Destabilizing Mutations Promote Membrane Protein Misfolding†

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ABSTRACT: In this work, the relationship between stability and propensity to misfold was probed for a series of purified variants of the polytopic integral membrane protein diacylglycerol kinase. It was observed that there was a strong correlation between stability and folding efficiency. The most common mutations that promoted misfolding were those which also destabilized the protein. These results imply that by targeting unstable membrane proteins for degradation, cellular protein folding quality control can eliminate proteins that have a high intrinsic propensity to misfold into aberrant structures. Moreover, the more rare class of amino acid mutations that promote misfolding without perturbing stability may be particularly dangerous because the mutant proteins may evade the surveillance of cellular quality control systems.

The efficiency of membrane protein folding is a matter of great relevance to human health and disease. Disease may result when mutations or other perturbations reduce the efficiency of assembly of a critical protein to below a minimum threshold required for the maintenance of that protein's normal function in the cell (*1*−*4*). Loss of protein function in some cases is compounded by cytotoxicity resulting from the failure of the cell to properly dispose of the misassembled protein. In eukaryotes, most membrane proteins fold early in the secretory pathway, a process that is monitored and facilitated by the quality control (QC)[1] system of the endoplasmic reticulum (*5*−*9*). Protein judged to be folding-defective is subjected either to further attempts at folding through the action of ER chaperones or is targeted for proteolytic degradation. It appears that bacteria have analogous quality control systems for monitoring membrane protein folding (*10*−*14*).

There is evidence that protein folding efficiency in the cell tends to correlate with protein stability (*15*−*21*). A possible explanation for this relationship is that protein folding quality control degrades unstable proteins because, in doing so, it preemptively eliminates proteins that have a high propensity to misfold and form potentially cytotoxic aggregates. For purified water soluble proteins, it has been shown that thermodynamic stability often inversely correlates with propensity for misfolding or aggregation (*15*, *22*−*28*). In the case of integral membrane proteins, the only work in this area is the study of Bowie and co-workers, who demonstrated that for diacylglycerol kinase (DAGK) there is a clear correlation between thermodynamic stability and resistance to thermal inactivation (*29*). Here, we extend their work by examining whether unstable forms of DAGK are more likely to misfold under the conditions of folding kinetic studies. The present work also complements recent progress in biophysical studies of membrane protein folding (*30*−*35*).

MATERIALS AND METHODS

Expression and Purification of Diacylglycerol Kinase (DAGK). Escherichia coli harboring plasmids encoding wild type and mutant forms of DAGK were generously provided by James Bowie of UCLA (*36*). DAGK mutants were overexpressed and then purified into detergent micelles, into 6.5 M urea plus 0.5% formic acid (no detergent or lipid), or into 8 M guanidinium chloride plus 0.5% formic acid (no detergent or lipid) as described previously (*37*). In some cases, DAGK was reconstituted from dodecylphosphocholine micelles into 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid vesicles, a process previously shown to result in the refolding of misfolded DAGK which may be present after purification (*38*). The POPC/DAGK mol/mol ratio in the resulting vesicles was always 120:1.

Measurement of Relative Thermodynamic Stability. The thermodynamic stability of the DAGK homotrimer was determined by first dispersing aliquots of DAGK in POPC vesicles to a concentration of 0.1 mg mL^{-1} into a buffer containing 75 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 6.8. The detergent β-*n*-decylmaltoside (DM) was then added to the vesicular solution resulting in mixed micelles in which the final DM to POPC molar ratio was 85:15. Samples were then titrated with SDS through a concentration range of 0−80 mol % (mol of micellar SDS/(mol of micellar SDS + mol of micellar DM + mol of POPC) × 100). At each titration point aliquots were removed and incubated at 30 °C for 30 min, followed by addition of glutaraldehyde to 25 mM. Samples were shaken vigorously for 16 h to allow cross-linking to reach completion (*39*) and

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[1] Abbreviations: Cysless: Cysteine-less mutant form of diacylglycerol kinase; DAGK: diacylglycerol kinase; DM: *n*-decyl-β-maltoside; ER: endoplasmic reticulum; GA: glutaraldehyde; PAGE: polyacrylamide gel electrophoresis; PIPES: piperazine-1,4-bis(2-ethanesulfonic acid); POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; QC: quality control; SDS: sodium dodecyl sulfate; WT: wild type.

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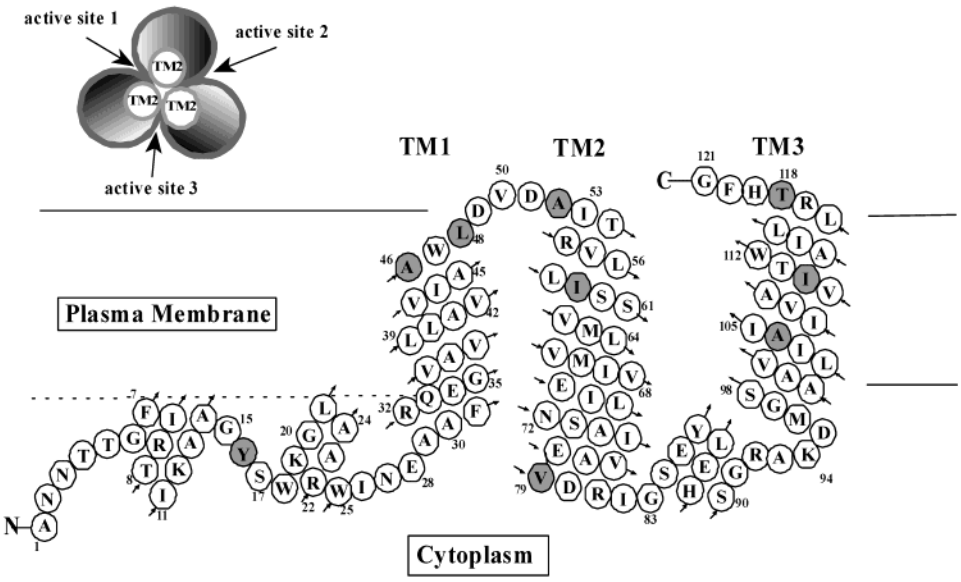

FIGURE 1: Model for the topology and secondary structure of *E. coli* diacylglycerol kinase based upon NMR and topology mapping experiments (*62*, *63*). DAGK functions only as a homotrimer, with its three active sites being located at the interfaces between subunits (*41*). The second transmembrane segment appears to form the 3-fold axis of symmetry at the center of the trimer (*64*). The sequence shown is for the cysteine-less (Cysless) mutant form of the enzyme (*36*). This mutant was subjected to cysteine replacements at the sites marked in gray to generate the single-cysteine mutants used in this work. The Cysless mutant differs from the true wild-type protein by virtue of the following mutations (*36*): C46A, C113A, W117R, and T118S. It should be noted that the C46A mutant is the Cysless mutant, but with one of the two wild-type Cys residues present (site 46).

analyzed by SDS−PAGE with Coomassie staining. To determine the mol % SDS at which conversion from trimer to monomer is 50% complete (SDS_{50}), gels were photographed with a Kodak (Rochester, NY) DC290 Zoom Digital Camera and bands intensities were determined using Kodak ID Image Analysis software (version 3.6). Intensities were corrected for the presence of background staining and the monomer bands were also corrected for the 0 SDS mol % band representing that fraction of DAGK that escapes conversion to trimer because of the less than 100% efficiency of the GA cross-linking reaction. For a given SDS titration, monomer and trimer band intensities were plotted, and the mol % SDS at the points of inflection for the sigmoidal curves of the monomer and dimer bands were determined and averaged. These average %SDS for the loss of trimer and appearance of monomer are here reported as SDS_{50}.

Kinetic Stability Measurements. The kinetic stability of DAGK in mixed micelles was determined by first diluting the enzyme in POPC vesicles into a buffer containing 2% DM, such that the final protein concentration was 0.2 mg mL^{-1}. Samples were then incubated at 80 °C. At various time points, aliquots were removed and assayed for catalytic activity in mixed micelles using a coupled assay system (*40*). For each mutant, these data were used to determine a $time_{1/2}$ for complete loss of DAGK activity.

Measurement of Folding Rates and Efficiencies for Purified DAGK in Model Membranes. Details of the spectrophotometrically based method for monitoring the functional assembly of DAGK in folding/insertion assays have been described elsewhere (*37*). Briefly, aliquots of stock DAGK in acidic 6.5 M urea or acidic 8 M guanidinium chloride were diluted 200-fold into 500 μL insertion assay mixtures containing POPC vesicles (2 mM POPC) or into mixed micelles containing 21 mM DM as the detergent component and 3 mol % cardiolipin as the lipid component. Productive folding was detected through the appearance of DAGK catalytic activity as a function of time. DAGK folding efficiency in these "folding assays" was determined by comparing the final plateaued activity with the known activity for the 100% active mutant under the same assay conditions. Overall rates of folding were estimated by measuring the time taken in each insertion assay for DAGK activity to reach half of its final plateaued activity in that assay. All of the folding efficiencies and $time_{1/2}$ reported in this work represent the average of at least three folding assays. A variety of controls establishing the validity of this method have been described elsewhere (*37*).

RESULTS

Choice of Diacylglycerol Kinase (DAGK) Variants. It has previously been shown that many mutant forms of DAGK (Figure 1) have a high propensity to misfold, forming kinetically trapped aberrant conformations before or during purification (*38*). For this work, we examined a subset of these mutants, for which sites labeled in gray in Figure 1 were individually substituted with cysteine. These mutants were chosen because they represent a cross-section in terms of propensity to misfold, stability, and location of the mutation site within the protein. No active site mutations were included; when properly folded, all of the chosen mutants exhibit catalytic activities which are at least 50% of the wild-type activity (*38*).

Stabilities of DAGK Variants. DAGK functions only as a homotrimer (*39*, *41*). As illustrated in Figure 2, the stability of each of 11 DAGK variants was determined by measuring the concentration of SDS required to dissociate trimers to monomers when the protein is solubilized in detergent micelles (SDS_{50}). To a good approximation, it has previously been shown that SDS unfolds DAGK in a reversible manner (*36*). In this work, we did not attempt to quantitatively measure stability, but instead used the SDS_{50} to rank the

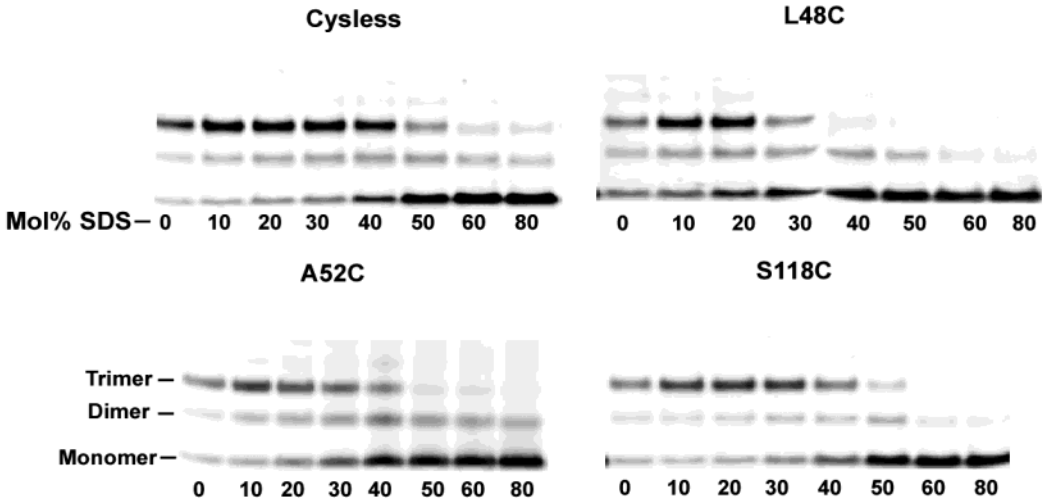

FIGURE 2: Determination of the relative thermodynamic stability of DAGK trimers. DAGK in nondenaturing DM micelles was titrated with SDS and subjected to nonspecific glutaraldehyde cross-linking and SDS−PAGE/Coomassie staining at each point. From these data SDS_{50} for conversion of trimers to monomers were determined. It should be noted that the presence of a monomer band even at 0% SDS does not mean that a fraction of each protein was monomeric. Rather, it has been shown that the GA cross-linking reaction is not 100% efficient for micellar (trimeric) DAGK, so that some monomer is inevitably observed even for stable forms of the enzyme.

Mutant	Thermodynamic Stability	Kinetic Stability	% Folded After Purification	Urea into POPC Efficiency	Urea into Micelles Efficiency	Guanidine into POPC Efficiency	Guanidine into Micelles Efficiency
Wild Type	++++	++++	++++	+++	++++	+++	++++
A46C	++++	++++	++++	++++	++++	++++	++++
Y16C	++++	+++	+++	+	++	+	+
Cysless	+++	++++	++++	++++	++++	+++	+++
S118C	+++	+++	+++	+++	++++	++	++
V79C	++	++	+++	++	+++	++++	++++
A52C	++	++	++	++	+++	+++	++
I110C	+	not tested	+	+	+++	+	+
A104C	+	+	++	+	++	+	++
L48C	+	+	+	+	+	+	+
I59C	+	+	+	+	+	+	+
V42C	not tested	+++	+++	not tested			
I11C		++	+++				
T111C		+	+				
L58C		+	+				
I75C		+	++				

FIGURE 3: Summary of stability and folding efficiency data for purified variants of DAGK in model membranes. Within each column, each mutant is ranked with regard to the property represented by that column relative to the other mutants. "++++" means high and "+" means low. The exact value ranges which lead to the groupings within each column can be inferred from the data of Table 1 or from data reported in the text. It should be emphasized that the measurements leading to assignment of a certain number of + in one column do not correspond quantitatively to the values leading to the same number of + in another column. For example, +++ is assigned to mutants which show a urea-to-mixed micelles folding efficiency of 10−15%, while in the guanidinium-to-POPC vesicles case +++ is assigned to mutants folding with 1−6% efficiency.

relative thermodynamic stabilities of the trimers (*29*). SDS_{50} were determined to be 51 ± 3, 50 ± 3, 50 ± 3, 45 ± 2, 41 ± 2, 36 ± 3, 35 ± 7, 30 ± 4, 29 ± 4, 27 ± 5, and 27 ± 3 mol % for A46C, WT, Y16C, Cysless, S118C, V79C, A52C, I59C, A104C, I110C, and L48C, respectively. These results are summarized in Figure 3.

Kinetic stabilities were assessed by measuring $time_{1/2}$ for irreversible loss of catalytic activity when the fully folded mutants were incubated in detergent micelles at 70 °C. Half times were observed to be 16, 16, 2.5, 2.5, 1.3, 1, <0.5, <0.5, and <0.5 min for Cysless, A46C, Y16C, S118C, V79C, A52C, L48C, I59C, and A104C, respectively. Measurements were also made for several other single cysteine mutants: V42C, I11C, T111C, T58C, and I75C, for which $time_{1/2}$ of 2.6, 1.4, <0.5, <0.5, and <0.5 min were observed. Relative kinetic stabilities are summarized in Figure 3; there is generally an excellent correlation between kinetic stability and the thermodynamic stability of DAGK trimers, as previously observed for other DAGK mutants (*29*). Indeed, when the data for the anomalous Y16C was neglected, the

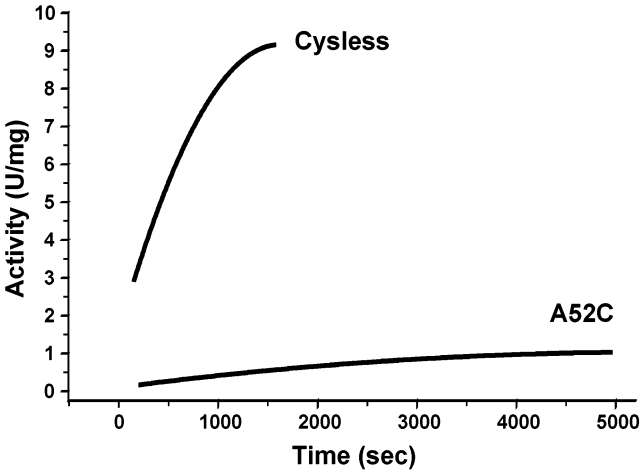

FIGURE 4: Examples of time traces from DAGK folding assays. The assays represented by these data were for DAGK folding/insertion into POPC vesicles following dilution from a urea stock solution of the enzyme. These particular DAGK variants each exhibited specific activities of about 30 U/mg when 100% folded under the specific conditions of this assay. Thus, the Cysless and A52C variants folded with ca. 30 and 4% efficiencies, respectively.

linear correlation coefficient between SDS_{50} values and $time_{1/2}$ was determined to be 0.65, indicating statistical significance at the 95% confidence level (*42*).

Folding Rates and Efficiencies. The DAGK variants being examined in this work were previously reported to contain substantial populations of kinetically trapped misfolded protein upon purification into detergent micelles (*38*), an observation that is summarized in the fourth column of Figure 3. To quantitate the folding/misfolding propensities of DAGK under well-controlled conditions, we have developed methods for generating lipid/detergent-free stock solutions of fully or partially unfolded DAGK. Wild type DAGK was previously shown to be a partially folded monomer in acidic 6.5 M urea, while in acidic 8 M guanidinium it appears to be completely unstructured (*37*). Small aliquots of these stock solutions were then introduced into either mixed micelle or lipid vesicle solutions, and the process of productive membrane insertion and folding was followed in real time through the appearance of DAGK enzyme activity (Figure 4).

Reciprocal rates and insertion/folding efficiencies are reported in Table 1. The rates are generally much more rapid for folding assays into detergent micelles than into lipid vesicles, as previously observed for the wild type enzyme (*37*). This indicates that for most DAGK variants the kinetic barrier to insertion into mixed micelles is much lower than into lipid vesicles, a barrier which in the latter case is probably rate-limiting for the overall assembly process. Also consistent with previous results for wild type, DAGK variants usually fold more efficiently in assays starting from urea stock solutions than in those initiated from guanidinium stocks. Evidently, DAGK variants are generally most susceptible to misfolding when fully unfolded, as is the case immediately following dilution into a folding assay mixture from a guanidinium stock. For folding assays involving mixed micelles rates were usually very rapid, suggesting that the rate-limiting step for productive folding under those conditions is not kinetically coupled to steps critical in determining partitioning between folding and misfolding. In the cases of assays involving POPC vesicles, there was generally a good correlation between folding rates and efficiencies, indicating either that misfolding commonly occurs at the stage of bilayer insertion and/or that factors that govern partitioning between folding and misfolding are also critical in determining the rate of bilayer insertion.

Table 1: Efficiencies and Overall Rates for Folding of DAGK Variants into Model Membranes

mutant	starting conditions	final conditions	efficiency (%)	$t_{1/2}$ (s)
wild type	urea	POPC vesicles	10 ± 2	390 ± 210
Cysless	urea	POPC vesicles	26 ± 5	370 ± 150
Y16C	urea	POPC vesicles	<0.5	ND[a]
A46C	urea	POPC vesicles	31 ± 8	750 ± 50
L48C	urea	POPC vesicles	<0.5	ND
A52C	urea	POPC vesicles	4.6 ± 3.1	2100 ± 600
I59C	urea	POPC vesicles	<0.5	ND
V79C	urea	POPC vesicles	2.6 ± 0.4	2600 ± 1500
A104C	urea	POPC vesicles	2.0 ± 0.9	2800 ± 1300
I110C	urea	POPC vesicles	1.4 ± 0.5	3300 ± 300
S118C	urea	POPC vesicles	16 ± 4	1500 ± 500
wild type	urea	mixed micelles	29 ± 7	4.4 ± 1.8
Cysless	urea	mixed micelles	45 ± 16	16 ± 6
Y16C	urea	mixed micelles	3 ± 1	4 ± 1
A46C	urea	mixed micelles	68 ± 10	10 ± 1
L48C	urea	mixed micelles	0.7 ± 0.3	9 ± 1
A52C	urea	mixed micelles	14 ± 2	8 ± 1
I59C	urea	mixed micelles	2.6 ± 1.3	8 ± 1
V79C	urea	mixed micelles	11 ± 2	8 ± 1
A104C	urea	mixed micelles	7.9 ± 1.1	8 ± 1
I110C	urea	mixed micelles	12 ± 3	8 ± 1
S118C	urea	mixed micelles	57 ± 6.	11 ± 2
wild type	guanidinium	POPC vesicles	1.5 ± 0.6	810 ± 50
Cysless	guanidinium	POPC vesicles	1 ± 0.5	1000 ± 100
Y16C	guanidinium	POPC vesicles	<0.2	ND
A46C	guanidinium	POPC vesicles	5.2 ± 0.8	320 ± 200
L48C	guanidinium	POPC vesicles	0.4 ± 0.2	2600 ± 800
A52C	guanidinium	POPC vesicles	1.2 ± 0.3	2300 ± 300
I59C	guanidinium	POPC vesicles	<0.3	ND
V79C	guanidinium	POPC vesicles	4.8 ± 0.9	700 ± 220
A104C	guanidinium	POPC vesicles	0.3 ± 0.09	3900 ± 400
I110C	guanidinium	POPC vesicles	<0.3	ND
S118C	guanidinium	POPC vesicles	0.2 ± 0.1	2600 ± 400
wild type	guanidinium	mixed micelles	4.4 ± 1.0	36 ± 8
Cysless	guanidinium	mixed micelles	3 ± 0.5	17 ± 9
Y16C	guanidinium	mixed micelles	0.4 ± 0.1	9 ± 1
A46C	guanidinium	mixed micelles	21 ± 2	9 ± 1
L48C	guanidinium	mixed micelles	0.1 ± 0.04	8 ± 1
A52C	guanidinium	mixed micelles	0.9 ± 0.1	10 ± 2
I59C	guanidinium	mixed micelles	0.5 ± 0.3	8 ± 1
V79C	guanidinium	mixed micelles	14 ± 1	9 ± 2
A104C	guanidinium	mixed micelles	0.7 ± 0.4	83 ± 8
I110C	guanidinium	mixed micelles	0.4 ± 0.2	8 ± 1
S118C	guanidinium	mixed micelles	0.75 ± 0.03	10 ± 1

[a] ND = not determined.

The detailed results of Table 1 are summarized in Figure 3. The efficiencies obtained from the denaturant-to-model membrane folding assays correlate well with trends in how much misfolded protein is found to be present following purification into detergent micelles. For each variant, there is usually good uniformity of relative efficiencies across the four sets of folding assay conditions. Of particular note, there is generally high correlation between the folding efficiencies and thermodynamic stabilities that extends across all four set of experiments. Excluding the results for Y16C, the calculated linear correlation coefficients (*42*) between folding efficiency and SDS_{50} were determined to fall the range of 0.6−0.8 for all four sets of conditions, with statistical significance being >95% in each case.

DISCUSSION

The most important result of this paper is that the kinetic and thermodynamic (trimer) stabilities of variants of purified DAGK are usually highly correlated with folding efficiency in model membranes (Figure 3). The most stable DAGK variants folded efficiently under all conditions, with the single exception being the Y16C mutant. All of the unstable mutants folded inefficiently under all conditions. Intermediate stability mutants tended to fold with intermediate efficiency. Because the variants examined represent a cross-section of mutation sites, general conclusions for DAGK can be derived from these results. Key among these are (i) that the most common class of DAGK mutations that lead to inefficient folding also lead to reduced stability for the properly folded protein, and (ii) that any mutation which reduces DAGK's stability will reduce its folding efficiency. These observations are consistent with the notion that the avoidance of misfolding may be an important selective factor contributing to evolutionary optimization of membrane protein stability.

E. coli DAGK is an established model system for studying membrane protein folding (*29*, *36*, *37*, *43*−*47*). However, further work will be required to determine if the instability/misfolding correlation observed for DAGK can be generalized to other membrane proteins. Despite uncertainty regarding this point, the potential relevance of the present results to eukaryotic membrane protein folding merits comment because of the role membrane protein misassembly plays in dozens of human diseases (*1*−*4*, *48*).

Most eukaryotic membrane proteins fold following insertion into the membrane of the endoplasmic reticulum. For soluble proteins that fold only after translocation into the lumen of the ER a correlation has been observed between protein stability and the efficiency of successful folding and trafficking on through the secretory pathway (*17*−*19*, *21*, *49*). The correlation between folding efficiency and stability has also been observed for a number of purified soluble proteins (*15*, *22*−*28*). While the relationship between stability and in vivo folding efficiency for eukaryotic membrane proteins has received only preliminary attention (*20*, *50*), it is probable that a similar correlation pertains, as supported by two lines of evidence. First, the quality control machinery of the ER most likely monitors the folding of both soluble and membrane proteins based on the similar structural biophysical principles, targeting both soluble and membrane proteins that are judged to be defective for degradation. Second, it is known that small molecular ligands that are able to access the ER and that bind to (and thereby stabilize) the folded forms of specific eukaryotic membrane proteins can dramatically enhance their efficiency of productive assembly (*51*−*58*). Thus, it is probable that for both soluble and membrane proteins, the decision by ER quality control of whether to degrade a nascent protein is based upon evaluation of some property that is closely related to stability. The observation of this work that membrane protein stability is highly correlated with propensity to misfold indicates that this *modus operandi* is sensible from the standpoint of cellular survival. By targeting unstable nascent proteins for degradation, quality control eliminates misfolding-prone proteins before they have actually had a chance to become entrapped in a potentially toxic aberrant conformation.

Another implication of the results regarding human membrane protein misassembly and disease concerns rare exceptions to the stability/efficiency correlation. One DAGK mutant was found, Y16C, which has wild type-like stability once properly folded, but nevertheless exhibited a very high propensity to misfold under all four sets of folding conditions examined. As described elsewhere (*43*), Y16C is a mutation that appears to perturb only folding/misfolding *pathways* without altering the stability of the correctly folded protein. Analogous mutations have previously been documented for soluble proteins (cf., *15*, *59*, *60*) and for a soluble domain of the CFTR protein containing the ΔF508 mutation that is the most common mutation leading to cystic fibrosis (*61*). The results of this work suggest that this class of mutation is rare compared to misfolding-promoting mutations which also reduce protein stability. If indeed cells evaluate the misfolding propensity of nascent proteins based on factors that are strongly coupled to intrinsic protein stability, then rare folding pathway-defective mutations such as Y16C in DAGK are potentially especially dangerous. The resulting mutant proteins might evade identification by quality control as being of high risk and would thereby escape the degradative fate normally assigned to unstable (misfolding-prone) proteins. Such proteins might then proceed to misfold and contribute to some of the dozens of "gain of function" disorders in which the loss of native function by the misfolded protein is exacerbated by a direct contribution of the misfolded protein to disease etiology or pathology (*1*−*4*).

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