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Review

Folding and stability of membrane transport proteins *in vitro* ☆

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ABSTRACT

Transmembrane transporters are responsible for maintaining a correct internal cellular environment. The inherent flexibility of transporters together with their hydrophobic environment means that they are challenging to study *in vitro*, but recently significant progress been made. This review will focus on *in vitro* stability and folding studies of transmembrane alpha helical transporters, including reversible folding systems and thermal denaturation. The successful re-assembly of a small number of ATP binding cassette transporters is also described as this is a significant step forward in terms of understanding the folding and assembly of these more complex, multi-subunit proteins. The studies on transporters discussed here represent substantial advances for membrane protein studies as well as for research into protein folding. The work demonstrates that large flexible hydrophobic proteins are within reach of *in vitro* folding studies, thus holding promise for furthering knowledge on the structure, function and biogenesis of ubiquitous membrane transporter families. This article is part of a Special Issue entitled: Protein Folding in Membranes.

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Contents

Abbreviations: MFS, Major Facilitator Superfamily; GalP, *Escherichia coli* galactose transporter; LacY, Lactose Permease; ABC, ATP Binding Cassette Protein; DDM, *n*-dodecyl β-D-maltoside; DS, decanoylsucrose; LDAO, Lauryldimethyl-*N*-amineoxide; PE, phosphatidylserine; NBD, Nucleotide Binding Domain; TMD, Transmembrane domain; DOPE, L-α-1,2-dioleoylphosphatidylethanolamine; DOPC, L-α-1,2-dioleoylphospatidylcholine; GuHCl, Guanidine Hydrochloride; SDS, Sodium Dodecyl Sulphate; CD, Circular Dichroism; ITC, Isothermal Titration Calorimetry; C12E10, Polyoxyethylene 10 Laurylether; SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; DSC, Differential Scanning Calorimetry; PTS, phosphotransferase system; PEP, phosphoenolpyruvate; DGK, diacylglycerol kinase; KcsA, potassium channel; DsbB, disulphide bond reducing protein; CopA, thermophilic copper transporter; EmrE, small multidrug transporter; CFTR, cystic fibrosis transmembrane conductance regulator; TDG, β,D-galactopyronosyl 1 -thio- β,D-galactopyranoside; POPG, 1-palmitoyl-2-oleoyl phosphatidylglycerol; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; NhaA, Sodium proton antiporter; AFM, Atomic Force Microscopy; SMR, Small Multidrug Resistance

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1. Introduction

1.1. Folding transporters

There is currently a large imbalance in protein folding studies, with a large body of knowledge relating to water-soluble proteins [1], whilst the membrane protein field remains largely a mystery. Folding studies to date have focussed mainly on small soluble proteins [2], with very few studies on larger, multidomain soluble proteins [3] and even fewer on integral membrane proteins [4–6]. Those few membrane proteins that have been studied have generally also been single domain, relatively well behaved, stable proteins. The significant, ubiquitous membrane transporter families comprise large multi-domain or multi-subunit proteins and are therefore a very under-represented group of proteins in the folding field. The principles of water-soluble protein folding cannot be applied wholeheartedly to membrane proteins due to the radically different environments of the two protein classes, where membrane proteins fold within a hydrophobic, anisotropic environment rather than a polar, aqueous solvent. Unlike water-soluble proteins, membrane proteins expose their hydrophobic residues to the membrane, instead of burying them in the protein interior. In contrast hydrophilic residues are found either on the surface of the protein outside the membrane, or neighbouring the lipid headgroups at the membrane edges, as well as sometimes on the inside of the protein structure, for example when forming a channel. Membrane proteins exist in a heterogeneous environment, interacting with lipid chains, headgroups and aqueous phases, and are also highly influenced by their lipid surroundings, which take an active role in topology of the protein and its function [5].

Membrane proteins, particularly transporters and receptors, have been the subject of many reviews [7–11]. Receptors have been studied in great detail, and are reviewed elsewhere [12]. Transporters have a high physiological relevance and represent over a third of all drug targets [13].The lack of information regarding transporters means that the molecular basis of action of many drugs is unclear. In addition, there are many diseases associated with membrane proteins [8,14–16], which are often as a consequence of mutation as in the case of cystic fibrosis [17]. Disease creating mutations are often far away from the active site [18], with an unknown mechanism of action but frequently influencing folding, stability and trafficking within the cell. Owing to the lack of information on membrane proteins, any further elucidation of the molecular basis of these diseases is very difficult, which in turn means many therapies out currently of reach until more information can be gained.

The current disparity in knowledge of water-soluble and membrane protein folding is in part a consequence of the fact that, as well as being more hydrophobic, membrane proteins are often much more dynamic than soluble proteins. Such dynamic behaviour is inherent to transport but makes membrane transporters very difficult to work with *in vitro*. In addition to this problem, membrane proteins can be challenging to express and purify in sufficient quantities *in vitro*, as their inherent hydrophobic nature means they have a high tendency to aggregate once removed from their native lipid environment. However, these barriers to folding research must be overcome in order to learn about the fundamental folding properties and thus maintain and study the correct structure and function of membrane proteins, and therefore ultimately to learn more about their physiological roles. Current folding work *in vitro* therefore focuses on elucidating how to stabilise and study relatively simple, usually prokaryotic, membrane proteins, with the aim of extending the knowledge gained to a wider range of physiologically relevant proteins in the future. *In vivo* folding of helical membrane proteins tends to involve co-translational insertion via a membrane-embedded translocon complex. Cellular studies are thus also undertaken, with notable examples including probing the role of lipids in transporter topology [19] or the role of the translocon in identifying transmembrane helices, and the order in which helices emerge from the translocon into the bilayer [20,21]. There are also studies on β barrel membrane proteins, as they are often comparatively easy to express as inclusion bodies which can then be dissolved in urea [22].

Transporters often have more than one domain or even multiple subunits, which create further difficulties *in vitro* in ensuring the correct assembly is being studied (see references [7,23] for reviews on transporter structures). Studying multidomain and multisubunit membrane proteins provides information on the interfaces between different domains and subunits, and the importance of these interactions in the overall stability and fold of the protein [24,25]. These interface regions are particularly important as they allow the flexibility needed for transporter function. It is also important to establish whether domains fold independently or cooperatively, as this may shed some light on assembly of membrane proteins *in vivo*, and the consequences of small alterations induced in assembly and stability by mutations.

1.2. Detergent or lipid?

In addition to the heterogeneous environment surrounding membrane proteins, there is the added complexity in that membranes in different cellular locations contain very different lipid compositions (for a review on these see reference [26]). It is becoming increasingly obvious that these diverse lipid compositions are important, with certain specific lipids being essential for correct protein function, topology, and overall fold [19,27]. There have been extensive reviews on the effect of lipids on the topology of secondary transporters [19,28,29]. There are other examples where the exact molecular mechanism of these lipid effects on transporters is unclear, with evidence varying as to whether these are due to bulk lipid effects [30–32], or specific interactions between the lipid and the protein [33]. Several studies show that the lipid curvature and lateral pressure in the bilayer alter the membrane insertion kinetics and folding rates of membrane proteins [30–32,34,35]. It is therefore important to consider these lipid effects for the protein in question, and whether the absence of, or an incorrect, lipid influence would create artefactual results.

The complex native membrane protein environment is challenging to reproduce and thus folding is studied in a simpler system *in vitro*. During purification, membrane proteins are usually purified into detergents or lipid vesicles, or liposomes, as a mimic of the lipid bilayer. The validity of this is obviously questionable, but simpler systems must first be used in order to gain sufficient information to move forward successfully to a more complex and realistic artificial system. Many *in vitro* membrane protein folding studies use detergent micelles, however the lack of curvature stress in micelles could alter the stability or structure of the protein [6]. Nonetheless the detergent micelles used *in vitro* provide a convenient and practical solution for membrane protein study and in numerous cases can sustain a stable, functional protein structure, highlighted in a recent review by Sanders and Mittendorf, which argues the case for using model

membrane systems *in vitro*. The similar structures seen between homologous membrane proteins across all organisms, and the differing lipid compositions experienced by a single membrane protein in its lifetime, both suggest that while the lipid environment is very important, membrane proteins clearly have a high tolerance for the surrounding membrane's composition. This is also illustrated by the fact that *in vitro* studies using different model membrane systems give similar results [36]. Therefore *in vitro* studies using non-physiological model membrane systems can still be highly useful.

1.3. Membrane protein folding in vitro and mechanistic models

Several models and hypotheses have been proposed for the folding mechanisms of alpha helices within the membrane, as outlined below.

The classic two stage model for folding was originally outlined in 1990 by Popot and Engleman [37]. This model is based on thermodynamic principles, and whilst being oversimplistic, has nonetheless often provided a useful framework for transmembrane helical proteins. The two-stage model is based upon the principle that transmembrane helices can be inherently stable, thus in stage one helices insert into the membrane, whilst in stage two they rearrange to form their native interhelical contacts and tertiary structure. This model is generally insufficient to explain transporter folding, especially in cases where there is more than one domain or subunit folding cooperatively. The two stage model was modified in 2003 to include a third stage [38], which could involve any number of events including higher order domain association, the binding of a prosthetic group, or the folding of extramembrane loops after insertion. This three stage model still contains the assumption that individual helices are stable independently. Equally, however, some transmembrane helices are often not stable entities, for example if they contain several polar amino acids, and there are cases where certain helices require the existence of a pre-formed helical bundle in the membrane to insert and fold.

The proposal that interactions between several helices occur in a single second step most certainly masks a number of smaller steps necessary to form these interactions. The early stage of α helical interactions within the membrane is thought to involve minor docking events, with few specific interactions between side chains, and little or no change in the degree of solvent bound to the structure [39]. A later folding stage would involve the formation of stabilising contacts, where helices come together and the solvent is forced away due to the higher stability of the helical contacts. Loops may be the final parts to fold, after being brought together by the helices, which can act as an initial scaffold.

This review will focus on the stability and folding of transporters, and will briefly discuss different mechanistic models for folding, then summarise the results from *in vitro* stability studies and the effects of varying lipid composition on stability. Some examples of the effects of mutation on folding and stability will also be discussed with respect to a variety of different families of transporter. We will commence with an overview of experimental approaches that are commonly applied in studies of transporter folding and stability.

2. Methods to study transporters *in vitro*

2.1. Techniques to measure folding and activity

The unavoidable presence of detergents and lipids in membrane protein samples increases the difficulty of *in vitro* work, due to the high level of scattering in spectroscopic methods, which can create artefacts in measurements [4,18]. The presence of lipids or detergent also creates a highly anisotropic environment, and in addition the usually relatively low quantities of protein make data interpretation difficult due to a low signal to noise ratio, and the high likelihood of artefacts in the data. Often there are only very small differences between the folded and unfolded state [18], which further adds to the signal *vs.* noise problem. The method of expression and purification of alpha helical membrane proteins is very important, as most membrane proteins have a high tendency to form insoluble aggregates when overexpressed, giving low yields (see reference [40] for a review on this subject). Table 1 gives an overview of the expression and purification methods of the transporters discussed in this review.

A novel method to measure a high affinity association of helices within the bilayer is to use a steric trap. The association of Glycophorin A helices has been measured using this method, a biotin acceptor peptide was fused to Glycophorin A, and the binding of streptavidin (mSA) was used to measure the dissociation constants of helix association. The binding sites for mSA can be engineered in such a way so that only one mSA can bind if Glycophorin A is a dimer due to steric clashing, therefore the binding parameters of mSA can be used to elucidate the dissociation constant of the dimer. This method has the advantage in that it enables the measurement of a high affinity association without the need to dilute the complex, which is usually necessary to dissociate high affinity interactions. This is an important tool for the investigation of multidomain membrane complexes, and to compare the stability of the complex in different systems. For example in this study, the dimer was much more stable in POPC bilayers than in detergent micelles [41].

2.2. Reversible and irreversible folding experiments

There are several methods to study folding *in vitro*. A folding reaction measured at equilibrium is fully microscopically reversible, *i.e.* removing the denaturant is sufficient for full refolding back to the native state, and equilibrium unfolding and refolding curves will overlay as folding and unfolding reactions follow the same pathway. Denaturation can be either chemical or thermal; therefore reversibility would involve either removing a chemical denaturant, for example by dialysis or dilution, or decreasing the temperature for thermal studies. Membrane transporters often unfold irreversibly by thermal

Table 1
Expression and purification systems for transporters.

Family	Transporter	Chromatograpy	Detergent solubilisation and experiments	Problems encountered?	Reference
MFS	GalP	C terminal 6-His tag, Ni^{2+}-NTA	DDM solubilised DDM/liposomes (DOPE/DOPC 60 mol% PE)		[43,92]
	LacY GLUT1	Biotin/Avidin	DM	Aggregation during refolding	[44]
SMR	EmrE	6-His/Ni^{2+}-NTA followed by gel filtration	DDM	6-His tag interferes with dimer formation	[34,35]
ATPase	CopA	Purified at 4 °C with C terminal 6-His tag, Ni^{2+}-NTA	C12E10 solubilised DDM/asolectin	Some detergents give inactive protein	[42,76]
PTS	II^{mtl}	6-His tag purified by Ni^{2+}-NTA	Decyl-PEG solubilised DM exchange DMPC for experiments		[25]

The purification systems used for some of the transporters discussed in this review.

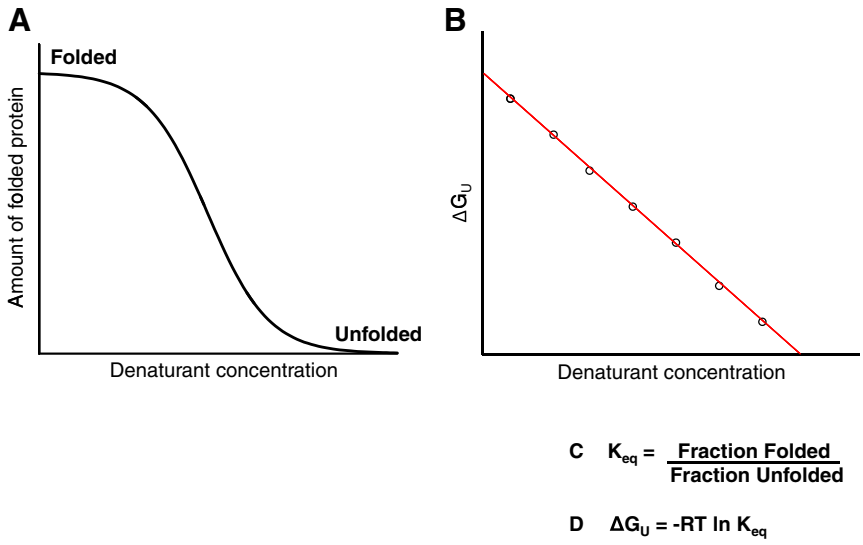

Fig. 1. Determination of unfolding free energy from a denaturation curve. In an unfolding experiment the amount of native protein structure is measured, for example by fluorescence or CD, at increasing concentrations of denaturant. The change in the amount of folded structure is then plotted against the denaturant concentration, giving a denaturation curve (A). Refolding curves are measured in the same way, by diluting the denaturant. If the folding and unfolding curves overlay, then it is a two state reaction, and the fraction of folded and unfolded protein can be calculated at each denaturant concentration. From this, the equilibrium constant (K_{eq}) can be calculated (see the equation in C), and free energy of unfolding (ΔG_U) can be calculated (see the equation in D) at each denaturant concentration, which is then plotted (B). The linear fit of these points can then be extrapolated to zero denaturant, which gives the free energy of unfolding in the absence of denaturant, ΔG_U^{H2O}.

denaturation, and tend to aggregate during the experiment. Chemical denaturation however, can often be reversible [34,42–44] (see Fig. 1). The reduction in native protein structure induced during titration with denaturant is measured to produce a denaturation curve, which for an equilibrium reaction can be exactly overlaid by the refolding curve, during which the denaturant is diluted. An equilibrium constant, K, can be measured for both the forward and reverse reactions (unfolding and refolding respectively), and the associated free energy of folding or unfolding, ΔG, can be calculated.

Unfolding can be fully reversible, but with a different reaction pathway for unfolding and folding. This would happen for example when lipids have been added after the removal of denaturant to promote refolding. No thermodynamic equilibrium information can be gained from these sorts of folding reactions, but it is useful as a measure of stability for investigating the effects of varying the lipid composition on refolding [31,32,34,35,43]. See Fig. 2 for a comparison of reversible folding at equilibrium, and reversible folding via a separate folding pathway.

Thermal denaturation can often be fit to a reaction scheme with an initial, reversible equilibrium folding step up to a certain temperature, and a subsequent irreversible step at higher temperatures. Thus, thermodynamic can still be obtained. In the case of membrane proteins, lipids and detergent properties change at different temperatures, which also affect the stability and the results seen.

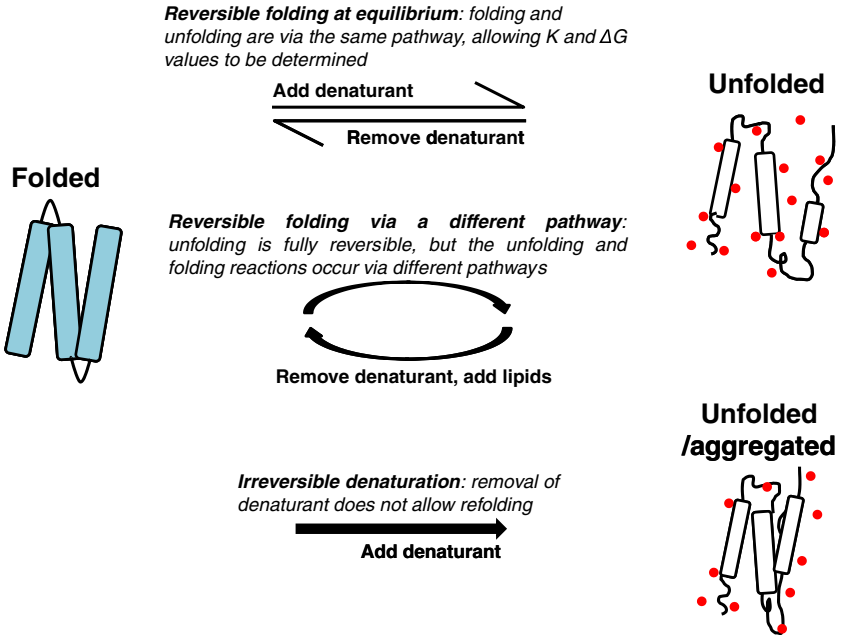

Fig. 2. Reversible and irreversible folding. Different types of *in vitro* folding reactions, reversible folding at equilibrium is shown at the top, reversible folding *via* different pathways in the middle and irreversible folding at the bottom. Denaturant is shown as red circles, with the location of the denaturant around the protein approximated. Detergents or lipids have not been shown for clarity.

2.3. Thermal denaturation

One of the earliest methods applied to probe transporter stability was thermal denaturation. The enthalpy of a protein is usually affected by temperature, and increases with increased temperature [45]. The method of choice is Differential Scanning Calorimetry (DSC), a highly sensitive technique giving quantitative thermodynamic information [46–48]. DSC involves comparing the heat generated by a sample in comparison to a reference cell at different temperatures. Detailed thermodynamic information can be gained via this method, including the temperature at which transitions occur (Tm), and the heat capacity of these transitions (Cp). The calorimetric enthalpy, ΔH^{cal}, can be determined directly obtained from a DSC experiment from the area under the heat absorption peak in the thermogram and is expressed on a molar basis. It is often useful to also determine the van't Hoff enthalpy, for which an effective equilibrium constant is defined, based on the progress of the reaction as obtained from the transition curve. However, this analysis is only valid if the sample is in thermodynamic equilibrium throughout the experiment. This is shown by the fact that the transition is reversible (as determined in a rescan of the sample) and independent of the scanning rate used in the experiment, which shows that the reaction is not under kinetic control. Although this is rarely the case for membrane proteins, this technique has been particularly useful for multidomain membrane proteins, as it gives insight into the interactions between different domains [25,46]. Data for most membrane proteins is fit to a model involving a reversible reaction followed by irreversible denaturation.

There are many problems associated with measuring the thermodynamic parameters of alpha helical membrane protein thermal denaturation. The detergent or lipid used and its effects are important to consider, as the interactions with the protein are likely to be affected at different temperatures. Phase transitions of the detergent or lipid can also occur, creating artefacts in the results. The altered interactions between the solvent and the detergent can also prevent the protein from being soluble, leading to aggregation during the experiment. Membrane transporter unfolding by thermal denaturation is usually only reversible up to a certain temperature, therefore irreversible transitions make DSC experiments and the subsequent data interpretation difficult, it is also fairly common to be unable to get replicable results. There have however been a few examples of alpha helical membrane proteins investigated in this way, most have been receptors and photosystems [46], but there have been a small number of transporters also, as explained in greater detail in Section 3 [25,45,47,48].

2.4. Reversible folding

The establishment of a two-state (or three-state) folding reaction *in vitro* greatly facilitates a complete understanding of folding, including thermodynamic stability. An experimental system is required in which a microscopically reversible folding reaction is observed at equilibrium. This is frequently established for helical membrane proteins using chemical denaturants such as urea or SDS, and renaturing micelles. Kinetic measurements have been used to confirm two-state equilibrium folding between unfolded and refolded states [49].

There are only a handful of transporters for which reversible folding has been successful. These come from several different families of transporter, and are summarised in Table 2. Briefly, these are the Major Facilitator Superfamily galactose and lactose transporters GalP and LacY [43,44], the Small Multidrug Resistance transporter EmrE [34], the thermophilic copper ATPase CopA [42], and the Na^+/H^+ antiporter NhaA [50]. These have been expressed and purified using a variety of methods using different detergents, and unfolded with different denaturants. As previous folding studies to date had been confined to small soluble proteins or single domain, experimentally amenable, helical membrane proteins and β barrels, these more recent studies on transmembrane α helical transporters represent a major step forward in the folding field.

2.5. A brief note on denaturants

SDS is a common denaturant used for α helical proteins, with several theories proposed for its denaturant action [39,51], whilst conversely it has been found to stabilise and induce helical structure [52]. SDS maintains a micellar environment by forming mixed micelles with the renaturing detergent in the system. Morever, although SDS causes reductions in tertiary structure and loss of function, at best it only causes partial reductions in helicity of transmembrane proteins and sometimes the native α helical content is maintained [53]. As a result, the unfolded state created by SDS contains significant helical structure (see Fig. 3). For more detailed information on the interactions between SDS and membrane proteins, see references [54,55]. Fully unfolded states of helical membrane proteins require strong

Table 2
Reversible folding methods.

Family	Transporter	How it was unfolded, and how it was measured	How it was refolded	How was the return of function demonstrated?	What did we learn?	Reference
ABC	Vitamin B12 transporter (BtuCD)	Unfolded in urea Measured by fluorescence, CD and loss of ATPase activity	Bound to Ni^{2+}-NTA column and eluted into DDM	Correct oligomeric state by gel filtration and SDS-PAGE, return of ATPase activity	More efficient re-assembly from unfolded subunits	[24]
MFS	GalP	Unfolded in urea or GuHCl Measured by fluorescence, CD or loss of ligand binding	Dilution into DDM or into liposomes	Return of ligand binding and transport	Liposome composition affects refold yield	[43]
	Lactose Permease (LacY)	Unfolded in GuHCl Measured by MIANS fluorescence and substrate protection	Dialysis into DM in presence of 3:1 POPE/POPG	Return of substrate protection against thermal denaturation	Presence of TDG did not accelerate refolding	[44]
SMR	EmrE	Unfolded in 10 M urea plus 5% or 10% SDS Measured by fluorescence, CD and cysteine labelling	Either by dilution or by binding to Ni^{2+}-NTA column and eluted into DDM	Return of TPP binding measured by ITC	Liposome composition affects refold yield	[34]
ATPase	CopA	Unfolded in 8 M GuHCl Measured by fluorescence, CD and loss of transport activity	Dilution into DDM and asolectin	Return of transport function	Unfolded state present undetected by spectroscopic methods	[42]
Na^+/H^+ antiporter	NhaA	Unfolded by AFM	Helices reinserted spontaneously	Not done	Helices are associated in pairs	[50]

The conditions and methods used for reversible folding of transporters, and how this reversibility and return of function was demonstrated. BtuCD has been included in this table, as while not strictly a two state reversible folding study, the re-assembly was specifically investigated from both unfolded and folded subunits.

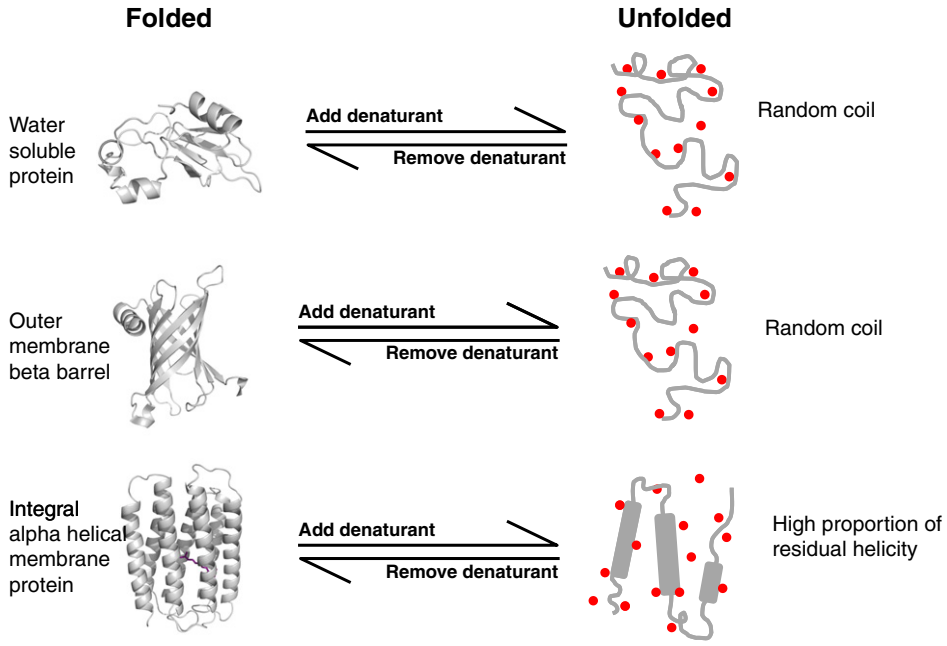

Fig. 3. Reversible unfolding of proteins. Alpha helical membrane proteins have a lot of residual helical structure remaining when reversibly unfolded, unlike β barrels and water soluble proteins which tend to unfold into a more random coil-like structure. Denaturant is shown as red circles, with the positions of the denaturant around each protein approximated. The denaturant is not necessarily the same for each protein. Detergents or lipids have not been shown around the β barrel and alpha helical proteins for clarity (PDB IDs 1B2X, 2BRD, 1THQ).

organic acids which are unsuitable to direct refolding into detergents or lipids, since they also destabilise these latter structures.

Chaotrope denaturants such as urea and guanidine hydrochloride (GuHCl) may preferentially bind to the unfolded state, stabilising it over the folded state, and altering the folding equilibrium. These denaturants may also solvate charged residues, or directly hydrogen bond with the backbone, disrupting native interactions [56]. This is a different mechanism of action from that proposed for SDS denaturation, thus limiting direct comparisons between these two methods of unfolding in that SDS can readily partition into micelles and destabilise lipid bilayers, whilst urea and GuHCl do not. Urea does, however, alter the dynamics and phase behaviour of detergents and lipids, for example by a change in the detergent critical micelle concentration. The relative inability of urea to denature micelles or bilayers means it often cannot denature helical membrane proteins, although in acidic conditions — as is the case for diacylglycerol kinase (DGK) — quaternary trimer interactions are perturbed in micelles but little helix reduction occurs. Urea can also denature aqueous extrinsic regions of helical membrane proteins. The flexibility of transporters with hydrophilic binding pockets may also provide access for urea into the protein interior for denaturation. Urea is however an effective denaturant for β barrel proteins, as seen in Fig. 3 [22].

2.6. What is the unfolded state?

The denatured state of a folding reaction is not a single state, but an ensemble of multiple unfolded conformations [19,42], all with many long range interactions still present. Therefore the unfolded state measured experimentally is an average representative state of this ensemble, so specific structural information is difficult to access. α helical membrane proteins tend to aggregate when unfolded. Moreover, as described in Section 2.5 and shown in Fig. 3, there is a considerable amount of structure remaining in the experimental unfolded state, in contrast with β barrels and water-soluble proteins [4,5]. This partly structured unfolded state may provide a critical helical core that is required as a scaffold for successful folding [5]. This is perhaps more physiologically relevant than a folding study from a completely denatured state, as a helical membrane protein is never in a completely random coil *in vivo* since they are translated directly into the translocon, and the helices are subsequently inserted laterally into the bilayer from the translocon [20,57].

3. Families of transporters

There are several examples of transporters which have been studied *in vitro* for stability and folding, both by irreversible thermal denaturation and reversible systems. These are summarised in Table 3, along with the methods applied to each transporter.

3.1. Major Facilitator Superfamily

The Major Facilitator Superfamily (MFS) is a very large group of transporters found in all organisms and is responsible for transporting a large range of substrates such as sugars, amino acids and peptides [9,10]. There are now several high resolution crystal structures, for a range of members and in different conformations with both bound and unbound ligand [58–62]. Each has a very similar structure, with 12 transmembrane helices divided into two helical domains and a large central cavity between them. There have been extensive studies into the transport mechanism of this family [63–65], the location and contacts within the substrate binding sites [61,62,66,67], and specific lipid effects on function [19,33], but only a handful of studies on stability as outlined below.

An MFS member to be investigated by thermal denaturation is the facilitative glucose transporter GLUT1, using DSC. Thermal denaturation of GLUT1 is irreversible, as demonstrated by the lack of structure when the sample was cooled. It was found that the GLUT1 substrate D-glucose specifically stabilises the protein, increasing the Tm of 68.5 °C to 72.6 °C. It is thought that substrate binds to the folded state and stabilises it, shifting the equilibrium between the folded and unfolded states. The calorimetric transition enthalpy was comparable with other membrane proteins, at 2.8 cal/g [45].

The only two state reversible unfolding study to date of an MFS member is on the *Escherichia coli* galactose transporter, GalP [43]. Reversible folding in urea was measured at equilibrium, with intrinsic tryptophan fluorescence indicating the exposure of tryptophan

Table 3
Summary of stability studies on transporters.

Family	Protein	Method	Results	Reference
MFS	GalP	Reversible folding by fluorescence and CD	Two state free energy of unfolding Effects of lipid composition on folding and insertion	[43]
	Lactose Permease (LacY)	Reversible folding by fluorescence	Demonstration of folding reversibility	[44]
	Glucose transporter 1 (GLUT1)	Thermal denaturation by DSC	Substrate binding increases thermal stability	[45]
SMR	EmrE	Reversible folding by fluorescence and with lipids, ligand binding measured by ITC	Demonstration of folding reversibility Effects of lipid composition on folding and insertion	[34]
ATPases	CopA	Reversible folding by fluorescence and CD	Reversible system and free energy	[42]
	NaK-ATPase	Thermal denaturation by DSC	Thermal stability	[48]
	Ca-ATPase	Thermal denaturation by DSC	Thermal stability	[47]
ABC	Histidine permease ($HisQMP_2$)	Re-assembly of subunits	Non-cooperative assembly of subunits	[86]
	Maltose transporter ($MalFGK_2$)	Re-assembly of subunits	Cooperative assembly of subunits	[87]
	Arginine transporter ($Art(MP)_2$)	Re-assembly of subunits	Assembly of subunits	[88]
	Vitamin B12 transporter (BtuCD)	Re-assembly of subunits from folded and unfolded states	Cooperative assembly of subunits	[24]
Na^+/H^+ antiporter	NhaA	Reversible folding measured by AFM	Demonstration of folding reversibility	[50]
PTS	Mannitol Permease (II^{mtl})	Thermal denaturation by DSC	Interactions of domains and contribution to stability	[25]
AE	Anion Exchanger 1 (AE1)	Thermal denaturation by DSC	Interactions of domains and contribution to stability	[46]

A summary table of all the transporters discussed in this review, what was measured, and the important findings from each.

residues to a more polar environment that is indicative of unfolding. Fluorescence studies on GalP are complicated to interpret however, due to the large number of tryptophans in a range of different environments. A more useful technique in this case is to monitor the reduction in secondary structure that is induced by urea through the use of circular dichroism (CD) spectroscopy. The free energy of unfolding in the absence of denaturant, ΔG_U^{H2O}, was found to be 2.5 kcal/mol ± 0.2 kcal/mol, with a linear dependence on denaturant. GalP has also been found to partially refold from 3.5 M GuHCl, a much harsher denaturant.

As discussed above, it is thought that urea is able to denature members of the MFS due to the large proportion of solvent accessible areas in comparison to other membrane proteins. There is currently no crystal structure for GalP, but it is thought to be similar to GlpT and as all the MFS structures solved thus far are very similar, it is likely that GalP also has large solvent exposed ends of helices and a large hydrophilic cavity that will allow access of the urea to large areas of the protein.

The most well characterised MFS member, Lactose Permease (LacY) has also been successfully refolded after denaturation in 3.5 M GuHCl [44], with the denaturant removed by dialysis into renaturing dodecylmaltoside (DDM), in the presence of the lipids palmitoyloleoyl phosphatidylethanolamine (POPE) and palmitoyloleoyl phosphatidylglycerol (POPG). The reversibility and recovery of function after refolding were demonstrated by a return of the substrate TDG protecting LacY from thermal denaturation, in a manner similar to GLUT1 as discussed above. It was necessary for refolding of LacY to be done at a very low protein concentration (0.05 mg/ml), in order to prevent aggregation during refolding.

LacY is well known for being very tolerant of mutations [68], with only six residues absolutely irreplaceable for transport activity. There is however an interesting example of how a single point mutation can have unexpected large consequences on transport activity and stability. The LacY C154G mutation is completely folded and able to bind ligand, but has highly impaired transport function. It is also much more thermally and chemically stable in comparison to the wild type [69]. It is believed, but still unclear, that altered packing interactions between helices account for this increased stability [70].

The effects of varying lipid composition on topology, stability and activity have been investigated for several members of the MFS with some very interesting findings. Most of the topology studies have been on LacY, where it has been found that when *in vivo*, and when reconstituted into bilayers *in vitro*, the topology is severely affected by the absence of the lipid PE, in that the entire N domain assumes an inverted topology. It is thought that this drastic topology change is not necessarily due to a specific requirement for PE, but is due to the high amount of negatively charged headgroups in the bilayer that would be the result of an absence of PE. This high negative charge of the bilayer may disrupt the "positive inside rule" and the normal topogenesis signals, causing an inversion of N domain topology. Nonetheless, this inverted LacY is still folded and able to catalyse downhill transport (see references [19,28,29] for detailed discussion on the effects of PE on transporter topology). To date, there is no evidence of an inverted topology when detergent micelles are used instead of lipid vesicles. In addition, the *Lactococcus lactis* multidrug MFS antiporter LmrP, which uses the proton gradient to transport cytotoxic compounds out of the cell, is an example of the requirement of a specific interaction between a transporter and lipid headgroups for correct function [71]. Hydrogen bonding with the lipid headgroup seems to be necessary for transporter function, although the exact mechanism is unclear [33]. The effects of varying lipid compositions on GalP folding and insertion have also been investigated. Refolding into liposomes has an optimum composition of 60 mol% DOPE, but is around 2.5 times less active when in this manner in comparison to when directly reconstituted into liposomes [43].

3.2. Na^+/H^+ antiporters

The maintenance of an internal cellular pH, and the homeostasis of sodium ion concentration is essential for cell viability. This is the role of Na^+/H^+ antiporters, and there are many homologues found across all organisms [11]. The most well characterised Na^+/H^+ antiporter, the *E. coli* NhaA, was unfolded by Atomic Force Microscopy (AFM). AFM uses a stylus attached to either the N or C terminal end of the protein, with an increasing force applied to sequentially unfold individual helices. This method has the advantage in that it can be done on single transporters embedded in membranes. The NhaA unfolding results showed that the transmembrane helices unfolded two at a time, suggesting that they are associated in pairs. It was also found that the whole protein is required to form native interactions. Unfolding of NhaA by AFM was reversible, as shown when the polypeptide chain was relaxed and the helices spontaneously re-insert ("snap in") in an immediate refolding step, followed by a slower refolding step of the remaining helices [50].

3.3. Phosphoenolpyruvate dependent phosphotransferase system

The phosphoenolpyruvate dependent phosphotransferase system (PTS) couples transport and phosphorylation of substrates, using phosphoenolpyruvate (PEP) as the energy source to overcome the substrate concentration gradient. The transporters in this system are known as enzyme II, and one of its members, mannitol permease (II^{mtl}), has been studied by DSC. This transporter, like the other

members of this family, has one transmembrane domain and two cytoplasmic phosphorylation domains, so DSC can give information on the interactions between the domains as well as their thermal stability. The constituent domains were expressed separately and their stability compared with that of the complete transporter. There was more than one calorimetric transition seen in the full transporter, with the cytoplasmic domains being responsible for the reversible transitions at around 60 °C, and the transmembrane domain being attributed to the irreversible transition at 76 °C. The Tm of the transmembrane domain decreases when the B cytoplasmic domain is not present, indicating that association is necessary for stability. The calorimetric transition enthalpy of 4.2 cal/g was found to be slightly higher than other membrane proteins investigated in this way [46], thought to be due to the degree of solvent exposure [25].

3.4. Anion exchangers

Anion Exchanger 1 (AE1), or Band 3, is a chloride-bicarbonate antiporter mainly found in eukaryotes [72], with a transmembrane domain and large cytoplasmic domains which interact with other cellular proteins and the cytoskeleton. The stability and domain interactions of AE1 have been studied by DSC, with the different domains giving separate peaks in calorimetric thermograms. The cytoplasmic domain transition was found to be pH dependent, with a Tm of 72 °C at pH6, and a Tm of 55 °C at pH8. The transmembrane domain transition was between 66 and 68 °C, with the calorimetric transition enthalpy varying depending on whether peripheral proteins were present [46], indicating that interactions with other proteins affect the stability of AE1. An early study of AE1 found that the membrane spanning domain is much more resistant than the cytoplasmic domain to GuHCl denaturation when measured by CD, as at 4 M GuHCl the cytoplasmic domain unfolded to a random coil, and the membrane spanning domain retained around 70% of its helical content. This further illustrates the notion that membrane spanning regions of proteins are more resistant to denaturation [73].

3.5. Small Multidrug Resistance Family

The Small Multidrug Resistance Family (SMR) is a subgroup of the Drug/Metabolite Transporter Superfamily. The members of the SMR family have four transmembrane helices, and function as dimers [74]. Some reversible refolding work has been done on the SMR transporter EmrE [34], which functions as a homodimer. EmrE exchanges two protons for a substrate, which is usually hydrophobic and cationic, but the transporter has a broad specificity and broad range for substrates. EmrE has very stable helices, with only a small amount of structure loss when denatured in a combination of 10 M urea and 5% SDS. From this EmrE can be refolded into both DDM micelles and lipid vesicles. CD has been used to monitor the loss of secondary structure, and fluorescence for the degree of tryptophan exposure. The loss of helix when unfolded is small and unquantifiable due to the high amount of urea, but it is believed that urea disrupts the dimer, as it has been found to be monomeric when visualised by SDS-PAGE.

EmrE has also been investigated in liposomes of different compositions, to elucidate the effects of different lipids on refolding and insertion efficiencies. It has been found that there is lower transport activity in bilayers of higher curvature stress, as seen by increasing the dioleoylethanolamine (DOPE) content in DOPE/dioleoylphosphatidylcholine (DOPC) liposomes [34,35].

3.6. Transmembrane ATPases

Transmembrane ATPases are transporters which use the hydrolysis of ATP as an energy source to transport substrates against their concentration gradient [8]. There are several types of ATPase, classified by the types of substrate they transport, with a high diversity in structure and oligomeric state between each transporter [16].

There are examples of P-Type ATPases which have been investigated by DSC, being the sarcoplasmic reticulum Ca^{2+}-ATPase and the Na^+, K^+-ATPase [47,48]. In these studies the protein used was isolated from native membranes, therefore the data is difficult to compare with other studies due to the lower purity of the sample.

CopA is a thermophilic alpha helical P-Type ATPase, which transports Cu^+ in *Archaeglobus fulgidus*. It belongs to the P_{IB}-Type ATPase family responsible for transporting heavy metals [75], and has an optimum transport activity at a temperature of 75 °C [42,76]. Mixed micelles of DDM and asolectin were used to unfold CopA in GuHCl. The change in tryptophan fluorescence was used to monitor the changes in tertiary structure, and the change in CD used to monitor the decrease in secondary structure. Unfolding was achieved through incubation in GuHCl for 2 h, with no further reduction in structure occurring if incubated overnight. CopA was refolded by dilution into renaturing micelles of DDM and asolectin. During unfolding the function of CopA was measured at each denaturant concentration, showing a loss of activity before any structural differences could be detected. This shows the existence an early unfolding state not detected spectroscopically, suggesting that a two state model is not applicable to the unfolding of this protein under the experimental conditions.

CopA was successfully refolded from 7.5 M GuHCl, showing a higher stability than the members of the MFS which are reversible only up to 3.5 M [43,44]. This higher stability could be due to its nature as a thermophilic protein. Nevertheless, the ΔG_U^{H2O} of 3 kcal/mol is still roughly of the same magnitude as GalP [43]. Similarly to the MFS transporters, CopA is accessible to water soluble chaotropes, and it also has a large extramembrane domain which may or may not account for the majority of structure loss seen.

3.7. ATP binding cassette transporters

Most membrane protein folding studies to date have focussed on monomeric proteins. A large number of membrane proteins however have multiple subunits, both within the membrane and in the periplasm or cytoplasm. ABC (ATP Binding Cassette) transporters are a class of transmembrane ATPase, and are a ubiquitous group of multisubunit membrane proteins responsible for transporting a range of substrates either into or out of the cell with the hydrolysis of ATP. They generally have two transmembrane domains, and two nucleotide binding domains in the cytoplasm (see reference [77] for a review on ABC transporters), and require molecular chaperones for their correct assembly in the membrane [78]. Prokaryotic ABC importers also have a periplasmic substrate binding protein associated with the complex, to deliver substrate for transport. Folding studies on ABC transporters are complicated by the possibility of cooperative interactions between the multiple subunits [24], and is almost certainly not as simple as a two state process, due to the necessity of the different subunits having to interact with each other.

The Cystic Fibrosis Transmembrane conductance Regulator (CFTR) is perhaps the most well known ABC transporter, and the most well known example of how a small mutation can have devastating consequences to transporter function. CFTR is a eukaryotic chloride ion channel gated by the hydrolysis of ATP. Like other eukaryotic ABC transporters, it is formed of two halves, each of these composed of a transmembrane spanning domain (TMD) and a nucleotide binding domain (NBD). There is also a cytoplasmic regulatory domain, R, which contains serine residues for regulation by phosphorylation. There are many cystic fibrosis causing mutations in this R domain, often leading to trafficking defects [79]. However, the mutation responsible for over 90% of cystic fibrosis cases is ΔF508 in NBD1, which lies at the interface between NBD1 and intracellular loop 4 (ICL4) of TMD2 [17].

There is evidence to suggest that the loss of F508 directly affects the folding of NBD1, both *in vitro* and *in vivo* [80], not due to the loss of side chain interactions but due to the deletion of backbone. This has been shown by the lack of an effect on isolated NBD folding caused by missense mutations [81,82]. ΔF508 may slightly slow the rate of folding, altering the equilibrium of chaperone binding and therefore causing more of it to be degraded by cellular quality control mechanisms [17]. Larger downstream effects on assembly due to ΔF508 may also occur, as the interaction of NBD1 with ICL4 is altered by this deletion [17], and the TMDs are not as stable in the bilayer when the NBDs are absent [81]. The missense mutations which did not affect NBD1 folding when in isolation were shown to lower the amount of mature full length CFTR, illustrating the importance of F508 interacting with ICL4 [81]. The ΔF508 mutation illustrates how general domain packing and domain interactions rather than specific residue effects are important, which is demonstrated by the ability of suppressor mutations in sites away from F508 partially reversing the folding defects of ΔF508 [17,80].

The bacterial homologue of CFTR, Yor1p, has several destabilising mutations, including an analogous ΔF deletion. It has been found that two suppressor mutations (F270S and R1168M) correct many of these mutations, and enable the protein to avoid the quality control machinery of the cell. These mutations do not directly correct folding defects, but it is thought instead that due to their location near the transmembrane domain, that they alter helix packing and bilayer insertion, giving some extra tolerance for the original mutation [83]. The mechanisms of why this should be are not understood, and highlight the need for further understanding of interactions between helices within the membrane.

Like other transporters, ABC transporter function is affected by the presence of specific lipids. HorA is an ABC multidrug transporter of *Lactobacillus brevis*, and has been found to require the presence of the native lipid PE for transport. When HorA was reconstituted into liposomes containing DOPC instead of DOPE, HorA was still able to catalyse ATP hydrolysis, but lost the ability to transport the substrate Hoechst 33342. In this case the lipid effects are thought to be the more general bulk properties of PE affecting transport function and orientation of transmembrane helices, as detected by Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR) [84]. Another ABC transporter affected by general bulk lipid effects is the yeast *Candida albicans* multidrug transporter Cdr1p, with a higher flippase activity recorded bilayers of increased fluidity, but a corresponding lower transport activity [85].

3.8. Re-assembly of ABC transporters

There are a few examples of ABC transporters which have been re-assembled *in vitro*, in order to learn about the specific interactions between subunits, and to gain insight into assembly of these large complexes within the cell. Re-assembly requires the individual expression of each subunit, or failing this, the removal of subunits from each other once expressed as a whole. These subunits are then tested for a lack of transport activity, to demonstrate the requirement of a correctly re-assembled complex for activity. The activity is then demonstrated to have returned once the subunits are mixed together and re-assembled. Table 4 gives the re-assembly conditions for those that have been re-assembled successfully *in vitro*. These re-assembly studies differ from the folding studies already discussed, as shown in Fig. 4, in that they often start from prefolded subunits (with the exception of BtuCD, [24]), and are not reversible systems.

The Type I ABC transporters Histidine Permease ($HisQMP_2$) [86], the Maltose Transporter ($MalFGK_2$) [87], and the thermophilic Arginine Transporter ($Art(MP)_2$) [88] have all been re-assembled *in vitro*, with slightly different findings from each study. $HisQMP_2$ was found to be uncooperative in assembly, with the two soluble NBD subunits recruited separately [86]. In contrast, the recruitment of the two NBDs was found to be highly cooperative in $MalFGK_2$ [87]. In both these transporters it was necessary to express the entire complex, and remove the NBDs with urea during purification, indicating the need for interactions between the TMDs and NBDs *in vivo* for the correct fold. In contrast however, the subunits in the transporter $Art(MP)_2$ were expressed entirely separately, and re-assembled without aggregation problems [88].

To date, the only Type II ABC importer that has been used in a refolding and re-assembly study is the *E. coli* vitamin B_{12} transporter BtuCD. The re-assembly of BtuCD was investigated from starting states both unfolded and refolded, with the results indicating that the most efficient transporter re-assembly occurs when the subunits have been partially unfolded prior to re-assembly, indicating some cooperativity in folding and assembly. The results also indicated that hydrophobic interdomain contacts increase the likelihood of co-operative folding between subunits, perhaps explaining cooperativity has not been observed during folding of all ABC transporters studied [24].

The ability for each of these ABC transporters to re-assemble *in vitro* from separate components illustrates that assembly does not

Table 4
Purification, expression and re-assembly of ABC transporters.

Transporter	ABC Type	System for overexpression	Extraction of NBD from TMD	Re-assembly	Lipid or detergent used	What problems were encountered?	Reference
His permease ($HisQMP_2$)	Type I	*E. coli* TA1889	NBD HisP extracted with 15 mM ATP,15 mM $MgSO_4$, 6.6 M urea	Subunits mixed on ice for 30 min and centrifuged, re-assembled complex pellets with liposomes	Solubilised with OG and *E. coli* lipids	Subunit HisP has a high aggregation tendency	[86]
Maltose transporter ($MalFGK_2$)	Type I	*E. coli* BL21(DE3)	NBD MalK extracted with 4–6 M urea	TMD subunit MalFG attached to a Co^{2+}, column, only NBD subunit MalK binds	Complex solubilised in DDM, PC liposomes used for transport assays	Aggregation of MalFG if expressed separately	[87]
Arginine transporter ($Art(MP)_2$)	Type I	*E. coli* Rosetta2 for TMD ArtM (22 °C overnight) *E. coli* Rosetta for NBD ArtP (37 °C)	Subunits expressed separately	Tagless TMD ArtM and NBD ArtP incubated at 4 °C for 12 h and reapplied to TALON resin for removal of any unbound ArtP	Complex solubilised in DS, DDM used for re-assembly Liposomes used for transport assays using *Geobacillus stearothermophilus* total lipids	ArtM does not stain well, giving inaccurate quantification of re-assembly	[88]
Vitamin B12 transporter (BtuCD)	Type II	*E. coli* BL21 (DE3) (20 °C induction)	NBD BtuD extracted with 3 M urea	Tagless NBD BtuD and TMD BtuC incubated for 30 min on ice, followed by binding to Ni^{2+} -NTA column and elution	BtuCD solubilised in LDAO, exchanged into DDM during purification	Aggregation of subunit BtuC if expressed separately	[24]

Overexpression and re-assembly conditions for the Type I ABC transporters $HisQMP_2$, $MalFGK_2$, $Art(MP)_2$, and Type II BtuCD. All of the ABC transporters in the table were purified with a poly His tag. The "extraction of NBD from TMD" column gives details on the separation of the NBD and TMD subunits from each other if they were expressed as a complete transporter.

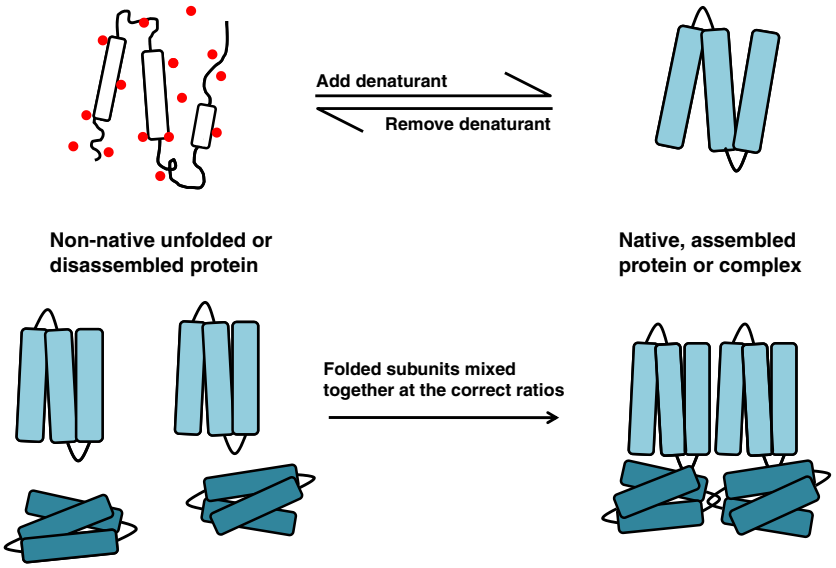

Fig. 4. The difference between re-assembly and refolding. The reaction at the top shows a reversible folding system, with the unfolded and folded states at either end of the folding equilibrium, and the denaturant shown as red circles. The reaction at the bottom shows the re-assembly of a multisubunit protein from individual subunits. Re-assembly and disassembly occur via different pathways under different conditions, with no denaturant involved.

need to occur cotranslationally for formation of an active complex. There in some variation however in whether or not there is cooperativity in assembly, with each giving different results. BtuCD and $MalFGK_2$ are highly cooperative in assembly, whereas there is no evidence for it with $HisQMP_2$. These different results between illustrate the need for more information on ABC transporters before generalisations and patterns emerge in their folding behaviour.

4. Comparison of transporter stability with other membrane proteins

A common feature of the thermal denaturation studies discussed in Section 3 is that the transmembrane regions of the transporters have a higher Tm than the cytoplasmic domains [25,45,46]. The higher stability of transmembrane regions is thought to be due to their environment in the lipid bilayer shielding the helices, although this does depend on the system used in the experiment.

While there are only few examples of transporters and two state folding reactions, there are also only a handful of examples of other membrane proteins that have been characterised in terms of the free energy of folding. These include bacteriorhodopsin (bR, 20 kcal/mol,[51]), diacylglycerol kinase (DGK, 16 kcal/mol, [53]), the potassium channel KcsA (30.5 kcal/mol, [89]), disulphide bond reducing protein DsbB (4.4 kcal/mol, [90]), the β barrel outer membrane protein PagP (14.3 kcal/mol, [91]) as well the transporters CopA (3 kcal/mol, [42]) and MFS GalP (2.5 kcal/mol, [43]). Interestingly, most of these former examples are similar to soluble proteins, in that the free energy stability is proportional to the size of the protein, as in smaller proteins are less stable due to less small stabilising forces and *vice versa*. The ΔG_U^{H2O} for small soluble proteins has been found to be around 0.06 kcal/mol/residue [49], with a few membrane proteins, for example bR (+0.08 kcal/mol [49]), DGK (+0.13 kcal/mol, [53]), and PagP (+0.09 kcal/mol, [91]) following this trend. However, the transporters CopA and GalP have very low values of 0.004 kcal/mol [42] and 0.005 kcal/mol [43] respectively, suggesting that this linear relationship with size is not always the case. These lower per residue stability values more likely reflect the fact there is much more flexibility and solvent exposure in these two than in the average membrane protein, giving more accessibility to denaturant. The ΔG_U^{H2O} is perhaps more related to exposed surface area than to the actual size of the protein. It should also be noted that although all above values of ΔG_U^{H2O} are obtained via extrapolation of a linear free energy relationship to zero denaturant and thus are in principle comparable, they relate to different detergent/lipid folding systems as well as different degrees of residual structure induced by different denaturants (*e.g.* urea or SDS).

Determining the kinetics of a reaction is particularly important, as it is the essential piece of information needed to understand the mechanism of any reaction, and gives information on the transition state of the reaction. There are only three cases where a full kinetic analysis of reversible folding has been measured for a membrane protein; the β barrel outer membrane protein PagP [91], and the α helical proteins bR [51] and DsbB [39], all of which are stable one domain proteins. The kinetics of a flexible multidomain or multisubunit protein would therefore significantly advance the membrane protein folding field.

5. Conclusions

Folding studies of integral membrane proteins have become well established since the pioneering work of Khorana, Engelman and others in the 1980s on bR. Investigations into larger, flexible transporters are now coming of age. In spite of the renowned challenges of hydrophobic membrane proteins, these transporter studies are also forging new ground in folding studies in general, through investigations of intricate, dynamic multidomain and multisubunit protein assemblies. Major experimental obstacles have been overcome, most notably in reversible folding reactions of transporters and the determination of folding free energies. This paves the way for further fundamental studies on transporter folding. Nonetheless, significant barriers remain to be overcome that will require intensive work and novel approaches if investigations are to progress to lipid bilayers and beyond to cellular and misfolding studies.

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