Misfolding of a bacterial autotransporter

JESPER E. MOGENSEN,[1] JÖRG H. KLEINSCHMIDT,[2]
M. ALEXANDER SCHMIDT,[3] AND DANIEL E. OTZEN[1]

[1]Department of Life Sciences, Aalborg University, DK-9000 Aalborg, Denmark
[2]Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany
[3]Institut für Infektiologie, Zentrum fur Molekularbiologie der Entzündung (ZMBE), Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany

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Abstract

The adhesin involved in diffuse adherence (AIDA) is an autotransporter protein that confers the diffuse adherence phenotype to certain diarrheagenic *Escherichia coli* strains. It consists of a 49 amino acid signal peptide, a 797 amino acid passenger domain, and a 440 amino acid β-domain integrated into the outer membrane. The β-domain consists of two parts: the β_1-domain, which is predicted to form two β-strands on the bacterial cell surface, and the β_2-domain, which constitutes the transmembrane domain. We have previously shown that the β-domain can be folded from the urea-denatured state when bound to a nickel column during purification. It has not been possible to achieve proper refolding of the β-domain in solution; instead, a misfolded state C is formed. Here, we characterize this misfolded state in greater detail, showing that despite being misfolded, C can be analyzed as a conventional conformational state, with cooperative unfolding in urea and SDS as well as showing simple exponential kinetics during its formation in the presence of lipid vesicles and detergent micelles. The kinetics of formation of C is sensitive to the lipid composition in vesicles. We have also attempted to identify biological factors that might aid folding of the β-domain to the properly folded state. However, no purified periplasmic or cytosolic chaperone was found to increase folding yields, and no factor in a periplasmic extract was identified that could bind to C. We conclude that it is the exposure to the unique spatial arrangement of the bacterial cell that leads to proper refolding of the β-domain.

Keywords: outer membrane protein; misfolding; autotransporter; periplasmic chaperones; thermal stability; thermodynamic stability; protein–lipid interactions

Reprint requests to: Daniel E. Otzen, Department of Life Sciences, Aalborg University, Sohngaardsholmsvej 49, DK-9000 Aalborg, Denmark; e-mail: dao@bio.aau.dk; fax: (0045) 98141808.

Abbreviations: AIDA, adhesin involved in diffuse adherence; ANS, 8-anilino-1-naphthalenesulfonic acid; ATP, adenosine 5′-triphosphate; $C_{10}E_7$, heptaethylene glycol monodecyl ether; CD, circular dichroism; DDPC, 1,2-didecyl-*sn*-glycero-3-phosphocholine; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DLPE, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine; DLPG, 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]; DM, *n*-dodecyl-β-D-maltoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleyl-*sn*-glycero-3-phosphocholine; DPC, dodecyl-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; LPS, lipopolysaccharide; OG, *n*-octyl-β-D-glucoside; OM, outer membrane; OMP, outer membrane protein; oPOE, octyl-polyoxyethylene; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, TN buffer, 50 mM Tris (pH 8), 100 mM NaCl.

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Knowledge about the thermodynamic stability and folding mechanism of membrane proteins lags far behind that of their water-soluble counterparts. This is largely due to their inherent hydrophobicity, which presents considerable challenges to their study in vitro. Low expression yields and difficulties in purification limit the amount of available material. Moreover, the refolding yield is often very low due to the formation of off-pathway products caused by aggregation and precipitation. In order to advance our knowledge of the biophysical properties of membrane proteins, we need to identify new candidate model proteins and establish reversible conditions for their study.

All characterized membrane proteins to date belong to only two different structural classes: proteins whose transmembrane segments are composed only of one or

more α-helices, and proteins that are composed of one or more β-barrels. The latter are found exclusively in the outer membranes of Gram-negative bacteria, mycobacteria, mitochondria and chloroplasts and are termed outer membrane proteins (OMPs) (Wimley 2003). The former are found in the cytoplasmic (or inner) membranes of bacteria and in all membranes of eukaryotic cells including the outer membranes of mitochondria and chloroplasts (von Heijne 1999; Taylor and Pfanner 2004). These are termed inner membrane or α-helical membrane proteins. Although they generally are easier to handle than inner membrane proteins (Allen et al. 2004), due to their relatively low hydrophobicity, which is cryptically encoded in their sequence, there are actually very few OMPs for which proper refolding conditions have been established. Here, refolding conditions refer to experimental conditions in which the protein can be reversibly unfolded. OmpA from *Escherichia coli* is the best studied OMP in terms of folding and stability (Kleinschmidt 2003; Tamm et al. 2004). It can successfully fold into a range of model membranes of very different phospholipid compositions in terms of different head group structures and hydrophobic chain lengths (Kleinschmidt 2003). However, the folding yield typically only reaches 100% when the pH approaches 10, probably because the protein is sufficiently negatively charged at this pH to reduce aggregation and unproductive binding to the vesicle surface. At pH 7, the refolding yield falls to ~70% (Surrey and Jähnig 1995). Other OMPs for which reversible refolding conditions have been established are OmpG from *E. coli* (Conlan and Bayley 2003) and the PorB class 3 protein from *Neisseria meningitidis* (Minetti et al. 1998). Examples of OMPs that do not refold quantitatively are common and include OMPLA (Dekker et al. 1995) and OmpF from *E. coli* (Surrey et al. 1996), Toc75 from the outer membrane of chloroplasts (Rogl et al. 1998), and porin from *Rhodopseudomonas blastica* (Schmidt et al. 1996).

In recent years a number of periplasmic chaperones have been shown to be important for OMP folding in vivo (Mogensen and Otzen 2005) as their deletion leads to reduced levels of properly folded proteins in the outer membrane (Chen and Henning 1996; Lazar and Kolter 1996; Rouviere and Gross 1996; Dartigalongue and Raina 1998). The "17 kDa protein" (Skp), the "survival protein A" (SurA), and DegP are part of a functional network that is vital for proper protein folding and degradation in the cell envelope (Rizzitello et al. 2001). DegP and Skp are in one pathway, whereas SurA belongs to a separate, parallel pathway. This means that if one component of either pathway is lost, the other pathway is still functional, however, losing one component of each pathway simultaneously results in a lethal phenotype because both chaperone pathways have been compromised (Rizzitello et al. 2001). Also, deletion of either Skp or SurA leads to increased levels of DegP in the cell to degrade or capture misfolded OMPs that accumulate in the periplasm (Lazar and Kolter 1996; Missiakas et al. 1996). In general, it seems that many periplasmic chaperones function in the process of OMP folding by acting on a mostly unstructured state preventing misfolding and aggregation. However, it is still an open question of how these early OMP folding intermediates become targeted and integrated into the outer membrane to reach their native functional state, although the recently identified Omp85 protein may turn out to be the translocon of the outer membrane (Voulhoux et al. 2003; Pfanner et al. 2004).

We have undertaken a biophysical study of the OMP domain of the autotransporter AIDA from *E. coli* to characterize its stability and folding behavior. AIDA is a 1286-residue protein that due to its adhesive properties is responsible for the attachment of certain pathogenic *E. coli* strains to the intestinal lining (Benz and Schmidt 1989). The protein consists of the adhesin AIDA-I (α-domain), which mediates intestinal adherence, and the translocator domain (β-domain), which anchors the adhesin domain in the bacterial outer membrane (Benz and Schmidt 1992; Suhr et al. 1996). The β-domain consists of two parts: the β_1-domain, which is predicted to form two β-strands on the bacterial cell surface, and the β_2-domain, which constitutes the transmembrane OMP domain (Konieczny et al. 2001).

We previously found that the AIDA β_2-domain in solution can fold only when the β_1-domain is present and only with 50% yield (Mogensen et al. 2005). However, 100% folding yield of the β_2-domain, with or without the β_1-domain, can be obtained in the presence of a solid support. Refolding attempts in the absence of a solid support result in the formation of an oligomeric, misfolded state both in the absence and the presence of detergent (Mogensen et al. 2005). The inability to refold in solution may reflect a requirement for periplasmic chaperones in vivo. In this study, we have characterized the misfolded states of AIDA in more detail to learn more about their stability, which might be relevant to understand the challenges relating to the folding of such states in vivo, given that outer membrane proteins have to traverse the periplasmic space prior to insertion in the outer membrane. Furthermore, we examined whether periplasmic chaperones (either purified or from a raw extract) could substitute for a solid support in the refolding of AIDA in vitro.

Results

Folding kinetics of AIDA DT4 on Ni-NTA-agarose beads

We routinely fold and purify urea-unfolded DT4 from inclusion bodies by incubating it with oPOE and then

adding it to Ni-NTA beads, from which it is eluted in oPOE and imidazole after washing away the urea. We use the constructs DT4 expressing residues 951–1286 (β_2-domain) including an N-terminal His_6-tag and MS12 expressing residues 842–1286 (~β-domain) including an N-terminal His_6-tag (see Mogensen et al. 2005 for details). Since the folding kinetics of AIDA cannot be measured directly in solution (Mogensen et al. 2005), we investigated the kinetics of formation of the native protein in the presence of Ni-NTA-agarose beads by adding the beads to DT4 in 4 M urea/125 mM oPOE and withdrawing samples for SDS-PAGE analysis. Folding of DT4 on the beads is relatively fast as >75% folded protein is reached within 11 min and >90% after 120 min (Fig. 1). Fitting the data to a single exponential function yields a k_f of 0.21 min^{-1}.

Before addition of beads, DT4 in 4 M urea/125 mM oPOE is routinely incubated overnight at 4°C for refolding and purification. We noted that if the beads were added immediately after mixing DT4 in urea with oPOE, the folding yield was much lower (data not shown). This could indicate that a slow structural rearrangement takes place after dilution of the solubilized inclusion bodies with oPOE; such a transition is however invisible by tryptophan fluorescence spectroscopy (data not shown). These experiments also showed that it is not possible to add oPOE after binding the protein in 8 M urea to the beads and obtain folded protein even if the bound protein is incubated in the presence of detergent for 24 h (data not shown). The conclusion of these experiments is that DT4 must assume a folding-competent state before binding to the beads, and this state is (1) only attained in the presence of 125 mM oPOE, (2) formed slowly over time but (3) spectrally identical to the state initially formed upon mixing with 125 mM oPOE. However, once the folding-competent state is formed, the native state forms relatively fast on the beads.

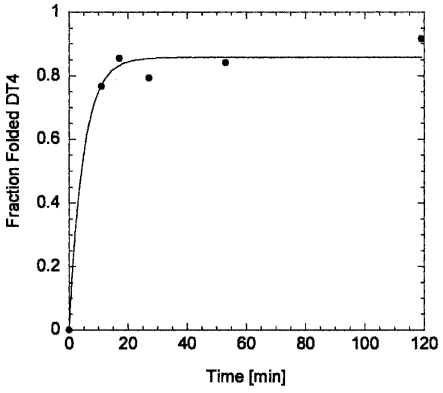

Figure 1. AIDA DT4 refolding kinetics on Ni-NTA-agarose beads. Folding was initiated by adding Ni-NTA-agarose beads to DT4 in 125 mM oPOE/4 M urea. The folding reaction was stopped at different time points by elution with 350 mM imidazol in 12.5 mM oPOE. Fraction folded protein was determined from SDS-polyacrylamide gel by densitometric analysis. Because of the steps involved, it was not possible to obtain reliable data points before 10 min into the reaction. The solid line represents the best fit to a single exponential function yielding a folding rate on the beads of 0.21 min^{-1}.

AIDA is water soluble and binds ANS at low pH

Having demonstrated the rather complex conditions required by DT4 for correct refolding, we now turn to a description of the misfolded states of DT4, which accumulate under other circumstances. When we attempt to refold urea-denatured DT4 in solution by diluting it out in the presence of detergent, a state C_d is formed, which is soluble but nonnative. The evidence for this is that unlike the properly refolded state, C_d remains protease-sensitive and unable to undergo band-shift on SDS-PAGE upon boiling (Mogensen et al. 2005). A state with similar properties, termed C_w, is formed when DT4 is diluted out in the absence of detergent. Neither of these states can be folded to the native state without backtracking to the urea-denatured state (Mogensen et al. 2005). Both C_w and C_d are oligomeric states (Mogensen et al. 2005). While C_w has a high tendency to form higher order aggregates, it remains fully water soluble at low μM concentrations from pH 1 to 13 except in the range of pH 3.5–7, where the solubility reaches 0 at pH 4.9 (see Fig. 2A). The insolubility is asymmetrically distributed around p*I*, which is 6.7 for DT4. Outside pH 3.5–7, the protein becomes prone to precipitation above ~100 μM (data not shown). Far-UV CD scans reveal that from pH 2.1 to pH 10.3, C_w has the same content of secondary structure except in the range of pH 3.5–7 where the protein no longer is in solution (Fig. 2A,B). Above pH 10.3, C_w loses secondary structure and shows a random-coil-like spectrum (Fig. 2A,B). We tested whether C_w contains exposed hydrophobic patches using the dye ANS, which has affinity for partially folded states (Stryer 1965). From pH 13 down to pH 7, ANS fluorescence is very low (Fig. 2A). Around pH 6 it starts to increase dramatically until it peaks at pH 3, from which it steeply decreases down to pH 1 where it is 50% of the intensity at pH 3. Thus, ANS is apparently able to prevent the precipitation of AIDA, probably because the affinity of ANS for the protein shifts the equilibrium away from precipitation. Taken together, these data show that C_w at acidic pH binds ANS indicating the formation of a species with exposed hydrophobic surface area. This state is different from C_w at pH 7, since no ANS binding takes place here.

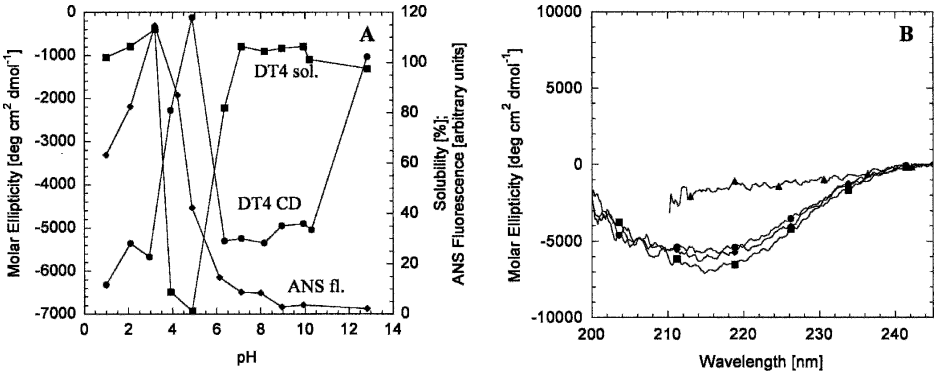

Figure 2. Solubility, secondary structure, and ANS binding ability of the DT4 C_w state. (*A*) Solubility of 3.5 μM (■) and molar ellipticity of 5 μM (●) urea-purified DT4 as a function of pH in 25 mM buffer as determined from centrifugation experiments and far-UV CD, respectively. The ellipticity at 220 nm is shown. ANS fluorescence (◆) as a function of pH in the presence of 5 μM urea-purified DT4 in 25 mM buffer. ANS was excited at 350 nm; the emission at 475 nm is shown. The intrinsic fluorescence of ANS vs. pH was subtracted as blank. The solid lines are drawn only to guide the eye. (*B*) Far-UV CD spectra of 5 μM urea-purified DT4 at pH 1.0 (■), 2.1 (◆), 8.2 (●), and 12.9 (▲) at 25°C.

Both the DT4 C_w and C_d states unfold cooperatively, and detergent does not affect stability significantly

We found earlier that the C states unfold cooperatively in thermal denaturation experiments. Here, we extend these experiments by employing a range of different detergents to further examine how the nature of the amphiphilic environment affects the stability of the C states. CD-monitored thermal scans of urea-purified DT4 in a range of different detergents and micelle-forming phospholipids lead to T_m values between ~65°C and 73°C (Fig. 3A; Table 1). This means that detergent and phospholipid chain length and head group has little impact on the thermal stability of the C_d state. C_w has a T_m of 70.2 ± 0.0°C (Fig. 3A; Table 1), showing that binding of the C state to micelles/vesicles does not affect stability of this partly folded state significantly. This agrees well with our working model that the protein is not integrally inserted into the hydrophobic phase of the micelles but merely associates superficially with them.

Given that the C_w and C_d states are oligomeric, what structural transition is being monitored in the thermal scans? If the protein molecules were tightly bound to each other in the aggregates, we would expect T_m to vary with the protein concentration due to the stabilizing effect of association. However, we do not see a change in T_m for C_w covering protein concentrations from 1 μM to > 30 μM DT4 (data not shown), indicating that the interactions between the monomers are rather weak and unspecific. Even if the dissociation constant for oligomer formation was well below 1 μM at room temperature, we would still expect to see a change in T_m with changing protein concentrations if unfolding occurred via a stable oligomer, because the dissociation constant will increase with temperature, eventually leading to dissociation. Thus, we are likely monitoring a true denaturation of individual monomers, although it cannot be ruled out that there is a spectroscopically silent dissociation step prior to the actual unfolding step.

To determine the thermodynamic stability of the C_d state, we titrated C_d in DM as a function of urea concentration between 10° and 40°C. Figure 3B shows a representative denaturation curve in 20 mM DM at 20°C, which displays a sharp transition with a midpoint at 2.43 M urea confirming the cooperative nature of C_d unfolding. Using Equations 2 and 3, a $\Delta G_{D\text{-}N}$ value of 2.75 ± 0.49 kcal/mol is obtained (see Table 2). This is in the very low end for natively folded globular proteins (Pace 1990) but is reasonable for a compact intermediate state (Spence et al. 2004). Urea-based unfolding in the detergents oPOE and $C_{10}E_7$ gave similar values of $\Delta G_{D\text{-}N}$ (see Table 2). For comparison, we also titrated C_w with urea (monitored by far-UV CD spectroscopy) at 25°C (Fig. 3B). This gives rise to a stability of 3.03 ± 0.65 kcal/mol, which is similar to the value found for the C_d state, again showing that the binding of the DT4 oligomers to detergent micelles does not stabilize the protein.

Complementary experiments in DM were performed using SDS as denaturant. SDS by itself acts as a denaturant, but when it forms mixed micelles with a nonionic detergent such as DM, the denaturing potency of the mixture depends on the mole fraction of SDS. It has been suggested that there is a linear relationship between the free energy of unfolding and the mole fraction of SDS (Lau and Bowie 1997; Otzen 2003; Faham et al. 2004). We observe a sharp transition when the CD signal is plotted versus the mole fraction of SDS (Fig. 3C). When the data are fitted to Equation 2, we obtain a $\Delta G_{D\text{-}N}$ value of 4.20 ± 0.25 kcal/mol at 25°C, which is

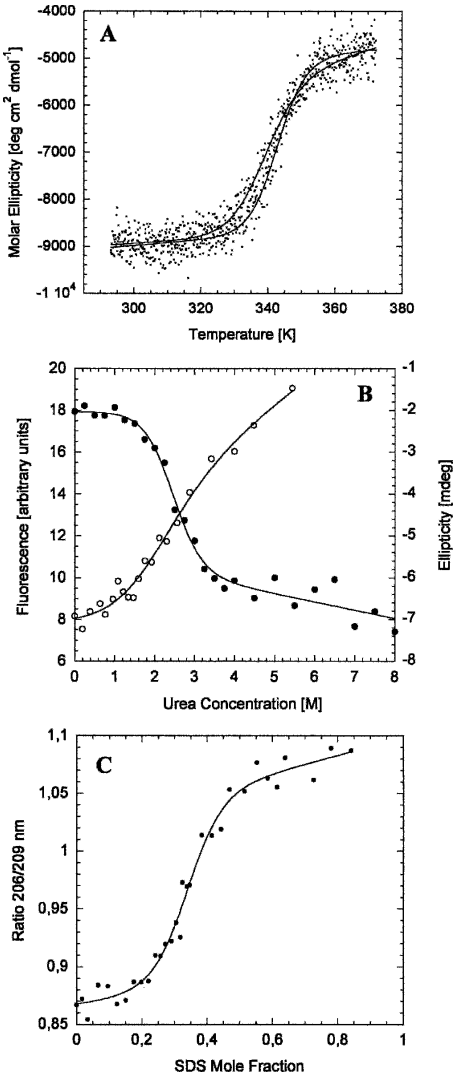

Figure 3. C unfolds cooperatively. (*A*) Thermal scans of 6.5 μM urea-purified DT4 in 10 mM TRIS (pH 8) (curve with *rightmost* transition) and in 20 mM DM in 10 mM TRIS (pH 8) (curve with *leftmost* transition) as monitored by far-UV CD at 220 nm. The solid lines represent the best fit to Equation 4, yielding T_m values of 70.2 ± 0.0°C and 65.4 ± 1.3°C, respectively. (*B*) Urea denaturation of 0.5 μM urea-purified DT4 in 20 mM DM in 25 mM sodium phosphate (pH 8), 100 mM NaCl at 20°C as monitored by fluorescence spectroscopy at 330 nm (●) and of 5 μM urea-purified DT4 in 10 mM TRIS (pH 8) at 25°C as monitored by far-UV CD at 220 nm (○). The solid lines represent the best fit to Equation 2, yielding $\Delta G_{D\text{-}N}$ values of 2.75 ± 0.49 and 3.03 ± 0.65 kcal/mol (Equation 3), respectively. (*C*) SDS denaturation of 2 μM urea-purified DT4 in 8 mM DM at 25°C as monitored by far-UV CD. The solid line represents the best fit to Equation 2, yielding a $\Delta G_{D\text{-}N}$ value of 4.20 ± 0.25 kcal/mol (Equation 3).

similar to the value obtained from the urea denaturation experiments.

Taken together, the denaturation data show that despite its oligomerization properties, DT4 unfolds cooperatively and independent of protein concentration. With the caveats mentioned above, the tertiary interactions in the individual protein molecules must therefore be stronger than the quaternary interactions in the oligomers. The stability of C_w and C_d is low and largely independent of the presence of detergent.

Folding of C_d is sensitive to the lipid environment

Given that C_d is significantly more structured than the urea-denatured state and forms in an apparently cooperative fashion, data on the kinetics of its formation could provide some insights into the mechanism by which it forms. For simplicity we refer to this process as "folding," with the caveat that we are not monitoring the formation of native structure. Stopped-flow fluorescence experiments in which AIDA DT4 was diluted from 8 M urea into detergent in the absence of Ni-NTA-agarose beads revealed three relaxation phases over a period of 100 sec (of which the slowest has a half-life of ~50 sec), suggesting that a number of conformational transitions were taking place on the way to the C_d state (Fig. 4A). However, to simplify our analysis and monitor the formation of stable structure, we have carried out a double jump assay, in which denatured DT4 was diluted out into conditions favoring folding (low urea concentrations and high concentrations of phospholipids or detergents). At various time points, samples were withdrawn and unfolded again in urea. Only structure that has developed during the refolding period will subsequently unfold in urea, and thus the unfolding amplitude is a measure of the extent of folding. Under these refolding conditions, C_d accumulated in the course of minutes to hours (Fig. 4B) without any significant lag time. The folding rate was sensitive to the nature of the amphiphilic environment. Folding occurred most rapidly in DM (with a half-time of ~2 min, in good agreement with the stopped-flow data; cf. Fig. 4A), followed by DLPC (approximately half as fast), and was significantly slower in lipids with other chain lengths (Fig. 4C). At low protein concentrations (~1

Table 1. *T_m -values as determined from CD thermal scans for the C_d state in different detergents*

Condition	T_m (°C)
20 mM DM	65.4 ± 1.3
35 mM OG	65.8 ± 1.0
25 mM oPOE	73.0 ± 1.0
20 mM $C_{10}E_7$	68.9 ± 1.9
65 mM DHPC	65.9 ± 3.8

Experiments performed in TN buffer. The error stated is the standard error of the mean of three experiments. DPC denatures DT4 and no thermal denaturation is observed. For comparison, T_m for C_w is 70.2 ± 0.0°C. Thermal unfolding of C_d is mostly reversible, whereas for C_w it is completely irreversible (data not shown).

Table 2. *Thermodynamic stability of the C_d and C_w states at different temperatures determined from urea denaturation experiments*

Condition	Temperature (°C)	[urea$^{50\%}$][a] (M)	$m_{D\text{-}N}$[a] (M^{-1})	$\Delta G_{D\text{-}N}$[b] (kcal/mol)
20 mM DM	10	2.25 ± 0.11	0.83 ± 0.15[c]	2.85 ± 0.52
20 mM DM	20	2.43 ± 0.08	0.83 ± 0.15[c]	2.75 ± 0.49
20 mM DM	30	2.28 ± 0.13	0.83 ± 0.15[c]	2.57 ± 0.48
20 mM DM	40	1.94 ± 0.28	0.83 ± 0.15[c]	2.20 ± 0.50
20 mM $C_{10}E_7$	20	2.26 ± 0.14	0.56 ± 0.09	2.02 ± 0.30
25 mM oPOE	20	3.33 ± 0.16	0.69 ± 0.12	3.13 ± 0.39
Buffer	25	2.17 ± 0.34	1.03 ± 0.65	3.03 ± 0.65

The error stated is the error of the fit.
[a] Obtained from fit to Equation 2.
[b] Calculated from fit to Equation 3.
[c] We used an average of the four $m_{D\text{-}N}$-values as they showed no systematic variation with temperature.

μM), folding occurred in a single step, i.e., with a single exponential phase. However, at higher protein concentrations, a second slower phase was also seen, suggesting two parallel pathways to the C-state. The fraction of the protein population folding by the fast phase decreased significantly with protein concentration (Fig. 4D), suggesting that protein association was competing with and slowing down folding.

We also tested the effect of alterations in the vesicle composition on the folding reaction by carrying it out in the presence of PC vesicles with increasing amounts of PE. PE has been shown to reduce the refolding yield of bacteriorhodopsin in mixed PC-PE vesicles (Curran et al. 1999). This has been rationalized by an increase in lateral pressure in the lipid chain region of the bilayer. Consistent with this, we observed that increasing concentrations of PE led to a decrease in the fraction of AIDA folding by the fast phase (Fig. 4E). Interestingly, we also observed that the larger the vesicles, the faster the folding. As the size of the extruded vesicles increased from 50 nm to 200 nm, refolding rates doubled from ~0.044 min^{-1} to 0.11 min^{-1}, while the rate was 0.26 min^{-1} in unextruded large unilamellar vesicles (data not shown). The total amplitude of folding remained constant irrespective of vesicle size, indicating that there was no change in the overall folding yield. This suggests that conditions approaching a planar bilayer favor the formation of C_d.

Effect of Skp on the refolding of AIDA DT4 and MS12

Since we were not able to refold AIDA under a wide array of solution conditions without a solid support, we attempted to use periplasmic chaperones from *E. coli* as an alternative. Chaperones are known to recognize partially folded protein states, which would make both C_d and C_w (as well as states formed at earlier stages in the "misfolding" sequence) good candidates for substrates. The chaperone Skp has been found to interact with OmpA together with LPS and facilitate its folding in solution (Bulieris et al. 2003). We therefore tested whether Skp would have an effect on the refolding of AIDA DT4 and MS12 in solution. Urea-unfolded AIDA was diluted with Skp in the presence and absence of LPS and mixed PC/PE/PG vesicles with lauroyl or oleoyl chains. The samples were incubated at 40°C for days to weeks, and were subsequently run on SDS-polyacrylamide gels. Heat shift on the gel was used as evidence for refolding. A representative gel for DT4 is shown in Figure 5A. No faster migrating, i.e., folded protein, band was observed either in the absence or presence of LPS and the presence of Skp and vesicles, showing that Skp cannot promote folding of AIDA. For MS12, similar results were obtained (data not shown).

To test for possible binding between Skp and nonnative AIDA, we also titrated DT4 and MS12 with Skp. Skp contains no tryptophan residues and therefore binding can be conveniently followed by the intrinsic fluorescence of AIDA. We saw no change in tryptophan fluorescence as a function of Skp concentration covering Skp/AIDA molar ratios up to 100 (data not shown). This indicates that Skp does not bind to AIDA; however, failure to bind might be caused by the high aggregation tendency of AIDA (Mogensen et al. 2005) occurring in competition with binding. We therefore bound DT4 to the Ni-NTA-agarose beads before addition of Skp. Skp was found to coelute with DT4 from the column; however, the control protein lysozyme, which is not expected to bind AIDA, also coeluted with DT4, and therefore we cannot ascribe this to a specific effect by Skp (data not shown). Also, addition of phospholipid vesicles to the eluted Skp-AIDA sample did not result in native protein (data not shown). In conclusion, we have no evidence that Skp either binds to AIDA or promotes the refolding of the protein in solution.

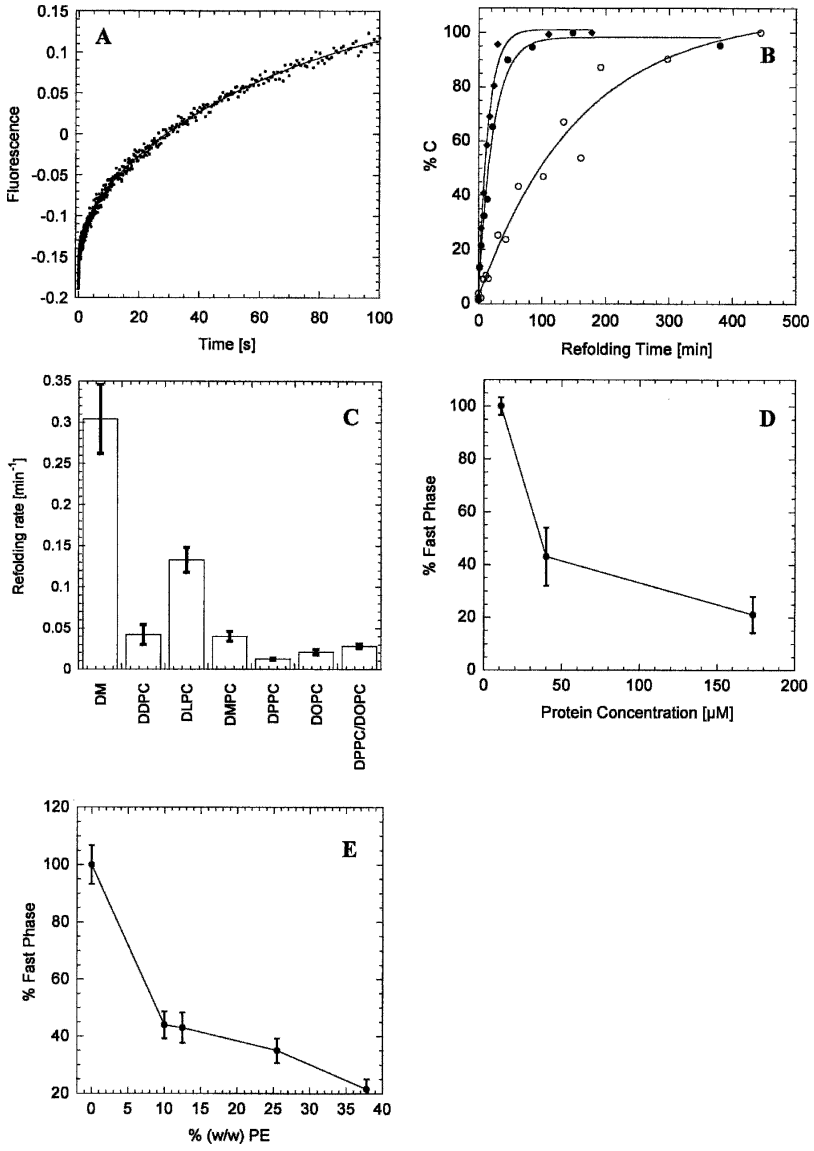

Figure 4. Folding of C is sensitive to the lipid environment. (*A*) Stopped-flow fluorescence time profile of the transfer of urea-purified DT4 in 8 M urea into 25 mM DM (residual urea concentration 0.72 M) at 25°C in TN buffer. The final protein concentration is 4 μM. The solid line represents the best fit to a triple-exponential function. (*B*) Double-jump data for refolding of 5 μM urea-purified DT4 from 6 M urea into 200 mM DM (◆), 60 mg/mL DLPC (●), and 60 mg/mL DOPC (○) . Data are fitted to a single exponential function. (*C*) Refolding rates from double-jump experiments of urea-purified DT4 into different amphiphilic environments. Same conditions as in *B*. (*D*) Fraction of DT4 folding via the fast folding phase into DLPC as a function of protein concentration. The solid lines are only drawn to guide the eye. The error bars represent the error of the fit. (*E*) Fraction of DT4 folding via the fast folding phase in mixed DLPC-DLPE vesicles as a function of the weight fraction of DLPE. The solid lines are only drawn to guide the eye. The error bars represent the error of the fit.

Effect of SurA on the refolding of DT4 and MS12

We also tested the periplasmic chaperone SurA for promoting AIDA refolding in solution. Conditions were basically the same as described for Skp although incubation was only up to 24 h. Again, heat shift on SDS-polyacrylamide gels was used as evidence for refolding. A representative gel of DT4 is shown in Figure 5B. As for Skp, in no cases were a faster migrating species observed on the gels, showing that SurA cannot promote refolding of AIDA DT4 or MS12 in solution.

Effect of DegP on the refolding of DT4 and MS12

We tested whether the periplasmic protease DegP, which also acts as a chaperone, could induce refolding of DT4 in solution using the same experimental approach as with

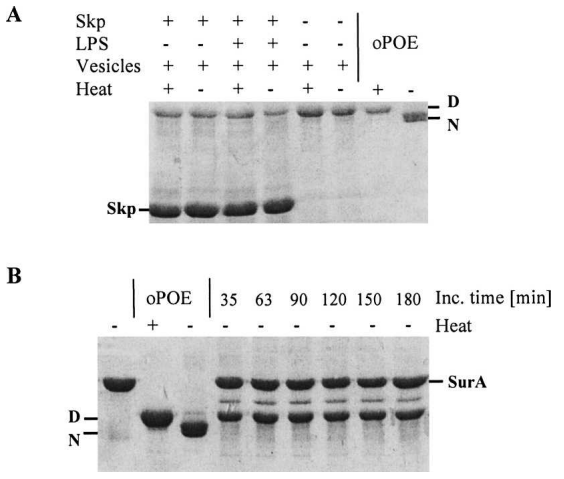

Figure 5. Refolding experiments with Skp and SurA. D and N denote denatured and natively folded AIDA, respectively. See Materials and Methods for details. (*A*) SDS-polyacrylamide gel of DT4 incubated with Skp under various conditions at 40°C for 2 d using DLPC/DLPE/DLPG vesicles. (*B*) SDS-polyacrylamide gel of DT4 incubated with SurA and DLPC/DLPE/DLPG vesicles at 37°C for up to 3 h.

Skp. DegP switches between protease and chaperone activity in a temperature-dependent manner with proteolytic activity dominating at high temperature (Spiess et al. 1999). Consequently, we incubated the samples at different temperatures namely 25°C and 37°C. Incubation times were 3 h. Representative SDS-polyacrylamide gels are shown in Figure 6, A and B. It can be seen that at all temperatures tested, DT4 is completely degraded by DegP. A kinetic experiment showed that at 25°C, complete degradation was reached within 10 min (data not shown). This means that irrespective of temperature, nonnative AIDA is a very good proteolytic substrate for DegP.

We wanted to rule out that the protease function masked a possible chaperone activity of DegP toward AIDA. By mutating the active site serine (S210) to an alanine, proteolytic activity is abolished with chaperone activity being retained (Spiess et al. 1999). We repeated the incubation experiments with $DegP_{S210A}$ (Fig. 6C). However, after incubation times of up to 5 d at 37°C there was no effect on the refolding yield of DT4. This firmly establishes that DegP does not promote the refolding of AIDA in solution.

Effect of GroEL on refolding

Although GroEL has no known biological relevance in OMP folding it has been shown to assist the refolding of the major outer membrane protein from *Campylobacter jejuni* in vitro (Goulhen et al. 2003). Furthermore, an effect of GroEL has also been observed for bacteriorhodopsin (Deaton et al. 2004). To investigate whether GroEL has a similar effect on AIDA, we mixed DT4 with GroEL and 10 mM ATP in AIDA/GroEL molar ratios of 10:1, 1:1, and 1:10. The samples were incubated at either 25°C or 37°C for up to 2 d. As revealed from SDS-polyacrylamide gels of the samples, no AIDA migrated with a mass of 30 kDa showing that refolding of the protein did not take place (data not shown). We conclude that GroEL is not able to promote refolding of AIDA DT4.

No proteins that bind nonnative DT4 could be identified from an E. coli *periplasmic extract*

In a final attempt to identify a candidate protein that can aid in the refolding of AIDA in vitro, we subjected AIDA DT4 bound to Ni-NTA-agarose beads to an *E. coli* periplasmic extract. The rationale was that a protein displaying such a function will bind to unfolded or misfolded AIDA and thereby be retained

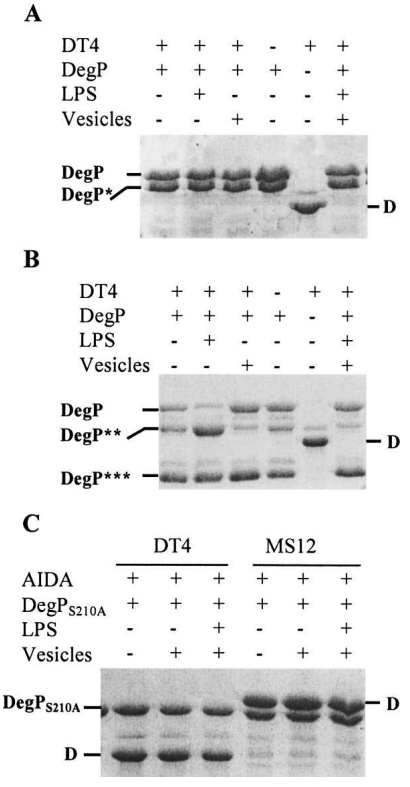

Figure 6. Refolding experiments with DegP. D and N denote denatured and natively folded AIDA, respectively. See Materials and Methods for details. DegP*, DegP**, and DegP*** represent different autodegradation products of DegP. (*A*) SDS-polyacrylamide gel of DT4 incubated with DegP under various conditions at 25°C for 24 h using DLPC/DLPE/DLPG vesicles. All samples were nonboiled. (*B*) As in *A* except using 37°C. (*C*) SDS-polyacrylamide gel of DT4 and MS12 incubated with $DegP_{S210A}$ under various conditions at 37°C for 24 h using DLPC/DLPE/DLPG vesicles. All samples were nonboiled.

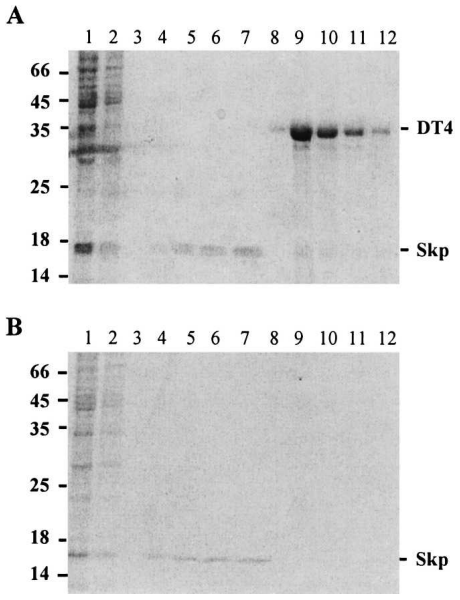

Figure 7. Incubation of resin-bound AIDA DT4 with *E. coli* Periplasmic Isolate. SDS-polyacrylamide gels showing the eluted fractions in the presence (*A*) and the absence (*B*) of DT4. One protein corresponding to the size of Skp is retained on the AIDA-bound beads; however, this is also the case in the absence of AIDA, showing that Skp binding is an unspecific effect. Lane *1*, flow-through, periplasmic fraction; *2*, first wash after periplasmic fraction; *3*, last wash after periplasmic fraction; *4*, first elution in 250 mM NaCl; *5*, second elution in 250 mM NaCl; *6*, first elution in 750 mM NaCl; *7*, second elution in 750 mM NaCl; *8*, first elution in 0.5 M imidazol; *9*, second elution in 0.5 M imidazol; *10*, third elution in 0.5 M imidazol; *11*, fourth elution in 0.5 M imidazol; *12*, fifth elution in 0.5 M imidazol. The numbers to the *left* of the gels denote molecular weight markers.

on the column. A periplasmic isolate from *E. coli* BL21(DE3) containing an Skp expression plasmid giving rise to a moderate leak expression of Skp was applied to the column containing resin-bound DT4 in buffer. After an extensive washing step in buffer, elution was performed first with NaCl in buffer to elute possible candidate proteins from AIDA and last with 0.5 M imidazol in buffer to elute AIDA and candidate proteins that bound very tightly to AIDA. The eluted fractions were run on SDS-polyacrylamide gels. As seen from Figure 7A, only one protein was retained on the column. This protein has a molecular mass of ~17 kDa and represents the slightly overexpressed Skp. In the experiments with the purified Skp, we found this binding to be an unspecific effect. Consequently, we performed the experiment with the periplasmic isolate in the absence of AIDA as a control. As seen in Figure 7B, the same protein is retained on the column, again showing that Skp binds to the Ni-NTA-agarose beads unspecifically. In conclusion, we could not identify a periplasmic protein that binds nonnative DT4 from an *E. coli* periplasmic extract.

Discussion

We have shown that the membrane-integrated β_2-domain of AIDA can only be refolded by binding it to a Ni-NTA-agarose column, and that all attempts to refold it in solution lead to partially structured states which are clearly nonnative. This is manifested by lack of heat-modifiability and protease protection, lower thermal stability, low sensitivity of thermal stability to the nature of the amphiphilic environment, and less β-structure compared to the native state (see also Mogensen et al. 2005). In the following, we discuss the properties of these states and their relation to the inability to bind to chaperones that might otherwise facilitate folding. Figure 8 gives an overview of the folding possibilities for AIDA DT4.

C_w is oligomeric and unfolds cooperatively

What is the structure of C_w? Since it is water soluble, except around p*I*, and does not bind ANS to any significant extent at neutral pH, it must be significantly hydrophilic on the outside. However, it is present as large aggregates, indicating that the monomers have

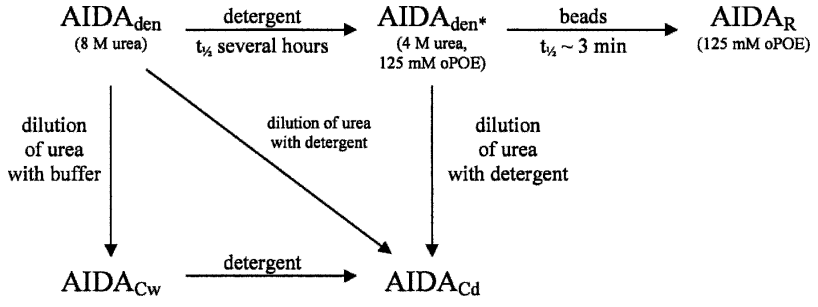

Figure 8. The folding possibilities of AIDA DT4. Schematic of the possible folding routes for AIDA DT4. $AIDA_{den}$ is the denatured state in 8 M urea; $AIDA_{den*}$ is the denatured but folding competent state in urea/detergent; $AIDA_R$ is the refolded state in detergent; $AIDA_{Cw}$ is the oligomeric, misfolded state in water; and $AIDA_{Cd}$ is the oligomeric, misfolded state in detergent.

some hydrophobic character on their surface (Mogensen et al. 2005). Water-soluble states have also been described for the outer membrane proteins OmpA, OmpF, and OmpG (Surrey and Jähnig 1995; Surrey et al. 1996; Conlan and Bayley 2003). In contrast to AIDA, both OmpA and OmpG bind ANS at neutral pH (Surrey and Jähnig 1995; Conlan and Bayley 2003). In the case of OmpG, ANS was suggested to bind to the interfaces of the oligomers or to the core of the protein molecules, thereby being shielded from water. OmpG also unfolds cooperatively in water when chemically denatured. The far-UV CD spectrum of OmpG in water resembles that of DT4 in water, with a minimum ~215 nm and crossing zero ellipticity ~200 nm (Mogensen et al. 2005). The far-UV CD spectrum of the OmpA water-soluble state is different, as it shows a minimum at 208 nm indicating a higher α-helical secondary structure content (Surrey and Jähnig 1995). Thus, the water-soluble states of AIDA and OmpG are similar, although they differ in ANS binding capacity at neutral pH.

C_d binds to phospholipid vesicles and becomes partially integrated into the bilayer

The C-state is able to bind to phospholipid vesicles, since it co-precipitates with the vesicle fraction in ultracentrifugation experiments, which is not the case in the absence of vesicles (data not shown). It is also possible that it even becomes partially integrated into the membrane, since its rate of folding is sensitive both to phospholipid chain length and head group composition. If it was only peripherally bound to the vesicle surface, the presence of the smaller PE head groups would be expected to encourage binding via the lowered lateral pressure in the interfacial region, which is opposite to what we observe. Rather, increased lateral pressure in the lipid chain region induced by PE may impede folding and partial insertion, leading to aggregation or some other conformational partitioning of an increasing proportion of the protein population. However, C_d is probably not able to penetrate the bilayer entirely; otherwise its stability in lipid vesicles and detergents would be higher than that of C_w. In addition, bilayer penetration would be encouraged by reducing the vesicle size to increase curvature and introduce weak points on the surface, which would be focus for insertion. This is contrary to the observation that larger vesicle sizes actually lead to an increase in folding rates. Another indication for lack of penetration is that C_d cannot perturb the bilayer enough to cause release of calcein from calcein-containing vesicles (data not shown).

The low stability of C_d (~3–4 kcal/mol) is not atypical for partially structured states. For globular proteins, stabilities of intermediates are ~3 kcal/mol for barnase (Dalby et al. 1998), 3.5 kcal/mol for p25α (Otzen et al. 2005), 1.5–2.5 kcal/mol for apomyoglobin (Fink et al. 1997), 2.7 kcal/mol for lysozyme (Parker et al. 1995), and 3.4 kcal/mol for the N-terminal domain of phosphoglycerate kinase (Parker et al. 1995). The low stability implies energetic accessibility to more denatured states, so that it should be possible for chaperones to unfold the protein even further in the course of binding if necessary. Furthermore, the C_d and C_w states are reached over a relatively long timescale through a series of steps which probably involve other less structured states. Such states, to which the chaperones are exposed in our assays, should also be good substrates for chaperone binding. The fact that the chaperones do not bind any of these states implies that there are other structural requirements for recognition in addition to a nonnative and destabilized conformation (see below).

Skp, SurA, and DegP do not promote folding of AIDA in vitro

The inability of the β-domain to refold upon transfer from urea to refolding conditions points to the importance of keeping the protein in an appropriate conformation prior to folding that is capable of inserting into the bilayer to reach the native state. In vivo, such a function is likely carried out by periplasmic chaperones that are able to bind to OMPs at a very early stage during the folding process, thus preventing premature folding or aggregation. The *E. coli* chaperone Skp is the best known candidate of such activity and, therefore, we tested its effect on AIDA. However, we did not see any effect on refolding yield in our incubation experiments and could not detect binding from fluorescence experiments in the absence of vesicles contrary to the case of OmpA (Bulieris et al. 2003). In vivo, Skp binds to the periplasmic side of the cytoplasmic membrane (Harms et al. 2001). Thus, it is ready to grab the OMP when it emerges from the translocon and maintain it in a soluble conformation. We tried to mimic this situation in vitro by binding unfolded AIDA to Ni-NTA-agarose beads, thus presumably maintaining the protein in a monomeric form even when the denaturant is removed. In this setup, we found Skp to elute with AIDA from the column; however, this was also the case with lysozyme, suggesting that binding was an unspecific charge effect. Furthermore, when adding phospholipid vesicles to the eluted Skp-AIDA sample, still no native protein was obtained. Thus, this setup does not provide a means for an in vitro chaperone-assisted pathway to fold the AIDA β-domain.

We also tested the periplasmic chaperones SurA and DegP but did not see an effect on the refolding of AIDA. Likewise, for the outer membrane protein TolC from *E.*

coli there was no effect of Skp, SurA, or LPS in vivo on the trimerization process and folding (Werner et al. 2003). TolC, which forms a homotrimeric β-barrel with each monomer contributing four β-strands to the barrel, like AIDA, belongs to a different structural class of OMPs than the classical porins, e.g., OmpC and OmpF, and OmpA. The fact that none of these well-known periplasmic chaperones assist in the folding of AIDA makes it possible that some unknown factor is required to fold the AIDA β-domain in vivo. Such a factor is probably not AIDA-specific because the AIDA β-domain can be produced in the native form in *E. coli* cells that do not contain the native AIDA plasmid (Benz and Schmidt 1989). However, we were not able to identify such a factor by incubating nonnative AIDA DT4 with an *E. coli* periplasmic extract, as no proteins were retained on the column to which AIDA was bound except for Skp. The latter's retention is probably fortuitous as outlined above. The critical need for a solid support to refold the AIDA β-domain might suggest that other components in the periplasmic space are involved in the folding rather than conventional chaperone-mediated folding.

Materials and methods

Materials

Octyl-polyoxyethylene was from Bachem AG; pentaethylene glycol monodecyl ether was from Fluka; 1,2-diacyl-*sn*-glycero-3-phosphocholines, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamines, and dodecyl-phosphocholine were from Avanti Polar Lipids; *n*-dodecyl-β-D-maltoside and *n*-octyl-β-D-glucoside were from Anatrace Inc.; Ni-NTA-agarose beads were from Qiagen; imidazole was from Acros Organics; SDS-PAGE low molecular weight markers were from MBI Fermentas; ATP was from Applichem; LPS R60 (R2 core type, $M_w \approx 3900$ g/mol) was isolated as reported (Muller-Loennies et al. 1994) from the rough mutant F576, which was cultivated as described (Vinogradov et al. 1999). All other chemicals were from Sigma Chemical Co.

Data analysis

Nonlinear least-squares regression analysis was carried out with Kaleidagraph, version 3.5 (Synergy Software). Raw CD data were converted to molar ellipticity, [θ] (expressed in deg cm^2 dmol^{-1}), using the relationship (Woody 1995):

$$[\theta] = \frac{100\ \theta}{l\,C} \tag{1}$$

where θ is the measured ellipticity [deg], l is the cuvette path length [cm], and C is the protein concentration times the number of amino acids [mol/L]. Chemical denaturation experiments (urea or SDS) were fitted to the following equation, assuming a linear dependence of the pre- and post-transition baselines on denaturant concentration (Pace 1986; Clarke and Fersht 1993):

$$Y_{\text{obs}} = \frac{\alpha_N + \beta_N[\text{urea}] + (\alpha_D + \beta_D[\text{urea}])10^{m_{D-N}([\text{urea}]-[\text{urea}^{50\%}])}}{1 + 10^{m_{D-N}([\text{urea}]-[\text{urea}^{50\%}])}} \tag{2}$$

where Y_{obs} is the observed signal; α_N and α_D denote the signal at 0 M denaturant for the native and the denatured states, respectively; β_N and β_D are the slopes of the baselines of the native and the denatured states, respectively; [urea$^{50\%}$] is the denaturant concentration where 50% of the protein is denatured; and $m_{D\text{-}N}$ is a constant. From the fit to Equation 2, the thermodynamic stability, $\Delta G_{D\text{-}N}$, of AIDA in the absence of denaturant is calculated as follows (Clarke and Fersht 1993):

$$\Delta G_{D-N} = -RT\ln(10)m_{D-N}[\text{urea}^{50\%}] \tag{3}$$

Thermal scans were fitted to the following equation, assuming a linear dependence of the pre- and post-transition baselines on temperature, to obtain T_m, the midpoint of denaturation (Yadav and Ahmad 2000):

$$\theta = \frac{\alpha_N + \beta_N T + (\alpha_D + \beta_D T)e^{\left(\frac{-\Delta H_{vH}}{R}\left(\frac{1}{T}-\frac{1}{Tm}\right)\right)}}{1 + e^{\left(\frac{-\Delta H_{vH}}{R}\left(\frac{1}{T}-\frac{1}{Tm}\right)\right)}} \tag{4}$$

where θ is the observed ellipticity; α_N and α_D are the ellipticities of the native and the denatured state, respectively, at 298 K; β_N and β_D are the slopes of the native and denatured state baselines, respectively; T is the temperature; ΔH_{vH} is the van't Hoff enthalpy change of unfolding; and R is the gas constant. Thermal unfolding of C_d is mostly reversible, whereas for C_w it is completely irreversible due to subsequent aggregation (data not shown). However, for simplicity, we assume that the steps prior to aggregation are essentially reversible. This does not affect our conclusions, since we carry out these scans only to compare the combined kinetic and thermodynamic stability of AIDA in different detergent/lipid compositions.

Production and purification of proteins

AIDA DT4 and MS12 were produced and purified as described (Mogensen et al. 2005). Skp was expressed from plasmid pSkp in *E. coli* CAG16037, and produced and purified as described (Bulieris et al. 2003). SurA was expressed from plasmid pASKSurA in *E. coli* CAG44102, and produced and purified as described (Behrens et al. 2001). DegP and the inactive mutant DegP$_{S210A}$ were expressed from plasmids pCS20 and pCS21, respectively, in *E. coli* KU98, and produced and purified as described (Spiess et al. 1999).

Preparation of phospholipid vesicles

Generally, lipids were suspended in buffer by at least 30 sec of vortexing and subjected to five rounds of freezing in liquid N_2 and thawing at 55°C before extrusion on a 15-mL thermobarrel extruder (Northern Lipids). Lipid mixtures were dissolved in chloroform and dried on a Speed-Vac at low pressure for at least 6 h before resuspension in buffer. Lipid suspensions were extruded five times through 100-nm filters followed by extrusion eight times through 50-nm filters at least 10°C above their melting temperature. Specifically, lipids for mixed PC/PE/PG vesicles were dissolved in chloroform and mixed to a molar

ratio of 50% PC, 30% PE, and 20% PG. The lipid mixtures were first dried under a stream of nitrogen and subsequently in a desiccator for at least 3 h. Lipids were then hydrated in buffer and dispersed by vortexing. Small unilammellar vesicles were prepared by sonicating the lipid dispersions with a Branson W450D ultrasonicator for 40 min with a 50% pulse cycle.

Folding kinetics of AIDA DT4 on Ni-NTA-agarose beads

The DT4 folding kinetics experiments on beads were essentially performed as described for the purification of the protein in oPOE (Mogensen et al. 2005); 500 μL DT4 in 8 M urea in 50 mM TRIS (pH 8), 100 mM NaCl (TN buffer) (solubilized inclusion bodies) was mixed with 500 μL 250 mM oPOE in TN buffer and incubated overnight at 4°C. At time 0, 100 μL Ni-NTA-agarose beads were added and at subsequent time points 200 μL samples were removed and applied to an empty Pharmacia PD-10 column. The beads were washed with 500 μL 12.5 mM oPOE in TN buffer and subsequently eluted in 350 mM imidazole in 12.5 mM oPOE in TN buffer. The dead time of the experiment was ~2 min. The eluted fractions were run on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. The fraction of folded protein was determined by densitometry using ImageJ V1.31v (Research Services Brand, NIH).

Characterization of the water-soluble state C_w of AIDA DT4

Solubility in buffer vs. pH

The solubility of AIDA DT4 was determined as a function of pH by centrifuging samples at 16,000*g* for 15 min. The protein concentration was 3.6 μM in 25 mM buffer containing 13 mM residual urea. The concentration was measured spectrophotometrically at 280 nm before and after centrifugation, and the solubility was expressed in percent by dividing the protein concentration after centrifugation with the concentration before centrifugation. Buffers used were as follows: pH 1, 0.1 M HCl; pH 2–3.5, glycine; pH 3.7–4.7, sodium acetate; pH 6, MES; pH 7, MOPS; pH 8–9, TRIS; pH 10–11, glycine; pH 13, 0.1 M NaOH.

Secondary structure vs. pH

Far-UV CD scans of 5 μM urea-purified DT4 were recorded as a function of pH in 25 mM buffer at 25°C on a JASCO J-715 spectropolarimeter (Jasco Spectroscopic Co. Ltd.) equipped with a JASCO PTC-348WI temperature control unit. The ellipticity was measured in the wavelength range of 195–245 nm and six accumulations were averaged to yield the final spectrum. The contribution of buffer to the measured ellipticity was subtracted. A 0.1-cm path length cuvette was employed. The buffers used were as described above.

ANS binding experiments

Urea-purified DT4 (5 μM) was incubated with 5 μM ANS for 30 min in 50 mM buffer before measurement at 25°C on a RTC2000 Spectrometer from Photon Technology International equipped with a 75W Xenon Arc Lamp and a temperature control unit. ANS was excited at 350 nm. All experiments were recorded as the average of five emission scans. Excitation and emission band paths were both 5 nm. A 3-nm path length cuvette was employed. The contribution of buffer and protein to the measured fluorescence was subtracted. The buffers used were as described above.

Characterization of the micelle-associated state C_d of AIDA DT4

Thermal denaturation

Urea-purified AIDA DT4 (6.5 μM) in 10 mM TRIS (pH 8) was mixed with different detergents and allowed to equilibrate for 30 min. Thermal unfolding was monitored by far-UV CD at 220 nm with a scan rate of 60°C/h. The contribution of buffer to the measured ellipticity was subtracted. The unfolding curves were fitted to Equation 4 to obtain T_m.

Urea denaturation

For unfolding experiments in detergent, 0.5 μM urea-purified DT4 was incubated in 0–8 M urea in 25 mM sodium phosphate (pH 8.0), 100 mM NaCl containing 1% (w/v) detergent at 10°–40°C. Unfolding was monitored by fluorescence with excitation at 295 nm and emission at 330 nm, where the greatest intensity difference between the folded and the unfolded state was observed. For unfolding experiments in buffer, 5 μM urea-purified DT4 was incubated in 0–5.5 M urea in 10 mM TRIS (pH 8) at 25°C. Unfolding was monitored by far-UV CD at 220 nm. The contribution of buffer to the measured fluorescence or ellipticity was subtracted. Data were fitted to Equation 2.

SDS denaturation

Urea-purified AIDA DT4 (2 μM) was mixed with 20 mM DM in 10 mM phosphate buffer (pH 7). After 30 min, SDS was added at various concentrations and the samples equilibrated for another 30 min before measurement. Far-UV CD scans were recorded at 25°C and the contribution of buffer to the measured ellipticity was subtracted. Unfolding curves, expressed as the ratio between the ellipticity at 206 nm and at 209 nm (which gave the largest signal change upon unfolding), were fitted to Equation 2, using the mole fraction of SDS as the independent variable instead of denaturant concentration (Lau and Bowie 1997).

Double-jump experiments to monitor formation of C_w

Double jump experiments were carried out by diluting AIDA DT4 (denatured in 6 M urea) 11-fold into lipid or detergent, typically to a final concentration of 30 mg/mL lipid and 4 μM AIDA in TN buffer at 25°C. Each solution was equilibrated at 25°C for several minutes prior to mixing. At appropriate time points, 130 μL aliquots were withdrawn and mixed with 2270 μL 6 M urea preincubated for several minutes at 40°C. The emission intensity at 350 nm (excitation at 280 nm) was followed for ~10 min and fitted to a single exponential function with drift. To obtain the refolding rate, the plot of the amplitude versus time was fitted to a single exponential function. Under some circumstances (high protein concentration or high content of PE in the vesicles), an additional slower phase was observed when amplitude was plotted versus time. In this case, the fraction of DT4 folding via the fast phase was estimated as the amplitude of the fast phase relative to the total amplitude.

Periplasmic chaperone experiments

Refolding experiments with Skp

The folding experiment was initiated by rapidly mixing 1 μL of urea-purified AIDA DT4 or MS12 in 8 M urea first with 12 ηL of 10 mM glycine buffer, (pH 8.5), and then 2 μL of Skp followed by the immediate addition of 5 μL of LPS and then by the addition of 30 μL of preformed phospholipid vesicles. The final concentrations were 5 μM AIDA, 160 mM urea, 100 μM Skp, 5 mM lipid, and 75 μM LPS. When experiments were performed in the absence of Skp, LPS, or lipid vesicles, the corresponding solutions were replaced by the same volume of buffer. Samples were incubated at 40°C for up to several weeks and run on an SDS-polyacrylamide gel to test for heat modifiability.

Binding experiments with Skp

Skp binding to AIDA DT4 and MS12 was studied in a titration experiment by fluorescence spectroscopy using urea-purified protein. Emission spectra were recorded on a Spex Fluorolog-3 spectrofluorometer with double monochromators in the excitation and emission pathways. Excitation and emission band paths were 3 nm. Samples were excited at 295 nm. Background spectra of Skp were subtracted, although Skp only contributes little as it does not contain tryptophan. Skp concentrations ranged from 0 to 25 μM with an AIDA concentration of 0.25 μM in 1000 μL sample volume. Experiments were performed at room temperature in 10 mM glycine (pH 8.5).

Refolding experiments with SurA

The folding experiment was initiated by rapidly mixing 0.5 μL of urea-purified AIDA DT4 or MS12 in 8 M urea first with 64.5 μL of 10 mM HEPES (pH 7), and then 5 μL of SurA followed by the immediate addition of 10 μL of LPS and then by the addition of 20 μL of preformed phospholipid vesicles. The final concentrations were 2 μM AIDA, 40 mM urea, 15 μM Skp, 5 mM lipid, and 75 μM LPS. When experiments were performed in the absence of SurA, LPS, or lipid vesicles, the corresponding solutions were replaced by the same volume of buffer. Samples incubated at 37°C for up to several days and were run on an SDS-polyacrylamide gel to test for heat modifiability.

Refolding experiments with DegP

The folding experiment was initiated by rapidly mixing 2 μL of urea-purified AIDA DT4 or MS12 in 8 M urea first with 8 μL of glycine buffer (10 mM [pH 8.5], 150 mM NaCl, 1 mM EDTA) and then 20 μL of DegP followed by the immediate addition of 10 μL of LPS and then by the addition of 60 μL of preformed phospholipid vesicles. The final concentrations were 5 μM AIDA, 160 mM urea, 30 μM DegP, 5 mM lipid, and 75 μM LPS. When experiments were performed in the absence of DegP, LPS, or lipid vesicles, the corresponding solutions were replaced by the same volume of buffer. Samples incubated at 37°C for up to several days and were run on an SDS-polyacrylamide gel to test for heat modifiability.

GroEL experiments

Essentially, the same conditions as described by Goulhen et al. (2003) were used. 0.5, 5, or 50 μg GroEL in refolding buffer (7.5 mM oPOE, 5 mM $MgCl_2$, 5 mM KCl, 20 mM NaCl, 100 mM TRIS [pH 8], and 10 mM ATP) in the presence or absence of 0.3 mM SDS was mixed with 5 μg urea-purified AIDA DT4 in 8 M urea in a final volume of 10 μL. Samples incubated at room temperature or 37°C for 1.5 h or 2 d. The reaction was stopped by addition of 10 μL SDS-PAGE loading buffer. Samples were run on 12% SDS-polyacrylamide gels to test for heat modifiability.

Incubation of Ni-NTA-agarose-bound DT4 with E. coli *periplasmic extract*

Isolation of periplasmic fraction

The periplasmic fraction from *E. coli* BL21(DE3) containing the plasmid pTONYSKP was isolated as follows: Cells were grown in 0.5-L LB medium at 37°C to an OD_{600} of 1.0 and harvested by centrifugation at 7000*g* for 10 min at 4°C. Cells were resuspended in sucrose buffer (100 mM TRIS [pH 8], 20% [w/v] sucrose) and equilibrated on ice for 10 min. After centrifugation, cells were resuspended in sucrose buffer containing 10 mM EDTA and 10 μg/mL lysozyme and incubated for 30 min on ice. Spheroplasts were sedimented at 7000*g* after the addition of 20 mM $MgSO_4$, and the resulting supernatant was used directly on the column.

Column experiments

Four hundred microliters of urea-purified DT4 in 8 M urea (~250 μM) were mixed with 75 μL of Ni-NTA-agarose beads and incubated for 1 h at room temperature to allow for binding of AIDA to the resin. The sample was applied to the column (empty Pharmacia PD10 disposable column) and extensively washed in TN buffer (20 mM TRIS [pH 8], 40 mM NaCl). One milliliter of the periplasmic fraction was applied to the column followed by extensive washing in TN buffer. Elution of possible proteins binding to AIDA was performed in 2×200 μL 250 mM NaCl in 20 mM TRIS (pH 8) followed by 2×200 μL 750 mM NaCl in 20 mM TRIS (pH 8). After extensive washing in TN buffer, AIDA was eluted in 5×200 μL 0.5 M imidazol in TN buffer. The different fractions were run on 12% SDS-polyacrylamide gels.

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