Kinetic Study of Folding and Misfolding of Diacylglycerol Kinase in Model Membranes†

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ABSTRACT: Despite the relevance of membrane protein misfolding to a number of common diseases, our understanding of the folding and misfolding of membrane proteins lags well behind soluble proteins. Here, the overall kinetics of membrane insertion and folding of the homotrimeric integral membrane protein diacylglycerol kinase (DAGK) is addressed. DAGK was purified into lipid/detergent-free urea and guanidinium solutions and subjected to general structural characterization. In urea, the enzyme was observed to be monomeric but maintained considerable tertiary structure. In guanidinium, it was also monomeric but exhibited much less tertiary structure. Aliquots of these DAGK stock solutions were diluted 200-fold into lipid vesicles or into detergent/lipid mixed micelles, and the rates and efficiencies of folding/insertion were monitored. Reactions were also carried out in which micellar DAGK solutions were diluted into vesicular solutions. Productive insertion of DAGK from denaturant solutions into mixed micelles occurred much more rapidly than into lipid vesicles, suggesting that bilayer transversal represents the rate-limiting step for DAGK assembly in vesicles. The efficiency of productive folding/insertion into vesicles was highest in reactions initiated with micellar DAGK stock solutions (where DAGK maintains a nativelike fold and oligomeric state) and lowest in reactions starting with guanidinium stocks (where DAGK is an unfolded monomer). Moreover, the final ratio of irreversibly misfolded DAGK to reversibly misfolded enzyme was highest following reactions initiated with guanidinium stock solutions and lowest when micellar stocks were used. Finally, it was also observed that very low concentrations of detergents were able to both enhance the bilayer insertion rate and suppress misfolding.

A number of common diseases are associated with the aberrant folding and assembly of membrane proteins (*1*, *2*), including cystic fibrosis (*3*), Charcot−Marie−Tooth disease (*4*), and common forms of diabetes (*5*), blindness (*6*, *7*), and deafness (*8*). The development of a thorough understanding of the mechanisms and energetics of membrane protein folding pathways therefore represents a goal of particular importance. Previous studies in this area can be divided into two general classes. First, much effort is being placed upon unraveling the protein machinery which is involved in membrane protein insertion, folding, and quality control in living cells or in vitro cellular extracts (*3*, *9*−*12*). A second class of studies involves studies of membrane protein folding under "test tube" conditions using purified proteins and model membranes. Included in this latter class are studies of the thermodynamics of membrane protein assembly, stability, and protein−bilayer interactions (*13*−*16*). Less well developed are investigations of membrane protein folding pathways and kinetics under test tube conditions, although important contributions have been made regarding bacteriorhodopsin (*17*−*25*), the outer membrane porins (*26*−*29*), and other proteins (*30*, *31*). In this contribution, we explore the membrane insertion and folding of purified diacylglycerol kinase (DAGK)[1] as a function both of its initial structural state and of the nature of the target model membrane. A novel emphasis of this study is an effort to glean insight into *misfolding* processes which compete with productive insertion/folding of the enzyme.

Prokaryotic DAGK (Figure 1) functions as a homotrimer of 13 kDa subunits (*32*, *33*), each of which has three transmembrane segments (*34*). DAGK does not have an obvious signal sequence, and little is known about how it is inserted into the cytoplasmic membranes of bacteria in vivo. Using purified wild-type and mutant forms of DAGK the Bowie laboratory (UCLA) has initiated an extensive study of its stability and its mechanism of thermal inactivation (*35*−*37*). Once correctly folded, DAGK is a rather stable protein, even in detergent micelles (*35*). However, it is also

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; BCA, bicinchoninic acid; BOG, *n*-octyl glucoside; BR, bacteriorhodopsin; CD, circular dichroism; CFTR, cystic fibrosis transmembrane regulator; CL, cardiolipin; CMC, critical micelle concentration; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DBG, dibutyrylglycerol; DM, *n*-decyl maltoside; DPC, dodecylphosphocholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; GA, glutaraldehyde; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LDH, lactate dehydrogenase; LHC, light harvesting complex; NADH, nicotinamide adenine dinucleotide, reduced form; NMR, nuclear magnetic resonance; OmpA, outer membrane porin A; OmpF, outer membrane porin F; PA, phosphatidic acid; PEP, phosphoenolpyruvate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PK, pyruvate kinase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UV, ultraviolet.

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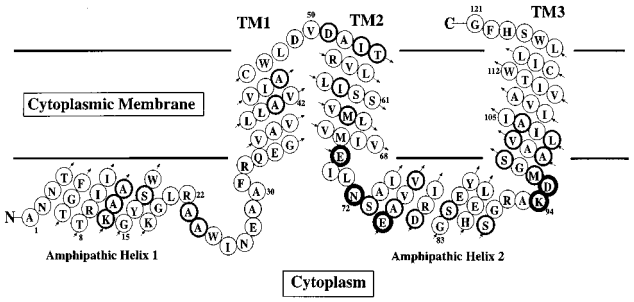

FIGURE 1: Model for wild-type DAGK's topology, secondary structure, and probable helical orientations based upon a variety of experimental data (*32*, *34*, *40*). The bold-circled residues are those which were judged to be essential on the basis of high conservation among natural isozymes and/or on results of the extensive mutagenesis study of Wen et al. (*69*). The five residues in extra bold are those which have been observed to be absolutely conserved both in the mutagenesis study *and* in all available wild-type microbial DAGK sequences.

known that DAGK has a high propensity to misfold, which can be enhanced by single site mutations at many different sequential locations (*38*). The qualitative structural properties of the misfolded form of one particular DAGK mutant were recently characterized (*39*). Thus, DAGK may be a particularly suitable model system for studying the fundamental basis for partitioning of membrane proteins between folding and misfolding pathways.

We have previously shown that DAGK can be purified as a lipid/detergent-free preparation in urea solutions and that the efficiency of insertion from such solutions into preformed lipid vesicles was significant (*40*). However, the method used for monitoring insertion in that study did not allow the time dependency of the insertion process to be monitored, and so it was impossible to measure insertion rates. In this contribution, a new method for monitoring DAGK insertion is presented which allows the folding/insertion process to be examined in a time-resolved manner. This has enabled examination of the kinetics of insertion of DAGK into either vesicles or mixed micelles starting from more than one structurally distinct set of conditions. These studies shed light upon critical factors determining the rate of productive folding and insertion into model membranes. Also illuminated are factors determining the partitioning of the enzyme between productive folding/insertion pathways and pathways leading to kinetically or thermodynamically trapped nonfunctional forms. These nonproductive pathways and their end points are collectively referred to in this paper as "misfolding".

METHODS

Overexpression and Purification of DAGK. *Escherichia coli* overexpressing a recombinant DAGK variant with an N-terminal polyhistidine tag was supplied by the laboratory of James Bowie (UCLA; *35*). The C46A,C113A double mutant form of this construct was used in the studies of this work. This mutant was chosen because it has no Cys residues which could form disulfide bonds and potentially complicate these studies but is known to be similar to the wild-type enzyme.[2] Cell growth and DAGK purification using metal ion chelate chromatography have been described previously (*32*, *35*, *38*).

Membrane proteins were extracted by adding the detergent empigen (Calbiochem) to whole cell lysate to a concentration of 3% and mixing for 30 min, followed by incubation with Ni−NTA resin (Qiagen) for 1 h at 4 °C (1.2 mL of resin/1 g of original packed cells). Resin was transferred to a column, and all non-DAGK proteins were eluted by washing the resin with 40 mM imidazole and 1.5% empigen in buffer A: 40 mM HEPES and 0.3 M NaCl, pH 7.8. Resin was then equilibrated with 1% dodecylphosphocholine (DPC) in H_2O, and DAGK was eluted with 0.3 M imidazole and 1% DPC, pH 7.8. The purified DAGK/DPC pool was then mixed with a DPC/POPC stock solution such that the POPC:DAGK molar ratio was 120:1 and subjected to a "reconstitutive refolding" procedure which leads to properly folded DAGK in POPC vesicles, as described elsewhere (*38*). DAGK thus refolded maintains its native structure even when the resulting POPC/DAGK vesicles are redissolved by detergent. To prepare micellar stock solutions for insertion reactions, DAGK/POPC vesicles were dissolved by a 1% decyl maltoside solution such that the DM:POPC molar ratio was 40:1.

For experiments requiring lipid/detergent-free DAGK in urea or guanidinium, the enzyme was prepared as follows. The Ni(II)−agarose resin with which pure DAGK is associated (following the 40 mM imidazole "wash" step described above) was reequilibrated with 3% empigen in buffer A, followed by 2 column volumes of a 1:1 mixture of a urea solution (6.5 M urea, 150 mM NaCl, 10% buffer A) and 3% empigen in buffer A. The column was then exhaustively reequilibrated with 12 successive column volumes of 6.5 M urea and 150 mM NaCl. DAGK was then eluted with 6.5 M urea, 150 mM NaCl, and 1% formic acid. Guanidinium solutions of DAGK were prepared as for urea above, except that 8 M guanidine hydrochloride was substituted for urea at all steps.

DAGK concentration was determined by measuring A_{280} using an extinction coefficient of $\epsilon_{280,0.1\%} = 1.8$. DAGK activity was determined using a standard mixed micellar assay described elsewhere (*41*). One unit of DAGK activity is equal to 1 μmol of DAG converted into PA per minute.

Glutaraldehyde Cross-Linking. Cross-linking of DAGK in POPC vesicular solutions following insertion/folding reactions was carried out by diluting mixtures to a DAGK concentration of 0.15 μg/mL in 20 mM phosphate, 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT, pH 7.5, followed by GA addition to 25 mM and incubation with shaking for 16 h. Cross-linked products were analyzed via SDS−PAGE using 4−12% Bis-Tris NuPage gels (Invitrogen, Carlsbad, CA). Cross-linking of urea-solubilized DAGK was accomplished by dilution to 10 μM in 6.5 M urea, 1% formic acid, and 150 mM NaCl and incubation with 25 mM GA for 16 h. Analogous cross-linking reactions were run in 8 M guanidinium in place of urea. Because guanidinium ions form insoluble salts with dodecyl sulfate, these latter reaction mixtures were desalted to remove guanidinium before running SDS−PAGE.

[2] Two insertion/folding assays were carried out using the true wild-type DAGK. The $t_{1/2}$ and efficiency for insertion from a urea stock into POPC vesicles were 350 s and 42%, respectively. When a guanidinium stock was used, the $t_{1/2}$ was 600 s and the efficiency was 12%. These results can be compared to the corresponding measurements for Cysless presented in Table 1. It can be seen that while the results are qualitatively the same, the efficiencies for both reactions involving the true wild type were higher than for Cysless.

Circular Dichroism of DAGK. Near-UV CD spectra of various 1 mg/mL DAGK solutions were acquired using a Jasco J810 spectropolarimeter. Scans were taken spanning 250−310 nm at a rates of 20 nm/min. A total of 10 scans were taken for each sample. The scans were averaged, and the resulting spectrum was baseline-corrected using the spectrum from a protein-free blank. Spectra were acquired at 30 °C using a 1 cm path-length cell.

Size Exclusion Chromatography of DAGK in Urea or Guanidinium Solutions. Aliquots (0.6 mL) of stock guanidinium or urea stock DAGK solutions were applied to 1 × 45 cm columns of Sephacryl S-200 gel (Amersham-Pharmacia, Piscataway, NJ) and eluted under conditions of gravity flow with 150 mM NaCl and 1% formic acid, plus either 6.5 M urea or 8 M guanidine hydrochloride. The absorbance of the eluate was monitored at 280 nm.

Insertion of DAGK into Preformed Lipid Vesicles. Vesicles were formed by dispersing POPC at nominal concentrations of 20 mM in an insertion assay mixture containing 75 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PEP, 0.5 mM NADH, 3 mM ATP, 15 mM Mg(II), and 3 mM dibutyrylglycerol (DBG), pH 6.8. Unilamellar vesicles were formed by repeated extrusion through a 50 nm polycarbonate membrane (Avestin, Toronto, Canada). The resulting vesicles were diluted into a solution of the above assay mixture but also including 37 units/mL PK and 52 units/mL LDH, such that the final POPC concentration was 2 mM.[3] Aliquots (2−10 μL) (usually 2.5 μL) of stock DAGK in urea, guanidinium, or mixed micelles were then added to 500 μL insertion reaction mixtures in tubes such that the final DAGK concentration was 40 nM. Following mixing, 200 μL of each reaction in progress was transferred to microwell plates for monitoring absorbance at 340 nm for 3 h at 30 °C using a Molecular Devices (Sunnyvale, CA) ThermoMax microplate reader. Control tests verified that the enzymic reaction coupling systems remain viable over the time span of the longest reactions examined in this study (100 min) and that possible interference from vesicle light scattering does not significantly impact assay results.

Reactions were also carried out in which denaturant stock solutions of DAGK were diluted 200-fold into mixed micelles containing 1% (21 mM) DM as the detergent component, 3 mol % CL as the lipid component, and dihexanoylglycerol (5 mol %) as the phosphoryl acceptor.

The insertion of DAGK into vesicles or mixed micelles was monitored by following the insertion/folding-dependent appearance of DAGK activity as reflected by the decrease in A_{340} as NADH is oxidized through coupling to the DAGK reaction. The data were then processed as follows using the program Axum 4.1 (MathSoft, Cambridge, MA). A fourth-order polynomial was used to smooth the A_{340} vs time experimental data. The first derivative of the resulting polynomial function was then calculated and used to generate a $\Delta A_{340}/\Delta$time vs time curve which was then converted into a activity/time plot. From these plots, final (plateau) activity, initial insertion rate, and $t_{1/2}$ for maximal insertion could be measured. Insertion efficiency was determined by dividing the final plateau activity observed following insertion of denaturant-solubilized or detergent-solubilized protein by the known standard activity for 100% inserted/folded DAGK under the same reaction conditions.

[3] The vesicle concentration was calculated on the basis of the 2 mM lipid concentration, the 7850 nm^2 surface area of a 50 nm diameter sphere, and the assumption that each lipid occupies a surface area of ca. 60 Å^2.

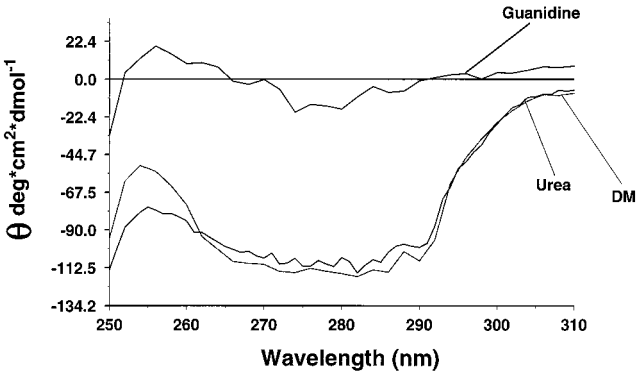

FIGURE 2: Near-UV circular dichroism spectra of DAGK (1 mg/mL) solubilized in either DM micelles, acidic 6.5 M urea, or acidic 8.0 M guanidinium at 30 °C. Ten scans were taken for each sample, averaged, and subtracted from protein-free blanks. These spectra were found to be reproducible.

Sucrose Density Gradient Ultracentrifugation. Sucrose gradients (30 mL, 3%−30%) were formed in Beckman Ultra-Clear centrifuge tubes in 75 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, and 0.1 mM EGTA, pH 6.8. Samples (1 mL) were loaded onto the top of the gradients, which were then spun at 89000*g* at 4 °C for 16 h in an SW28 rotor (Beckman). Fractions were collected and analyzed for DAGK activity using the standard assay described above. Protein concentration was determined using a standard colorimetric assay (BCA, Pierce). Phospholipid concentration was also determined using a colorimetric method (*42*).

RESULTS

General Structural Properties of DAGK in Guanidinium, Urea, and Detergent Stock Solutions. The goal of this work was to conduct studies of spontaneous bilayer insertion, folding, and misfolding of DAGK as a function of structurally distinct starting conditions for the enzyme. The ideal set of conditions would lead to DAGK stock solutions for folding/insertion studies in which the enzyme is either (i) denatured, (ii) a folded monomer, or (iii) a folded homotrimer. DAGK solutions which to some degree satisfied these criteria were prepared using variations of metal ion chelate chromatography to yield final pure enzyme in either (i) acidic 8 M guanidine hydrochloride, (ii) acidic 6.5 M urea, or (iii) 1% decyl maltoside micelles at neutral pH.

It has previously been shown that DAGK in DM micelles maintains a nativelike tertiary structure and oligomeric state (*32*, *38*). The near-UV circular dichroism spectrum of DAGK in DM micelles is shown in Figure 2 and provides a "fingerprint" pattern indicative of DAGK's nativelike tertiary structure, as previously demonstrated (*32*, *33*). DAGK in DM micelles is fully active either upon addition of activating phospholipid or upon transfer to mixed micellar assay mixtures.

DAGK has previously been shown to be highly soluble in acidic 6.5 M urea solutions, even in the absence of detergent or lipid. Solubility in urea is reduced at neutral pH. For this study, we sought verification that urea-purified DAGK was truly detergent/lipid-free by acquiring a 600 MHz

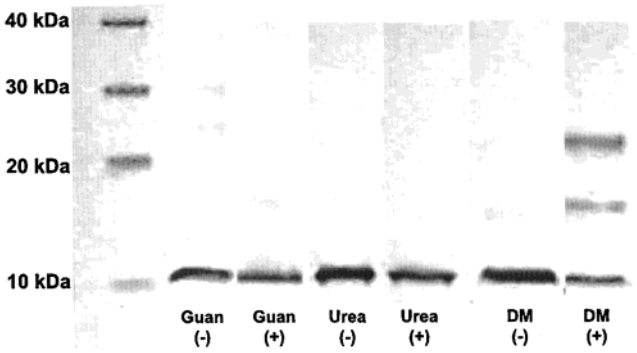

FIGURE 3: SDS−PAGE results comparing stock DAGK in acidic guanidinium and urea solutions before **(−)** and after **(+)** glutaraldehyde cross-linking for 16 h. The two lanes labeled DM represent control DAGK (fully folded trimer) in micelles under the same acidic conditions as for the urea stock. It should be pointed out that GA cross-linking of trimeric DAGK leading to covalent homotrimer is not generally 100% efficient, even under ideal conditions (cf. refs *32* and *38*). The monomer/dimer/trimer pattern observed for the cross-linked control sample is typical.

proton NMR spectrum of DAGK purified in 6.5 M perdeuterated urea/D_2O. No lipid or detergent peaks could be detected (data not shown), indicating that if these solutions contained any residual lipid or detergent, it was either present at an insubstantial concentration or was tightly bound to DAGK. No attempt was made to assess the secondary structure in acidic urea. However, DAGK's near-UV CD spectrum was observed to be similar to the spectrum of the DM-solubilized enzyme (Figure 2), indicating that urea-solubilized DAGK maintains a tertiary structure which is similar to its nativelike structure present in DM micelles. The oligomeric state of urea-solubilized DAGK was assessed by random cross-linking using glutaraldehyde, a method which has previously been used to assess the oligomeric state of DAGK under various conditions (*32*, *38*). The SDS−PAGE pattern observed following cross-linking in acidic urea (Figure 3) shows that DAGK did not maintain its native trimeric oligomeric state but was dissociated to a monomeric form. Results from size exclusion chromatography are consistent with these observations. Only a single protein peak eluted from the column using acidic 6.5 M urea as eluent (not shown), as expected for a monomer existing in a single conformational state. To see if the DAGK monomers could be converted back into functional trimer, aliquots of stock acidic urea/DAGK solutions were diluted into detergent micelles, followed by enzyme activity assay under mixed micellar conditions. It was observed that the refolded enzyme never exhibited a specific activity of great than 70% that of fully active protein. Failure to recover full activity most likely reflects irreversible misfolding which occurred during dilution of the enzyme back into nondenaturing conditions. However, our results cannot absolutely rule out the possibility that a fraction of the DAGK in acidic urea stock solutions is in the form of an irreversibly misfolded monomer which cannot be distinguished from the partially folded monomer by near-UV CD, cross-linking/SDS−PAGE, or size exclusion chromatography.

DAGK in 8 M guanidine hydrochloride plus 1% formic acid yielded only a very minimal CD spectrum in the near-UV region (Figure 2), indicating a lack of tertiary structure. SDS−PAGE following GA cross-linking of an acidic guanidinium stock solution indicated that the enzyme was completely dissociated to the monomeric oligomeric state (Figure 3). DAGK was also passed over a size exclusion chromatography column eluted with 8 M guanidinium plus 1% formic acid and was observed to yield only a single peak, as would be expected for the unfolded monomer (data not shown). When DAGK in acidic guanidinium stock solutions was diluted into micelles or mixed micelles and then used to initiate standard mixed micellar enzyme assays, it was observed that the recovery of enzyme activity was only 10%−35% of full activity, depending on exact conditions. This suggests that once DAGK is completely unfolded in the absence of detergent or lipid, it cannot readily be refolded. This may not be completely unexpected given that many membrane proteins have evolved to fold in vivo in a highly facilitated manner (*9*−*11*). However, an alternate interpretation cannot be absolutely ruled out—namely, that a sizable fraction of DAGK in acidic guanidinium stock solutions might already be in the form of an irreversibly misfolded monomer which cannot be distinguished from unfolded monomer by CD, size exclusion chromatography, or cross-linking/SDS−PAGE.

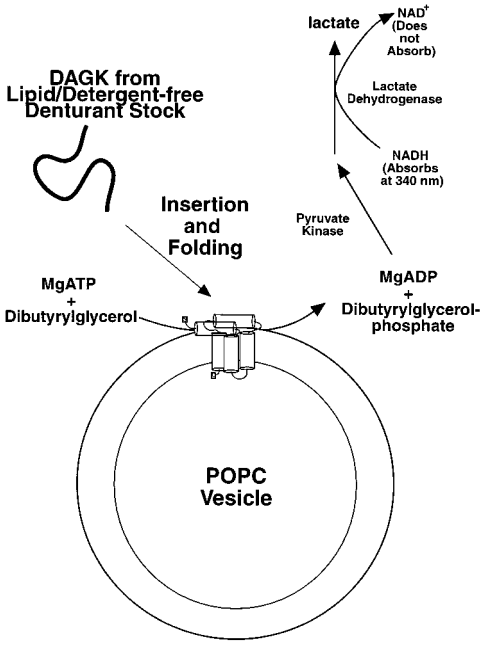

FIGURE 4: Time-resolved method for following the insertion of DAGK into preformed POPC bilayers. For the sake of convenience, the assembled protein is depicted as a monomer but is, in fact, a trimer (*32*, *33*).

A New Assay for Monitoring Folding and Insertion of DAGK in Lipid Vesicles. It has previously been shown that it is possible for DAGK to assemble into preformed liposomes when small aliquots of stock enzyme/detergent or enzyme/urea solutions are added to POPC unilamellar vesicles (*40*). For the present study, a method for following this process in real time was developed, as summarized in Figure 4. Folding/insertion reactions were initiated by 200-fold dilution of aliquots of stock DAGK into vesicular solutions, such that the final DAGK concentration was ca. 40 nM and the vesicle concentration was roughly 80 nM.[3] Following dilution of the enzyme stocks, the denaturant concentrations were 33−40 mM, well below the point at which urea or guanidinium could maintain DAGK solubility

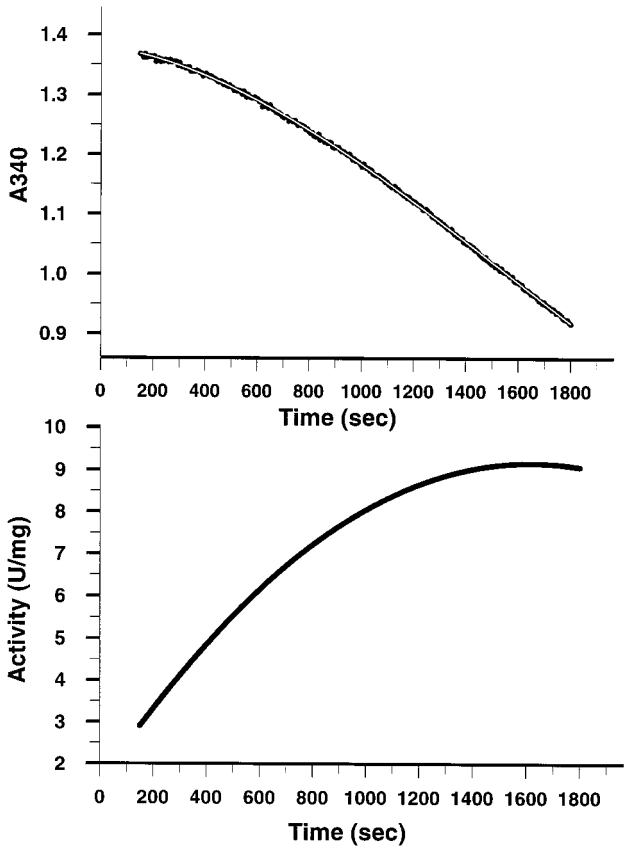

FIGURE 5: Example of an A_{340} vs time and the derived DAGK activity vs time plots representing DAGK insertion into preformed POPC vesicles following dilution from a urea stock. In the A_{340} vs time plot, both the original data (points) and the resulting polynomial fit to these data (white curve) are illustrated. This particular reaction was characterized by a $t_{1/2}$ of 370 s and reached an efficiency of ca. 30% (9.5 units mg^{-1}/32.5 units $mg^{-1} \times 100$).

or significantly influence DAGK's structure. In the case of DM/DAGK stock solutions, 200-fold dilution led to DM concentrations which were well over an order of magnitude lower than its 2 mM critical micelle concentration. At such low concentrations, DM is expected to dissociate from DAGK/detergent complexes. The residual urea, guanidinium, or DM is not expected to disrupt the integrity of the vesicles in the insertion assays. It has previously been shown that even high concentrations of urea and guanidinium do not permeabilize lipid vesicles (*43*, *44*) and that DM is an extremely poor POPC solubilizing agent even at high concentrations (*40*; unpublished observations). It was also confirmed that addition of the stock detergent or denaturant solutions did not perturb the light scattering by the vesicles, as monitored at 600 nm.

Once any of the three stock DAGK solutions was diluted into the vesicular solutions, the enzyme had the potential of undergoing bilayer insertion and folding to reach its catalytically viable structural state. To directly monitor the appearance of this fully assembled form of the enzyme, the production of ADP by active DAGK was coupled to spectrophotometrically detectable NADH oxidation (Figure 4). Key to this method is the use of a water-soluble form of diacylglycerol, dibutyrylglycerol (*45*). Only by use of this soluble form of DAG can high enough concentrations be employed to allow reactions to proceed over long periods of time without disrupting the lipid vesicles, which must be maintained at a low concentration to minimize unwanted light scattering. It was verified that all DAGK which reaches its catalytically active form following one of these assays was physically associated with POPC vesicles using sucrose density gradient equilibrium ultracentrifugation (data not shown).

The original A_{340} vs time data acquired during insertion assays were transformed into activity vs time plots as described in Methods. Examples of both the original and the transformed data appear in Figure 5. These data specifically document a reaction in which a small aliquot of urea/DAGK was added to POPC vesicles. The time trace exhibits no lag phase in the initial phase and eventually plateaus. Curves for insertion reactions which involved urea, guanidinium, or DM stock solutions were generally similar and did not exhibit lag phases. However, we found that the exact shape of each observed activity vs time curve depended upon the method used for smoothing the original A_{340} vs time data and upon the method used to transform the original data into activity vs time. We have therefore not attempted to fit kinetic models to the data. It was observed, however, that $t_{1/2}$ for reaching the maximal rate and the final maximal (plateau) rate were not dependent upon the details of data processing and could be reliably measured. We therefore focused upon these parameters. The final activity observed for each reaction at long times can be divided by the known activity for 100% folded and active DAGK under identical conditions to yield the efficiency of the insertion process. The known activity of fully active DAGK in vesicles at the same substrate concentrations as used in the insertion assays is about 32 units/mg, based on direct assays of reconstitutively refolded DAGK. Thus, by way of example, the trace shown in Figure 5 indicates that the efficiency of folding/insertion during this particular reaction was about 30%, with the remainder of the enzyme undergoing misfolding to inactive form(s).

Insertion assays were also carried out in which the process of DAGK insertion from 200-fold diluted urea or guanidinium stock solutions into DM/cardiolipin mixed micelles was monitored. Correctly folded DAGK is highly active in DM/CL mixed micelles. The method used to monitor folding/insertion under these conditions was completely analogous to that used for the vesicular insertion assays.

Folding and Insertion of DAGK into Vesicles or Mixed Micelles: Variation of Starting Conditions. The efficiencies and $t_{1/2}$ characterizing each DAGK insertion/folding reaction were measured as a function of the composition of both the stock DAGK solution (guanidinium, urea, or micelles) and the target model membrane (vesicles or mixed micelles). Small aliquots of stock DAGK solutions were added to 0.5 mL solutions containing either POPC vesicles or DM/CL mixed micelles. For each set of conditions, reactions were run and analyzed in triplicate, with results being reported in Table 1. These data and some follow-up experiments can be summarized as follows.

(1) In no case was efficiency 100%. The transfers of DAGK out of stock denaturant solutions into mixed micelles or vesicles and the transfers of DAGK from detergent solutions into vesicles were accompanied by misfolding of varying fractions of the total DAGK population which fail to fold and insert to attain a catalytically functional structural state.

Table 1: Efficiencies and $t_{1/2}$ for Productive Folding/Insertion of DAGK at 30 °C

starting state	final state	efficiency (%)	$t_{1/2}$ (s)
urea stock	POPC vesicles	26 ± 5^a	370 ± 150^a
urea stock	mixed micelles	46 ± 13	16 ± 6
guanidinium stock	POPC vesicles	1.3 ± 0.5	1000 ± 100
guanidinium stock	mixed micelles	6 ± 4	17 ± 9
DM stock	POPC vesicles	79 ± 22	6 ± 4
BOG stock	POPC vesicles	54	<20
SDS stock	POPC vesicles	95	<20

[a] All values reported in this table which include standard deviations represent the mean value of at least three separate measurements. Standard deviations are not reported in cases where the results are given as an upper limit or where only a single measurement was carried out.

(2) The highest efficiencies were for micelle → POPC reactions, followed by the urea reactions, followed by the guanidinium reactions.

(3) From both urea and guanidinium, the efficiencies were higher into mixed micelles than for the corresponding transfer into POPC.

(4) The urea → DM/CL and guanidinium → DM/CL insertion rates were much higher than for the corresponding urea → POPC and guanidinium → POPC reactions.

(5) The DM → POPC insertion rate was much higher than for urea → POPC and guanidinium → POPC reactions. This observation prompted additional experiments to distinguish which of the following possible interpretations might explain these data: (i) that folded trimeric DAGK has a much higher intrinsic rate of insertion than monomeric, partially unfolded DAGK, (ii) that DM from the stock solution does not, contrary to assumption, dissociate extremely rapidly from DAGK and directly facilitates proteins insertion, or (iii) that DM dissociates from DAGK but interacts with the vesicles to induce some sort of perturbation which facilitates DAGK insertion, despite being present at a concentration well below its CMC. This last possibility was ruled out by running a reaction using twice as much DAGK/DM stock and observing that the efficiency of insertion actually was reduced at the higher detergent concentration, contrary to expectations if DM facilitates DAGK insertion by perturbing the vesicles. To distinguish between possibilities i and ii, we monitored insertion of DAGK into vesicles from other micelle types, one of which (SDS) is known to denature DAGK (*35*). As shown in Table 1, efficiency and rates of folding/insertion from both BOG and SDS were high. This rules out possibility i, suggesting that all three detergent types are able to play a facilitating role in DAGK folding/insertion.

(6) The urea → POPC efficiency observed in this work (ca. 25%) is much higher than the ca. 4% value reported previously (32). This reflects the very different conditions under which the present measurements were made compared to those for the previous work (different temperature, different vesicle and enzyme concentrations, and different forms of DAG used).

Additional Data on Misfolding. Three additional experiments were carried out to further illuminate DAGK misfolding. The first experiment was to examine the time course of irreversible activity loss for different insertion reactions. This was tested by running POPC insertion reactions and then removing aliquots at 1, 5, 10, 20, and 60 min, which were immediately subjected to standard mixed micellar DAGK assays. In the case of a DM/DAGK stock being added to POPC vesicles the activities of aliquots removed at the various time points were constant and were equal to the activity of fully folded standard DAGK. This indicates that the modest degree of misfolding which occurs during DM → *POPC* insertions is completely reversible. In the case of urea/DAGK being added to vesicles, the recoverable enzyme activity after 1 min was ca. 55%, a value which remained constant for subsequent time points. This suggests that 45% of the DAGK misfolds rapidly and irreversibly upon dilution of urea/DAGK into vesicles. The 55% recoverable DAGK activity appears to represent two DAGK populations. One is the ca. 25% population which correctly folds and inserts into vesicles. The remaining 30% is DAGK which misfolds and never reaches its functional state in vesicles but which is *reversibly* misfolded in that its activity is recovered upon transfer to mixed micelles. The case of vesicle insertion reactions initiated with guandinium/DAGK was qualitatively similar to that of urea: at 1 min the recoverable activity was only 6% and remains constant thereafter. Thus, 94% of the enzyme rapidly and irreversibly misfolded, about 5% reversibly misfolded, and 1% productively folded and inserted.

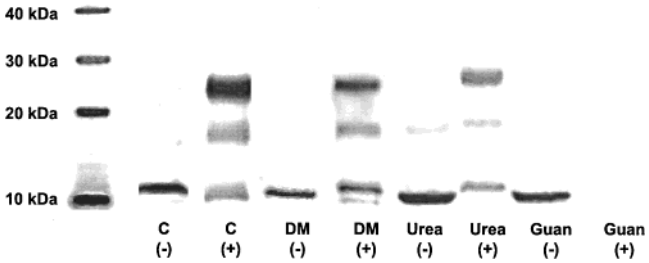

FIGURE 6: SDS−PAGE of DAGK which was cross-linked following vesicular insertion/folding reactions. DM-solubilized and denaturant-solubilized protein (20 μg/mL) were added to POPC vesicles, and the insertion reaction proceeded at 30 °C for 3 h. Aliquots were then removed and subjected to GA cross-linking. SDS−PAGE was then run on both non-cross-linked samples **(−)** and cross-linked samples **(+)**. The total amount of DAGK added to each lane of this gel was the same. Control samples C involved 100% correctly assembled DAGK prepared by reconstitutive refolding into POPC vesicles (see Methods). DM represents an insertion reaction mixture which was initiated using micellar (DM) stock DAGK, from which insertion was observed to be of high efficiency (see Table 1). Urea represents a reaction mixture which was initiated using a urea stock solution of DAGK, from which insertion was of moderate efficiency. Guan represents a reaction mixture which was initiated using a guanidinium stock solution of DAGK, from which insertion was of low (ca. 1%) efficiency.

A second follow-up experiment sought insight into the nature of the structural state adopted by misfolded DAGK. GA cross-linking/SDS−PAGE of DAGK was carried out following the completion of various insertion/folding reactions. As shown in Figure 6, the guanidinium reaction which led to ca. 95% irreversible misfolding did not yield detectable bands. This indicates that the minimal (ca. 1%) population of correctly inserted DAGK was simply too small to detect and that the irreversibly misfolded population apparently was in the form of very large aggregates which did not migrate into the gel at all following cross-linking. It is difficult to ascertain from the quantitative intensities of the bands observed in the DM and urea lanes of the gel in Figure 6 whether reversibly misfolded DAGK was absent due to formation of large nonmigratory aggregates or whether this population yields bands following cross-linking which are

not resolved from the bands representing productively folding/inserted population of DAGK.

Finally, sucrose density gradient equilibrium ultracentrifugation of DAGK/vesicle mixtures following insertion reactions was carried out to see if misfolded DAGK might possibly form a band which was lipid-free. This was not the case: all fractions which contained DAGK also contained lipid. Whether or not some DAGK misfolding occurs in a manner which is independent of model membrane interactions is not established by this result.

DISCUSSION

DAGK Folding and Insertion Can Be Initiated from Multiple Starting Structural States. This work complements previous studies of DAGK's folding and stability (*35*−*39*) and represents a first step toward establishing DAGK as a model system for studying the kinetic aspects of membrane protein folding and misfolding under test tube conditions. Aliquots of DAGK from one of three different starting solutions were diluted manyfold into either mixed micelles or into lipid vesicles to start the folding process. The overall rates for productive insertion/folding and the overall folding efficiencies were in each case measured. This general approach is perhaps most similar to that taken by the Tamm and Jahnig laboratories to study the folding of the OmpA and OmpF porins into lipid vesicles starting from urea stock solutions (*26*−*29*). One aspect of the present work which is distinct from the Omp studies and from other previous studies of membrane protein folding kinetics (*17*−*25*, *30*, *31*) is the focus upon the dependency of folding kinetics and efficiency upon DAGK's starting structural state. It is well-known from studies of water-soluble proteins that the nature of the starting structural state is extremely relevant to interpretation of folding kinetic data (*46*).

The results established three very distinct sets of conditions for the DAGK stock solutions used to initiate folding reactions. The first set of conditions was provided by nonionic detergent micelles in which DAGK retains its native trimeric oligomeric state and tertiary structure. DAGK was found to be very soluble in both concentrated urea and guanidinium solutions under acidic conditions. It was observed that DAGK in acidic 6.5 M urea is dissociated into monomers but that the monomers retain considerable tertiary structure. DAGK in acidic 8 M guanidinium was also observed to be monomeric but was observed to have greatly reduced tertiary structure compared to the urea case.[4] Differences in DAGK's structural state in urea vs guanidinium contrast the case of β barrel outer membrane porins, which appear to be fully denatured in either of these media (*29*, *47*, *48*).

The observation that DAGK was monomeric in both urea and guanidinium solutions but retained some degree of tertiary structure in urea is consistent with a previous observation (*33*) that SDS-induced denaturation of DAGK in micelles is multiphase, with loss of quaternary structure occurring at low mole fraction levels of SDS. This indicates that DAGK's tertiary structure is more stable than its quaternary structure and that its tertiary structure is to some extent uncoupled from its quaternary structure, contrary to a previous suggestion (*32*). Bowie and co-workers have also presented evidence that DAGK's transmembrane domain is much more stable than its extramembrane domain (*33*). On the basis of this latter observation, it seems probable that the residual tertiary structure present for DAGK in urea involves the transmembrane domain. Indeed, this conclusion is consistent with the near-UV CD results of this work, since DAGK's near-UV CD signal (which is retained in urea) arises from its aromatic side chains, the majority of which are located in the transmembrane domain.

Rates of DAGK Assembly into Model Membranes. It was observed that the unimolecular rates of DAGK insertion/folding from urea and guanidinium were qualitatively similar: in both cases DAGK assembled rapidly into mixed micelles ($t_{1/2}$ <20 s), while exhibiting much slower assembly into lipid vesicles ($t_{1/2}$ on the order of 5−15 min).

Assembly of DAGK into lipid vesicles from urea or guanidinium was in each case much slower than insertion into mixed micelles by at least an order of magnitude. It is unlikely that this difference represents a slower rate of association of DAGK with vesicles than with micelles. The rate of initial bilayer or micelle association is probably diffusion-limited. Instead, the bilayer versus mixed micellar insertion rate differences most likely reflect the barrier to bilayer transversal of DAGK's three transmembrane helices (Figure 1). Since both the hairpin loop and the C-terminus contain charged residues, it is expected that there would be a considerable energy barrier to insertion into vesicles given the low dielectric constant and anhydrous nature of the interior of lipid bilayers. The much more rapid insertion of DAGK into mixed micelles may be accounted for by several possible factors, including the higher degree of hydration of the interiors of micelles compared to the interior of bilayers, the lower overall order of detergent molecules in micelles compared to lipids in bilayers, the rapid exchange of detergent monomers between solution and micellar phases, and the greater variety of feasible routes to protein−micelle coassembly compared to protein−bilayer coassembly.

In contrast to insertion into vesicles from denaturant solutions, DAGK was able to insert very rapidly into vesicles from detergent stock solutions following dilution to well below the detergent critical micelle concentration. The efficiency and rate of productive insertion/folding from micelles into vesicles were high, even when a denaturing detergent (SDS) was used. This observation and related control experiments suggest that detergent plays a direct role in DAGK insertion as a result of the kinetic persistence of some DAGK-associated detergent. DAGK−detergent interactions would only need to persist for times on the order of the time scale of diffusion-limited bilayer−DAGK collisions following mixing. Given that a DAGK trimer−micelle complex contains 150 detergent molecules (*32*), each of which presumably dissociates in a noncooperative fashion as a monomer upon dilution to well below CMC, this does not seem unlikely. Detergents could play a facilitating role in membrane protein insertion through highly localized and transient membrane destabilization upon collision of the

[4] As noted in the first section of the Results, the available structural data on DAGK in acidic guanidinium stock solutions do not absolutely rule out the possibility that some of the DAGK in the stock guanidinium solutions could exist in the form of a kinetically trapped misfolded monomer.

protein−detergent complex with the membrane or might facilitate conformational rearrangements within the protein which are necessary for insertion and which may occur much more slowly for bilayer surface-associated protein in the absence of detergent. It is interesting to note that in at least some bacteria there are lipids which are detergent-like in the sense that they form micelles when purified and dispersed in water (*49*). Whether such lipids might possibly play a special role in membrane protein insertion in vivo is unclear but perhaps worth considering in light of the observations for DAGK.

The rates for DAGK folding from denaturant solutions into mixed micelles and from micelles into vesicles at 30 °C were rapid enough to be physiologically relevant (see below). However, rates of folding/insertion from denaturant solutions into vesicles were characterized by $t_{1/2}$ on the order of 5−15 min. These rates may be compared with those measured for the insertion of membrane proteins and toxins into model membranes and with the rates for membrane protein folding in vivo. The $t_{1/2}$ observed for OmpA and OmpF insertion into vesicles from urea at 30 °C were on the order of tens of minutes, with the rate-limiting step to assembly being bilayer transversal (*26*−*29*). These observations mirror the conclusions of this paper regarding DAGK insertion from denaturant solutions into vesicles, despite the structural dissimilarity of the porins and DAGK. Folding of apoBR in mixed micelles from SDS micelles occurs with a $t_{1/2}$ of about 1 min, with the rate-limiting step believed to be a conformational change after insertion (*21*−*23*). DAGK folds much more rapidly than BR either from detergent into vesicles or from denaturants into mixed micelles, indicating the lack of any required BR-like slow conformational change in the process of assembly to a catalytically functional state.

A variety of toxin proteins integrate into membranes from solutions with $t_{1/2}$ of 10−200 s (*50*−*53*). DAGK's $t_{1/2}$ for insertion into vesicles from urea is only slightly longer than the upper end of the range for toxins, which may be surprising given the very different evolutionary restraints placed upon DAGK−membrane interactions versus toxin−membrane interactions.

While in vivo biosynthesis/insertion/folding $t_{1/2}$ are available for outer membrane porins (roughly 30 s, including translation), these rates include the transport step from the inner to the outer membrane (*54*−*57*). Pulse/chase studies of the biosynthesis of mostly helical bacterial plasma membrane proteins indicate that biosynthesis/membrane insertion generally occurs rapidly [$t_{1/2}$ <30 s (*56*−*60*)] and very likely approaches the translational rate for protein biosynthesis in *E. coli* (ca. 15 residues/s; *61*). It should be noted that pulse/chase $t_{1/2}$ will underestimate the mean time for folding of proteins which must undergo relatively slow conformational changes following membrane insertion to reach a functional fold. The $t_{1/2}$ for biosynthesis and membrane integration of ca. 100 residue fragments of BR into purple membranes have been measured to be roughly 3 min (*62*). We see from the above survey that DAGK insertion/folding into *mixed micelles* is rapid by any standard, underscoring our observation that the rate-limiting step for DAGK insertion into vesicles from denaturant solutions most likely is bilayer transversal. Bilayer transversal for DAGK starting from urea or guanidinium solutions therefore occurs 1−2 orders of magnitude slower than the in vivo biosynthesis/membrane integration rates summarized above. This 1−2 order of magnitude difference may actually be regarded as being *surprisingly small*, given the complexity of the membrane protein processing/insertion machinery present in virtually all living cells. Indeed, this modest difference in test tube and in vivo folding rate is completely obviated for DAGK when insertion reactions are initiated using micellar stock solutions because of the facilitative role of the residual detergent (see above). Of course, it is possible that DAGK is atypical and that much lower test tube insertion rates would be observed for most other membrane proteins, if measured. On the other hand, perhaps DAGK and other "modern" membrane proteins are descended from ancient progenitors which evolved autoinsertion mechanisms due to the lack of any membrane insertion machinery in early life forms. If so, then it may be that many membrane proteins, like DAGK, maintain some vestigial ability to autoinsert into bilayers. Moreover, the in vivo role of membrane protein insertion machinery may be as much to provide a way to avoid membrane protein *misfolding* as it is to enhance the rate of insertion and folding (see next section).

The kinetic results of this paper require us to correct a conclusion drawn from an earlier study of DAGK insertion into vesicles (*40*). It was previously concluded that the free energy barrier to bilayer insertion of DAGK was very low (only a few kilocalories per mole). The observation in this work that the $t_{1/2}$ for DAGK insertion from urea into vesicles is on the order of 5 min is not consistent with such a low rate barrier. Insertion rates were not measured in the previous work because of the crude nature of the insertion assay then employed. The former conclusion was based upon insertion efficiency (rather than upon rates), combined with the assumption that the free energy barrier(s) associated with misfolding pathways was (were) extremely low. The results of this paper indicate that this assumption was incorrect.

Misfolding of DAGK. Misfolding in this paper is used broadly to describe all forms of failure by DAGK to eventually achieve a functional structural state under the conditions of the various insertion reactions. The degree of misfolding associated with any particular set of conditions is therefore inversely related to the measured efficiency of productive folding/insertion. We observed that misfolding was most severe for insertion/folding reactions which were initiated using guanidinium stocks. Moreover, it was observed that the majority of the misfolded DAGK was irreversibly misfolded and that irreversible misfolding occurred rapidly (complete in <1 min) after the stock solution was diluted into vesicles. This is in stark contrast to vesicular reactions which were initiated using DM micellar stock solutions; in this case the degree of misfolding was much lower, and misfolding was completely reversible. Urea represented an intermediate case in terms of both the overall degree of misfolding and the fact that misfolding was about equally distributed between reversible and irreversible forms. Thus, both the overall degree of misfolding and the "severity" of misfolding (proportion of irreversible misfolding) strongly correlate with the degree to which DAGK's native structure has been disrupted in the stock solution used to initiate a given folding/insertion reaction. Given that the degree of water-exposed hydrophobic surface area will be highest for completely unfolded membrane proteins, this observation is not surprising. These results also imply that irreversible

misfolding (regardless of the exact nature of this process) is rapid enough to compete well with the formation of nativelike tertiary structure.

The results regarding DAGK's efficiency of folding/insertion can be compared to the sparse data available for other membrane proteins. The efficiency of folding for BR which is inserted into mixed micelles or into vesicles starting from an SDS solution has been consistently observed (*21*−*24*) to be very high (>95%), similar to SDS → vesicle results for DAGK, but considerably higher than for virtually all other folding/insertion conditions tested. The insertion of OmpA into vesicles or mixed micelles starting from urea has also been observed to be quite efficient (80%−100%) under a variety of conditions (*28*, *29*). However, both BR and OmpA are monomeric proteins, unlike the homotrimeric DAGK. It is therefore interesting that the efficiency of assembly of the homotrimeric OmpF from urea into vesicles is much lower than for OmpA. This lower efficiency has been ascribed to difficulties in trimer formation for OmpF (*29*). It has also been observed (*29*) that the efficiency of OmpF insertion into mixed micelles from urea is somewhat more efficient than into vesicles, an observation which also holds for DAGK insertion from urea into vesicles versus mixed micelles.

The results of this study set the stage for more detailed studies of DAGK misfolding which may shed light upon fundamental mechanisms of membrane protein misfolding, a matter of considerable biomedical and basic biological relevance. All cells have a complicated array of molecules dedicated to assisting the folding of both soluble and membrane proteins and also to the suppression or correction of misfolding when it occurs (*2*, *9*−*12*). Recent data, however, indicate that bulk protein synthesis in vivo is surprisingly inefficient, with a substantial fraction of all nascent proteins undergoing misfolding (*63*, *64*). Nonproductive biosynthesis/assembly may be especially prevalent for membrane proteins (*1*). It is known, for example, that the wild-type CFTR chloride transporter of mammalian epithelial cells and the wild-type peripheral myelin protein 22 undergo inefficient biosynthesis and trafficking, so that only a fraction of newly synthesized protein ever reaches the cell surface (*1*, *3*, *65*). Missense mutations in either of these proteins further reduce folding/trafficking efficiency, leading to specific phenotypes of cystic fibrosis (*3*) and Charcot−Marie−Tooth disease (*4*). Missense mutations in lactose permease are also known to lower the efficiency of biosynthesis and membrane integration of this protein in *E. coli* (*66*). Moreover, some of these low-efficiency mutants are also toxic to the *E. coli* host. Despite the importance of misfolding to human health and disease, very little is known regarding the detailed structural/chemical biology of misfolding processes or the factors which promote or suppress misfolding. DAGK may serve as an appropriate model system for membrane protein misfolding. As shown in this paper, DAGK undergoes misfolding with considerable efficiency under a variety of conditions. Moreover, several dozen DAGK mutants are available for future studies which are known to exhibit a higher propensity to misfold than the wild-type-like variant which was examined in this study (*38*). Finally, the genetics and molecular biology of DAGK in *E. coli* have been well developed (*67*−*69*) so that test tube observations may ultimately be tested for relevance to misfolding in vivo.

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