larger SDS micelles can decrease tumbling rate and thus increase anisotropy.	~10 times higher than Trp fluorescence (requires polarised light).	[280]
Clustering on the protein	Pyrene, Nile red, rhodamine B, dimethylaminoazobenzene (DMAB)	These compounds undergo change in fluorescence upon transfer from aqueous to hydrophobic phase such as a bulk micelle or a protein-bound surfactant cluster.	Same as Trp fluorescence (pyrene solubility limit is ~1 μM).	Pyrene [282] Nile red and rhodamine B [283], DMAB [284]
Protein compactness and surfactant binding	Capillary electrophoresis	Mobility depends on charge and hydrodynamic radius. Can be used to determine whether SDS unfolds proteins or not.	Few μl of ~1 mg/ml protein.	[39,285,286]
	Denaturing gradient gel electrophoresis	Mobility depends on charge and hydrodynamic radius. Requires gradient to be maintained while running.	~100 μl of 1 mg/ml protein.	[287]
Stoichiometry of binding	Eluent gel permeation chromatography	Column is equilibrated with a certain surfactant concentration. Injection of protein with different concentrations of surfactant will either decrease or increase the amount of surfactant eluting after the protein.	Few hundred μl of 0.5–1 mg/ml protein.	[288]
	Isothermal titration calorimetry	Binding of surfactant to proteins is associated with heat-flow, (exothermic for electrostatic interactions and endothermic for unfolding). Individual binding steps displace to higher surfactant concentrations with higher protein concentrations.	1.5 ml of protein in suitable concentration range, e.g. 0.2–2 mg/ml	[32,39–41,50]
	Equilibrium dialysis	Thermodynamically sound. Slow equilibration (several days). Surfactant concentration must be determined by reliable method. Solvent ionic strength must override charge compensation by surfactant.	Typically several mg/ml in several ml.	[135]
Shape and structure of protein-surfactant complexes	Small-angle X-ray scattering	Contrast between solvent and protein/surfactant molecules makes it possible to reconstruct average shape of protein–surfactant complexes.	0.5–10 mg/ml in 100–400 μl.	[29,39,86]
	Small-angle neutron scattering	Use deuterated surfactants or deuterated proteins to get information about either component. Major contrast between solvent D_2O and micellar hydrocarbon core. Requires access to neutron source, e.g. Swiss Pallation Source.		[51,57,84]
Mechanisms of surfactant-induced conformational changes	Kinetics and stopped-flow kinetics	Follow changes in Trp fluorescence over time when protein is mixed with surfactant. Changes in polarity usually occur within deadtime of mixing (5 ms in stopped-flow). Changes in CD signal too small and too noisy.	150 μl of a 0.1–0.5 mg/ml solution per time profile.	[38–42,65,73,112,119,152]

detected and quantified by isothermal titration calorimetry (Fig. 5). Here surfactant is titrated into a solution of protein and the heat flow associated with binding is monitored [49]. All binding processes are accompanied by an enthalpic change that can very often be measured as a heat flow, though this heat flow is also temperature-sensitive and will therefore be negligible at some temperatures [50]. Surfactant is injected into the protein solution at high concentrations (>cmc) and diluted to well below the cmc, which means that there will also be contributions from the heat of demicellisation until the final concentration of free surfactant equals or exceeds the cmc (providing an excellent probe-free method to determine the cmc). ITC titration with SDS is typically performed around 22 °C where the demicellisation process has a very low enthalpy [51]. ITC is faster, more convenient and more informative than e.g. equilibrium dialysis in this regard, since it provides the full picture of the different steps of surfactant binding, which will typically differ in terms of the magnitude and sign of the enthalpic change. This change is typically exothermic for electrostatically driven binding reactions and endothermic when binding is coupled to protein unfolding [52,53], analogous to the large endothermic unfolding peak observed for protein denaturation in differential scanning calorimetry [54].

4.4. Determining the stoichiometry of binding

Interesting as the magnitude of these enthalpic changes may be, the practical value of ITC lies elsewhere: To determine the stoichiometry of binding (surfactant molecules per protein molecule) at each step, the titration is repeated at different protein concentrations ([*P*]). Increasing protein concentrations will shift each binding step or transition to higher surfactant concentrations ($[S]_{transition}$), and this may be quantified by the simple linear relationship:

$$[S]_{transition} = [S]_{free} + N * [P] \quad (1)$$

where $[S]_{free}$ is the concentration of unbound surfactant and *N* is the number of surfactant molecules bound per protein molecule at a given transition. ITC can monitor binding reactions right up to the stage where all binding sites on the protein are saturated; this occurs when

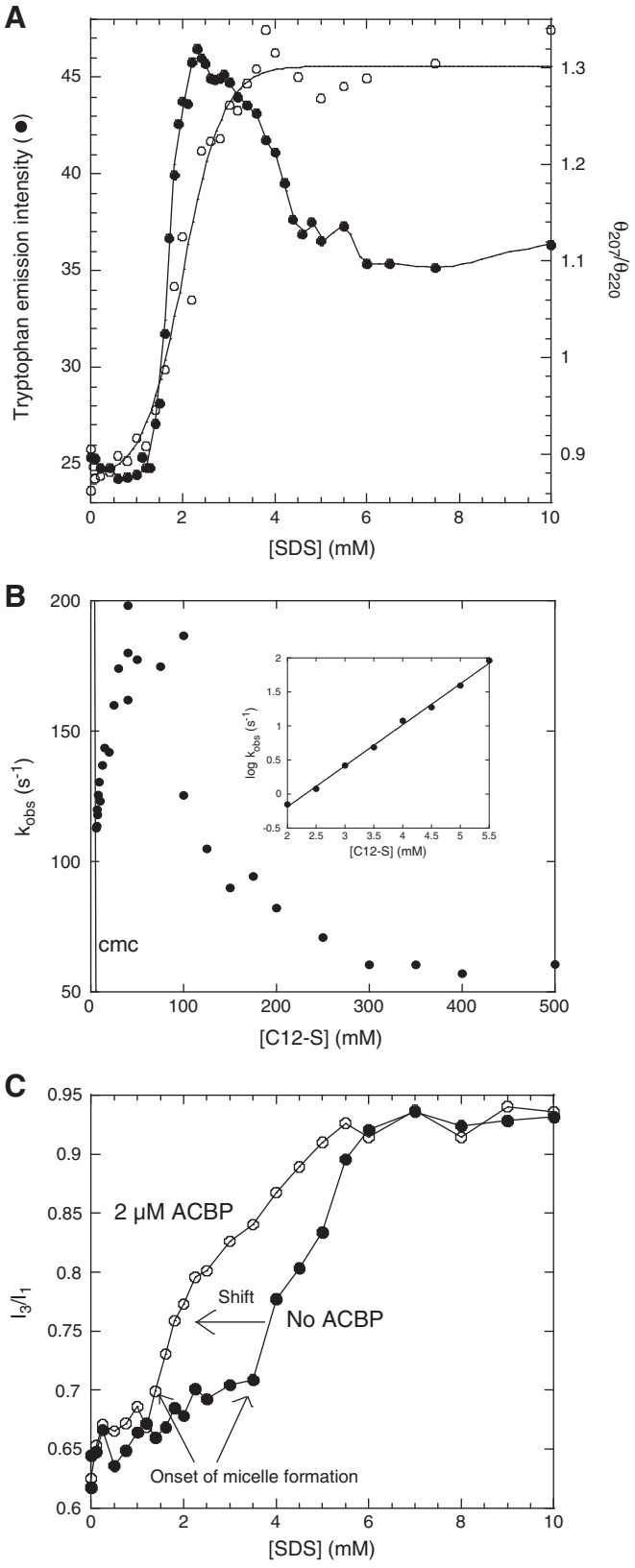

Fig. 4. Examples of spectroscopic analysis of protein–surfactant interactions, using the protein ACBP and SDS. (A) Changes in Trp fluorescence (emission intensity at 345 nm) and far-UV circular dichroism (ratio of ellipticity at 207 and 220 nm) follow in parallel. Lines provided to guide the eye. (B) Kinetics of unfolding of ACBP. Data follow a single exponential decay with a rate constant k_{obs}. Insert shows the linear increase in log of k_{obs} versus [SDS] at low SDS concentrations. (C) Pyrene fluorescence used to monitor formation of SDS micelles. This occurs earlier in the presence of protein.
Data in panels A and C from Ref. [39], data in panel B from Ref. [65].

bulk micelles start to form since $[S]_{free}$ has reached the cmc. Given that most proteins bind around 1.4 g SDS per gram protein [55], the cmc will be shifted well above that which is observed in the absence of protein [56].

4.5. Small angle scattering techniques to determine complex shapes

These stoichiometry values are essential in the last stage of the binding-and-unfolding analysis, namely the structure of the protein–surfactant complexes. Such complexes defy all attempts to crystallise them because of their inherent flexibility and sampling of different conformations, ruling out X-ray crystallography; NMR is also out of bounds because of the complexes' large size and multiple conformations. However, small-angle scattering techniques are ideal for this type of analysis, in particular Small Angle X-ray Scattering (SAXS), which can be set up using small-scale in-house X-ray sources such as rotating anodes, rather than the full-blown neutron source required for Small-Angle Neutron Scattering (SANS). Traditionally, SAXS and SANS have been used to study the overall size and shape of species in solution, providing information on the average micelle size and its aggregation number, the fractal dimension (which relates end-to-end length of chain to number of amino acid residues), the correlation length giving the extent of the unfolded polypeptide chains, and the numbers of micelle-like clusters in the complex [57,58]. However, recent developments in *ab initio* (typically Monte Carlo based) modeling have made it possible to construct relatively detailed albeit low-resolution structures of macromolecular complexes (for reviews see [59–61]). Contributions from proteins and surfactants can to some extent be distinguished because the alkyl chains scatter less than the slightly heavier and more electron-rich protein atoms and also less than the solvent. Although increasingly robust, this approach still relies on constraints provided by complementary techniques. In the first step, an indirect Fourier transformation [62] provides model-independent information about the overall size and general features of the sample. In the second modeling-based step, knowledge about the stoichiometry of protein: surfactant complexes, as provided by ITC, can guide model building. This was instrumental in determining the dimerisation of ACBP at low surfactant:protein ratios [39] by eliminating alternative structures.

5. An overview of the different binding steps in surfactant–protein interactions

For water-soluble proteins, interactions with surfactants can broadly be split up into two regions: below and above the cmc. The situation is more complicated for membrane proteins which have an absolute requirement for a contiguous amphiphilic region to shield their hydrophobic transmembrane regions, and therefore aggregate below the cmc. Interesting phenomena arise just around the cmc for β-barrel membrane proteins, see Issue 8. The following discussion relates only to water-soluble proteins.

Below the cmc, water-soluble proteins undergo a series of conformational changes as it binds to increasing numbers of ionic surfactants (In contrast, there are only very weak interactions with non-ionic surfactants in this concentration range). Bhuyan's conclusion that "tertiary structure unfolding in the submicellar and chain expansion in the micellar range of SDS concentrations are the two major and discrete events in the perturbation of protein structure" [63] sums it up well to a first approximation.

5.1. Binding as monomers, shared micelles and full micelles

Binding of ionic surfactants is complex and proceeds in many steps. Often there is a "baseline region" at very low surfactant proteins where surfactants bind without eliciting conformational changes. The first binding steps involve individual monomers binding via electrostatic and hydrophobic interactions [24]. For anionic surfactants, this involves

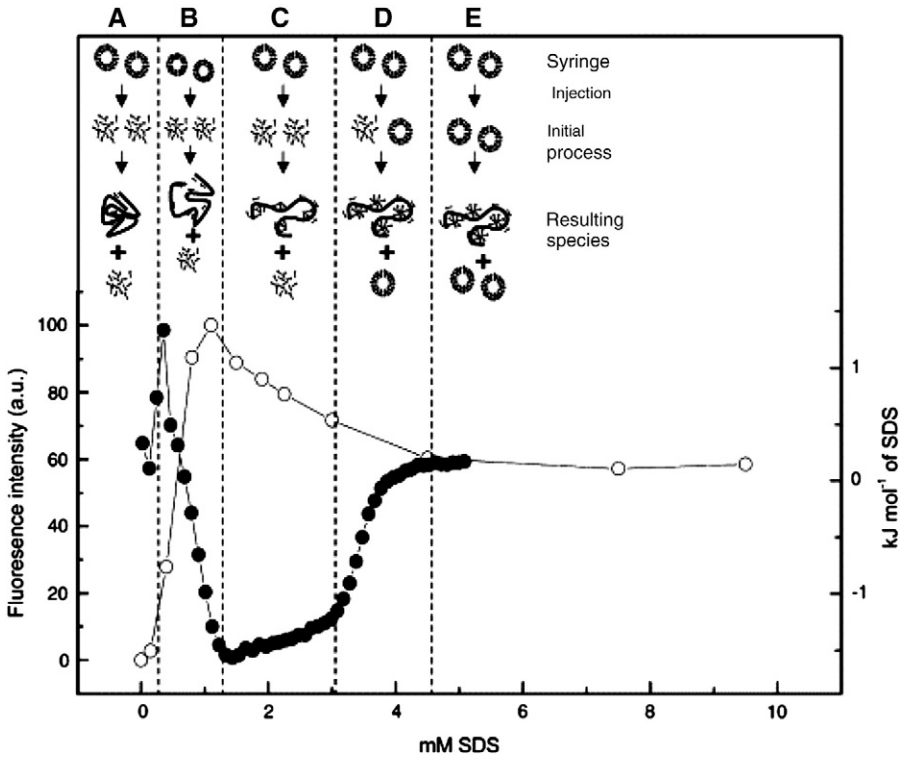

Fig. 5. Juxtaposition of the change in fluorescence (empty circles) and ITC enthalpogram (filled circles) describing the interaction between SDS and *H. insolens* cutinase. The enthalpogram is based on a single titration series. Each ITC point denotes the total enthalpy change associated with injection of a single aliquot of SDS leading to the cumulative SDS concentration described on the x-axis. The stippled lines mark each region, defined at each boundary by a characteristic transition in the enthalpogram. These transitions agree well with the fluorescence change. Reprinted from Ref. [50] with permission.

cationic side chains (Lys, Arg, His) while cationic surfactants bind to complementary anionic side chains (Glu, Asp). In both cases, the alkyl chains will bind to nearby hydrophobic patches [64]. At higher concentrations when the initial binding sites are saturated, binding of more surfactants can lead to clusters that start to unfold the protein. Such clusters are often missed in simple binding isotherms but nevertheless play an absolutely critical role in the properties of protein–surfactant complexes at low surfactant:protein ratios. These clusters may be stabilised by the association of several protein molecules [39], leading to small protein complexes driven by the formation of shared micelles. These shared clusters only form in a relatively small concentration window, however. Higher (but still sub-cmc) surfactant concentrations provide sufficient surfactant to allow each protein to form a cluster on its own. The critical issue in this sub-cmc concentration range is not so much absolute surfactant concentration as the ratio between protein and surfactant, as this will determine how much surfactant as available per protein molecule. In this regime, there is typically a linear increase in the logarithm of the unfolding rate constant versus surfactant concentration, similar to plots of log unfolding rates versus chemical denaturants [43]. Thus at this stage the surfactant behaves like a classical denaturant, though many orders of magnitude stronger than chemical denaturants. In some cases, such as ACBP [65], but not in others such as S6 [42], the kinetics and equilibria of this unfolding step are sensitive to mutagenesis in the same way as classical unfolding in chemical denaturants. The rate of unfolding tends to level off around the cmc. The protein concentration is usually so low for the spectroscopic techniques used here that the bulk cmc is not affected by the small proportion of surfactant molecules sequestered by the protein.

5.2. Surfactants as precipitants

Monomer binding can also have pronounced effects on protein solubility. It has been conjectured that proteins below their isoelectric point behave as cationic polymers, and can therefore be precipitated by anionic surfactants at relatively low surfactant:protein ratios through simple charge neutralisation [66–68], whereas the complexes are fully soluble above the pI and can therefore change topology and conformation [69]. The reality is a little more complex. Submicellar SDS can in fact promote protein aggregation well above the proteins' pI [70]. This probably occurs through the formation of shared micelles as described below and in a recent review [71]. Furthermore, although charge neutralisation is indeed a prominent factor in many cases, we have shown that numerous proteins with different pI values share a tendency to precipitate at sub-cmc concentrations around pH 5 and below [42]. This includes lysozyme, which has a pI around 11. We ascribe this to the neutralization of the negatively charged Asp and Glu side chains which would otherwise repel SDS from binding [38]. Although these side chains titrate around pH 3.5–4 in water, the presence of a hydrophobic environment and the increase in local pH through the anionic SDS environment increases the pK_a by up to 2 units [38]. These phenomena require a degree of clustering of SDS to create a quasi-micellar environment, and this can presumably drive protein association through shared micelles. In contrast, monomeric SDS can prevent aggregation by binding in low numbers to exposed hydrophobic patches of dissociated proteins such as the normally tetrameric strepatividin [72].

5.3. Micellar interactions are best analysed by kinetics

Above the cmc, it becomes very difficult to follow changes in the equilibrium structures of the protein–surfactant complexes. Scattering techniques cannot filter out background contributions from "empty" micelles, there are no major rearrangements in the protein conformation at secondary and tertiary levels according to spectroscopic techniques and it is not possible to discern additional binding events by calorimetry as the binding sites appear to be generally saturated at the typical Tanford ratio of 1.2 g SDS/g protein. The only technique that can provide additional information about changes in

protein–surfactant interactions in this concentration range is kinetics, i.e. the rate at which structural changes occur when proteins are mixed with surfactants. Kinetics can provide insight about the way in which the micelles bind to and denature proteins, e.g. the number of steps involved (though it can be very difficult to decide whether several exponential decays indicate sequential unfolding steps or parallel unfolding pathways) and how they are affected by variables such as pH, salt, temperature, intrinsic protein stability (probed through protein engineering) and changes in micellar properties such as shape. In practice a great diversity of behaviour is observed. Above the cmc, the rate of unfolding may decline, particularly for cationic surfactants [38], but also for anionic surfactants, typically at low salt concentrations [38]. In other cases, it remains at a plateau which is sensitive to mutagenesis [73], but in a different way than for chemical denaturants. This provides an opportunity to reconstruct a picture of the unfolding mechanism and identify the initial sites of attack by SDS micelles [73]. Finally, at very high (several hundred mM) concentrations of surfactant, the rate of unfolding in some cases [38,73] increases steeply, leading to a power-law relationship between refolding rates and surfactant concentration.

6. Structures of protein–surfactant complexes: anything goes?

6.1. SDS–protein complexes as rod-like structures

The structure formed by proteins in complex with SDS under micellar conditions has been the subject of many different studies. There is a practical angle to this: SDS–PAGE is the most wide-spread application of protein–surfactant interactions, and the analysis is based on the sieving of the protein–SDS complex in the acrylamide matrix [74]. Obviously this will depend on the overall hydrodynamic properties of the complex and thus its shape. When keeping the protein:SDS weight ratio constant at 1:1.4, there is good linearity between the log of the protein molecular weight and the log of the protein Stokes radius on calibrated gel filtration columns [75]. Such a relationship is also seen using protein denatured in 6M guanidinium chloride (GdmCl) [75], though the relationship differs in SDS and GdmCl because of the different shapes of the complexes in the two solvents. Based on the seminal work by Reynolds and Tanford who measured intrinsic viscosities [23,76], the shape in SDS is generally depicted as a "rod-like" prolate ellipsoidal protein–surfactant aggregate. The dimensions of the complex reflect both protein and surfactant properties. The length, which will define its migration speed in the polyacrylamide gel matrix, depends on the protein molecular weight, and the width of ~18 Å corresponds to the SDS alkyl chain length. The strength of the model is that it provides a structural explanation for the predictable size-dependence of protein migration by SDS–PAGE. It does not try to resolve the complex structure in more detail, and so neatly straddles at least two possibilities that exist for such a structural rearrangement. In principle the extended protein could wrap around the micelles (the decorated micelle model) or the micelles could form on different parts of the extended protein (the pearl necklace or necklace-and-beads model). This has been discussed in more detail by Jones [24], and a brief summary must suffice.

6.2. Decorated micelles versus necklace and beads

The decorated micelle model [58,77–79] is supported by results from viscometry [56,80], dynamic light scattering [81], NMR [82] and fluorescence spectroscopy [78] and is similar to the complexes formed between surfactants and polymers [77]. It has also received detailed structural support from SANS [83–85] which at concentrations well below saturation of binding (corresponding to 1 SDS per 2 residues) identified 3 well-separated micelles on the chain of the 452-residue N-5′-phosphoribosyl anthranilate isomerase. At even lower concentrations (1 SDS per 4 residues), two micelles coalesce, leading to a two-micelle complex. However, the same SANS technique has been used to model the complex between BSA and lithium dodecyl sulfate as globular micelles randomly decorating the polypeptide backbone [57,58], i.e. necklace-and-beads. The BSA–SDS complex has also been modeled as a necklace-and-beads complex by SAXS [86]. Indirect evidence for this model has been provided, albeit much more speculatively, by Ferguson plots of complex mobility in gels of different acrylamide concentration [87].

6.3. One size does not fit all: different complexes in different conditions

However, rather than try to rigorously eliminate or exalt any particular model, it may be more instructive to bear in mind that protein–surfactant interactions are too diverse to be simply accommodated in one model. As we will discuss in more detail below, the structures and energetic of protein–surfactant complexes will depend on a whole host of factors. The protein sequence (including hydrophobicity, charge and propensity for different types of secondary structure which will be more or less compatible with a micellar environment) will determine the nature of the binding sites for surfactant in conjunction with the type of the surfactant (head group charge, length of alkyl chain) and the ratio between protein and surfactant. Solvent conditions such as pH, ionic strength and temperature will also affect the extent of electrostatic attractions and the stability and shape of micellar structures. The pearl-and-necklace model features electrostatic repulsion between individual (well exposed) micelles as a driving force for protein denaturation, whereas the decorated micelle has the micellar charges more or less sequestered by the protein. In practice the distinction may be subtle, because the degree of electrostatic compensation from cationic side chains may be similar. The same protein can indulge in different types of complex formation depending on conditions. This is truly a movable feast.

6.4. Examples of the same protein forming different complexes

For N-5′-phosphoribosyl anthranilate isomerase (Fig. 6), halving the surfactant:protein ratio led to a coalescence of two of the three micelles [85]. By increasing the ratio to make it approach conditions where free micelles can form in solution, rather than requiring proteins to stabilise the micelles, it is quite conceivable that the complex could change from a pearl-necklace form to a decorated micelle complex. For ACBP, we observed both a shared micelle stabilised by two protein chains and a one-protein-per-micelle structure depending on the SDS:ACBP ratio [39]. Changes in the stoichiometry also alter the nature of the complex formed between α-synuclein and SDS [29]. For the natively disordered 140-residue protein α-synuclein, shared micelles between up to 4 different protein molecules provide the nucleus for a polymeric "beads-on-a-

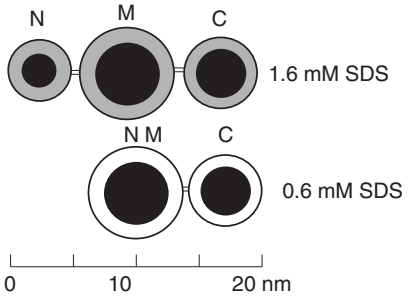

Fig. 6. Structure of N-5′-phosphoribosyl anthranilate isomerase in complex with SDS at 1.6 and 0.6 mM SDS, based on SAXS data. Three independent micelles (N, M and C) are connected by flexible 5–6 residue linkers, but coalesce to two micelles at lower SDS concentrations. SAXS also detects a difference in contrast between the dodecyl chain core (black) and surrounding protein/sulfate shell (white or gray).
Redrawn from Ref. [85].

string" species rich in amyloid structure [29]. For larger proteins such as the 452-residue N-5′-phosphoribosyl anthranilate isomerase, the length of the polypeptide chain means that there will be enough binding sites on one polypeptide chain to allow micelles to form by linking up different parts of the protein [85]. Thus, for larger proteins, shared micelles do not necessarily stimulate aggregation but can lead to intramolecular reorganisation as the surfactant:protein ratio changes. I have treated the role of shared micelles in sub-micellar surfactant-stimulated protein association more extensively in a recent review [71]. Shared micelle-driven protein association has not been reported for other protein surfactant complexes as yet, but the growing use of SAXS in this field will likely remedy the situation.

6.5. Future research on protein–surfactant complex structures

Although structural analyses of denatured protein–surfactant complexes may seem "old hat", wizened by decades of debate which have not yielded any truly universal structural model, the kinetic approaches briefly mentioned above and discussed in more detail below, reveal many different structural transitions at high micelle concentrations. There is more than ever a need to understand the dynamics and dimensions of SDS-denatured membrane proteins, since the use of SDS in mixed micelles with non-ionic surfactants is turning out to be an excellent way to determine membrane protein stability (see Issue 8). One of the interesting challenges of the future will be to develop medium-resolution approaches to further explore protein conformations in this high surfactant concentration range. While SAXS is limited by the large background contributions from micelles (which will be dominated by micelles free from proteins), SANS is able to differentiate between deuterated and non-deuterated species, given that deuterated species will lead to much more scattering [88]. The use of non-deuterated proteins in deuterated surfactants may eventually allow SANS to shed light on the overall protein shape at these concentrations.

7. Issue 1: are proteins denatured by monomeric surfactant molecules, micelles or both?

7.1. Stage A: binding of monomers to the native state as stabilising ligands

The very ability of surfactants to form higher order structures in the form of micelles suggests that this self-assembling structure should play a large role in surfactant-induced protein denaturation. Near or above the cmc (typically at ratios of at least 100 surfactant molecules per protein), cooperative binding of surfactant molecules disrupts the native structure and compromises enzyme activity [73,78,89,90]. However, not least because it is easiest to analyse protein–surfactant interactions below the cmc, there has been a great deal of attention towards this region of the concentration range, and this naturally highlights the role of the monomer surfactant molecule. In fact a review [91] celebrates the monomer by concluding that "Little further binding is observed upon increasing the free detergent concentration beyond the cmc [55,92,93]." While the reality is more complex, it is true that ionic surfactants bind as monomers with very high affinity to protein molecules. The very early binding steps rely on specific interactions with the native state [64], so that the surfactant molecules under those conditions assume the role of a conventional ligand that by simple linkage relationships will stabilise proteins against denaturation, as seen for BSA [94,95]. The longer the chain length, the greater the degree of binding (provided there are appropriate binding sites on the protein) and the greater the stabilisation [96]. BSA is in a class of its own due to its large size and natural "vacuum-cleaner" role as plasma transporter of small amphiphilic or hydrophobic molecules. Twelve binding sites for sulfonate half esters have been identified in the native state of BSA [97–99], so the surfactants are stabilising at BSA:surfactant ratios up to around 1:12. This balance will tip at higher stoichiometries, where unfolding gradually becomes more favourable as the unfolded state has more binding sites. Other proteins, while they may have a built-in binding capacity for small amphiphiles, only tolerate a smaller number of surfactant molecules. β-lactoglobulin [96] and Bet v 1 [100] have one binding site in the native state which for β-lactoglobulin increases thermal stability at low surfactant concentrations [101]. ACBP, which has a binding site for acyl-modified CoA, also binds 1–3 SDS molecules without structural changes [39] and S6 and α-lactalbumin can bind 8 and 3–4 SDS molecules, respectively, with a similar lack of consequences [32,42]. Nevertheless, stabilising binding to the native state is not a universal phenomenon. Myoglobin is monotonically destabilised by increasing SDS concentrations, even at 1:1 ratios [40].

7.2. Stage B: cooperative binding of surfactants leads to (shared) micelles and protein denaturation

After this stabilising and usually spectroscopically silent binding stage, additional binding of anywhere between 4 and 16 SDS molecules is associated with cluster formation and denaturation at the level of both secondary and tertiary structure.

7.2.1. Two stages of binding and unfolding

Anionic surfactants can aggregate to form clusters on polymers above a certain concentration of surfactant [102–104], leading to an apparent lowering of the cmc [52]. The use of ITC to quantify the number of SDS molecules involved in clustering has been carried out for many proteins, including cutinase [50], S6 [42], α-lactalbumin [32], ACBP [39], myoglobin [40], TII27 [41] and β-lactoglobulin (J.G. Hansted and D.E.O, data not shown). In some cases, this unfolding occurs in two stages. The first stage is usually coupled to an endothermic transition in the ITC profile, indicating actual loss of protein structure rather than the complementary electrostatic binding typical of earlier stages in binding. The second stage does not involve a change in secondary structure but only in the Trp environment, usually a decline in the emission intensity, leading to the appearance of an overshoot in the titration profile. This second stage is coupled to the additional uptake of a substantial number of extra SDS molecules (e.g. 26 for ACBP [39], 24 for S6 [42] and 16 for myoglobin [40]) and a reduction in ACBP mobility by anisotropy [39]. Cyt *c* also undergoes two conformational transitions [105,106] which both involve the update of ~20 SDS molecules [106,107], though this has not been analysed by calorimetry. Another heme-binding protein, myoglobin, shows considerably more complex unfolding transitions than proteins without covalent co-factors. There is no endothermic peak for unfolding, and it is not possible to determine the stoichiometry of binding around the stage where clustering occurs. This may reflect the contribution of heme-surfactant interactions.

7.2.2. Shared micelles and protein association

The two general stages in SDS-induced denaturation have been analysed in particular detail for ACBP [39]. At the end of the first stage, 16 SDS molecules are bound per ACBP. Remarkably, dimerisation of ACBP leads to the formation of a shared micelle involving (according to SAXS estimates) ~38 SDS molecules. Thus micelle formation in this context drives protein association and concomitant unfolding. This work also demonstrates that we may have to revise preconceived (though not always fully articulated) notions about cooperative binding of surfactants to proteins: SAXS data for both ACBP and α-synuclein (see below) indicate that the SDS clusters are not just small "hemimicellar" aggregates consisting of a dozen or so surfactant molecules on the protein surface, but proper quasi-spherical micelles of a size approaching those in solution. Interestingly, only part of ACBP is directly associated with the micelle; at least half of the protein is modelled to form a disordered structure which extends away from the

protein (Fig. 7). Only the additional uptake of around 26 more SDS molecules per ACBP in the second stage provides enough SDS molecules to allow one micelle per ACBP [39]. The last stage in titration measurable under equilibrium conditions is the formation of bulk micelles in solution. These micelles probably confer additional rearrangements, although they generally do not lead to spectroscopic changes for the protein, which is now fully exposed to the micellar environment.

7.2.3. Shared micelles are often but not always the driving force for unfolding

Protein unfolding is intimately coupled to formation of clusters which in most cases are shared micelles. Independent documentation is provided by the lipase TlL which is activated by sub-cmc concentrations of anionic surfactants (see below), which bind *without* forming clusters (H. Wang and D.E.O, data not shown). TlL does not denature at pH 8; only when the pH is lowered and SDS can bind with higher affinity, do clusters form and the protein actually unfolds. Thus the question of whether monomers or micelles unfolds proteins cannot be answered with a simple yes or no. Unfolding typically occurs already below the cmc (thus there are no free micelles but only monomers in solution), *but* requires the formation of micelles on the protein. Not all proteins unfold below the cmc, however. A nice twist is the comparison of SDS-induced denaturation of two structurally similar β-sheet proteins, Tnfn3 and TII27 [41]. TII27 is unfolded by SDS with a midpoint of ~3 mM, well below the cmc of 5 mM; in contrast, Tnfn3 only unfolds around the cmc. For TII27 there is cluster formation in the sub-cmc region and an early exothermic binding event by ITC, but for Tnfn3 cluster formation and stoichiometric binding only occurs at the cmc. A map of the electrostatic potential of these two proteins (Fig. 8) identifies several positive patches with surrounding hydrophobic areas on TII27 but hardly any on Tnfn3 [41]. Clearly micelles or clusters are required to denature both proteins, but for Tnfn3 the lack of monomeric binding sites prevents the protein from "kick-starting" the clustering and associated denaturation process before ready-made micelles are available in solution. Further confirmation of the driving force of micelles is provided by using mixed micelles: If SDS monomers are "mopped up" from solution by providing micelles of dodecyl maltoside (which has a ~40-fold lower cmc than SDS; the cmc remains around 0.2–0.3 mM even in up to 75% cmc [41]), we reduce the SDS monomer concentration well below the threshold required for unfolding of TII27, so the only driving force for unfolding is provided by micelles. Using micelles containing 75% SDS, TII27 still unfolds at significantly lower (though super-cmc) concentrations than Tnfn3. This difference can be rationalised by the increased electrostatic attraction of whole micelles rather than clusters of monomeric surfactant molecules [41]. Nevertheless, the use of micelles containing 75% SDS and 25% DDM leads to a four-fold increase in the absolute concentration of SDS required to unfold TII27 [41], indicating that micelles are much *less* potent denaturants than sub-cmc clusters.

7.2.4. Uncharged surfactants as chaperones

A different angle on the discussion of monomer-versus-micelle is provided by uncharged surfactants. Given that electrostatics is the most important driving force for binding of monomeric and sub-cmc ionic surfactant molecules to the protein surface, it is no surprise that uncharged surfactants have little impact below the cmc. When non-ionic surfactants do have an impact on protein stability, it is mostly due to their chaperoning ability to prevent protein aggregation [108,109]. Although the non-ionic surfactant Tween 40 binds to the native state of the human growth hormone (hGH), it shows a particularly high affinity for the molten globule state of hGH, binding to the contiguous hydrophobic regions exposed in this state and in this way inhibiting the (essentially irreversible) aggregation that otherwise leads to an apparent destabilisation of the protein [108,110]. Other proteins such as IgG are not stabilised by Tween 40 and interferon-γ is actually slightly destabilised [111].

7.2.5. Cutinase destabilisation by monomers below the cmc

More detailed aspects of non-ionic surfactant interactions with proteins have been revealed by cutinase and α-lactalbumin. At concentrations above their cmc, zwitterionic surfactants such as short-chain phospholipids strongly retard the refolding of cutinase from the chemically denatured state, trapping the protein in an inactive state which only regains activity over hours to days [112]. Remarkably, the thermal stability of cutinase is reduced by these surfactants well *below* the cmc. The destabilisation decreases with increasing chain length, which sounds counterintuitive unless one takes into account that increasing chain length reduces the cmc and thus lowers the concentration of monomer. There is no cluster formation below the cmc (consistent with these surfactants' relatively low affinity for proteins), indicating that the monomer is actively involved. We have proposed a destabilisation model [112], in which monomers bind to various sites on the native state (some of which have been identified around the active site where amphiphilic substrates bind), and thus prime it for interaction with micelles in a destabilising fashion. In contrast, only the micelles halt the refolding process, and we believe this to be due to the absence of such (presumably contiguously hydrophobic) monomer-binding sites in the denatured state.

7.2.6. The combined micelle-monomer strategy of uncharged surfactants: α-lactalbumin unfolding by monomers and micelles

This monomer–micelle cooperation model has been vindicated by the unfolding of the apo-form of α-lactalbumin, which unlike cutinase is sensitive enough to be unfolded by both zwitterionic and non-ionic surfactants [32]. ITC detects specific binding of surfactants above but not below the cmc, and denaturation only sets in around the cmc (Fig. 9). Kinetics provide a much clearer view of the mechanism. Low levels of (incomplete) unfolding can be detected below the cmc, but around the cmc the unfolding rates increase remarkably. The end-plateau level of unfolding decreases with increasing chain length for uncharged surfactants but remains the same for ionic (both anionic and cationic) surfactants [32]. These data are consistent with a model in which uncharged monomers cooperate with micelles to denature the protein, although the monomers cannot by themselves affect significant unfolding below cmc. In this way a fundamental difference is revealed between the unfolding strategies adopted by charged versus uncharged surfactants: charged surfactants bind either in the monomeric or the micellar form and the concentration of monomer does not affect the efficiency of micellar denaturation, whereas an intimate collaboration between monomer and micelle forms of nonionic surfactants promotes binding and denaturation (Fig. 10).

7.2.7. Ionisable surfactants and pH effects

An additional level of complexity is introduced if the surfactant changes ionisation over a pH range relevant for the protein. In this case, small changes in pH can have profound effects on protein–surfactant interactions. Lack of space prohibits detailed treatment of this topic, but an excellent example is provided by lauryldimethylamino-N-oxide, which at pH values slightly above its pK_a of ~5.8 induces microscopic phase separation when complexed with the bacterial photosynthetic Reaction Center [113]. There is most likely a mutual charge neutralisation which leads to reduced electrostatic solubility and formation of stable microemulsions.

7.3. Stage C: the transition to micelles as revealed by kinetics

7.3.1. Log-linear relationships between unfolding rate constants and sub-cmc surfactant concentration

Kinetic measurements are the only way to analyse protein–surfactant interactions above the cmc. A proper appreciation of these

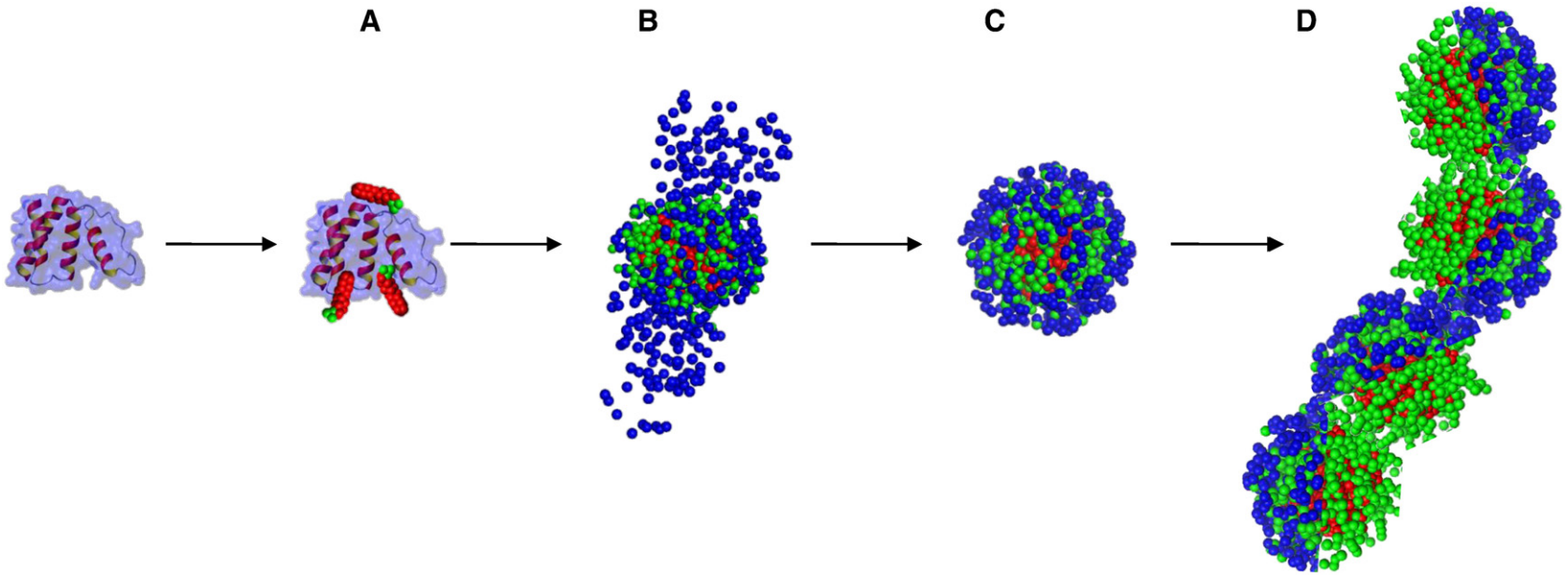

Fig. 7. Four different stages in the binding of SDS to ACBP. Schematic representation of the different stages of ACBP denaturation. In stage A, ACBP binds between 1 and 3 SDS molecules without losing the native structure. Stage B involves the formation of a decorated micelle of 37 SDS molecules that binds 2 ACBP molecules. Further binding of SDS to a total of 40 in stage C leads to monomeric ACBP with a shell-like structure of SDS. The structure presented in stage D is a speculative "beads on a string" model.
Reprinted from Ref. [39] with permission.

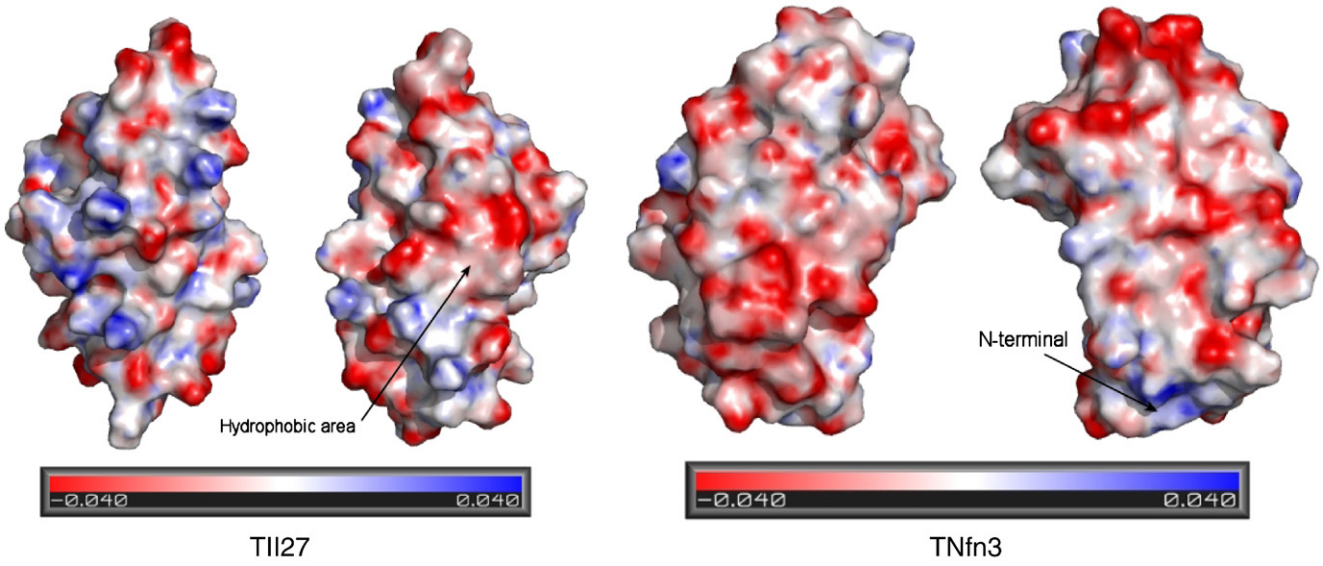

Fig. 8. Electrostatic potential of TII27 and Tnfn3, providing a possible basis for TII27's higher sensitivity to monomeric SDS. TII27 shows several areas with positive potential where SDS' sulfate headgroup could bind, some with neighboring hydrophobic areas that could interact with the detergent alkyl chain. TNfn3 has fewer positively charged sites, which moreover are surrounded by negatively charged residues.
Reprinted from Ref. [41] with permission.

kinetics requires us to compare them with what happens below the cmc. There have been a few reports describing concentration-independent unfolding kinetics in SDS, but these have focused on very low sub-mM concentrations and have not been particularly quantitative [114]. In the sub-micellar region, the general trend is that the kinetics of unfolding show a linear increase of the log of the major unfolding rate constant versus SDS concentration and then reaches a plateau around, or slightly above, the cmc. This has been reported for numerous proteins, including BSA [115], bromelain [116], ACBP [65], α-lactalbumin [32] and S6 [42]. Such a log-linear correlation is also seen for unfolding kinetics in conventional chemical denaturants, though at ~1000-fold higher concentrations. Over this concentration range, both ACBP and S6 undergo two conformational transitions according to spectroscopy, ITC and (for ACBP) SAXS, corresponding to the formation of different kinds of protein–micelle complexes. This complexity is not reflected in the uninterrupted linearity of the kinetics (although S6 has two additional minor relaxation phases in the first transition which disappear in the second), indicating that both types of micelles utilise the same cluster-promoted driving forces in denaturation of these two proteins. But for α-lactalbumin, the situation is more complicated, since the two unfolding transitions monitored by equilibrium titration correspond to two distinctly different linear stretches in the semi-log plot of unfolding rates versus SDS concentration [32]. All three proteins have comparable levels of SDS uptake in the two transitions. Thus the same level of cluster formation can have different mechanistic consequences in different proteins, and the strong affinity of SDS for proteins leads to specific rather than general effects in denaturation. Cationic surfactants such as TTAC show the same linear plots below cmc for denaturation of α-lactalbumin [32] and β-lactoglobulin [117].

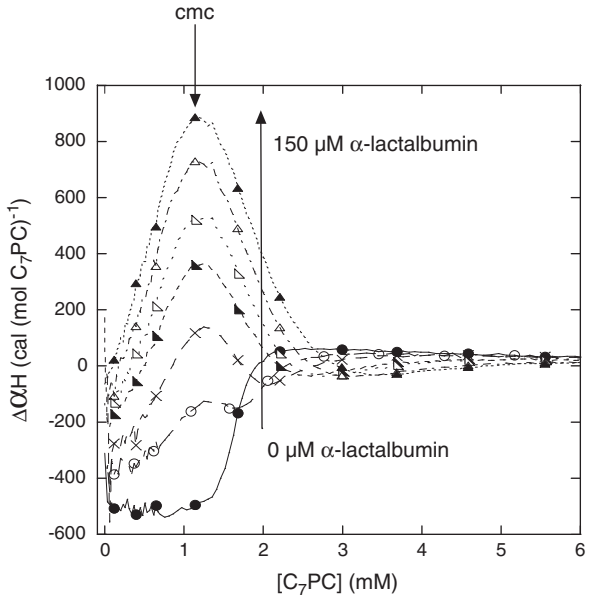

Fig. 9. Enthalpogram for titration of the zwitterionic surfactant di-heptanoyl-phosphatidyl choline into α-lactalbumin. The large denaturation peak occurs at the cmc and does not shift with protein concentration, indicating that there is not a large number of monomers binding to the protein prior to the onset of micellisation.
Reprinted from Ref. [32] with permission.

7.3.2. A plateau region in the unfolding plot

The extent to which the linearity levels out to a plateau varies from protein to protein. For ACBP [65], S6 [42] and α-lactalbumin [32], the plots depart from linearity right around the cmc but a plateau is only reached at 2–3 cmc values in SDS (Fig. 11); in shorter chain lengths, there is an abrupt decline in kinetics right at the cmc. Bovine carbonic anhydrase continues to increase its unfolding kinetics well above the cmc [70] (Fig. 4C). The heme-protein cyt *c* unfolds in two phases below the cmc; both phases follow an essentially linear increase in the unfolding kinetics determined by the change in heme absorption up to the cmc and then the fast phase abruptly flattens while the slower one disappears [105,118]. Note here that cyt *c*'s kinetics of unfolding followed by Trp fluorescence show a more gradual levelling out above the cmc. This reflects a partial uncoupling between global conformational changes (Trp fluorescence) and the heme binding pocket (heme absorbance). Another heme-binding protein, myoglobin, reaches a plateau for the fast unfolding phase around the cmc, whereas the intermediate phase's rate constant declines above the cmc [40]. The unfolding kinetics of both Tnfn3 and TII27 (Fig. 11) continue to rise well above the cmc [41], and the same is observed for the cellulase Cel45 where the surfactant concentration required to

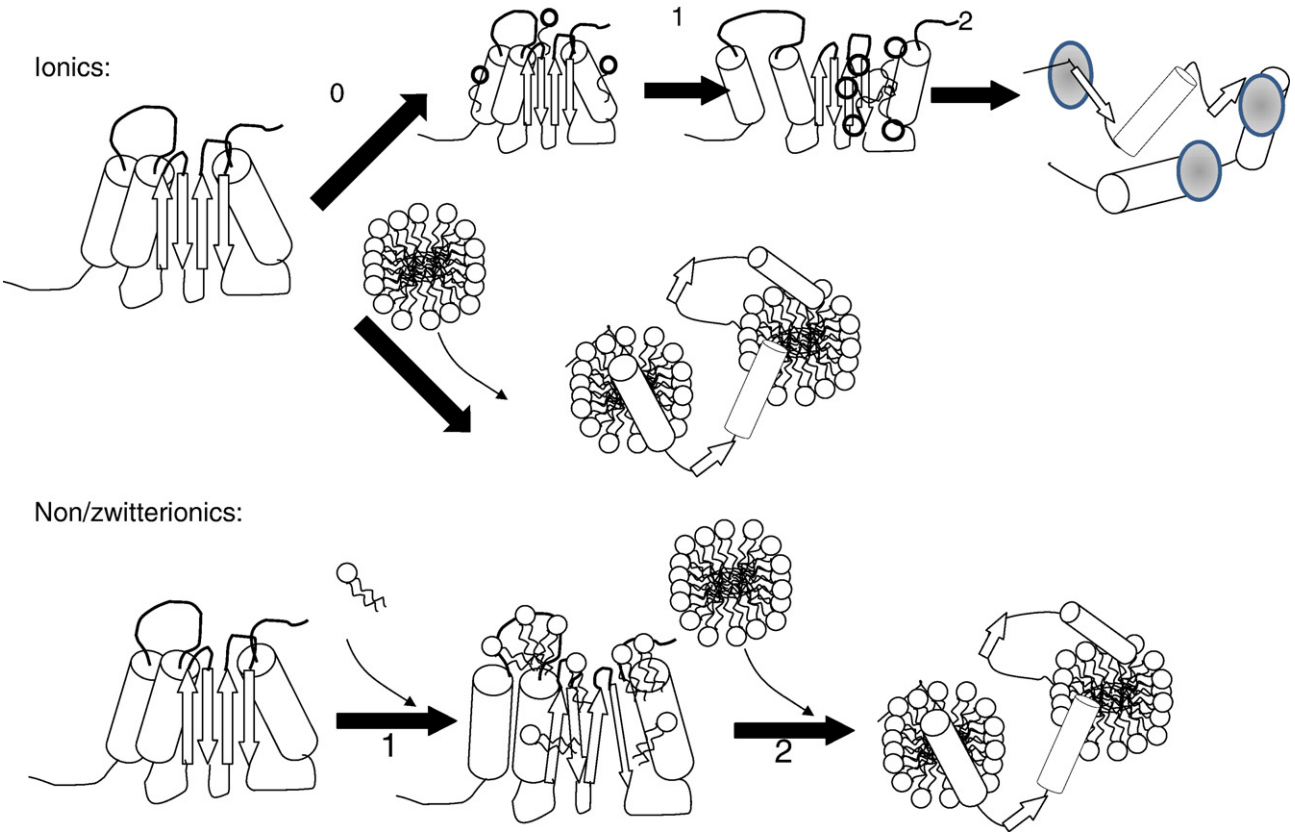

Fig. 10. Model summarising different strategies of attack by ionic and nonionic surfactant molecules. Reprinted from [32] with permission.

reach saturation is sensitive to mutagenesis [119]. δ-chymotrypsin's denaturation kinetics in SDS [120] show a completely different course: the first structural transition, complete around the cmc of 5 mM SDS, leads to binding of 80 molecules of SDS but extremely slow and apparently constant unfolding kinetics (in a linear–linear plot).

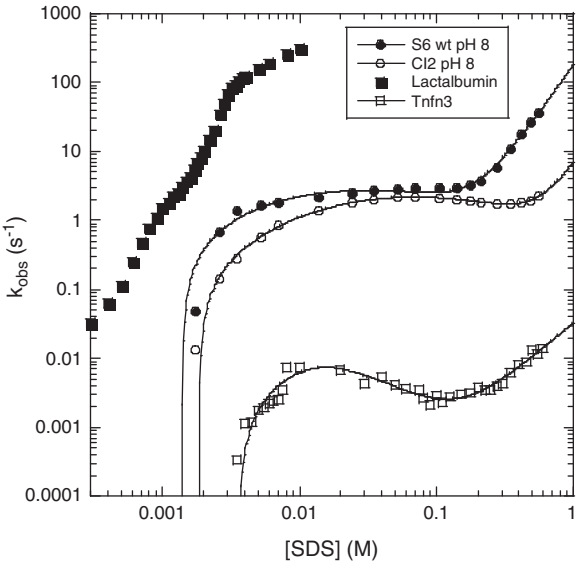

Fig. 11. Kinetics of unfolding of S6, CI2, Tnfn3 and α-lactalbumin in SDS. The lines are best fits to a model involving saturation-level binding and unfolding combined with inhibition of unfolding and log–log unfolding at higher SDS concentrations. Data from Ref. [32,38,41].

The kinetics then rise dramatically up to 12 mM SDS, after which they level out.

7.3.3. Evidence for the role of micelles in denaturation

The plateau observed for many proteins, which is reached only slightly above the cmc, does not imply that bulk micelles (rather than the micelles induced by the proteins) do not take an active part in the denaturation. The levelling out can also be seen and modelled as a binding type reaction with high affinity for the micelle, where the rate limiting step becomes the subsequent conformational change of unfolding after micelle binding. In this model, the micelles bind to S6 within the dead time of mixing to form a partially expanded state that is primed to undergo a major conformational change [73]. There is a great deal of evidence that increasing the surfactant concentration well beyond the cmc can accelerate unfolding kinetics. The log of unfolding kinetics scales with the log of the micellar SDS concentration up to several hundred mM SDS for aprA-subtilisin [121], horse cytochrome *c* [106], protease Q [122], S6 and CI2 [38,73] and the β-sheet proteins Tnfn3 and TII27 [41]. Furthermore, many proteins only unfold well above the cmc, such as methemerythin [123], urease [124] and β-galactosidase [125]. Although the authors of a study on the unfolding of endoglucanase III in alkyl sulfates concluded that monomers were the active species in unfolding because unfolding was slowed at higher ionic strength where the cmc is reduced [126], the stabilising effect of salts on unfolding kinetics was not taken into account in the analysis; it is also noteworthy that sodium decyl sulfate was only able to unfold the endoglucanase above its cmc.

7.3.4. Complex relationships between unfolding rates and surfactant concentration suggest multiple binding modes

The intriguing linear relationship between log k_{unf} and log [SDS] at high surfactant concentrations has prompted speculation [38,65,73].

Alkyl sulfates are known to undergo changes in micellar structure at higher concentrations, where they tend towards more cylindrical or elongated micellar structures [127,128]. The most convincing evidence that this micellar change affects unfolding kinetics is that an increase in the ionic strength, which decreases the concentration of surfactant required for formation of cylindrical micelles [129], promotes the transition from the plateau region to the log–log region for the unfolding of S6 in SDS in a similar manner [38]. Ionic effects on S6 itself cannot account for these changes at such relatively low salt concentrations. Furthermore, the log–log relationship is not seen for unfolding of S6 in cationic surfactants which do not form these cylindrical structures [38]. We have suggested that the elongated micelles denature the proteins in a quasi-denaturant type of interaction where they bind preferentially to the transition state for unfolding and thus accelerate unfolding at higher concentrations, unlike the spherical micelles. Nevertheless, it should—as always—be pointed out that these effects can be modulated in a complex manner by the protein itself. CI2, which unlike S6 does not unfold in cationic surfactants [38], undergoes the transition from plateau to the log–log region at a higher SDS concentration than S6 [73]. For ACBP, the onset of the formation of cylindrical micelles, probed by the reporter acridine orange [130], coincides with a pronounced *decrease* in the rate of unfolding, both in pure SDS micelles and in mixed micelles also containing the non-ionic surfactant dodecyl maltoside [65]. For the micelles only containing 25% SDS, there is actually a transition to a log–log region at higher surfactant concentrations (much higher than the onset of cylindrical micelles), but we attribute this to the formation of new and weak binding sites [65]. This receives indirect support from the fact that myoglobin actually shows a log–log increase in unfolding kinetics in CTAC above a region where the unfolding rate constants decline [40]. Since CTAC does not form cylindrical micelles, the log–log region is here best explained by the formation of new and weak binding sites.

7.3.5. Decline in unfolding kinetics: co-existence of different binding sites?

This brings us to a discussion of the second characteristic phenomenon in super-cmc unfolding kinetics, namely the decline in unfolding kinetics. In addition to ACBP unfolding in SDS and shorter chain alkyl sulfates (see above), this has been observed for S6 and CI2 to a small extent at very low ionic strength in SDS [38], for β-lactoglobulin in DTAC just above the cmc [117], for Tnfn3 but not the structurally related TII27 [41] and for oxidised cytochrome *c* in SDS and 50 mM phosphate buffer [131]. It is probably hazardous to assign a unifying principle for these phenomena. It appears inconsistent that the onset of cylindrical micelle formation should be the reason for the decline in unfolding rates of ACBP in decyl and octyl sulfate, which starts right above their respective cmcs [65], while the decline sets in further above the cmc in SDS. Short-chain alkyl chains are expected to need even higher concentrations than their longer-chain counterparts to form cylindrical micelles. Furthermore, DTAC does not form cylindrical micelles. Formally, the decline can be modelled as the binding of micelles to a second binding site on the protein [38,73], or direct "protective" partitioning of the protein into micelles inhibiting subsequent unfolding [117]. This seems counterintuitive in view of the established denaturing potency of micelles, but it may be the best explanation when combined with structural changes in the micelles with increasing surfactant concentration.

7.3.6. Heme proteins and decline in unfolding rates

For cyt *c*, the decline has been attributed to SDS micelles blocking the access of non-native heme ligands (His33 and His 26) to the heme binding site [131]. Here unfolding is conceived to occur in two stages. At low SDS concentrations, the native to B2 high spin state transition may require SDS monomers (or at most relatively small clusters of SDS molecules) to bind to the heme pocket. This occurs slightly above the cmc. In fact, the very high protein concentration used (100 μM), combined with the knowledge that 20–40 molecules of SDS bind at this stage [106], suggests that no free micelles are found in solution but only on the protein surface. In the second transition (well above the cmc and thus in the presence of free micelles), the B2 high spin state converts to the B2 low spin state, but this requires reorientation of the peptide chain to allow His 33/26 to bind to the heme and could be blocked by bulk micelles. This provides a nice illustration of the potentially conflicting roles of monomers and micelles when complex conformational rearrangements are required, but it is doubtful whether these specific considerations apply to proteins without co-factors such as heme. In this context it is interesting to note that Tnfn3, which does not bind monomeric SDS, is inhibited from unfolding by micellar SDS but not TII27, which *does* bind monomeric SDS [41]. For myoglobin, the decrease of the intermediate and slow rate constants occur above the cmc and at such low protein concentrations that the cmc is not affected [40], so no monomer/cluster/micelle competition can justify this. Nevertheless, the fact that many cationic side chains line the heme pocket [132] indicate that one can indeed envisage different binding sites for micelles which could interfere with heme-linked rearrangements.

7.3.7. β-lactoglobulin and cyt c unfolding data cannot be interpreted as simple folding-unfolding plots

There remain other intriguing phenomena in the kinetics of surfactant-induced unfolding: at very low DTAC concentrations (up to ~3 mM), there is actually an (uncommented) decrease in the rate constant for a conformational change in β-lactoglobulin before the rate constant starts to increase again up to the cmc [117]. A decline in rate constants is also seen for globular proteins in urea and GdmCl, but this is only when measuring *re*folding rate constants where the protein starts from an unfolded state and is then allowed to refold in the presence of increasing concentrations of denaturant [43,45]. In contrast, β-lactoglobulin is in the native state before mixing with surfactant. In this particular case, one possible explanation is that low concentrations of DTAC transform β-lactoglobulin from a dimer to a monomer, and the measured rate constants reports on binding to the dimer. Clearly interpretation of these kinetics relies heavily on a detailed understanding of both protein and surfactant properties.

A decline in the unfolding kinetics of oxidised cyt *c* at low SDS concentrations has also been noted by Bhuyan [63] up to 0.3 mM SDS (well below the cmc of 1.5 mM under those buffer conditions), followed by a rise and yet another decline up to 8 mM SDS, after which kinetic rates gradually increase again. These data are interpreted as two separate chevron plots for a very complex folding scheme involving a large number of different species. However, the same reservations apply as for β-lactoglobulin: chevron plots are conventionally constructed by combining refolding and unfolding data, so that the starting point for the reaction differs depending on which part of the plot is being analysed, and this is clearly not the case for cyt *c* in this study, since all the data are based on changes starting from the native state. There is no doubt that a number of different species accumulate at different SDS concentrations, as demonstrated by Trp and ANS fluorescence and CD spectroscopy [63], but rather than focus exclusively on the protein itself, it is necessary to combine spectral and kinetic data with ITC, pyrene and SAXS analyses to determine the extent of SDS binding, clustering and possible dimer formation at the different stages. Simple conversion from native to more and more unfolded species, increasingly stabilised by SDS (analogous to chemical denaturants), would lead to a monotonic increase in unfolding rates. Such an approach cannot explain the observed decrease in kinetics over some concentration ranges, but need to be linked to an understanding of other types of binding equilibria. Micelle-induced unfolding inhibition and additional weak binding sites can explain the chevron-like plot at high SDS concentrations but not conclusively prove it. Ultimately this may be

resolved by more detailed structural analyses of the protein–surfactant complexes at high surfactant concentrations.

7.3.8. BSA and chain-length dependent binding sites

This somewhat esoteric discussion about the subtleties of denaturation kinetics in surfactant has a very interesting link to a very mundane issue, namely the physiological interaction of BSA with fatty acids of different chain lengths [133]. C_8–C_{10} fatty acids only bind on one site in subdomain IIIA whereas C_{12} and C_{14} acids bind to both subdomain IB and IIIB [134]. It may be expected that these interactions will be mirrored by the corresponding alkyl sulfates. Thus BSA, both wildtype and the many mutants that affect different binding sites, provides a unique opportunity to investigate how the shift in initial binding sites will affect the kinetics of denaturation, and could even shed light on the conformational transitions that occur at higher surfactant concentrations.

8. Issue 2: how does unfolding of proteins in surfactant compare with "proper" unfolding in chemical denaturants?

8.1. Limited proteolysis reveals similar intermediate structures in different denaturing conditions

The high affinity of SDS and other ionic surfactants for proteins can be expected to lead to different denaturation mechanisms compared to weakly binding chemical denaturants. Unlike these denaturants, electrostatic interactions play a large role in the affinity of ionic surfactants, and the removal of positive charges by mutagenesis [119] or the blocking of cationic groups by acetylation or other chemical approaches [70,135,136] can significantly reduce sensitivity towards SDS denaturation. The end-point of the denaturation process is also very different. Chemical denaturants typically lead to random coil structures [137], whereas the SDS-denatured state is rich in α-helices, albeit highly dynamic. A simple way to compare the two states is by limited proteolytic cleavage, which exploits the fact that the first site (s) of attack by proteases are the flexible regions of a protein [138,139]. When α-lactalbumin and myoglobin were exposed to this treatment by the SDS-stable proteases savinase and alcalase and the cleavage sites identified by N-terminal sequencing, well-defined fragments were obtained, indicating that flexibility is limited to certain regions of the protein [7]. Remarkably, these sites correspond to regions identified in other studies as being partially unfolded at low pH or in organic solvents [140], indicating that different denaturants can exploit the same strategy for denaturing a protein. However, this simple relationship seems to work best for proteins that unfold via stable intermediates. The situation is more complex for proteins which do not form partially folded structures, such as Tnfn3. Here cleavage sites can to some extent be rationalised from the structure of the protein's folding transition state and the position of loops in the native state [7].

8.2. Multiple strong binding sites lead to more complex log k_{obs}–[SDS] relationships

If we simply compare the way that unfolding kinetics vary with SDS or GdmCl concentration, the behaviour appears much more complex in SDS. Chemical denaturants generally show very weak binding to proteins [141], and there is a simple linear relationship between the log of a given microscopic rate constant and denaturant concentration [43,45]. The linear increase of log k_u with urea can be interpreted as a simple jump from the native state to the transition state of unfolding. At most one observes some curvature at high concentrations of denaturant, which can be interpreted either as the formation of unfolding intermediates or a moving transition state [142,143]. Only very rarely are there kinks and additional curved regions, and these can be explained by the inclusion of additional unfolding pathways [144]. In contrast, the corresponding plots with SDS can show many different types of curves as discussed before, cfr. The complex curves reported for proteins as diverse as S6 [38,73], ACBP [65], β-lactoglobulin [117] and cytochrome *c* [63]. These complex curves likely reflect changes in the way SDS binds to the protein rather than actual changes in the transition state(s) of unfolding. Although there is often a linear relationship between log k_u and [SDS] at low SDS concentrations, this must involve much stronger interactions than those involving denaturants. A specific ligand binding model would lead to a linear–linear (single site binding) or log–log (multiple site binding) relationship, but this is generally not observed. This illustrates that surfactants such as SDS occupy a unique position midway between ligands and chemical denaturants in terms of the mechanism of interaction. It is possible that the denaturant-like log-linear relationship derives from cluster formation in conjunction with high affinity binding, though that remains to be modelled in more detail.

8.3. Protein engineering studies highlight differences between unfolding in surfactant and denaturant and the dramatic consequences of charge mutations

A more direct analysis of the unfolding mechanism can be carried out by comparing the unfolding kinetics of different mutants of a given protein in SDS versus chemical denaturants. For proteins unfolding from the native state via a rate-limiting transition state (TS), changes in the unfolding kinetics will reflect changes in the activation barrier to unfolding, i.e. the energy difference between the native state and the TS [145,146]; by taking into account the change in the stability of the native state, it is possible to infer changes in the stability and thus the structure of the transition state, which can be taken as a direct measure of the unfolding mechanism. The first report of this type [119], using 16 mutants of the cellulase Cel45 employed in the detergent industry for colour brightening, showed no clear correlation between unfolding rates in GdmCl and in the industrially used surfactant LAS (linear alkyl benzene sulfonate) and thus suggested fundamental differences in the unfolding mechanism. This was particularly evident when comparing charge-altering mutants such as the introduction or removal of Arg residues. The data are consistent with a simple electrostatic-hydrophobic binding-and-denaturation model, where denaturation potency is increased by longer alkyl chains and the introduction of positive charges [119]. However, it is possible to have too much of a good thing: the introduction of 3 proximal Arg groups decreases LAS sensitivity, possibly because the high polarity of that binding site interferes with binding of the alkyl chain. This was borne out by double mutant cycles [147] that suggest antagonistic effects between the Arg side chains during SDS denaturation but not GdmCl denaturation [119].

8.4. Using protein engineering to measure the degree of integrity

In the Cel45 study, 10 of the 16 mutants studied involved charge changes. Another study of the ribosomal protein S6 employed 19 hydrophobic truncation deletions in order to filter out the very strong electrostatic effects and focus on the inherent stability of the protein [73]. Unfolding rate constants in SDS increase to a binding plateau above the cmc. Accordingly, we used a binding-and-denaturation unfolding model, in which initial binding of micelles to the denatured state was required before the actual unfolding step. This defines the ground state of unfolding as an encounter complex between S6 and SDS micelles (formed in the burst phase of mixing), rather than the native state. Dead time spectra indicated an altered tertiary structure in the burst phase complex. To elucidate the structure of this complex, we introduced the term *degree of integrity* (DI), which compares the destabilisation ($\Delta\Delta G$) of the protein by the mutant in SDS normalised to that in GdmCl [73]. $\Delta\Delta G$ values were calculated by comparing

unfolding kinetics in 4M GdmCl versus the plateau levels of unfolding. DI-values of 1 suggested that the side-chain in the SDS burst phase was just as folded as in the GdmCl burst phase (which is considered identical to the native state because there is no evidence for any conformational changes in the mixing deadtime in GdmCl). In contrast, if the mutated side chain is more dynamic and flexible because of partial unfolding caused by binding of SDS, it will be less sensitive to mutation, leading to DI values below 1. Using this approach, we identified the two α-helices of S6 as the most dynamic parts of the burst phase complex and thus presumably the primary sites of attack by SDS, in good agreement with the affinity of SDS for helical regions. When the same approach was used to analyse unfolding kinetics in the log–log region of the unfolding plot, it became apparent that the region of attack in this concentration range has been expanded to include parts of the β-sheet, while 3 residues define a "pivot region" around which the helices can rotate to expand out during the attack (Fig. 12). These two unfolding pathways in SDS are fundamentally similar but differ from that in GdmCl. The DI values correlate poorly with the ϕ-values that describe the degree of structure in the transition state [45], and the hydrophobic core, which is the last part of the protein to unfold in GdmCl [148], is considerably weakened by SDS. All in all, this study emphasises differences in unfolding in surfactants versus denaturants.

8.5. Proteins with the same unfolding transition state in surfactant and denaturant

While Cel45 and S6 show considerable differences in their unfolding mechanisms in SDS and GdmCl, the analysis of a smaller number of mutants of the β-sheet proteins TII27 and Tnfn3 by the same approach reveals a closer similarity between the two unfolding pathways [41]. There is a good correlation between unfolding rates in k_{unf} in micellar SDS and GdmCl for both proteins, and the two different unfolding modes (plateau-region unfolding versus the log–log region) also correlate well. This lack of difference between SDS and GdmCl could be attributed to the fact that the two proteins lack any α-helical structures which could conceivably form the first line of attack for SDS micelles as seen for S6. However, that neat theory is robustly refuted by data for the all-α-helix ACBP, which show a clear correlation between unfolding in SDS and GdmCl, both as regards equilibrium unfolding and unfolding rate constants [65]. This correlation requires us to ignore three outliers whose maverick behaviour (smaller SDS sensitivity than we would predict from GdmCl data) may be related to their proximity to the (amphiphilic) active site and thus potential initial site of attack by SDS, which could make it more flexible and less sensitive to mutagenesis. The complexity of these correlations is emphasised by the fact that the first α-helix of ACBP, though more dynamic than the other 3 α-helices according to H–D exchange experiments [149], is not more sensitive than the others to SDS denaturation [65].

8.6. A versatile NMR-based approach to compare unfolding in different denaturing conditions

We have very recently developed an NMR-based approach to directly compare the unfolding pathways of a protein under different denaturing conditions, namely GPS (Global Protein folded State) NMR [150]. ^{1}H-liquid state NMR spectra of the protein during titration with different denaturing agents (*e.g.* surfactants, chemical denaturants, organic solvents, extreme pH, temperature) are analysed by Principal Component Analysis to identify principal components that can be used to describe the variation in these spectra. By mapping out how the values of the major two or three principal components vary with each other, we obtain a trajectory of protein unfolding in two- or three-dimensional space, where each linear segment represents one transition (Fig. 13). Many different denaturation processes can be

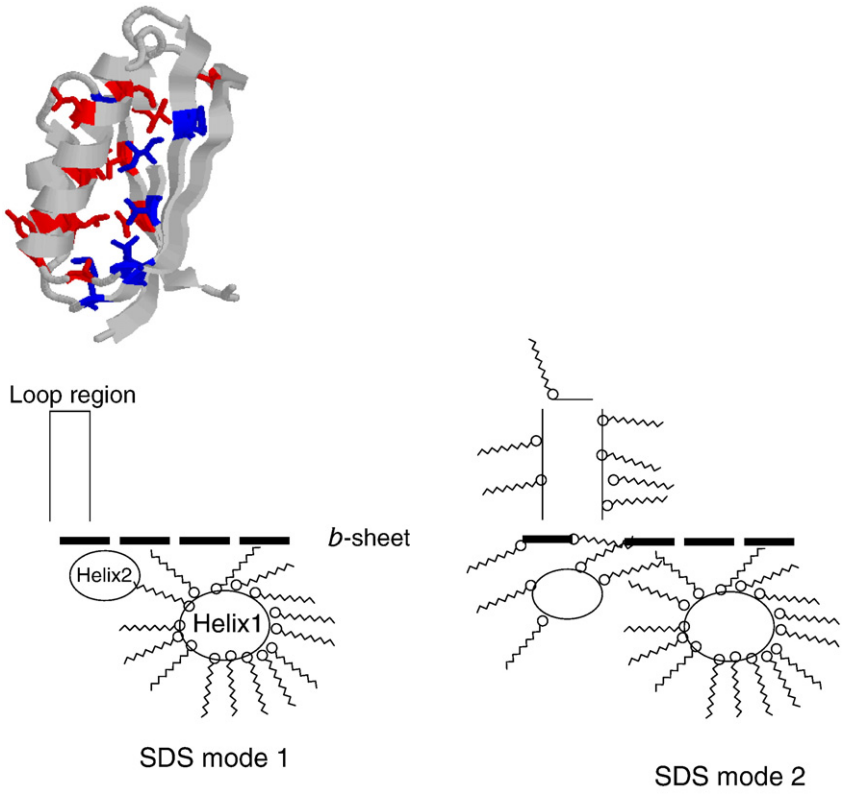

Fig. 12. Points of attack of SDS on S6. The cartoon structure (reprinted from [73] with permission) shows regions predominantly attacked by SDS in the burst-phase of unfolding (red); regions unaffected by SDS in the burst phase are in blue. The two diagrams show how the region of attack by SDS expands from mode 1 (saturation-level binding at relatively low SDS concentrations) to mode 2 (log–log region above 200 mM SDS).

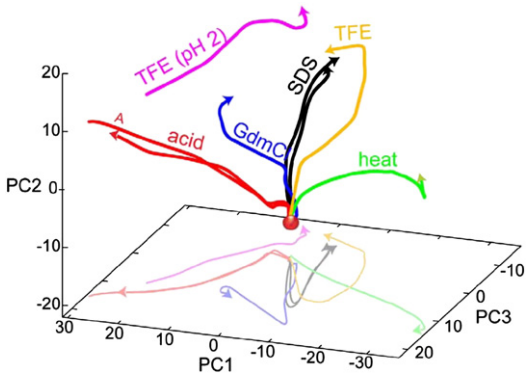

Fig. 13. Using GPS (Global Protein Folding State mapping) NMR to compare pathways of unfolding of α-lactalbumin in different denaturation processes. The diagram shows the evolution through geometric space of coordinates corresponding to the three main principal components from NMR spectra recorded under different denaturing conditions. Reprinted from Ref. [150] with permission.

plotted in the same graph. This approach reveals that unfolding of α-lactalbumin proceeds in (at least) two steps in SDS, but only one step in zwitterionic surfactants, and this single step is not the same as the first step in SDS [150]. By contrast, the initial SDS unfolding step in α-lactalbumin overlays nicely with the first part of the GdmCl denaturation pathway [150]. This suggests that SDS acts like a conventional denaturant at low concentrations, which agrees nicely with the linear increase of log k_u with SDS concentration in this concentration range [32]. GPS-NMR requires only low concentrations of protein (which does not have to be isotopically labelled) and is very robust towards broad spectra. It represents a very promising general analytical approach, and further comparison of other well-characterised proteins may consolidate these observations.

9. Issue 3: are there non-denaturing roles for surfactants?

9.1. Anionic and nonionic surfactants can activate proteins

Given the multiple ways in which surfactants can bind to proteins, it is to be expected that they can have other effects on proteins besides denaturing them or (at low concentrations) stabilising them as ligands. Many proteins benefit from the binding of ionic surfactants. A good example is the lipase from *Thermomyces lanuginosus* (TlL), which normally degrades (hydrophobic) triglycerides. TlL is activated by binding to the water-oil interface, which opens the lid covering the active site [151]. SDS and the cationic surfactant TTAB both elicit such a response at concentrations below the cmc, and the effect persists well above the cmc but then declines [152]. Uncharged surfactants also activate TlL well below cmc, but the effect peaks and then disappears before the cmc is reached [152]. Remarkably, clustering is *not* observed for any surfactants, and the protein migrates as a smear on an SDS polyacrylamide gel, indicative of multiple interconverting populations with different levels of SDS bound [152]. The effect can be modelled from enzymatic data through the binding of surfactants to TlL at different levels; low levels activate the protein while high levels inhibit it. Activation is divorced from the stability effects of the surfactants, since non-ionic surfactants have no effect on TlL stability whereas the ionic ones reduce it (though the protein is not denatured) [152]; in contrast, classical chemical denaturants uniformly inactivate TlL [152]. The lower the pH, the easier it is for SDS to denature the protein by cluster formation, and the narrower the window of opportunity for activation [152]. The surfactant-mediated activation mechanism is different from that at the water-oil interface, since the surfactants do not form any clusters and thus no contiguous interface. Presumably they bind as amphiphiles in the active site, similar to the binding of surfactants to cutinase [112], allowing them to act both as facilitators and blockers of substrate access in a manner that is promoted by increased chain length.

9.2. Activation and inhibition by SDS

Activation is not restricted to TlL. SDS is a strong activator of *S. solfataricus* β-glycosidase [153]; β-galactosidase is slightly activated by 35–70 mM SDS but inactivated at higher (350 mM) concentrations [125], while *Aspergillus niger* catalase is activated by around 180% by SDS (at a stoichiometry of around 150 molecules/protein) around neutral pH [154]. It has not been investigated whether these examples of activation require cluster formation, but most likely the effects will differ among the proteins, simply because there are so many different binding options. Nor is the effect restricted to SDS. Triton X-100 activates glucose-6-phosphatase [155] while sodium deoxycholate activates phospholipase [156]. The cationic surfactant AOT is a competitive inhibitor of alcohol dehydrogenase [157] and α-chymotrypsin but a non-competitive inhibitor of *C. viscosum* lipase [158]. There are also many examples of how SDS can act as enzyme inhibitor, targeting α-chymotrypsin [158] as noncompetitive inhibitor, amino acid oxidase as mixed inhibitor [159], and also reducing the enzyme activity of fatty acid synthase [160], α-glucosidase [161] and zinc-binding aminoacylase [162,163]. These effects take place at concentrations well below those where conformational changes and loss of native structure occur, indicating that SDS here binds as a ligand rather than a self-assembling and denaturing amphiphile.

9.3. Activation linked to destabilisation

Mushroom tyrosinase, which hydroxylates mono- and di-phenols, normally exists as 98–99% latent and is activated by intermediate levels of various denaturants (acid, surfactants, alcohols or proteases) [164], presumably because optimal activity in this case requires an optimal (and regulatable) level of flexibility. Thus SDS shows a sigmoidal activation profile [165–167], activating well below the cmc and showing a lag time of several hundred seconds at the optimal concentration. Higher SDS concentrations lead to shorter lag times but also reduced activity, while even higher concentrations lead to unfolding [165]. This is an excellent example of how the multiple binding steps of SDS can be used to fine-tune several different levels of unfolding of the protein with obvious practical applications.

9.4. The phenomenon of SDS-resistant proteins

These activation/inhibition effects are not coupled to major conformational changes, otherwise activity would be lost. Some proteins are simply resistant to SDS denaturation. It was shown early on that binding of SDS is typically all-or-none: either proteins bind 1.4 g SDS per g protein at saturation (in the absence of disulfide bonds) or none at all [168]. Examples of non-SDS-binding proteins include papain and pepsin [168], glucose oxidase near neutral pH [90] and bacterial catalase [154] which at room temperature remain in the native state. This does not imply that the proteins cannot interact with SDS at all. It is possible to "crank up the stress" by combining SDS with boiling to denature these proteins. Upon cooling in the presence of SDS, the proteins typically do not revert to the native state, indicating that SDS resistance is a kinetic rather than a thermodynamic issue and likely reflects a higher rigidity of the native state, which is typically found in β-sheet rich and oligomeric proteins [169,170]. It is simply a question of lowering the activation energy for unfolding to allow the SDS to have access to the denatured state for which it will always have higher affinity. For the same reason, kinetically stable proteins are typically very difficult to refold in the presence of SDS (though refolding proceeds readily once SDS has been removed by e.g. cyclodextrins [109]). This makes it very simple to identify kinetically stable proteins by comparative SDS–PAGE [171] or capillary

electrophoresis [172] of boiled and non-boiled samples, as the native state will have a different (and usually higher) mobility than the denatured state. Moreover, as SDS freezes the protein in whatever conformation it had when the SDS-containing loading buffer was added, these gels allow us to get a very precise snap-shot of the degree of folding of kinetically stable proteins at a given time in a refolding reaction.

10. Issue 4: how reversible is unfolding in SDS?

10.1. Refolding from the SDS-denatured state: dilution is not always the solution

For practical applications, SDS is often used to unfold proteins irreversibly [173]. Although SDS binds reversibly to proteins, it can be difficult to get rid of it rapidly because of its high binding affinity. Traditional methods include equilibrium dialysis [174], but this is a very slow process because micelles are too large to migrate across the semi-permeable membrane. A simpler approach is to dilute out SDS to concentrations below the critical aggregation concentration. This approach is not without complications since a certain range of SDS concentrations (typically 1–2 mM) can promote aggregation through e.g. formation of shared micelles (as reviewed in [71]), in this way trapping aggregation-prone intermediates and making equilibrium studies unfeasible, as demonstrated for carbonic anhydrase [70]. If this is not an issue, or if the aggregation occurs on a much slower time scale, this method provides an interesting approach for refolding studies. For e.g. cyt *c*, enough SDS remains bound to the protein to leave it in a partially structured state which is poised to refold, making refolding faster than from the GdmCl-denatured state [106]. SDS has also been used to trigger very fast folding of cyt *c*, since the reduced state is significantly more stable than the oxidised state, and so conditions can be found (0.65 mM) where the oxidised state is unfolded while the reduced state remains folded. Thus a rapid photoreduction will trigger conversion to the folded state [175].

10.2. Refolding by transfer to mixed micelles or stripping with cyclodextrin

Folding may also be effected by diluting SDS into non-ionic micelles, but in this case refolding is slowed down by an order of magnitude compared to refolding from the chemically denatured state, most likely because the mixed micelles interfere with the refolding process [48]. Mixing of micelles is not expected to be rate-limiting due to the very rapid dynamics of surfactants in micellar reconfiguration [176]. Much more rapid removal of SDS can be effected by simply mixing the SDS-denatured state with a stoichiometric amount of cyclodextrin, which complexes with SDS within milliseconds, emphasising the rapid dynamics of SDS binding and release [177]. Unlike the state with residual SDS or mixed micelles, this SDS-stripped state corresponds in the case of the protein CI2 to all intents and purposes to a random coil, since it refolds at exactly the rate expected from extrapolated refolding data in GdmCl and shows the same dynamics of *cis–trans* peptidyl-prolyl isomerisation as the acid- or GdmCl-denatured state [177]. Although this approach has been demonstrated for 3 different small proteins, larger proteins can show more complex behaviour (D.E.O, unpublished observations), possibly because of complications from potential aggregation of different domains. Nevertheless, equilibrium complexation of SDS with cyclodextrins has been exploited as part of an artificial chaperone strategy to avoid aggregation during refolding; the protein is first transferred to SDS and cyclodextrin is then added to remove the SDS and allow the protein to refold [178,179]. This approach has recently been extended to cationic [180] and non-ionic [181] surfactants. In all cases, 2 equivalents of cyclodextrin are needed per surfactant and it is best to add surfactant early in the folding process to avoid formation of aggregates that are difficult to solubilise [180]. Interestingly, proteolysis experiments with aspartate aminotransferase indicate that the SDS-bound state formed prior to the addition of cyclodextrin is similar to the state in which the protein binds to the biological chaperone GroEL, i.e. a partially unfolded state [109]. This illustrates how surfactants can be considered simple chaperones, although obviously unable to go through the multiple refolding cycles that GroEL can perform [182] unless this is done manually (or possibly by microfluidic approaches) by cyclic addition of SDS and cyclodextrins.

11. Issue 5: how do solvent conditions affect the way in which surfactants denature proteins?

11.1. pH modulates SDS interactions and vice versa

Ionic strength can indirectly affect protein–surfactant interactions by altering micelle structure [38] as well as directly modulating the electrostatic and hydrophobic interactions which promote ionic surfactant binding to protein [64]. Temperature effects are less dramatic [38], provided the experiments are performed above the Kraft temperature, below which surfactant solubility decreases dramatically. pH provides greater scope for modulation. It might be expected that a decrease in pH will increase the degree of protonation and thus increase the affinity of SDS for proteins, making unfolding occur more rapidly. The reality is more subtle. The kinetics of unfolding of S6 do increase as we lower the pH [38], but the apparent pK_a for this effect is around 5.6, which does not correspond to the pK_a of the acid residues Asp and Glu in water (3.5–4.0). However, the negative electrostatic potential of SDS micelles increases the local proton concentration sufficiently to lower the pH of the micellar environment by more than 2 units [183], nicely rationalising the observed shift in pK_a [38]. Under pH-sensitive conditions these effects can be quite dramatic. For example, the heme-binding properties of the membrane protein cytochrome b_{559}' are optimal between pH 6 and 7 in pure dodecyl maltoside micelles and start to decline at pH 8.0 [184]. However, inclusion of intermediate concentrations of SDS into these micelles lowers the pH sufficiently to restore heme binding; at higher concentrations of SDS (or higher pH values) this effect is overridden by the denaturing effect of SDS.

11.2. Combination with other denaturants opens up the conformational landscape

Although SDS has traditionally been used to investigate the unfolding of native proteins, it is also possible to explore other regions of the conformational landscape by combining SDS with other denaturants. Although the complexity of these new interactions generally makes it more of an academic curiosity than a practical asset, it is an excellent illustration of the structure inducing properties of surfactants and their ability to expand the conformational landscape. Although SDS reduces the α-helicity of native BSA, it can restore some of the lost secondary structure in urea-denatured BSA, though this new state is of course non-native [185]. Note that urea increases the cmc of cationic [186] and anionic [187] surfactants due to increased solubilisation of the monomer and replacement of water in the micellar interface region [188], so distinctions between sub- and super-micellar conditions have to take this into account. Similarly, cyt *c* can be "pre-denatured" in urea [189], acid [190,191] or alkali [63], after which secondary structure can be induced by addition of SDS and the process followed by kinetics or equilibrium techniques. These lead to the formation of new species which often correspond to molten globule type species with intermediate levels of structure with well-defined stabilities and rates of interconversion. For these different types of denaturation, it will be very rewarding to compare denaturation mechanisms by GPS-NMR, as this allows a direct juxtaposition of different pathways in the same graph [150].

12. Issue 6: do anionic surfactants such as SDS mimic biological membranes?

12.1. SDS as an inducer or destroyer of structure

Although many studies of protein–SDS interactions are sought justified as a mimic of protein–membrane interactions, this is generally not warranted for globular proteins, since tertiary structure is rarely sustained in SDS micelles. If the protein is without well-defined structure in the absence of SDS, e.g. small peptides such as monellin [192] or Aβ [193] or a natively unfolded protein such as α-synuclein [194], SDS can induce local secondary structure and thus increase the rigidity of the protein in a manner reminiscent of a membrane, but this does not qualify as true folding to a single well-defined state. In addition, it is clear from the previous sections that the interaction of SDS with proteins relies heavily on features which are not found in membrane vesicles, namely the existence of a large reservoir of monomeric surfactant molecules which can bind individually or assemble as quasi-micellar structures on the protein surface and the ability of micelles to sculpt themselves around the protein surface. I will provide some examples in this section.

12.2. Cytochrome c

cyt *c* participates in electron transfer processes in membranes in the respiratory chain of aerobic organisms [195]. However, it also has a second function: the activation of apoptotic pathway [196], where cyt *c* is transferred across the negatively charged mitochondrial membrane into the cytosol where it binds to the apoptosis protease activation factor 1 (Apaf-1) to initiate the apoptotic reaction cascade [197]. The interaction with SDS could therefore conceivably mimic the protein's binding to the mitochondrial membrane. However, in many cases protein interactions with the membrane require small defects to facilitate contact [198,199], and in the absence of such defects (which are typically only found in small vesicles with high intrinsic curvature), little interaction is observed. Therefore surfactants can exaggerate the potential structural changes that a membrane can induce, particularly when considering zwitterionic vesicles which have very low levels of interaction with most proteins.

12.3. Bet v 1

A good example is provided by the globular protein Bet v 1, which only binds to zwitterionic vesicles if they are sonicated *and* the pH is low, whereas binding to anionic vesicles requires either sonication or low pH [200]. The micelle-forming lysolipids lysoMPC (zwitterionic) and lysoMPG (anionic) have essentially the same influence on Bet v 1 as sonicated anionic vesicles (Fig. 14), provided they are studied in the micellar form; below the cmc, they have no interaction with Bet v 1 [200]. Thus these surfactant lipids are only good mimics because they do not interact with Bet v 1 as monomers, which is not the case for SDS.

12.4. FtsY

The protein FtsY, which ferries membrane proteins to the membrane from the cytosol, interacts more strongly with lysolipids than the diacylated membrane phospholipids, leading to uncooperative thermal transitions above the cmc but no effect below it [201]. The lysolipid interactions with FtsY define a state with properties distinctly different from those of states formed in the presence of different vesicles [201].

12.5. α-Lactalbumin

α-Lactalbumin forms a molten globule structure with "sticky" exposed regions in solution at pH 4.2 and below. In this state, it binds to both zwitterionic [202] and anionic [203] vesicles. It can also bind to vesicles when the solution pH is 4.5 and the protein is nominally in the native state [204]. Presumably this occurs because the local pH is even lower around the micelle interface, lowering the pH to 4.2 or less and allowing partial unfolding to occur. In contrast, when α-lactalbumin is denatured by zwitterionic surfactants, the monomer population clearly plays a role in the denaturation process [32], and this has no parallel on membranes. The amphipathic C-terminal helix has been implicated in the binding to membranes, leading to increased flexibility and detachment from the rest of the protein [205], but our proteolytic analysis of the protein's structural changes in zwitterionic surfactants does not indicate any change in flexibility around that part of the protein [7].

12.6. Lactadherin

Another caveat about equating micelles with membranes in general is provided by the milk protein lactadherin. This 409-residue multidomain protein binds strongly to cellular membranes containing the phosphatidyl serine (PS) head group [206], with a specificity for the right stereo-isoform of PS [207]. Binding can be recapitulated *in vitro* using synthetic phospholipid vesicles containing PS [207], and kinetic studies based on simple changes in Trp fluorescence indicate a two-step binding mechanism with an absolute requirement for PS groups (D.E.O, data not shown) However, if the PS lipid is integrated as a "minority surfactant" into surfactant micelles of dodecyl phosphocholine, the specificity is lost, since similar binding signals

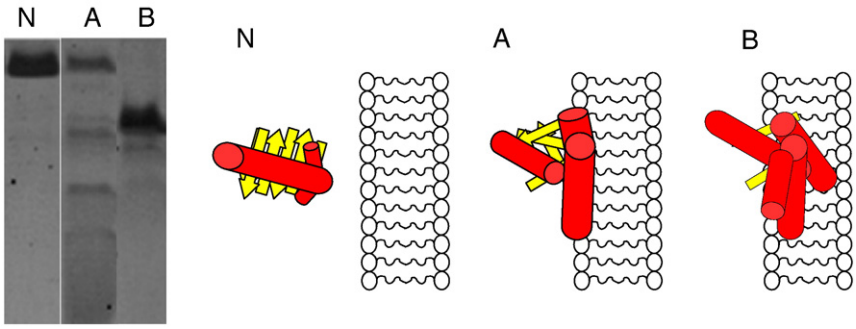

Fig. 14. Left: The protein allergen Bet v 1 is resistant to trypsin in buffer (lane N), but is degraded extensively in the presence of lysoMPC micelles at pH 7.2 (lane A) and less extensively by pepsin at pH 4.2 in lysoMPC micelles. The right panel depicts a representation of the different levels of insertion into the micelle surface (here depicted flat to emphasise that the same two partially inserted states are found in the presence of vesicles).
Reprinted from Ref. [200] with permission.

are observed when PS is substituted for other head groups such as the anionic phosphoglycerol or even the zwitterionic phosphocholine. Clearly the stable and regular membrane surface provides a different way of presenting potential anchor points for globular proteins than micelles than the fluctuating and dynamic micelle surface.

There is, however, one aspect where surfactants are useful membrane mimics, namely as solubilisers for membrane proteins. Mixed micelles consisting of both zwitterionic and anionic surfactants show "tunable" denaturation potency towards membrane proteins. Before we embark on that topic, let us first discuss the interaction of globular proteins with mixed micelles.

13. Issue 7: how do mixed micelles interact with globular proteins?

13.1. Using mixed micelles to reduce the cmc

Mixed micelles are the natural environment of the detergent enzyme, which is exposed to a mixture of both anionic and non-ionic surfactants [5]. Mixed micelles also constitute an interesting transition to a "proper" (i.e. complex and non-denaturing) membrane environment and as such they are relevant for membrane proteins. Unlike membrane proteins, water-soluble proteins do not as a rule interact with uncharged surfactants (with a few exception such as cutinase and α-lactalbumin, as detailed in Issue 1). There are two reasons for this. Firstly, these surfactants lack the charged head groups whose electrostatic attraction to oppositely charged side chains on membrane proteins is a powerful contributor to charged surfactant affinity binding to proteins. Secondly, the same absence of charge in the head groups removes the electrostatic repulsion at the micellar interface between water and hydrocarbon interior, which markedly increases the cmc of charged surfactants compared to their uncharged counterparts. In water, the cmc of SDS (~8 mM) is around 40-fold higher than its maltoside counterpart DDM (~0.17 mM). At the same time, this means that mixed micelles will have a cmc markedly lower than that of the charged surfactant alone, since they space out the individual charged surfactant molecules and thus reduce the repulsion, making it easier to incorporate monomeric anionic surfactant molecules into micelles [208]. This effectively removes most of the active monomeric surfactant from solution and allows us to focus on micellar effects (cfr. discussion on TII27 in Issue 1).

13.2. Absolute surfactant concentration versus mole fraction

For membrane proteins, it has been possible to establish a linear relationship between the free energy of unfolding and the mole fraction of SDS in mixed micelles, as will be detailed below. For this class of proteins, the absolute concentration of surfactants is not significant, since it does not affect the local environment of the membrane protein which always needs to be bathed in a micellar coat anyway. However, there is no such simple relationship for globular proteins, simply because they can bind stably (*i.e.* without aggregation) to the mixed micelles to different extents, ranging from no binding at all (in the absence of surfactant) to the completely denatured state at high surfactant concentrations. Thus denaturation of globular proteins like ACBP [65] and Tnfn3 [41], as well as natively unfolded proteins like α-synuclein [29], is achieved by increasing the absolute concentration of surfactant micelles, and ACBP's log of maximal k_u at a given surfactant mole fraction does not increase linearly with the SDS mole fraction [65]. Rather, the use of mixed micelles with different mole fractions of anionic versus uncharged surfactants appears to lead to different pathways for binding and unfolding (clearly something that should be explored more systematically by GPS-NMR [150]). This is apparent in all the cases that have been investigated.

13.3. α-Synuclein aggregation is abolished by mixed micelles

The natively unfolded protein α-synuclein can be aggregated optimally at half the bulk cmc value in neat SDS through the formation of shared micelles [29]. This effect is retained in the presence of decyl maltoside (DecM), which has a slightly higher cmc than SDS in PBS buffer, allowing significant monomeric concentrations of SDS to exist in the presence of DecM. A mole fraction of at least 85% DecM is required to significantly increase the concentration at which α-synuclein starts to form surfactant clusters; below this mole fraction, cluster formation is unperturbed by DecM. DecM shifts the optimal concentration for aggregation to higher surfactant concentrations to a smaller degree than the decline in absolute SDS concentration, suggesting that the absolute concentration of negative charge is not critical for cluster-induced aggregation [29]. However, the aggregation effect is completely abolished by 1:1 SDS–DDM mixed micelles which obviously prevent such sub-cmc cluster formation and also induce somewhat lower levels of α-helicity compared to those seen in neat SDS [29].

13.4. Tnfn3 unfolding is attenuated by mixed micelles

For the β-sheet protein Tnfn3, which only unfolds in the presence of bulk SDS micelles [41], a simple decrease in SDS mole fraction from 100% to 75% (with 25% DDM) leads to a significantly less cooperative unfolding transition and a four-fold increase in the total SDS concentration required for 50% denaturation, and at 50% and 25% SDS denaturation remains incomplete, even at SDS concentrations up to 25–50 mM [41]. Its structural analogue TII27, which can be denatured slightly below the cmc and forms pyrene-detected clusters under these conditions, also shows weakened denaturation in mixed micelles.

13.5. Mixed micelles mimic surfactants with short chain lengths in ACBP unfolding

A different situation arises for ACBP, which denatures very far below the cmc; the midpoint of denaturation is around 1.9 mM SDS, which is only 30% of the total cmc [65]. Adding as little as 25% DDM decreases the midpoint denaturation concentration almost 4-fold, though this value stays constant at 50% and 75% DDM [65]. Interestingly, denaturation occurs in the same two-stage fashion as in neat SDS, in which both secondary and tertiary structure undergo changes in the first transition but only tertiary structure changes in the second. However, the second transition becomes less pronounced at higher DDM mole fractions just as the first transition becomes less cooperative, and this provides two hints that the less charged micelles bind more weakly. It is particularly instructive to focus on the kinetics of ACBP's unfolding in these SDS–DDM micelles [65]. In all cases, an initial increase in unfolding rates is followed by a decline, but the decline sets in earlier as the SDS mole fraction decreases (Fig. 15A). The strong correlation between the concentration of the onset of decline and the maximal unfolding rate suggests that the absolute concentration of SDS in the strong or linear binding mode (prior to the onset of decline) is limiting for unfolding. Shortening the alkyl chain length has the same effect as increasing the mole fraction of DDM: an overall reduction in unfolding rates, an earlier onset of the decline in unfolding rates and the appearance of log k_{unf} –log [surfactant] unfolding behavior [65]. In both cases, we weaken the denaturing potency of the micelles (by reducing electrostatic and hydrophobic interactions, respectively). We speculate that these weakened attractions in both cases lead to the formation of new binding sites on different sites of the protein which alter the general surfactant–protein interactions and change the actual unfolding pathway.

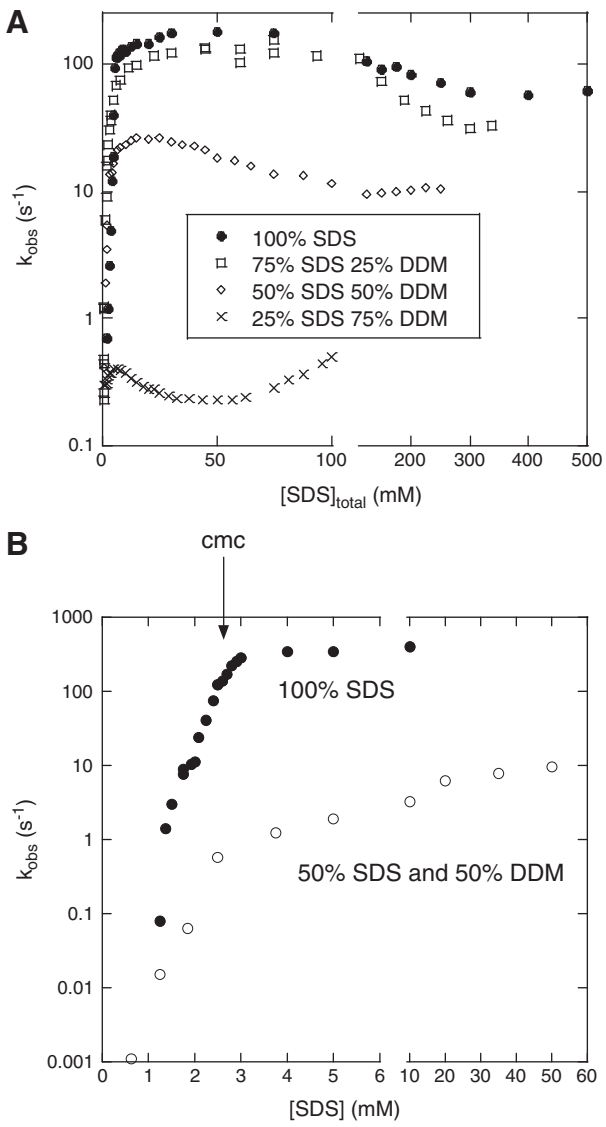

Fig. 15. Effect of mixed micelles on unfolding of (A) ACBP and (B) BSA in different mole fractions of SDS and DDM.
Panel A reprinted from Ref. [65] with permission.

13.6. Dramatic decrease in unfolding kinetics of BSA in mixed micelles

More support for this is provided by BSA, which shows a simple linear relationship between log k_{unf} and the SDS concentration below cmc, which sharply transforms to a plateau around the cmc [115]. However, in 1:1 SDS:DDM micelles (where all unfolding occurs in the presence of micelles), the unfolding rate constant not only decreases more than hundred-fold, but also rises hyperbolically with micelle concentration, indicating a saturation effect in binding (Fig. 15B). ACBP's hyperbolic relationship between log k_{unf}^{max} and SDS mole fraction [65] suggests that there is a mole fraction saturation effect, so that above a certain SDS mole fraction the micellar aggressiveness does not increase further because the maximal number of binding sites has been reached.

14. Issue 8: how can mixed micelles be used to measure the stability of membrane proteins?

14.1. Early use of surfactants for membrane proteins studies

Let us now contrast this idiosyncratic behaviour with the use of mixed micelles to study the folding and unfolding of membrane proteins, i.e. proteins integrated into the cellular membrane through at least one transmembrane domain. Nonionic surfactants, particularly alkyl glucosides and maltosides, have been used to extract membrane proteins such as rhodopsin and opsin [209,210] for many years in order to solubilise them properly and provide well-defined amphiphilic media. Although membrane proteins are generally less stable in surfactants such as digitonin than in membranes composed of phospholipids [211], the stabilising properties may be increased by increasing alkyl chain lengths (in the case of diacyl glycerate kinase for example increasing the melting temperature by 25 °C on going from an eight- to a ten-carbon chain [212]) and at the same time increasing solubility by expanding the head group from glucoside to maltoside [213]. On the other hand, lower chain lengths lead to higher cmc values, making it easier to remove the surfactant by dialysis. The solubilisation of phospholipid membranes by surfactants is a complex process (reviewed in [214,215]), which involves several stages of solubilisation and ultimately ends up with membrane proteins in mixed micelles containing surfactants and varying amounts of phospholipids [209]. In this situation, phospholipids are reduced to the role of "minority surfactants" and should be treated as such.

14.2. Thermal scans and denaturants are unsatisfactory approaches to membrane protein denaturation

While the use of surfactants to solubilise membrane proteins has been known for many years, it is only comparatively recently that the combination of nonionic and ionic surfactants has been used to systematically shift the equilibrium from the native to the denatured state for these proteins. Prior to this, the conventional way to assess membrane protein stability was to use thermal denaturation, as practiced on bacteriorhodopsin [216], photosystem II [217], cytochrome *c* oxidase [218], the *Rhodobacter sphaeroides* reaction center membrane protein [219] and erythrocyte band 3 [220], but this invariably led to an irreversibly aggregated state, precluding thermodynamical analyses, let alone folding/unfolding kinetics. Conventional denaturants such as GdmCl and urea typically fail to denature membrane proteins properly because of an inability to completely solubilise the very hydrophobic core of these proteins, though aggregation may be avoided in combination with low pH [221]. They generally lead to irreversible unfolding, even when surfactant micelles are included [222], and provide poor starting points for reconstitution into micelles or vesicles [221], though an exception was recently reported for the 804-residue CopA copper transporter, which unfolds rapidly and reversibly in GdmCl [223]. Nevertheless, even this protein loses its activity well below the overall loss of structure and retains some residual structure upon denaturation [223]. Excellent reviews on membrane protein stability determination are provided by Stanley and Fleming [224] and Hong et al. [225].

14.3. Simple linear relationships are provided by mixed micelles

In contrast, SDS has always been known to have good potential for reversible unfolding of membrane proteins. It combines strong membrane solubilisation properties with an ability to stepwise denature proteins, *e.g.* the sodium-potassium ATPase whose ATPase activity is lost at SDS concentrations sufficient to solubilise it, while retaining the quaternary structure and ability to occlude Rb^+ and Na^+ [226]. Reversible unfolding of the SDS-denatured state of a membrane protein was first shown in two landmark papers by Khorana and coworkers [227,228], where bacteriorhodopsin was transferred from the SDS-solubilised state to phospholipid/cholate mixtures and regained the ability to bind retinal. This was elegantly exploited by Booth and coworkers in a series of papers (starting with [229] and summarised in [230]) to follow the kinetics of refolding into these mixed phospholipid-detergent micelle systems. However, this remained a one-way street focusing on the refolding process, admittedly the most relevant but only

a part of the greater picture. The break-through came with the empirical observation by Lau and Bowie that the trimeric membrane protein Diacyl Glycerate Kinase (DAGK) could simply be unfolded (as monitored by a change in absorbance or the far-UV CD signal) by increasing the mole fraction of SDS in a mixed micelle system consisting of SDS and DDM [231]. Bowie and co-workers have subsequently used this approach to determine the stability of bacteriorhodopsin and mutants thereof [232–234].

14.4. Exploiting these linear relationships

The key observation was that the data can be fitted to a simple two-state unfolding mechanism involving a linear correlation between the free energy of unfolding and the mole fraction of SDS [231]. (The data for bacteriorhodopsin deviate slightly but systematically from this simple fit, but that can in principle be accommodated by expanding the unfolding process to include an intermediate unfolding step). Thus stability at any mole fraction of SDS can be obtained by simple extrapolation. This link to mole fraction is intuitively correct, as membrane proteins sense the environment through the micelle, and therefore should be sensitive to the composition of the micelle but—as shown by Lau and Bowie [231]—indifferent to the absolute concentration of micelles. This indifference requires that there are enough micelles to give on average significantly less than one protein molecule per micelle, avoiding complications with several proteins cohabiting the same micelle; complications arising from such a situation are described below.

14.5. Association of membrane proteins is sensitive to absolute surfactant concentration as well as micelle composition

The only exception to this mole fraction rule is when folding is combined with association. Here the prime example is dimerisation of the single transmembrane helix of glycophorin A (GpA) [235], which is mediated by specific side-chain interactions [236]. The higher the micelle concentration, the more glycophorin is diluted out, and the less favourable dimerisation becomes. Thus the folded-unfolded equilibrium can be displaced simply by increasing the absolute concentration of micelles, rather than by increasing the mole fraction of SDS. In fact, the glycophorin dimer is sufficiently stable to survive in SDS, providing the basis for SDS–PAGE analyses of the extent of dimerisation using chimeric GpA-nuclease constructs [237]. However, the dissociation constant K_d (measured more accurately by FRET using pyrene- and coumarin-labelled GpA) increases from 0.56 μM in 1.2 mM SDS to 10 μM in 25 mM SDS [238], while the K_d values are around 100-fold lower in dodecylphosphocholine [238], depending on concentration. These analyses are not trivial, since several molecular species co-exist in equilibria. To measure true dimerisation, it is necessary that surfactant molecules have the same affinity for monomer, dimer and (empty) micelle [238]. A very careful study of the thermodynamics of GpA dimerisation at different temperatures in SDS and lauryl-N,N-dimethyl-*N*-oxide [239] highlighted two opposing effects in monomer–dimer equilibrium: first; an entropic effect that is strongly dissociative, sensitive to detergent concentration, and relatively insensitive to head group chemistry. This is counterbalanced by a second associative enthalpic effect modulated both by head group chemistry and detergent concentration. This emphasises the complex nature of peptide-surfactant interactions. The situation becomes even more complicated when GpA dimerisation is combined with changing mole fractions of SDS and DDM [240], and here the free energy of dimerisation shows a linear dependence on the *logarithm* of the mole fraction of SDS, probably a reflection of the monomer–micelle–peptide equilibria superimposed on the change in micellar composition.

14.6. Bulk versus micellar composition differences can complicate matters

A critical aspect of the mixed-micelle approach is the assumption that the composition of the micelles mirrors the bulk composition. This is not always the case. Although surfactants such as SDS and DDM mix readily and rapidly [176], the very significant difference in their critical micelle concentrations implies that they are not taken up equally readily into micelles. Clint developed an approach to describe the ideal mixing of two surfactants and the composition of the micelle phase versus the monomeric phase [241], but in practice it is necessary to measure the cmc of a given molar composition to calculate the actual micellar and monomeric compositions [242,243]. We have elucidated the importance of using bulk versus micellar composition as a measure of denaturation potency by determining the melting temperature t_m of the β-barrel membrane protein AIDA, which unfolds irreversibly in ionic surfactants upon heating [244], in a large array of different mixed micelles [208]. In all mixed micelle pairs where there was significant difference between the bulk and micellar composition, we observed that only the micellar mole fractions gave a linear correlation with t_m [208], clearly demonstrating that it is the micelle composition *per se* that is important. Fortunately, there was little significant difference between the two types of compositions in the "canonical" SDS–DDM pair [208], supporting the simple use of bulk composition to determine SDS mole fractions.

14.7. Specific protein–surfactant interactions and specific binding of SDS to membrane proteins

Specific interactions between (in particular) SDS and the membrane protein can complicate the use of mixed micelles. In other words, does SDS behave as a general denaturant or a specific ligand? This question, forcefully put by Renthal in a recent review [245] goes to the heart of the matter in terms of how mixed micelles actually work. A naive pictorial interpretation is that the surfactant molecules, whether SDS or DDM, both bind as annular surfactants, i.e. forming a micellar belt around the protein with the alkyl chains in contact with the highly hydrophobic transmembrane parts of the protein. In this model, SDS would not be expected to interact through specific electrostatic contacts, and this should reduce the degree of ligand-type interactions in favour of a more general type of denaturation, presumably mediated by simple repulsion between different head groups. Evidence in favour of this is that electrostatic mutations in the transmembrane sections of membrane proteins such as bacteriorhodopsin [232] or DsbB [47] do not alter the general unfolding behavior and do not in fact have the effect that could be predicted from a simple electrostatic model (where more positive charge leads to greater SDS sensitivity). For example, D38A and K40A have essentially the same (small) effect on bacteriorhodopsin stability [232]. On the other hand, the solvent-exposed parts of the membrane proteins can be expected to have properties more akin to those of globular proteins and might therefore bind SDS in the same way through electrostatic interactions. This is counterbalanced by the fact that in mixed-micelle systems with membrane proteins, SDS is mainly present in the micellar form and not at the monomeric concentrations approaching that of SDS and globular proteins at sub-cmc conditions. Therefore the interactions will not involve shared micelle clusters but rather binding of bulk micelles.

14.8. Preferential binding of SDS micelles to extramembraneous protein regions can lead to anomalous thermodynamics

That this binding to the non-membrane bound parts of the membrane protein could still have an impact was demonstrated in a thermodynamic study of the unfolding of DsbB in mixed micelles [48], where the heat capacity of unfolding (going from the intermediate

state to the denatured state) decreases. This is surprising, because heat capacity is considered to be proportional to the amount of water molecules bound (primarily) to the hydrophobic surface [246,247]. Thus decreased heat capacity is normally associated with less exposed surface area. The interpretation we have proposed is that the denatured state, which is favoured at higher SDS mole fractions, binds surfactant micelles more extensively because micelles containing SDS are also able to bind to the periplasmic loops, unlike DDM micelles [48]. Binding of SDS will displace water molecules from the loops (implying that the loops are relatively unstructured and solvent-exposed in the native state, which is borne out by the solution structure [248]) and in this way decrease the heat capacity. There is nothing in this work that implies that there should be specific binding of SDS to membrane proteins which could obscure the thermodynamic analysis. Nevertheless, specific binding sites for anionic amphiphiles can interfere with the normal interpretation of mixed micelles, as we will now discuss.

14.9. Specific binding sites can interfere with linear relationships

The tetrameric potassium channel KcsA binds anionic phospholipids at a "non-annular" site [249], originally identified through crystallography [250] and mass spectrometry [251]. Both anionic lipids such as phosphatidyl glycerol and phosphatidyl serine and anionic surfactants such as alkyl sulfates stabilise the protein against denaturation proportional to the chain length and mole fraction of the anionic component [252]. This rationalises the resistance of KcsA to SDS-mediated denaturation. The degree of thermal stabilisation by (single-chain) alkyl sulphates is similar to that of the double-chain lipids, and outlines the major alkyl chain binding site in KcsA at the interface between different monomers [252]. Specific binding has called into question the use of mixed micelles as a generic approach to systematically probe the stability of membrane proteins [245]. However, the many examples of linear relationships between the degree or rate of folding and the mole fraction SDS suggest that this is not a general problem.

14.10. Towards a more detailed understanding of the SDS-denatured state

The preceding section also highlights that an important area of research in future will be the residual structure in the SDS-denatured state of membrane proteins. There are two reasons for this. Firstly, this state is the starting point for refolding studies of membrane proteins, and detailed interpretation of the effect of mutagenesis on protein stability requires us to understand the impact of the mutation not only on the structure and stability of the native state but also on that of the denatured state. A promising start has been made by Bowie and coworkers who model the denatured state by considering each helix in isolation [232]. In this way they have obtained a relationship between buried surface area and membrane protein stability that is surprisingly (and at present still inexplicably) similar to that for global proteins in the absence of surfactants. This approach has not yielded the same simple correlation for DsbB [47], but that could be related to differences in the nature of the denatured state. Secondly, the SDS-denatured state might also have some relationship to the state in which membrane proteins are inserted into the cell membrane helix by helix through the translocon [253]. The stability of individual membrane protein helices in isolation *in vitro* has suggested that membrane proteins can associate in stages by insertion followed by association. It would be very satisfying if it turned out that the SDS-denatured state, which has a level of α-helicity almost identical [46] or similar to [254] the native state, is essentially the native state minus the tertiary structure. Information on this is sorely needed. However, the reality is probably more complex, as suggested by DsbB [47]. One approach recently reported is to use laser pulses to oxidise Met residues and generate hydroxyl radicals to footprint accessible parts (*i.e.* not covered by micelles) of the protein [255]. This is limited by the availability of Met residues. So far it has suggested that helices A and D are partially unraveled in the SDS-denatured state of bacteriorhodopsin, whereas the other helices appear relatively intact [255]. An alternative will be to attempt hydrogen-deuterium exchange studies in combination with tryptic digestion and mass spectrometry to determine the rate of exchange in different parts of the protein and thus their relative dynamics. This is relatively simple for water-soluble proteins [256], but for membrane proteins it will be necessary to take the intrinsic effect of surfactant micelles on exchange rates into account.

14.11. Mixed micelles are useful tools for kinetic studies of membrane protein folding

The initial denaturation studies using mixed micelles focused on equilibrium transitions. For bacteriorhodopsin, the loss of retinal upon unfolding provides a convenient spectroscopic signal to follow the denaturation process. Equilibrium fluorescence studies do not provide unambiguous signals, since Trp fluorescence is sensitive not only to the conformation of the protein, but also the polarity of the micellar medium. Thus DAGK denaturation could not be monitored in this way [231]. Similar problems were encountered for the 4-transmembrane protein DsbB. The alternative to this is to use kinetic studies [46]. Given the rapid mixing of different surfactant molecules [176], any relaxations that can be picked up by conventional stopped-flow apparatus must reflect relatively slow conformational changes associated with protein folding/unfolding, while the baseline fluorescence levels are a composite of polarity effects and burst-phase conformational changes. Refolding experiments can be carried out by first unfolding DsbB in SDS and then diluting it out with appropriate amounts of DDM micelles, and *vice versa* for unfolding [46]. Provided the kinetics are simple (i.e. monoexponential), it is possible to incorporate the rate constants into an appropriate kinetic folding model analogous to that of folding/unfolding of globular proteins in chemical denaturants. In practice a plot of the log of rate constants versus mole fraction SDS will reveal a series of linear regions joined by different transitions, which are consistent with Bowie's linear relationship between free energy of unfolding and the mole fraction SDS [231]. For DsbB, measured over the entire mole fraction interval from 0 to 1, there are three linear regions (Fig. 16A) corresponding to refolding, unfolding from the native state and unfolding via an intermediate [46–48]. Practical limitations prevent a full exploration of bacteriorhodopsin refolding/unfolding beyond the range of ~0.4–0.9 mole fraction SDS [257,258], and thus restricts the analysis to a region of the plot where unfolding is two-state from native to denatured with a single transition state (Fig. 16B). However, for both proteins these plots have provided an opportunity to carry out systematic protein engineering studies à la Fersht [45,145] to identify key residues in the folding of bacteriorhodopsin [258] and DsbB [47] (Fig. 16C). While several methodological questions remain regarding the underlying mechanisms of action (just as the nature of chemical denaturation still remains a subject of debate [259]), mixed micelles have truly come of age as a standard tool to study folding and stability of membrane proteins.

14.12. Outer membrane proteins may be unfolded in denaturant

I shall not devote much attention to the unfolding of the other class of membrane proteins (OMPs), namely the β-barrel proteins typically found in the outer bacterial membrane. This mainly reflects a courtesy provided by these proteins at the sequence level: the alternating pattern of hydrophilic and hydrophobic residues inherent in the β-strand composition means that these proteins lack the uninterrupted stretches of hydrophobic residues found in α-helix membrane

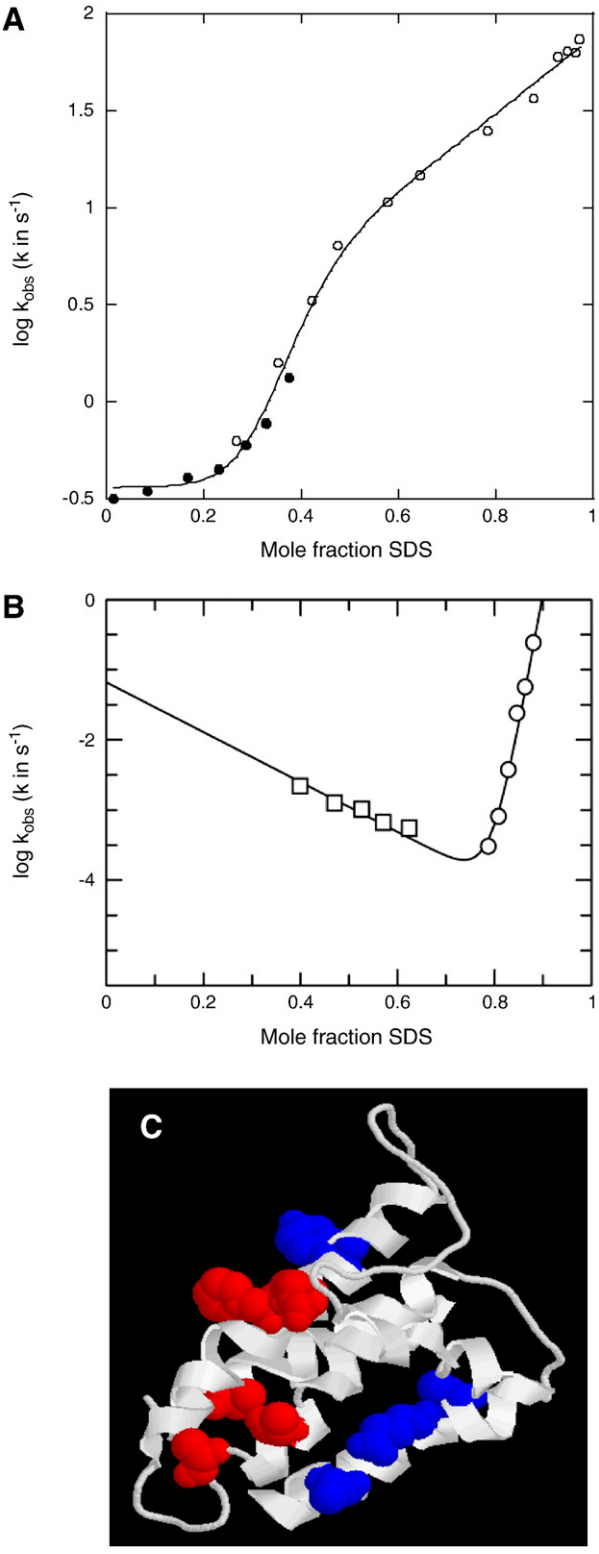

Fig. 16. The use of mixed SDS–DDM micelles to study membrane protein folding and unfolding. (A) Kinetics of refolding (filled circles) and unfolding (empty circles) of DsbB. Data are fitted to a three-state unfolding scheme, involving the native state equilibrating rapidly with an intermediate which then unfolds to the denatured state. Data taken from Ref. [46]. (B) Unfolding and refolding of bacteriorhodopsin. Data are fitted to a two-state unfolding scheme with a single transition state between the denatured and native state. Reprinted from [257] with permission. (C) Reconstruction of the folding intermediate of DsbB based on ϕ-value analysis of 12 mutants. Red indicates side-chains with significant levels of native-like structure in the intermediate ($\phi_I > 0.5$) while blue indicates side-chains with less structure ($\phi_I < 0.5$).
Reprinted from [47] with permission.

proteins. As a consequence, most outer membrane proteins, exemplified by OmpA from *Escherichia coli*, can be unfolded in chemical denaturants such as urea and GdmCl and refolded by simple dilution into either phospholipids [260] or surfactants [261]. Significant amounts of urea are even required to allow the OMP PagP to refold/unfold in the presence of phospholipid vesicles without aggregating [262]. Therefore there are few studies on the use of mixed micelles to refold OMPs. Privé and co-workers introduced a remarkable innovation by demonstrating that the crystallisation additive 2-methyl-2,4-pentanediol can refold SDS-unfolded OMPs such as PagP [263], presumably by "softening" SDS by reducing its cmc, aggregation number and surface charge density [264]. However, this has not been demonstrated to be a general approach to determine OMP stability or follow folding/unfolding kinetics.

14.13. Kinetic stability of outer membrane proteins complicates kinetic studies

Part of the reason for the limited amount of kinetic information on OMPs, apart from descriptions of the refolding at denaturant concentrations close to zero molar [265], resides in the considerable kinetic barriers to unfolding and refolding. Although OmpA can be unfolded into, and refolded from, high concentrations of urea, it is in our experience and that of others (K. Fleming, personal communication) essentially impossible at the experimentally accessible time scale to get a fully reversible unfolding curve, i.e. one in which the refolding transition (starting from the unfolded state diluted into lower urea concentrations) superimposes on the unfolding ditto (starting from the native state transferred to higher urea concentrations) (K.K. Andersen and D.E.O, data not shown). In the region of transition from predominantly folded to predominantly unfolded, equilibration kinetics take many days and rate constants in this region can only be obtained by extrapolation from regions where the kinetics are more rapid. Furthermore, unfolding and refolding proceed through multiple pathways which in the case of folding we interpret as being due to different levels of collapse prior to folding proper and in the case of unfolding depends on the amount of surfactant that is stripped from the protein prior to unfolding (K.K. Andersen and D.E.O, data not shown). This makes it very difficult to apply the principle of microscopic reversibility to OMP folding/unfolding. Even mixed micelles are not much help here. OmpA heat-denatured in SDS (and therefore not aggregated) can be refolded in the presence of higher than 0.7 mole fractions octyl-glucoside, but the process is very slow close to the transition midpoint of ~0.8 mole fraction, and refolding kinetics in neat SDS at 30 °C (estimated to have a half life of close to 4000 years) can only be obtained by extrapolation from very high OG mole fractions [266]. Conversely, unfolding in SDS can only be accomplished at high temperatures (64–78 °C) and extrapolation to neat SDS at 30 °C predicts a refolding half life of 3.3 years [266]. Thus, although the denatured state is stabler than the native state by a factor of ~1000 (corresponding to 4.4 kcal/mol), the activation barriers are enormous.

14.14. OMP solubility in denaturants opens up for controlled aggregation close to the cmc

Nevertheless, OMPs' relatively high solubility in denaturant also means that, although they require micellar concentrations of surfactant to refold [261], it is possible for them to remain soluble, if not completely folded, at concentrations close to the cmc. This opens up for an exploration of what happens under micelle-limiting conditions, analogous to the behaviour of globular proteins at sub-cmc conditions. OmpA forms a significant proportion of higher-order structures which correspond to apparently folded and unfolded oligomers (H. Wang and D.E.O, data not shown). One—as yet speculative—explanation for this behaviour is that the limiting

amount of micelles close to the cmc forces several protein molecules to share the same micelle. The spatial proximity of these molecules allows them to form intermolecular β-sheets, first through a relatively small number of strand contacts (the "unfolded" oligomeric state) and subsequently consolidated to more stable species as the proteins fold as oligomers, possibly interdigitating through domain swapping. Similar β-sheet promiscuity has been seen in the association of fragments of OmpA [267]. This behaviour is analogous to the folding and association of GpA, where micelle concentration is also the limiting factor in the formation of a given species (here the dimeric state), as well as the behaviour of globular proteins like ACBP and native unfolded proteins like α-synuclein, which also form higher order structures at micelle-limited conditions; for the helical protein ACBP, dimerisation is the highest association species whereas for α-synuclein, which aggregates via β-sheet structures, there are no upper limits to association and aggregation.

It seems fitting to end with this glimpse of the underlying grand theme of shared micelles as a driving force in surfactant-driven protein interactions, extending out to include membrane proteins.

14.14.1. Future perspectives in protein–surfactant interactions

14.14.1.1. The importance of understanding the surfactant-denatured state. The structures of denatured proteins at high surfactant concentration may help explain the many intriguing phenomena in the unfolding of globular proteins, as well as help rationalise stability changes for membrane proteins. A striking weakness of the protein–surfactant field is that at present there is no basis to make detailed predictions of how different proteins will interact with different surfactants and how e.g. site-directed mutagenesis can be expected to affect this. Experimentalists need to build closer links to simulators of protein–surfactant interactions. This requires sophisticated force fields that can take into account surfactants' self-assembling properties. The Sansom group has made pioneering contributions [268,269], which describe the increased flexibility of membrane proteins in the micellar environment, and also provide a useful approach to transfer structures solved in surfactant micelles to the lipid bilayer. Undoubtedly this work will be stimulated by a better understanding of how globular proteins are unfolded in surfactants. More detailed knowledge of the SDS-denatured state may ultimately provide the starting point for atomic-level simulations of the folding of membrane proteins from a denatured state. At present it may be an idea to use the relatively limited data base of mutant effects on surfactant-induced unfolding [73,119], which can serve to validate the results of the simulations.

14.14.1.2. A green vision: from petro-surfactants to biosurfactants. Currently used surfactants derive in large part from the petrochemical industry (derived from the sulfonation of hydrocarbon compounds) [270], and it is to be foreseen that we will have to adjust to surfactant produced from other sources as the era of unlimited fossil fuels draws to a close. Some demands may be met by replacing current alkanes with microbial biodiesel products and simply producing the same products using different raw products. However, there is also significant research in the use of alternatives such as rhamnolipids (glycolipids) which can be produced by *Pseudomonas* from various low-cost substrates and are both surface-active and antimicrobial [271]. It is unclear what environmental impact, e.g. in terms of perturbing microbial ecology and building up bacterial resistance, the bulk-level use of such bioactive products will have and whether they can be produced at quantities relevant for the detergent industry [272]. Nevertheless, should this venture take off in a big way, there will be a strong need to understand and control the types of interactions that these new classes of surfactants may form with proteins, in order to continue improving enzyme performance in this environment. It seems safe to conclude that there will be many opportunities to continue working with protein–surfactant interactions in the future.

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