Irreversible Misfolding of Diacylglycerol Kinase Is Independent of Aggregation and Occurs Prior to Trimerization and Membrane Association†

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ABSTRACT: *Escherichia coli* diacylglycerol kinase (DAGK) is a homotrimeric helical integral membrane protein in which a number of single-site mutations to cysteine are known to promote misfolding. Here, effects of other amino acid replacements have been explored using a folding assay based on the dilution of acidic urea/DAGK stock solutions into detergent/lipid mixed micelles. DAGK with an I110P or I110R mutation in the third transmembrane helix could not be purified because its expression was toxic to the *E. coli* host, most likely because of severe folding defects. Other mutations at Ile110 enhanced irreversible misfolding to varying degrees that generally correlated both with the polarity of the inserted amino acid and with the degree of protein destabilization. However, the I110W mutant was an exception in that it was highly misfolding prone while at the same time being more stable than the wild-type protein. This contrasts with I110Y, which also exhibited enhanced stability but folded with an efficiency similar to that of the wild type. For most mutants, the critical step leading to irreversible misfolding occurred for monomeric DAGK prior to trimerization and independent of association with mixed micelles. Misfolding of DAGK evidently involves the formation of incorrect monomer tertiary structure. Mutations appear to enhance misfolding by disfavoring the formation of correct structure rather than by directly stabilizing the misfolded state. Finally, when urea-solubilized DAGK was diluted into detergent/lipid-free buffer, it retained a significant degree of folding competency over a period of minutes. This property may be relevant to membrane protein folding in cells under conditions where the usual machinery associated with membrane integration is saturated, dysregulated, or dysfunctional.

Protein misassembly in the cell results in a loss of protein function and leads either to degradation or to the accumulation of misfolded protein. Mutations that result in protein misassembly may well be the most common class of mutations causing inherited human disorders and are also a common contributing factor to complex diseases (*5*−*9*). Nongenetic factors can also promote disease-linked protein misassembly. Many proteins that undergo disease-linked misassembly are integral membrane proteins, the basic cellular biology and trafficking of which remains a matter of intense scrutiny (*10*−*19*). Less effort has been devoted to structural biophysical characterization of membrane protein folding and misfolding. Among membrane proteins for which folding studies have been carried out under well-controlled model membrane conditions (see refs *16* and *20*−*39*), *Escherichia coli* diacylglycerol kinase (DAGK,[1] Figure 1) has emerged as a particularly suitable system for studying membrane protein misassembly. The lab of J. Bowie developed methods for characterizing the thermal and thermodynamic stability of DAGK (*4*, *40*) and also generated a large library of useful mutants, many of which were eventually shown to be highly prone to misfolding to form kinetically trapped structural states (*3*). In its high susceptibility to mutation-promoted misfolding, DAGK seems to resemble a number of human membrane proteins that each exhibit a spectrum of disease-linked mutations known to result in protein misassembly (*5*, *6*). We have developed a folding assay that allows folding kinetics to be monitored when aliquots of DAGK in acidic urea or guanidinium solutions are diluted into model membranes in which folding can potentially take place, an assay that also allows the partitioning ratio between productive folding and misfolding pathways to be quantitated (*2*, *41*). Studies employing this folding assay led to the observation that there is a strong correlation between DAGK folding efficiency and protein stability for a series of single-cysteine substitution mutants (*42*), although it was also observed that there are rare exceptions where stable mutants are nevertheless highly prone to misfolding (*41*). The possible implications of these observations for disease-related misassembly of human membrane proteins have been discussed (*5*, *42*). More

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[1] Abbreviations: CL, cardiolipin; cWT, cysteine-less wild-type-like mutant of diacylglycerol kinase; DAGK, diacylglycerol kinase; DM, *n*-decyl *β*-maltoside; DPC, *n*-dodecylphosphocholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene bis(oxyethylenenitrilo)tetraacetic acid; GA, glutaraldehyde; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis-2-ethanesulfonic acid; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; U, enzyme activity units; UV, ultraviolet.

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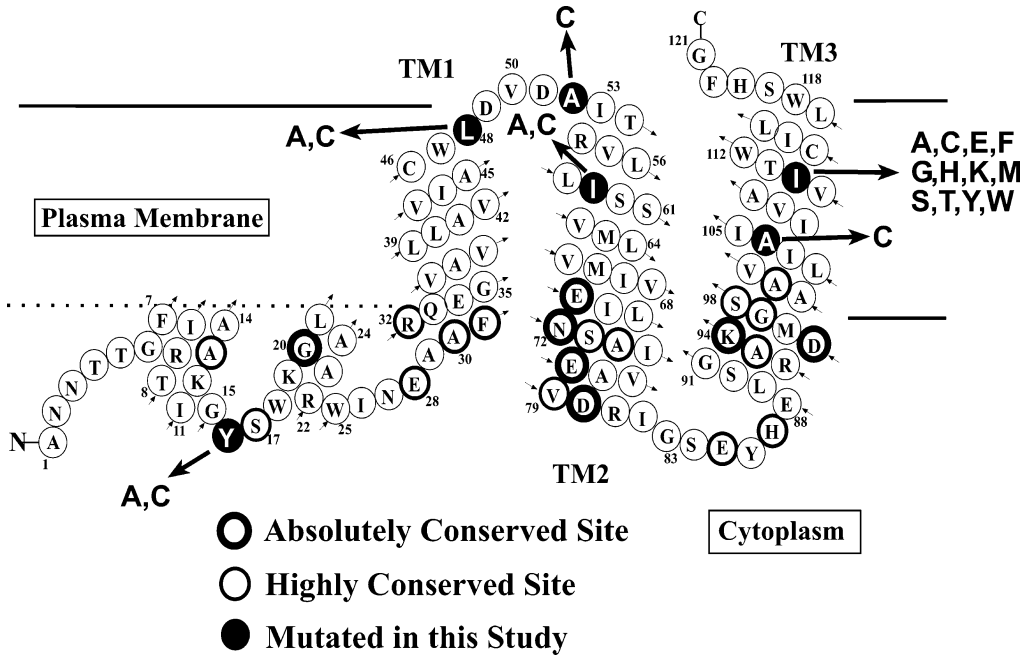

FIGURE 1: Experimentally derived (*45*, *46*, *68*) membrane topology and secondary structure of *E. coli* diacylglycerol kinase. DAGK is an almost exclusively α-helical membrane protein that functions as a homotrimer. Monomers are not active. Sites for which mutations were characterized in this study are highlighted, along with the amino acid replacements examined for each. Sites are also highlighted that are absolutely or highly conserved among a random subset of ca. 40 of the available prokaryotic DAGK sequences (C. R. Sanders, unpublished results). Unpublished NMR data indicate that I110 appears to be at least partially sequestered from the hydrocarbon phase of the membrane because of its placement at a protein−protein interface.

recently, the lab of P. Booth carried out a sophisticated rapid kinetic study that led to the proposal of a kinetic scheme to describe DAGK's folding and misfolding pathways under the conditions of the denaturant-into-model membrane folding assay (*1*).

In this study, the generality of the correlation between folding efficiency and protein stability observed for single-Cys mutant forms of DAGK was tested by replacing wild-type residues with a variety of amino acids besides cysteine. We also examined the consequences of a series of mutations at the isoleucine 110 site located near the middle of DAGK's third transmembrane segment. This not only further probed the relationship between folding efficiency and protein stability but also allowed the relationship between efficiency and amino acid polarity in a transmembrane segment to be assessed. Finally, the pathway(s) leading to misfolding was probed to determine when the committed step for irreversible misfolding occurs with respect to trimerization, aggregation, and association of the protein with model membranes.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of DAGK. A vector encoding the N-terminally His_6-tagged C46A/C113A/W117R/T118S mutant of DAGK was provided by the lab of J. Bowie (*40*). This cysteine-less form of the enzyme in this paper is termed cWT. cWT is very similar in most aspects to the true wild-type enzyme, except that cWT is somewhat less stable. All mutants used in this work were derived from cWT. Mutagenesis of the cWT gene was carried out using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The standard protocol was followed for primer design. Mutated plasmids were generated using *PfuTurbo* DNA polymerase, and template DNA was digested using the *Dpn*I enzyme. Following DNA sequencing, plasmids containing single-residue mutations were transformed into the WH1061 expression strain (*43*). Transformed cells were grown in Luria broth, from which glycerol stocks were generated and stored at −80 °C. Following growth of liquid cell cultures, induction of expression, harvesting, lysis, and detergent extraction, DAGK mutants were purified as described previously (*2*, *3*) using Ni(II) metal ion affinity chromatography into either 0.5% *n*-decyl *β*-maltoside (DM, Anatrace, Maumee, OH) in 250 mM imidazole (pH 7.5), 0.5% *n*-dodecylphosphocholine (DPC, Anatrace) in 250 mM imidazole (pH 7.5), or detergent-free acidic urea (6.5 M urea, 150 mM NaCl, and 1% formic acid). The method used in our lab to produce DAGK in acidic urea is slightly different from that used by the Booth lab (*1*) in that prior to equilibration of the enzyme on-resin in acidic urea, DAGK is extracted and purified on resin in the harsh detergent, empigen-BB, whereas Booth and co-workers used a much milder detergent, *β*-octyl glucoside. The DAGK concentration was determined by measuring the UV absorbance ($\epsilon_{280,0.1\%} = 1.8$). When required, DAGK was concentrated using centrifugal cartridges (Centricon Plus-20 PL 10, Millipore, Billerica, MA).

DAGK was sometimes purified into DPC and then "reconstitutively refolded" into 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, Avanti, Alabaster, AL) vesicles (*3*). This process results in correctly folded DAGK in lipid vesicles, with a POPC:DAGK molar ratio of 120:1. The activities of DAGK mutants prepared by this method were determined using a spectrophotometrically detected mixed micelle reaction system (*44*) that yields estimates of the specific catalytic activity (units per milligram) for each mutant under (ideally) near-100% folded conditions.[2] One unit (U) of DAGK activity equals 1 *μ*mol of diacylglycerol converted to phosphatidic acid per minute. The following

post-refolding activities (in units per milligram) were observed for the mutants that were tested: 113 for cWT, 92 for Y16A, 80 for Y16C, 74 for L48A, 74 for L48C, 83 for I59A, 87 for I59C, 103 for I110A, 95 for I110C, 36 for I110E, 83 for I110F, 87 for I110G, 109 for I110M, 89 for I110S, 109 for I110T, 85 for I110W, 63 for I110Y, 64 for I110H, and 12 for I110K.

Measurement of Folding Efficiency Using a Folding Assay. DAGK folding assays were carried out by adding mutant DAGK from lipid/detergent-free acidic urea stocks into mixed micelles composed of DM and bovine heart cardiolipin (CL, Avanti). For this study, the goal was not to monitor real-time folding but was instead to determine the final percentage of total DAGK that folded correctly. Folding assays were started by adding 15 μL of a 0.5 mg/mL DAGK stock (7.5 μg) in acidic urea (6.5 M urea, 150 mM NaCl, and 1% formic acid) to 500 μL of DM/CL mixed micelles [21 mM DM, 0.6 mM CL, 0.5 mM DTT, 75 mM PIPES, 50 mM LiCl, 0.1 mM EGTA, and 0.1 mM EDTA (pH 6.9)]. Assuming an aggregation number for DM of 104 (*45*), the concentration of mixed micelles in the folding assays was 190 μM, while the DAGK (monomer) concentration was 1.1 μM. Following introduction of DAGK, folding assay mixtures were incubated at 30 °C for up to 2 h to allow folding and misfolding to reach completion. Aliquots were removed and subjected to the standard spectrophotometric DAGK activity assay in DM/CL mixed micelles (*44*). The folding efficiency for DAGK equals the observed activity of DAGK from the folding assay mixture divided by the observed activity for the mutant following reconstitutive refolding (see the preceding section).

Measurement of Trimer Stability. Trimer stability was measured by determining the mole fraction of SDS in DM/POPC mixed micelles that was required to convert 50% of DAGK from its native trimeric form to monomers. The method is identical to that used previously (*42*), except for the manner in which the SDS_{50} was calculated. Previously, the reported SDS_{50} for each mutant was calculated as the average between the observed SDS_{50} for the loss of the trimer and the observed SDS_{50} for the appearance of the monomer. In this study, the reported SDS_{50} for each mutant is equal simply to the observed SDS_{50} for breakdown of the trimer to eliminate any complication arising from the possibility that some monomer formed was derived directly from the dissociation of aggregated protein.

Each DAGK mutant was purified and reconstitutively refolded into POPC vesicles. The DAGK concentration was adjusted to 0.2 mg/mL by diluting in buffer [75 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, and 0.1 mM EGTA (pH 6.9)]. DM was then added to the vesicles to form DAGK-containing mixed micelles at a DM:POPC molar ratio of 85:15. SDS was then titrated into the samples through a range of 0−80 mol % [mole percent SDS = moles of micellar SDS/(moles of micellar SDS + moles of micellar DM + moles of POPC) × 100]. The critical micelle concentration used for SDS was 3 mM, while the value for DM was 2 mM. Aliquots from each titration point were incubated at 30 °C for 30 min, followed by addition of glutaraldehyde to a final concentration of 25 mM and further incubation at 30 °C for 16 h with shaking at 300 rpm. The cross-linked aliquots were then mixed with SDS−PAGE loading buffer and analyzed by SDS−PAGE using Coomassie or silver staining. Novex 4 to 12% Bis-Tris gels were used (Invitrogen, Carlsbad, CA). Gel images were recorded and processed using ID Image Analysis Software 3.6 (Eastman Kodak, Rochester, NY). The trimer band intensities were plotted as a function of mole percent SDS. For each mutant, the mole percent SDS at the inflection point for the disappearance of trimer was determined and reported here as SDS_{50}.

Measurement of Thermal Stability. Thermal stability was assessed by monitoring the time dependence of irreversible inactivation when DAGK in mixed micelles was incubated at 70 °C. DM was added to folded DAGK in POPC lipid vesicles in 40 mM HEPES and 0.3 M NaCl (pH 7.5) so that the final DAGK concentration was 0.2 mg/mL, the DM:POPC molar ratio was 85:15, and the DAGK:POPC molar ratio was 1:120. Aliquots in thin-wall PCR tubes were placed into a preheated heating block, which was filled with light mineral oil in a 70 °C oven. Samples were incubated at 70 °C followed by removal of time point aliquots, which were then assayed for remaining DAGK activity using the standard spectrophotometric activity assay. In previous work (*42*), incubations were carried out at 80 °C and it was assumed that DAGK's inactivation with time was monoexponential such that only $t_{1/2}$ values were measured. In this study, incubations over much longer periods of time led to inactivation curves which clearly displayed two phases: an initial rapid inactivation of a significant fraction of the DAGK present (usually >50%) followed by a slower inactivation of the remaining active population. Rather than attempt quantitative model fitting, we chose a more simple approach to assess the kinetics of inactivation by measuring both $t_{1/2}$ for activity loss (reflecting the rate of the first phase of inactivation) and $t_{90\%}$, the time it takes for 90% inactivation (reflecting the second kinetic phase). We found an excellent linear relationship between measured $t_{1/2}$ and $t_{90\%}$ values, indicating that whatever the biophysical basis for the two-phase inactivation kinetics, both phases lead to the same conclusion regarding the relative thermal stability of each mutant. For this reason, we present only $t_{1/2}$ values in the Results and Discussion.

RESULTS

A Simplified Assay for DAGK Misfolding. In previous work, a real-time folding assay was used to monitor the kinetics of assembly of functional DAGK starting from detergent/lipid-free stocks into mixed micelles or vesicles (*2*). The focus of this paper is upon that fraction of DAGK which fails to fold properly and instead adopts an irreversibly misfolded state by the end of the folding assay, the point at which no further productive folding takes place. Reflecting this focus, the method used to quantitate misfolding was to add small aliquots of stock solutions containing detergent/lipid-free DAGK in acidic 6.5 M urea into DM/CL mixed micelles, followed by incubation for times long enough to

[2] For most mutants, it is probable that the observed post-reconstitutive refolding activity approaches the ideal value for the 100% correctly folded protein. Thus, the observed post-refolding activities were used as the standard in the calculation of folding efficiency. In the case of the I110E and I110K mutants, our results indicate that these mutants are unstable even following reconstitutive refolding, which explains their much lower observed activities following reconstitutive refolding relative to the other I110 mutants.

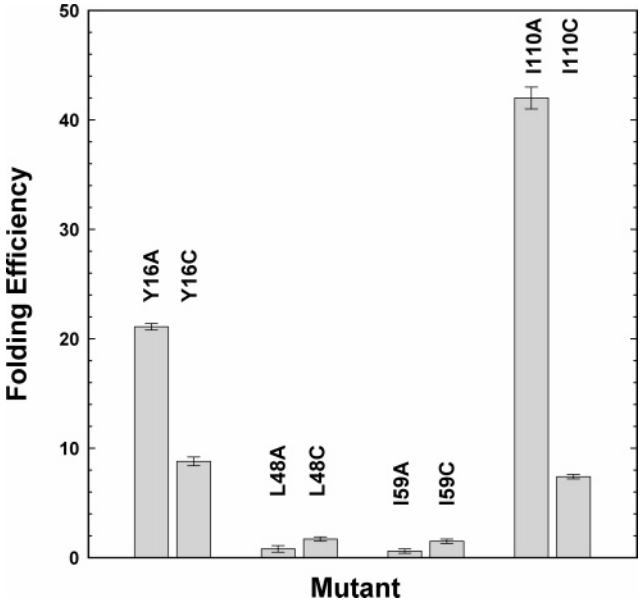

FIGURE 2: Folding efficiencies for selected misfolding-prone Cys replacement DAGK mutants compared with those of the corresponding Ala replacement mutants. Efficiencies were measured using the urea-into-mixed micelle folding assay. Each value represents the average of least three measurements, with standard deviations as shown. The activity values for properly folded forms of each mutant that were used in the calculation of efficiency are given in Materials and Methods.

ensure the completion of folding and misfolding processes, followed by measurement of DAGK's enzyme activity under the same mixed micelle conditions. In previous work, it was observed that folding and misfolding of DAGK in mixed micelles reach completion within seconds (*2*). This was confirmed in this work for cWT, L48C, A52C, and I110C, where it was observed for each that the amount of DAGK that reaches a properly folded state is invariant with time after the ca. 20 s dead time between dilution of the stock solution into mixed micelles and enzyme activity measurement (data not shown).[3] "Misfolded" DAGK is believed to represent a kinetically trapped structural state and does not involve chemical modification (*2*−*4*, *42*).

Promotion of Misfolding by Single-Amino Acid Replacements in DAGK Is Not Limited to Cysteine. Previous studies of the misfolding of DAGK have focused on the use of a library of DAGK mutants in which single wild-type residues were replaced with cysteine. Many of these mutants have previously been observed to be misfolding-prone, both following purification (*3*) and under conditions of folding assays (*42*). To test whether DAGK misfolding is uniquely promoted by mutations to cysteine, we chose four residues for which mutation to cysteine was previously shown to promote misfolding (Y16, L48, I59, and I110) and mutated each to alanine. These Ala mutants were prepared in acidic urea and subjected to the folding assay. As shown in Figure 2, two of these (Y16A and I110A) were less susceptible to misfolding than the corresponding Cys mutant. However, for the other two (L48A and I59A), mutation to alanine resulted in a degree of misfolding slightly higher than that observed for the corresponding Cys mutants. This indicates that cysteine is not unique in its ability to promote misfolding when introduced at specific (but common) sites in DAGK, a conclusion that is supported by the results in the following section.

Effect of Systematic Amino Acid Replacements at Isoleucine 110 on DAGK's Propensity To Misfold. Isoleucine 110 is a site that is located near the middle of DAGK's third transmembrane helix (*46*, *47*) where it is at least partially buried at a protein−protein interface (C. R. Sanders, unpublished NMR data). We tested the effect of a series of mutations at this residue on DAGK's expression in *E. coli* and also on the folding efficiency for each mutant using the urea-into-mixed micelles folding assay.

E. coli WH1061 was transformed with a series of rather leaky expression vectors that encode DAGK mutations at residue I110. For the I110R or I110P substitutions, we were unsuccessful in our attempts to obtain clones that contained the desired mutations. Viable transformants resulting from introduction of newly mutated double-stranded DNA into XL1-Blue cells contained either unmodified (cWT) DAGK or random mutations that resulted in truncated DAGK. It appears that the I110R and I110P mutations result in the recombinant protein becoming toxic to its host, most likely because of cell biological complications associated with severe folding defects for these mutants. However, the mechanisms by which folding problems led to cellular toxicity (possible translational arrest, inhibition of the protein membrane translocation system, etc.) are not established by our results. The other I110 mutants that were tested (see Figure 1) were successfully expressed and purified, although for I110K it was difficult to obtain stable transformants and expression yields were low.

Each purified I110 mutant was subjected to reconstitutive refolding (*3*) in an attempt to facilitate correct folding. For all I110 mutants, we observed (see the list in Materials and Methods) that the activities following refolding were more than 50% of the value of 113 ± 3 U/mg observed for cWT, except for I110E (36 ± 3 U/mg) and I110K (12 ± 2 U/mg). That high activity was observed even following nonconservative mutations such as I110G, I110H, and I110W indicates that Ile110 does not play a significant role in catalysis by DAGK. The lower activities observed for I110E and I110K appear to reflect extreme destabilization of the protein by these mutations (see the next section), which persists even following reconstitutive refolding into lipid vesicles.

Urea-into-mixed micelles folding efficiencies for each position 110 mutant are shown in Figure 3. Efficiency varied from mutant to mutant by factors as great as 20, with some mutants folding with the same ~50% efficiency as the parent cWT, while others folded with very low efficiencies (<3%). In general, folding became less efficient as mutations at position 110 became less conservative. These results confirmed that the promotion of DAGK misfolding by mutations at folding-sensitive positions is a property that is not unique to cysteine. A surprising result is that I110W folded with a very low efficiency, whereas the I110F and I110Y mutants folded nearly as efficiently as cWT.

[3] A few analogous assays were also carried out in which DAGK in acidic urea was diluted into intact POPC lipid vesicles, followed by incubation and then measurement of DAGK activity using the standard mixed micelle activity assay system. In this case, the final catalytic activity was derived not only from the DAGK that properly assembled after folding into lipid vesicles but also, in part, from protein that was reversibly misfolded in vesicles, but which rapidly refolds upon addition to mixed micelles (*1*, *2*).

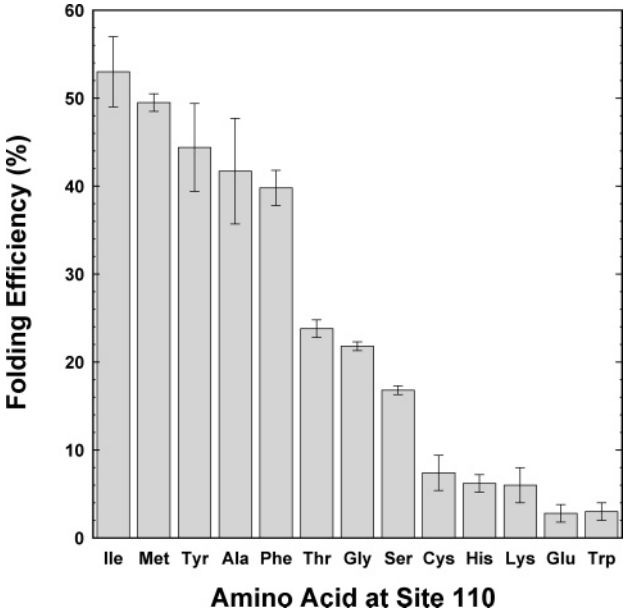

FIGURE 3: Folding efficiencies for the I110 mutant series under the conditions of the folding assay in which aliquots of DAGK in acidic urea were diluted into mixed micelles. Each value represents the average of at least three measurements, with standard deviations as shown.

Stabilities of Position 110 Mutants. For each mutant, we examined both the stability for the folded trimeric form and the kinetics of thermal inactivation. Trimer stability was assessed by measuring the mixed micelle mole fraction of the denaturing detergent, SDS, required to convert 50% of the DAGK trimers to monomers, a method derived from a procedure proposed by Lau and Bowie (*40*, *48*) for estimation of the thermodynamic stability of DAGK. As shown in Figure 4, the I110K and I110E mutants were so unstable that little trimer was present even at the beginning of the SDS titration. The trimeric forms of these mutants are evidently very unstable, an observation that is consistent with their low observed catalytic activities. SDS_{50} values for the other mutants are shown in Figure 5A. Most substitutions at I110 that introduced a hydrogen bond-forming side chain resulted in a protein that is less stable than cWT. I110W and I110Y are exceptions and formed trimers that are more stable than either cWT or I110F.

The thermal stability of each I110 mutant was evaluated by measuring the $t_{1/2}$ for irreversible inactivation when folded mixed micelle samples were incubated at 70 °C.

We observed that $t_{1/2}$ varied among the mutants by >2 orders of magnitude (from <30 to 4200 s) and that there was an excellent hyperbolic relationship between the $t_{1/2}$ and the SDS_{50} values (not shown). We therefore report thermal stability as $\log(t_{1/2})$, with results shown in Figure 5B. I110E and I110K exhibited very low thermal stabilities, while I110W and I110Y exhibited greater stability than either cWT or I110F.

Choice of Mutants for Studies of the Pathway(s) Leading to Irreversible Misfolding. As noted above, irreversible misfolding of DAGK reaches completion very rapidly (within seconds) during the folding assays in DM/CL mixed micelles. The final set of experiments of this study was dedicated to determining (i) whether misfolding occurs prior to oligomerization and/or aggregation and (ii) whether misfolding is influenced by the DM/CL mixed micelles present in the folding assays. To probe these issues in a general manner, studies were conducted using selected mutants from both the I110 series and the misfolding-prone single-Cys set.

Misfolding Occurs in a Manner Independent of DAGK Trimerization. Folding assays were initiated using acidic 6.5 M urea stock solutions that contained binary mixtures of the folding-proficient cWT and a misfolding-prone mutant (Y16C, L48C, or I110C), at varying cWT:mutant ratios. When pure cWT was subjected to the folding assay, a maximum DAGK activity of nearly 60 U/mg was observed, corresponding to a folding efficiency of ca. 55%. When folding assays were initiated with pure Y16C, L48C, or I110C, the activity approaches 0, as in previous results (*42*), indicative of very inefficient folding. When assays were initiated using binary mixtures of cWT and a mutant in acidic urea, the observed DAGK catalytic activities were directly proportional to the mole fraction of cWT relative to total moles of cWT and mutant DAGK in each mixture, with the results being exactly the same for all three binary cWT/mutant mixtures (Figure 6). The slight curvature of the data is probably due to an overestimate of the cWT concentration in the single solution used to make all cWT/mutant stock mixtures. The data depicted in Figure 6 strongly suggest that irreversible misfolding occurs in a manner independent of the formation of DAGK oligomers, since the presence of misfolding-prone mutant monomers does not inhibit formation of functional trimers by cWT, as would be expected if DAGK must first associate to form trimers before misfolding can occur. While one might question whether the results could reflect an energetic preference for cWT subunits to associate only with other cWT subunits, this is unlikely given that Y16C DAGK exhibits wild-type-like stability when properly folded (*41*) and would therefore be expected to form heterotrimers with cWT that also would exhibit wild-type-like stability. We cannot rule out the possibility that the presence of a single cWT subunit in a heterotrimer with misfolding-prone mutant subunits is sufficient to rescue the entire trimer from misfolding, but this seems improbable and is not consistent with additional observations recorded below.

Irreversible Misfolding Is Independent of the Concentrations of DAGK and the Mixed Micelles. For cWT, I59C, I110C, I110S, and I110W, folding assays in which the final DAGK concentration was varied over a range of 7.5−30 μg/mL at a constant mixed micelle concentration were carried out. In no case was a significant variation of folding efficiency observed with a changing level of the DAGK mutant (Figure 7A). Assays in which the DAGK concentration in the mixed micelle folding assay mixtures was held constant at the usual value of 15 μg/mL while the concentration of the mixed micelles was varied over a range of DM concentrations from 10 to 50 mM (at fixed DM:CL ratios) were also carried out. On the basis of the DAGK activities observed when aliquots from folding assay mixtures were then subjected to the standard catalytic activity assay (Figure 7B), it appears that in no case was the folding efficiency strongly dependent on the concentration of the mixed micelles in the folding assay mixtures. These results suggest that the degree of irreversible misfolding is largely independent of the protein:mixed micelle ratio, which supports the notion that most misfolding of DAGK occurs in a manner that is independent of the presence of the mixed micelles.

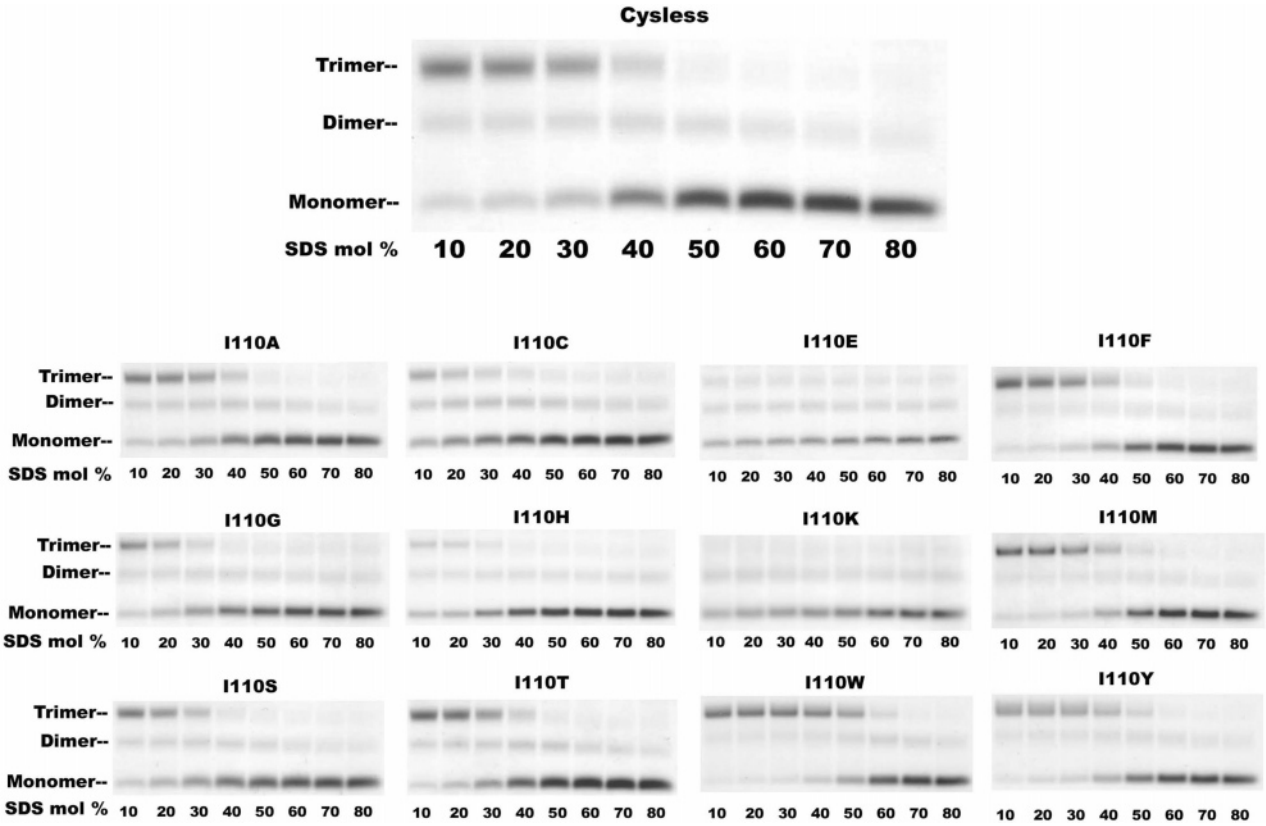

FIGURE 4: Data used for the determination of trimer stability. Each panel represents an SDS−PAGE gel (Coomassie staining) in which each lane represents one of the titration points when DAGK in DM/POPC mixed micelles was titrated with SDS. Aliquots from each of the time points were cross-linked with glutaraldehyde before being loaded onto the gel. Each lane was loaded with the same total amount of DAGK. The first titration point shown in each case is for 10 mol % SDS because interpretation of data for 0 mol % SDS lanes was sometimes complicated by the presence of reversibly aggregated DAGK (most likely aggregates of otherwise correctly folded trimers) that are converted to monodisperse trimers by 10 mol % SDS.

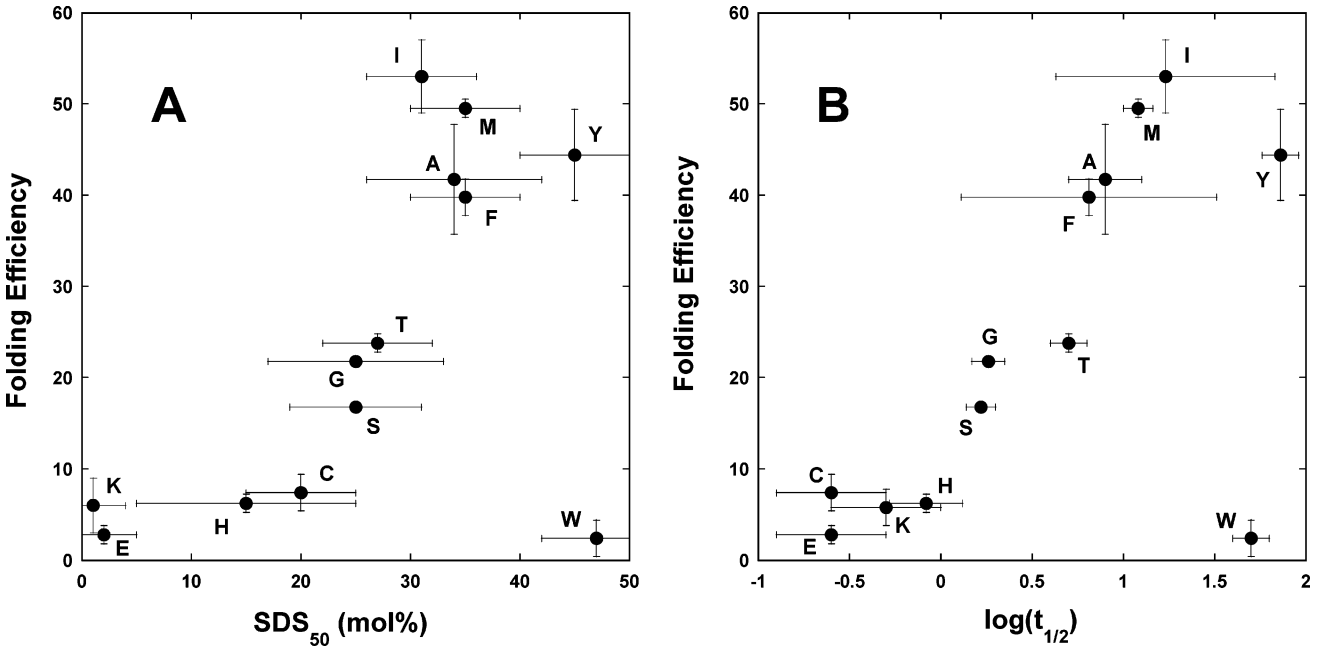

FIGURE 5: Correlation of folding efficiency with trimer stability (A) and thermal stability (B) for the I110 series of DAGK mutants. Folding efficiencies are as shown in Figure 3. (A) SDS_{50} values for trimer-to-monomer conversion were determined from the data in Figure 4. The experimental uncertainties in the SDS_{50} are shown as *X*-axis error bars. (B) Log($t_{1/2}$) values for thermal inactivation were determined for DAGK in DM/POPC mixed micelles by measuring time-dependent loss of catalytic activity during incubation at 70 °C. The experimental uncertainty in the log($t_{1/2}$) values is shown as *X*-axis error bars.

Misfolding of DAGK Mutants Appears To Be Independent of the Presence of Model Membranes. Assays in which acidic urea DAGK stock solutions were diluted into buffer that was completely free of detergent or lipid were carried out. Aliquots were then removed (within 30 s) and assayed for recoverable DAGK activity using the standard mixed micelle

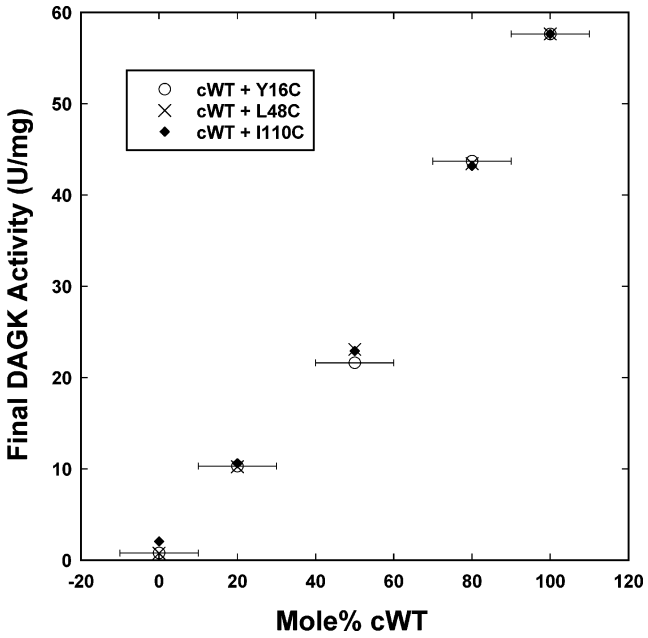

FIGURE 6: Folding efficiencies when binary mixtures of cWT and highly misfolding prone mutants in acidic urea were subjected to mixed micelle folding assays. Each reported activity is the average of three trials, with standard deviations (*Y*-axis) being smaller than the corresponding data points as plotted in this figure (such that error bars are not shown). The *X*-axis data uncertainties are shown only for the cWT/Y16C data but are the same for the corresponding data points of the other mutants.

enzyme assay. Results for cWT, I110C, and I110W are shown in Figure 7B (the DM = 0 points). Remarkably, the recovered DAGK activities following brief incubation in a detergent- and lipid-free solution approaches the activities observed for each mutant following a standard urea-into-mixed micelles folding assay (the 20 mM DM points in Figure 7B). We repeated this experiment for six other mutants and observed for each that the amount of recovered activity was similar to the final activity observed for a mixed micelle folding assay (recovered activity in units per milligram: 5 for Y16C, 4 for L48C, 35 for I110A, 3 for I110E, 28 for I110F, and 21 for I110T). This is surprising. It might have been predicted that DAGK would rapidly and irreversibly aggregate to near 100% after spending even a short time in a lipid- and detergent-free aqueous solution at neutral pH. It is also notable that for each mutant the percent of recovered DAGK activity is strikingly similar to the folding efficiency observed for each under the conditions of the standard folding assay (compare the following percent recoveries to Figures 2 and 3; 40 for cWT, 6 for Y16C, 4 for L48C, 34 for I110A, 8 for I110C, 8 for I110E, 34 for I110F, 19 for I110T, and 5 for I110W). In other words, the amount of irreversible misfolding that occurs when DAGK in a urea stock solution is diluted into detergent- and lipid-free buffer is comparable to the amount that occurs when urea/DAGK stocks are diluted into DM/CL mixed micelles. This strongly supports the conclusion of the above section that irreversible misfolding of DAGK under the conditions of the folding assay is independent of the presence of mixed micelles.

We next tested whether, upon dilution of DAGK from acidic urea into buffer, the amount of irreversible misfolding was dependent on the incubation time for the protein under detergent- and lipid-free conditions before it was transferred into mixed micelles for measurement of recovered activity. This was tested for cWT, L48C, and I110C. It was confirmed that the degree of irreversible misfolding that occurs during the dead time of the measurement (10−30 s incubation) is similar to the degree of misfolding that occurs during the urea-into-mixed micelle folding assays. However, a slower irreversible inactivation of the initially folding recoverable DAGK in buffer also occurs, with complete activity loss occurring over a period of minutes (for cWT) or tens of seconds (for L48C and I110C), as shown in Figure 8. This indicates that after rapid misfolding in buffer of a fraction of DAGK, the remaining folding-competent protein is slowly inactivated. This slowly inactivating fraction appears to correspond to the fraction of DAGK that folds correctly under conditions of the urea-into-mixed micelle folding assay. Glutaraldehyde cross-linking followed by SDS−PAGE confirmed that all the DAGK present in the detergent- and lipid-free mixtures eventually forms high-molecular weight aggregates (not shown).

Irreversible Misfolding of DAGK in Mixed Micelle Folding Assays Is Independent of Aggregation. Glutaraldehyde cross-linking of samples that contain aggregated DAGK results in the disappearance of DAGK bands from SDS−PAGE gels (because the cross-linked aggregates do not migrate into the gel). To test whether DAGK that misfolds under the conditions of the mixed micelle folding assay is aggregated, mixtures were subjected to GA cross-linking after completion of the assay. Results are shown in Figure 9. Assays in which the DAGK folded relatively efficiently are characterized by the presence of a strong trimer band after cross-linking, representing properly folded protein. Inefficient folders show little or no trimer band. Some inefficiently folding mutants appear to form aggregates, as reflected by the disappearance of DAGK bands after cross-linking (cf. I110H, I110K, and I110W). However, it is striking that a number of poorly folding mutants do not appear to form much aggregate, as judged by the presence of intense monomer bands following cross-linking (cf. L48C, I59C, and I110C). Moreover, while some evidence for aggregation can be detected in results for mutants that fold relatively efficiently (e.g., A104C), this does not appear to be the case for most mutants of this class (cf. cWT and Y16C). There is not a strong correlation between the degree of aggregate present following folding assays and the amount of misfolded protein determined to be present on the basis of folding assay measurements. To further probe this observation, DAGK folding efficiencies were measured in assays conducted under conditions where steps were taken to suppress aggregation.

We have observed that DAGK aggregates formed in the presence of mixed micelles can be at least partially reversed by the addition of SDS to a level of 10 mol %. 10 mol % SDS in mixed micelles neither denatures cWT DAGK nor inhibits its catalytic activity, a fact that was reconfirmed for most mutants used in this work (not shown). Folding assays were therefore conducted for cWT, I59C, I110C, I110S, I110H, I110K, and I110W under conditions where 10 mol % SDS was present in the DM/CL mixed micelle folding solution. We observed that the folding efficiencies for each mutant were identical to the corresponding folding efficiencies observed when no SDS was present; 10 mol % SDS neither promotes nor inhibits misfolding when DAGK from acidic urea stocks is diluted into mixed micelles. Glutaral-

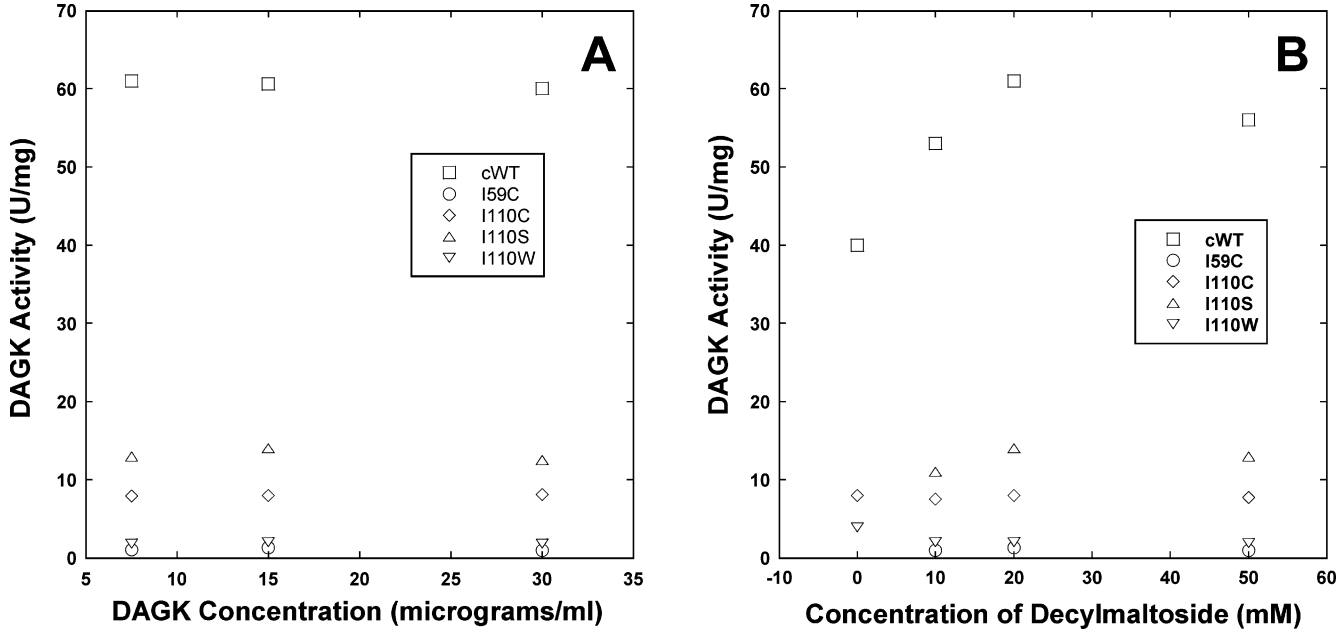

FIGURE 7: Final DAGK activities observed following the urea-into-mixed micelle folding assay as a function of (A) DAGK concentration at a fixed (standard) concentration of mixed micelles and (B) mixed micelle concentration at a fixed (standard) concentration of DAGK. The standard concentration of DAGK in the folding assays is 15 μg/mL, while the standard mixed micelle concentration is 20 mM micellar decyl maltoside plus 0.6 mM (3 mol %) cardiolipin. When the DM concentration was varied, the CL concentration was maintained at 3 mol %. The catalytic activities were measured when aliquots from the equilibrated folding assay mixtures were transferred to the standard spectrophotometric activity assay system (see Materials and Methods). Error bars are not shown, but the experimental uncertainty associated with each (single) activity measurement is estimated on the basis of extensive experience with this assay to be ±10% of the plotted value.

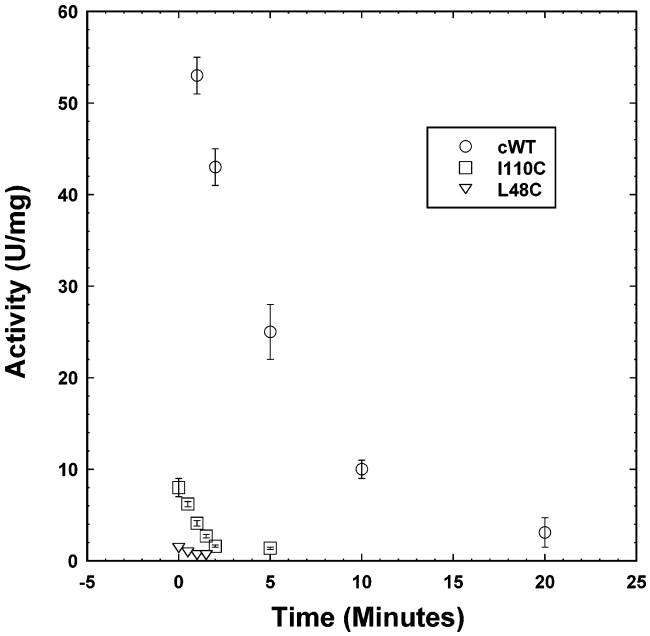

FIGURE 8: Recovered catalytic activity when DAGK was diluted from acidic urea into lipid- and detergent-free buffer, incubated for the indicated amount of time, and then transferred back into mixed micelles for measurement of enzyme activity. Each point represents the average of three measurements, with the standard deviations as shown.

dehyde cross-linking was then used to assess the oligomeric states and aggregation present following folding reactions carried out in the presence and absence of SDS. Results are shown in Figure 10. For cWT, we observed that the presence of SDS in the assay mixture leads to the same cross-linking/SDS−PAGE results as assays run in the absence of SDS. In the case of the misfolding-prone L48C, we observed that the monomer band dominates following cross-linking of a folding assay mixture, regardless of whether SDS is present, although the monomer band is more intense when SDS is present. Evidently, misfolding of L48C can lead to either monomer or aggregate. However, the fact that the degree of misfolding was exactly the same under conditions where aggregate was suppressed suggests that the committed step for misfolding occurs at the level of the monomeric protein, with aggregation being a secondary process. The results shown in Figure 10 suggest that for cWT, L48C, I59C, I110C, and I110S, misfolding takes place at the level of the monomeric protein. In the case of I110H, it appears that some, but not all, misfolding leads to aggregation, even in the presence of 10 mol % SDS in the folding assay. For I110K, it appears that nearly all misfolding that occurs in the presence of SDS leads to aggregation.

In total, the GA cross-linking experiments indicate that irreversible misfolding for a majority of the misfolding-prone DAGK mutants occurs for the monomeric protein, prior both to oligomerization and potential aggregation. This observation confirms the results of the subunit mixing experiment (Figure 6) and the results that showed the degree of misfolding is independent of DAGK/mixed micelle concentrations in the folding assays (Figure 7).

DISCUSSION

Promotion of Misfolding by Amino Acid Substitutions for Isoleucine 110. Previously, we observed that a single cysteine substitution at any one of many different residues in DAGK is sufficient to dramatically enhance the propensity of the protein to misfold (*3*, *42*). In this work, we first examined a few Cys-sensitive sites and confirmed that mutations to other amino acids besides cysteine can also promote misfolding. We then focused on a series of mutations for isoleucine 110.

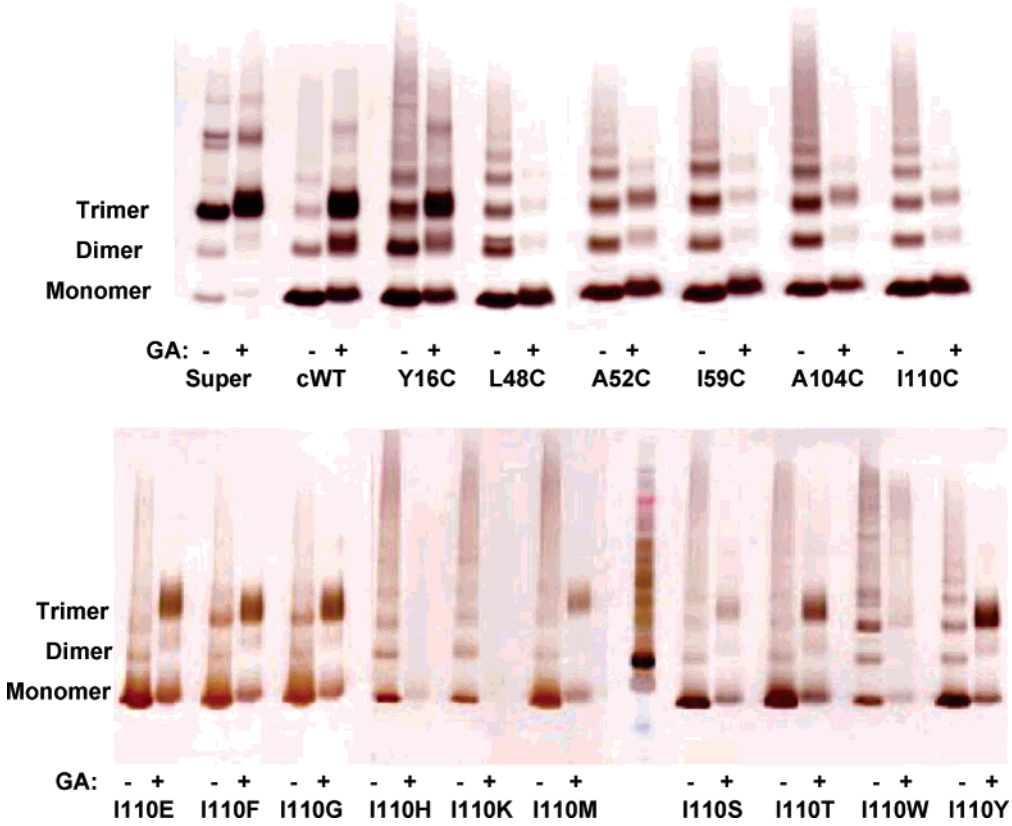

FIGURE 9: Silver-stained SDS−PAGE of aliquots from the end points of urea-into-mixed micelle folding assay mixtures that either were (+) or were not (−) cross-linked with glutaraldehyde prior to being mixed with SDS−PAGE loading buffer and loaded into the gel.

NMR studies have shown that Ile110 is located near the middle of DAGK's third transmembrane helix (*46*) and will therefore be deeply buried in the most hydrophobic part of the membrane bilayer. Additional NMR measurements (unpublished) indicate that Ile110 is at least partially shielded from direct protein−detergent interactions, indicating it is oriented at least partly toward a protein−protein interface. Ile110 is not part of DAGK's active site. Even nonconservative mutations at this site do not reduce catalytic activity, provided folding is maintained. Indeed, Ile110 is not well-conserved among prokaryotic DAGK homologues: Leu, Val, Ala, and Phe are commonly found at this site. Nevertheless, we observed that both folding efficiency[4] and protein stability can be profoundly compromised by mutations at this position.

I110P and I110R could not be expressed because they were toxic to their *E. coli* host. It is likely that this toxicity was derived from severe folding deficiencies. The most folding-defective of the mutants that was successfully purified was I110K, which also exhibited some toxicity to its host (transformed cells were obtained, but only with difficulty). I110K also was expressed the most poorly of all of the I110 mutants that were actually purified. Even though DAGK in native membranes is highly stable (*49*, *50*) and generally tolerant of mutations (*51*, *52*), it appears that introduction at a moderately conserved transmembrane site of a very polar amino acid (Arg or Lys) or one with the wrong conformational preferences (Pro) may lead to potentially lethal folding defects in vivo. It is interesting to note that Stewart et al. have documented *E. coli* cytotoxicity upon introduction of Pro and Arg into certain transmembrane sites of *lac* permease (*53*). Mutations to Pro and Arg are also the most commonly documented mutations that are linked to human disease (*6*, *54*, *55*).

Assayed folding efficiencies for the purified I110 series of mutants were observed to range from <3 to ~50%. Mutations involving potentially charged residues (I110C, I110E, I110K, and I110H) all folded inefficiently in the folding assay, even though they could be expressed. Not surprisingly, the most conservative mutations that were tested (I110A, I110M, and I110F) all folded with efficiencies approaching that of cWT. Plots of the folding efficiencies for the I110 series versus four different hydrophobicity scales (*56*−*59*) indicate a generally strong correlation between substituted amino acid polarity and misfolding propensity (not shown). However, no single scale exhibited a clearly superior correlation with the efficiency data. For all scales, results for Trp were always outliers. Further comment on this observation is offered below.

Correlation of Folding Efficiency and Stability for the Position 110 Mutant Series. A linear relationship was observed between the SDS_{50} for trimer dissociation and the $\log(t_{1/2})$ characterizing thermal inactivation of each DAGK

[4] We remind the reader that folding efficiency is defined as the percent of DAGK that adopts its functional catalytic state by the end of a folding assay relative to the total enzyme. "Irreversible misfolding" is used in this work to describe DAGK that does not spontaneously fold under mixed micelle conditions in which the folded form of the protein is stable and catalytically functional. This does not imply that the misfolded protein could not be refolded if it were to be denatured and then subjected to a refolding procedure (*3*, *4*). Under the experimental conditions of this work, misfolded DAGK almost certainly represents a kinetically trapped structural state.

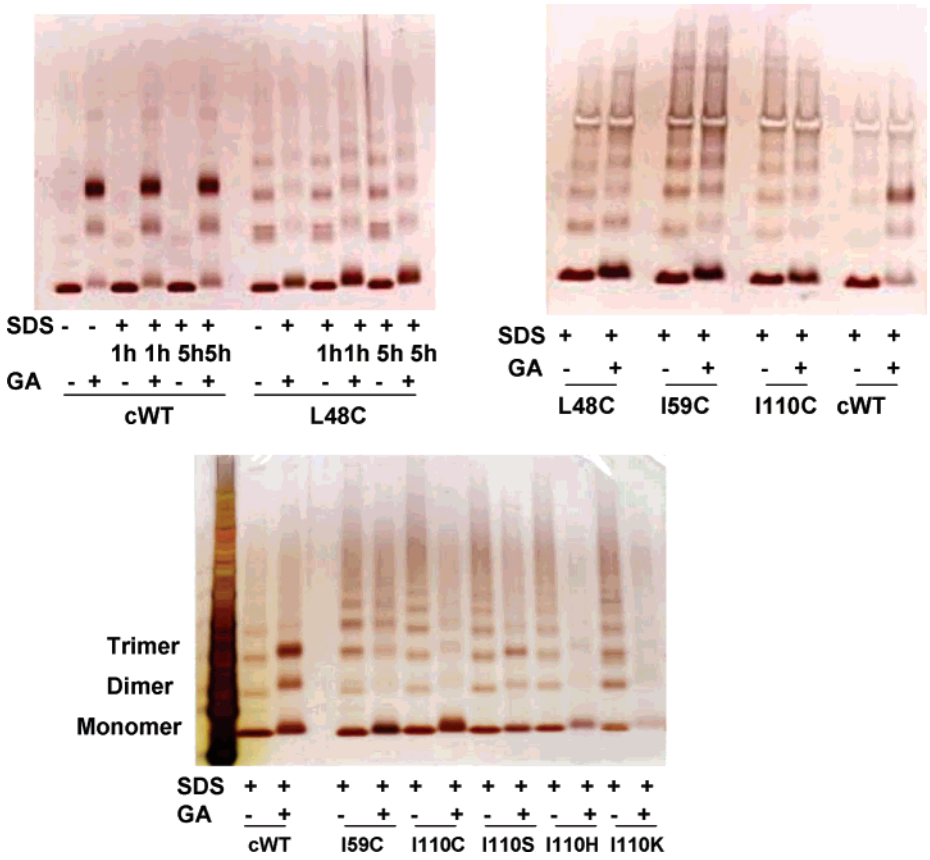

FIGURE 10: Silver-stained SDS−PAGE of aliquots from the end points of urea-into-mixed micelle folding assay mixtures that (in most cases) also contained 10 mol % SDS in the DM/CL mixed micelles. Aliquots either were (+) or were not (−) cross-linked with glutaraldehyde prior to being mixed with SDS−PAGE loading buffer.

position 110 mutant. This confirms the general proportionality originally documented by Bowie and co-workers between the thermodynamic and kinetic stability of mutant forms of DAGK (*4*, *48*), an observation that links the process of thermal inactivation of DAGK with subunit dissociation from the folded trimer.

The introduction of potentially charged amino acids into position 110 dramatically reduces the stability of the DAGK trimer and also makes it highly prone to thermal inactivation. Neutral hydrogen-bonding amino acids and glycine were also destabilizing, but to a lesser extent. In terms of stability, I110 was tolerant of mutations to other aliphatic or aromatic residues, regardless of side chain volume. I110Y and I110W mutations both led to a stability higher than that of the wild type, an observation that did not extend to I110F. While Bowie and co-workers previously showed that it is not unusual for mutations to confer greater-than-wild-type stability to DAGK (*48*, *60*), our results may be regarded as surprising because tyrosine and tryptophan are generally not found near the middle of transmembrane segments of wild-type membrane proteins but instead are most often located near the water−bilayer interface (*61*−*63*). While Ridder et al. recently showed that the introduction of buried Trp residues often enhances interactions between pairs of transmembrane helices (*64*), this effect is not well-understood, and it is not yet possible to explain why I110Y and I110W mutations result in stability enhancement for DAGK. Indeed, the possibility that introduction of Trp or Tyr at position 110 results in net stabilization of the protein primarily because each destabilizes the unfolded state cannot be ruled out. More data are required to illuminate these issues.

Panels A and B of Figure 5 indicate that for all I110 mutations except I110W, there is an good correlation between protein stability and folding efficiency. Unstable mutants are misfolding-prone. This correlation was previously observed for a series of Cys replacement mutants (*42*) and is now known to be general. For DAGK, the most common mutations that lead to misfolding are those that also destabilize the protein. We have previously reviewed a modest body of evidence that suggests protein folding quality control in the endoplasmic reticulum of higher organisms effectively probes the stability of nascent proteins and targets for degradation those judged to be unstable (*5*, *6*). If indeed this proves to be the case, then the stability−efficiency relationship observed for DAGK provides a teleological rationale (*5*, *6*, *42*): cells may choose to degrade unstable membrane proteins because unstable membrane proteins are misfolding-prone. Even though a protein may be stable enough to support near-100% physiological function, cells may prefer to degrade the protein if it is judged to be misfolding-prone. The resulting loss of protein function sometimes leads to disease if the function of the affected protein is critical to host physiology. The wide-ranging distribution of sites in DAGK for which mutations have been documented to lead both to destabilization and to the enhancement of misfolding propensity is very similar to the distribution of documented disease mutation sites in a number of human membrane proteins for which misassembly and

the resulting loss of function are thought to be etiologically critical (*5*, *6*).

I110W DAGK Exhibits a Paradoxical Folding Efficiency−*Stability Relationship.* The I110W mutant was unusual in that it is very stable, even more stable than cWT, yet highly prone to misfolding. I110Y is also very stable but folds efficiently. This indicates that a high degree of structural specificity must underlie the mechanism by which the Trp side chain promotes misfolding.

The I110W mutation enhances the pathway(s) leading to misfolding and/or interferes with the productive folding pathway, in either case without destabilizing or inactivating the folded state. We have previously documented another DAGK mutant with the same property, Y16C (*41*). In the case of Y16C, we observed that this mutant was misfolding-prone when folding assays were carried out in mixed micelles but that misfolding occurred to a much greater extent when folding was into lipid vesicles. From these observations, it was inferred that Y16 plays a specific role in DAGK's folding pathway by facilitating bilayer insertion. For I110W, the observed efficiency when the mutant was folded into POPC (not shown) was the same as for folding into mixed micelles (Figure 3), indicating that the mechanism by which the I110W mutation promotes misfolding is distinct from that for Y16C. Additional studies will be required to illuminate the mechanism for I110W. One wonders whether the proclivity of membrane-buried Trp to promote interactions between transmembrane helices (*64*) underlies the mechanisms by which it promotes both high stability and a high propensity for misfolding.

I110W and Y16C represent a rare class of misfolding−promoting mutations, those that promote misfolding without perturbing protein stability. It is possible that mutant membrane proteins of this class may be less readily detected as being folding-defective by folding quality control in the cell such that they are less likely to be degraded prior to misfolding (*5*). If so, mutants of this class may often be responsible for diseases in which a loss of membrane protein function is further exacerbated by the cytotoxicity of accumulated misfolded protein (*11*, *12*).

The Committed Step to Misfolding of DAGK in a Mixed Micelle Solution Is Independent of Oligomerization, Aggregation, or Micelle Interactions. For cWT and a majority of the mutants examined in this work, a series of results indicated that under the conditions of the urea-into-mixed micelle folding assay misfolding occurs when the protein is still monomeric and in a manner that is largely independent of the presence of mixed micelles. While aggregates may eventually form for some mutants, aggregation is a secondary process that follows the critical irreversible misfolding of monomers. In fact, conditions that suppressed the formation of aggregates during the folding assay did not reduce the degree of misfolding. These results are reminiscent of observations made by the Bowie lab with regard to the thermal inactivation of DAGK (*4*). On the basis of the observations that thermal stability is correlated with thermodynamic stability and that the rate of thermal inactivation decreases at higher DAGK concentrations, they proposed that subunit dissociation is the critical step leading to thermal inactivation, rather than aggregation.

It is surprising that aggregation is not an essential part of DAGK misfolding. For most soluble proteins, misfolding usually implies the formation of ordered or disordered aggregates, as typically staged from unfolded protein or from vulnerable folding intermediates (*7*, *65*, *66*). Aggregation seems most often to be driven by the formation of non-native intersubunit hydrophobic contacts. One might guess that most membrane proteins would be highly susceptible to misfolding via this mechanism, but DAGK does not appear to fit this mold. We suggest that upon dilution of partially unfolded DAGK from acidic urea into aqueous solution, the monomeric protein is particularly susceptible to the formation of inappropriate tertiary structural contacts that lead to rapid and irreversible inactivation. Because many of the mutations that were observed to promote misfolding involve aliphatic-to-hydrogen bonding amino acid substitutions, we also suggest that mutations that promote misfolding likely do so by disfavoring native tertiary and quaternary structure (rather than by actually lowering the free energy of the misfolded state). This is consistent with the excellent correlation observed between the propensity of mutants to misfold and the degree to which mutations destabilized the native trimer. The dominant forces responsible for stabilizing the misfolded state are likely to be inappropriate intramonomer hydrophobic interactions. Perhaps the high degree of hydrophobicity of helical multispan membrane proteins renders them particularly susceptible to unimolecular misfolding under conditions in which they are not threaded into the membrane via the highly facilitated process that is operative under cellular conditions.

Our conclusion that DAGK misfolds as a monomer may be used to illuminate some aspects of the complex kinetic model for DAGK folding and misfolding proposed by Lorch and Booth (*1*). However, there are complicating factors associated with the conditions used in the Lorch and Booth folding study which dictate that correlation of their results with those of this paper is difficult and perhaps better reserved for a future publication. First, different methods were used to characterize the folding and misfolding pathways in these two studies. The previous study focused on the use of time-resolved Trp fluorescence measurements, with the primary experimental variables being DAGK and vesicle concentrations. Second, the studies of Lorch and Booth were dedicated to studying folding of DAGK from urea into lipid vesicles, while this work focused on folding into mixed micelles. Key differences between micelles and vesicles that complicate direct comparison of data include the following. (i) DAGK monomer that inserts into a mixed micelle can still rapidly and freely exchange between different micelles (through micelle fusion, for example), whereas a lone monomer inserted into a lipid vesicle is unlikely to be able to exchange between vesicles on a relevant time scale. Because DAGK functions only as a trimer, the monomer is inactive without necessarily being misfolded. (ii) The energy barrier to insertion in lipid vesicles is much higher than it is in mixed micelles such that the productive folding process is much slower in vesicles (*1*, *2*, *42*). (iii) The focus of this study is on the cWT form of DAGK (see Materials and Methods) and derived mutants, whereas the Lorch and Booth study focused on the true wild-type enzyme, which is more stable than cWT.

Our data provide little insight into the structure of misfolded DAGK, other than that it misfolds as a monomer, which may sometimes go on to aggregate and/or associate

with model membranes. It is unclear whether misfolded DAGK is structurally homogeneous (for a given mutant), whether different mutants adopt the same misfolded form, or whether the misfolded protein retains the same mostly helical secondary structure as the native conformation. Addressing these issues will require future experimental results.

Naked DAGK in Aqueous Solution Remains Folding-Competent for Significant Periods of Time. When DAGK was diluted manyfold from lipid- and detergent-free urea solutions into lipid- and detergent-free buffer, a significant population of the protein retained the ability to fold spontaneously and correctly when aliquots were then transferred to mixed micelles. At short incubation times, the folding-competent population was roughly the same as that which would have folded if the original dilution had been into mixed micelles. At longer incubation times, the folding-competent population eventually undergoes irreversible aggregation. We speculate that the DAGK that is "naked" in aqueous solution yet remains folding-competent over a period of minutes may be in the form of reversible protein-only micelle-like aggregates that convert only slowly into irreversible aggregates. Lorch and Booth characterized the time dependence of DAGK's Trp fluorescence following dilution from acidic urea in detergent- and lipid-free buffer and observed a very rapid (30 s^{-1}) kinetic phase that was independent of DAGK concentration, followed by a somewhat slower (0.3 s^{-1}) phase that was second-order with respect to protein concentration (*1*). They attributed the first phase to solvent relaxation of monomeric DAGK upon dilution from the urea solution. The second phase was proposed to involve protein aggregation. Our results are not at variance with these observations, although it appears that the number of distinct structural species that evolve with time is higher than that which is evident from time-dependent Trp fluorescence.

The ability of DAGK to remain insertion- and folding-competent for short periods of time while naked in aqueous solution would not have been predicted from the highly hydrophobic nature of its sequence. It would be interesting to determine whether many other membrane proteins also possess this talent. It is conceivable that the ability to remain post-translationally folding-competent for short periods of time could reflect a vestige descended from primordial membrane proteins, which might have inserted into membranes and folded without the benefit of the membrane translocation system that is present in all modern cells (*67*). Alternatively, it is possible that DAGK's brief retention of folding competency in solution could reflect a route to folding and insertion that is still exploited by modern membrane proteins under cellular conditions as a route of last resort that is used only when the normal facilitated membrane insertion pathways are saturated, dysregulated, or dysfunctional. As such, it would be an example of biological pathway redundancy that has been developed (or retained by evolution) in the interest of making membrane protein assembly as robust as possible. In light of evidence that in vivo protein assembly (in general) and, more specifically, bilayer integration are often <100% efficient processes for membrane proteins (*5*, *14*, *68*), this seems plausible.

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