A Critical Residue in the Folding Pathway of an Integral Membrane Protein†

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ABSTRACT: Although a number of common diseases are a direct consequence of membrane protein misfolding, studies of membrane protein folding and misfolding lag well behind those of soluble proteins. Here it is shown that an interfacial residue, Tyr16, of the integral membrane protein diacylglycerol kinase (DAGK) plays a critical role in the folding pathway of this protein. Properly folded Y16C exhibits kinetic parameters and stability similar to wild-type DAGK. However, when unfolded and then allowed to spontaneously fold in the presence of model membranes, Y16C exhibits dramatically lower rates and efficiencies of functional assembly compared to the wild-type protein. The Y16C mutant represents a class of mutations which may be commonly found in disease-related membrane proteins.

Missense mutations in genes encoding membrane proteins often result in aberrant folding and/or mistrafficking which is linked to various human diseases (*1*−*3*). However, very little is known about the structural and energetic factors which govern the partitioning of nascent wild-type or mutant membrane proteins between folding and misfolding pathways. In this work, the folding kinetics of a wild-type membrane protein are compared to those of a folding-defective mutant.

Diacylglycerol kinase (DAGK,[1] Figure 1) is a homotrimeric integral membrane protein which catalyzes the phosphorylation of diacylglycerol by MgATP. When correctly folded, DAGK is a fairly stable protein, even when solubilized in detergent micelles (*4*). However, wild-type DAGK exhibits a tendency to become kinetically trapped in misfolded conformations (*5*). Moreover, as for a number of disease-related membrane proteins, mutations at any one of a variety of sites dramatically increase DAGK's propensity to misfold (*5*, *6*). Previously, we established well-defined conditions in which DAGK can be denatured in detergent/lipid-free form and then refolded in the presence of model membranes (*7*). In this study, we compare the folding rates and efficiencies of wild-type DAGK to those of the cysteine replacement mutant for tyrosine 16, a highly conserved residue. Results presented in this work indicate that Tyr16 plays an important role in protein folding which is not linked to potential roles in stability or catalysis.

MATERIALS AND METHODS

E. coli strains overexpressing the recombinant forms of DAGK with an N-terminal polyhistidine tag were supplied by the laboratory of James Bowie of UCLA (*4*). DAGK was purified into either detergent or denaturant solutions as previously described (*5*, *7*). For historical reasons (*4*) and in order to avoid the complications of possible disulfide bond formation during folding studies, the "wild-type" DAGK used in this study is actually a cysteine-less variant form of the protein containing C46A, C113A, W117R, and T118S mutations, with the Y16C mutant being derived from this parent. This "Cys-less" variant exhibits folding, kinetic, and structural properties which are very similar to those of the true wild-type DAGK.

DAGK activity and steady-state kinetic measurements were carried out using a spectrophotometric assay under mixed micellar conditions (*8*), where 1 unit of DAGK activity is equal to 1 μmol of DAG converted into phosphatidic acid per minute.

The thermodynamic stability of DAGK was assessed by first dispersing vesicular DAGK (prepared as described in *5*) to a concentration of 0.1 mg mL^{-1} in DAGK in a buffer containing 75 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, 0.1 mM EGTA, pH 6.8. Detergent (DM) was added from a stock solution in the above buffer such that the final DM:POPC molar ratio was 85:15. Varying amounts of SDS were then added to the samples to span a concentration range of 0−60 mol % (relative to total detergent+lipid concentration). Samples were incubated at 30 °C for 30 min followed by addition of glutaraldehyde to 25 mM. Samples were then shaken vigorously for 16 h and analyzed by SDS−PAGE.

Kinetic stability was determined by measuring the enzymatic activity of samples following incubation at 90 and 75 °C for varying amounts of time. For in-vesicle stability measurement, DAGK-containing vesicles were diluted to a final concentration of 0.1 mg mL^{-1} DAGK in a buffer containing 40 mM HEPES, 0.3 M NaCl, pH 7.5. Samples were heated at 90 °C for varying amounts of time (0−60 min). Immediately following incubation, samples were transferred to a standard mixed micellar assay mixture, and enzymatic activity was measured as previously described (*8*). For stability measurements in mixed micelles, refolded DAGK in POPC vesicles was diluted prior to incubation/

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[1] Abbreviations: DAG, diacylglycerol; DAGK, diacylglycerol kinase; DM, *n*-decyl-β-maltoside; FT-IR, Fourier transform infrared spectroscopy; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; near-UV CD, near-ultraviolet circular dichroism; NMR, nuclear magnetic resonance; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; WT, wild type.

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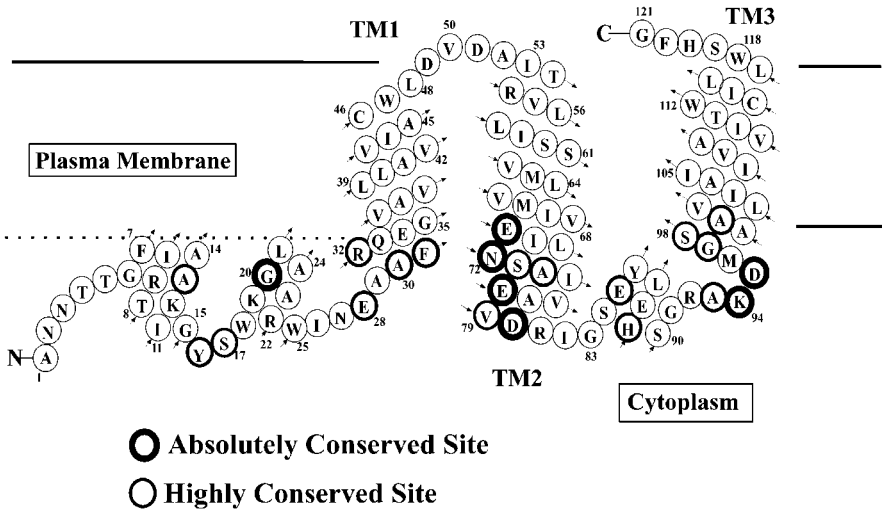

FIGURE 1: Topology and rough secondary structure of *E. coli* DAGK. The protein is largely helical as determined by CD and FT-IR, with the exact secondary structure of residues 1−54 having recently been determined by NMR spectroscopy (Sanders, unpublished experiments).

assay into a buffer containing 1% DM, 40 mM HEPES, 0.3 M NaCl such that the final protein concentration was 0.1 mg mL^{-1} and the final DM:POPC mol:mol ratio was 85:15. Samples were then incubated at either 90 °C or 75 °C for varying amounts of time (0−60 min), followed by assays.

Details of circular dichroism spectroscopy and the spectrophotometrically-based method for monitoring the functional assembly of DAGK in folding/insertion reactions have been described elsewhere (*7*).

RESULTS

Wild-Type and Y16C DAGK Exhibit Similar Stabilities and Catalytic Properties. NMR studies have shown that Tyr16 occupies a site in DAGK which is water-exposed near the water−bilayer interface in a short segment which links two helices (Sanders, unpublished results). The residue is also proximal to a confirmed active site residue, Ala14 (*9*). This latter observation prompted us to test whether Y16 might also be involved in DAGK's active site by comparing the steady-state kinetic parameters of the Y16C mutant form of the enzyme to those of the wild-type form. Under conditions where DAG was held at a saturating concentration, the wild-type enzyme exhibited Michaelis−Menten kinetics with a $K_{m,MgATP}$ of 1.6 ± 0.6 mM and a V_{max} of 109 ± 15 units/mg. Under these same conditions, the correctly folded Y16C mutant exhibited a $K_{m,MgATP}$ of 2.1 ± 0.6 mM and a V_{max} of 73 ± 8.8 units/mg. This similarity in kinetic parameters indicates that if Tyr16 plays a significant role in the active site of DAGK, its role is adequately filled by nonconservative replacement with a cysteine side chain.

The kinetic stability of Y16C was compared to that of WT DAGK. Both wild-type and Y16C retained full enzymatic activity after incubation in POPC vesicles for 60 min at 90 °C, indicating that both are very stable in bilayers. However, when vesicles were disrupted by the addition of decyl maltoside to form DM/POPC mixed micelles (85:15 mol:mol ratio), the half-life for the wild-type protein at 75 °C was 12 min while the half-life for Y16C was 20 min. Increasing the temperature to 90 °C under these same mixed micellar conditions lowered the half-life for wild-type protein to 1.25 min and that of Y16C to 1.15 min. These results

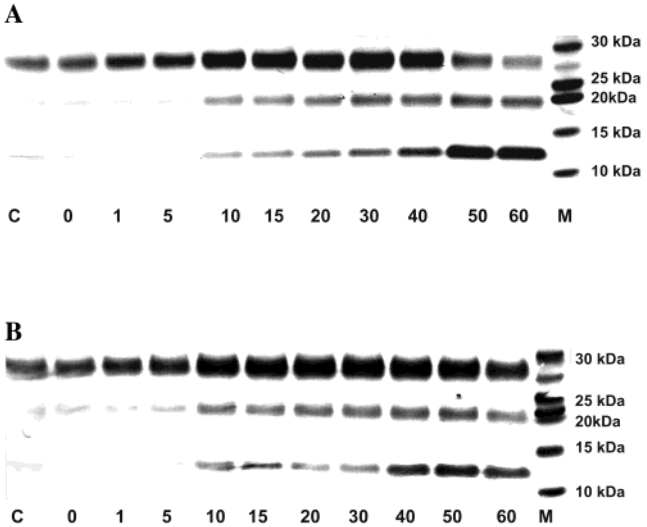

FIGURE 2: SDS−PAGE of cross-linked WT (A) and Y16C (B) DAGK. DAGK samples in DM/POPC mixed micelles were mixed with varying SDS concentrations (0−60 mol %), followed by cross-linking and SDS−PAGE. C: control lane (no SDS). For all numbered lanes, the numbers represent the mol % SDS added prior to cross-linking. M: molecular mass markers.

indicate that WT and Y16C DAGK have similar kinetic stabilities under a variety of conditions.

To compare the thermodynamic stability of Y16C to that of WT, DAGK was mixed with varying concentrations of SDS, a detergent which has been shown to reversibly denature the enzyme (*4*). Following incubation, samples were cross-linked with glutaraldehyde and analyzed via SDS−PAGE to monitor the loss of native trimeric structure which accompanies denaturation at high SDS concentrations. For WT, loss of trimeric state was noticeable at 50 mol % SDS, while for Y16C slightly higher concentrations of SDS were required to disrupt the trimer (Figure 2). Therefore, Y16C appears to exhibit a slightly higher thermodynamic stability than the wild-type enzyme.

Y16C and WT DAGK Exhibit Similar Structural Properties in Denaturant and Detergent Solutions. Previous folding and insertion studies employed WT DAGK stock solutions in which the enzyme was (i) a denatured monomer in lipid/detergent-free acidic 8 M guanidinium, (ii) a folded monomer

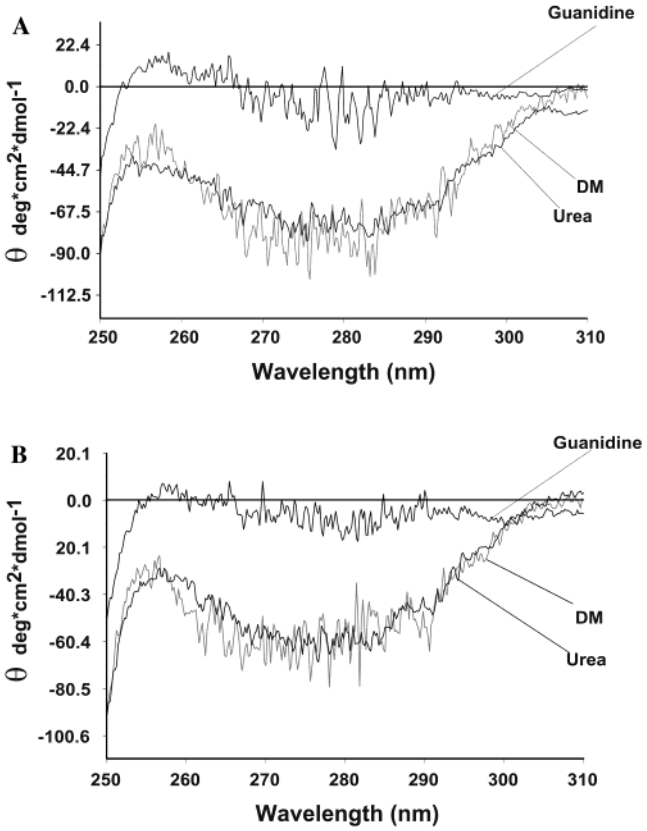

FIGURE 3: Near-UV CD spectra of 1 mg/mL WT (A) and Y16C (B) DAGK in stock solutions used to initiate folding/insertion reactions. Pairs of spectra for the WT and mutant enzyme under the same conditions are quite similar. Decrease in the absolute signal intensities from Y16C in the urea and DM cases relative to WT most likely results from the absence of the tyrosine 16 contribution to the signals from the Y16C mutant.

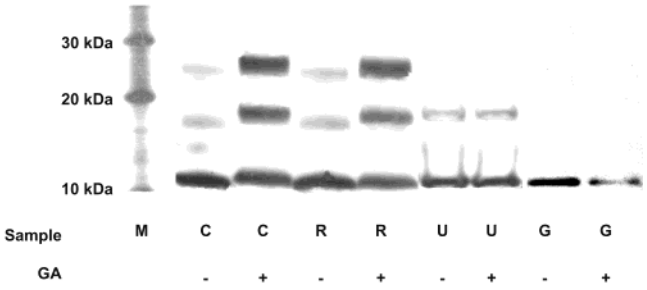

FIGURE 4: SDS−PAGE of various DAGK solutions in the absence (−) or presence (+) of GA cross-linking: WT in DM/POPC mixed micelles (C), Y16C in DM/POPC mixed micelles (R), Y16C in acidic urea (U), and Y16C in acidic guanidinium chloride (G).

in lipid/detergent-free acidic 6.5 M urea, or (iii) a folded trimer in 1% decyl maltoside micelles (*7*). For this work, Y16C was also purified in these same conditions. Near-UV CD results indicate that Y16C, like WT, maintains considerable tertiary structure in urea and DM solutions, but lacks tertiary structure in guanidinium (Figure 3). Chemical cross-linking indicates that Y16C is also similar to WT in maintaining its trimeric oligomeric state in DM micelles but not in urea or guanidinium solutions (*7*) (Figure 4). These results confirm observations that Y16C and WT DAGK have similar stabilities and indicate that any differences observed in their folding kinetics (see below) do not reflect grossly differing properties for the two forms of the enzyme in the various stock solutions.

Folding and Insertion of Y16C into Model Membranes. To monitor DAGK folding and insertion, stock detergent/protein or denaturant/protein solutions were diluted 200-fold into mixed micelles or POPC vesicles. The appearance of correctly folded and inserted DAGK was monitored as the appearance of catalytic activity with time (Figure 5). We have previously described a series of control experiments which show that the final denaturant or detergent concentrations originating from stock solutions in these "folding reactions" are well below the levels at which they could maintain DAGK solubility, affect its structure, or disrupt vesicle integrity (*7*). The $t_{1/2}$ values for the appearance of DAGK activity to plateau were calculated from the activity versus time plots and are directly proportional to the inverse of the overall folding rates. The efficiency of each reaction was determined by dividing the final (plateau) activity by the known activity for 100% correctly folded DAGK under the same conditions. Results are reported in Table 1.

For all four sets of reactions initiated with denaturant stock solutions, the efficiencies of productive Y16C assembly were much lower than for the corresponding reactions involving WT DAGK. This reflects a perturbation in the partitioning of Y16C between folding and misfolding pathways in favor of misfolding. For reactions involving folding/assembly into mixed micelles from denaturant solutions, the rates of productive assembly by both WT and Y16C were quite rapid. This indicates that the rate-limiting step for productive assembly into mixed micelles has not been perturbed for Y16C and that the enhanced misfolding observed for this mutant under these conditions evidently arises from an enhancement of the folding:misfolding partitioning ratio in favor of misfolding at a non-rate-limiting step early in the folding pathway.

Reactions for both WT and Y16C involving insertion into vesicles from denaturant solutions exhibited much lower productive folding/insertion rates than the corresponding reactions into mixed micelles, indicating that for vesicular reactions the rate-limiting step for productive folding/insertion is bilayer transversal by the transmembrane segments. In the case of reactions involving the insertion of urea stock solutions into lipid vesicles (and most likely for guanidinium-to-vesicle reactions as well), not only is the efficiency of the Y16C reaction extremely low, but the rate is also much slower than for WT. For Y16C, the process of productive bilayer insertion has evidently been impaired, in addition to an enhancement of its propensity to misfold prior to that step. The fact that WT exhibits dramatically slower folding/insertion rates into vesicles as compared with mixed micelles while exhibiting only 2−3-fold reductions in efficiency indicates that slowing down the productive assembly process does result in some additional misfolding, but that most misfolding occurs independently of the rate-limiting bilayer transversal step.

For both WT and Y16C, the efficiencies of reactions initiated using guanidinium stocks are much lower than the corresponding reactions using urea stocks. This suggests that for both WT and Y16C the enzyme is most susceptible to misfolding when starting from a fully denatured state (guanidinium) as opposed to reactions started with DAGK which is already partially folded (urea).

Finally, both WT and Y16C insert rapidly and with fairly high efficiency into POPC vesicles when small aliquots of

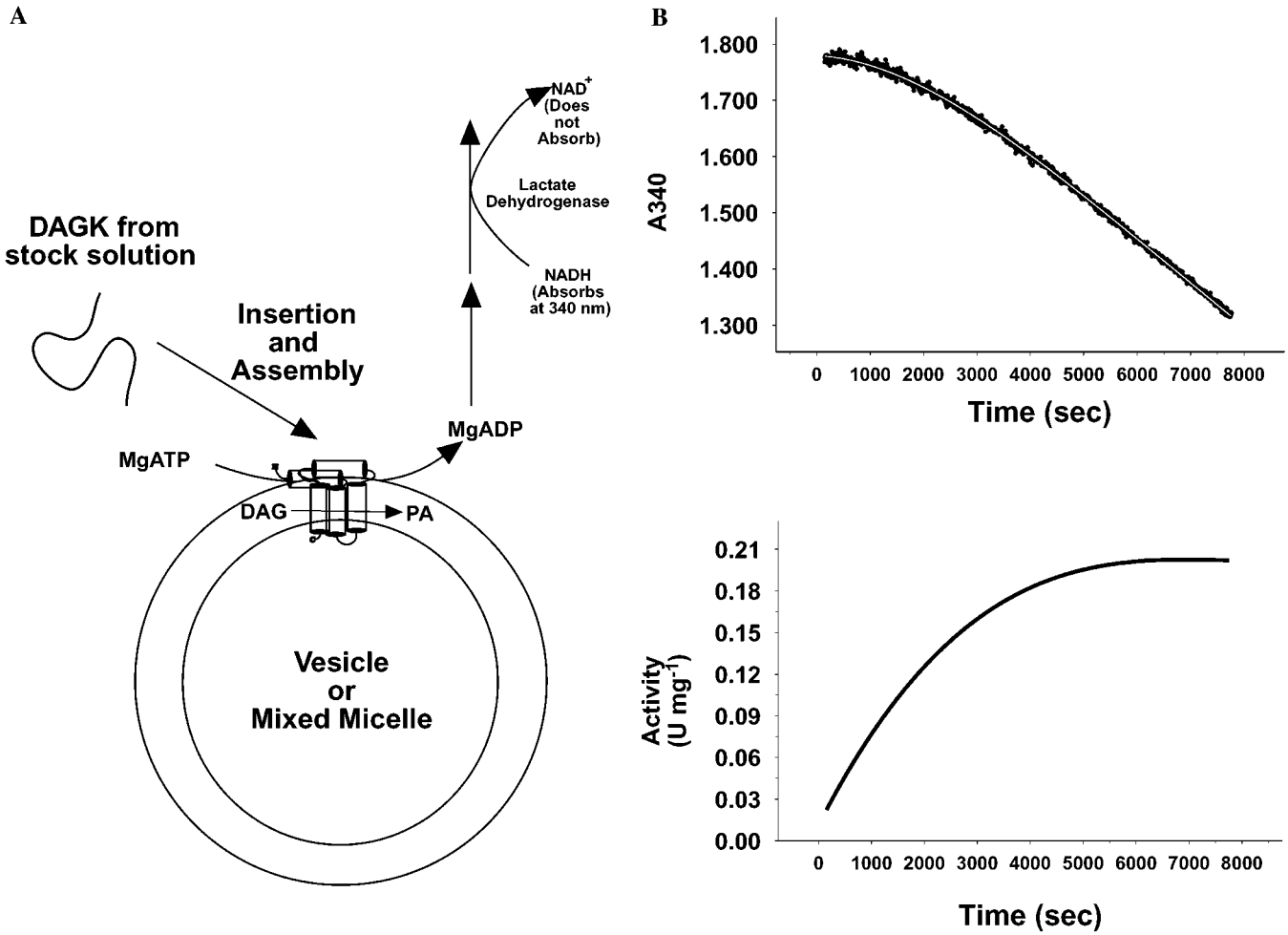

FIGURE 5: (A) Assay for monitoring DAGK folding and assembly in mixed micelles and POPC bilayers based on the appearance of catalytic activity representing correctly assembled DAGK (*7*). (B) Representative time trace for an insertion reaction involving addition of an aliquot of guanidinium-solubilized WT into POPC vesicles at time = 0. The top panel shows the spectrophotometric data representing the oxidation of NADH. The raw data (black circles) have been fit to a fourth-order polynomial (black line). The bottom trace shows a DAGK activity versus time plot derived from transforming the A_{340} vs time curve. From such plots, the final plateaued activity (reflecting insertion efficiency) and $t_{1/2}$ (proportional to the inverse of the overall folding/insertion rate) were measured.

Table 1: Comparison of WT and Y16C Insertion/Folding Rates and Efficiencies

		efficiency (%)		$t_{1/2}$ (s)	
starting state	final state	WT	Y16C	WT	Y16C
urea	mixed micelles	46 ± 13	3 ± 1	16 ± 6	4 ± 1
guanidine	mixed micelles	3 ± 1	0.4 ± 0.1	17 ± 9	9 ± 1
urea	vesicles	26 ± 5	$<0.5^a$	370 ± 150	3500^a
guanidine	vesicles	1.3 ± 0.5	$<0.2^a$	1000 ± 100	ND^a
DM micelles	vesicles	79 ± 22	61 ± 8	6 ± 4	4 ± 2

[a] Under standard reaction conditions, the efficiency of Y16C folding/insertion from either denaturant solution into vesicles was too low to measure within the sensitivity limit of the coupled assay system. When the quantity of Y16C urea stock used to initiate the reaction was increased 10-fold, it was possible to measure an efficiency of vesicle insertion of 1.5% and a $t_{1/2}$ of 3500 s (as reported). In the guanidinium to vesicles case, even a 10-fold increase in the amount of Y16C stock used to initiate the reaction did not result in the appearance of a detectable active DAGK population, even after an hour or more. ND: not determined because no detectable activity appeared.

DM/DAGK stock solutions were used to initiate the folding reactions. As described elsewhere for WT (*7*), such high rates and efficiencies appear to be the result of direct detergent participation in the bilayer insertion process even though the final concentration of DM is well below its critical micelle concentration. Residual DM apparently acts both to suppress misfolding and to enhance the rate of bilayer transversal by transmembrane segments.

DISCUSSION

Site 16 is highly conserved as an aromatic residue among prokaryotic DAGK orthologs. The results of this paper indicate that if high conservation is in any way reflective of a role in stability or catalysis, such a role is not revealed upon mutation of the native tyrosine to cysteine. The Y16C mutant is highly active and is at least as stable as the wild-type protein. However, our results suggest a role for Tyr16 in DAGK's folding pathway. In the absence of chaperone-like residual detergent or other folding/insertion facilitators, the Y16C mutant form of DAGK exhibits impaired folding behavior compared to WT. Specifically, this mutation promotes misfolding early in the folding pathway and also lowers the rate by which the protein can productively insert into lipid bilayers. This reduced insertional propensity is specific to bilayers and is not apparent when mixed micelles are the target model membrane.

While there is an emerging body of membrane protein folding kinetic data (*10*), we are unaware of another

documented case of a membrane protein mutation which appears to result in impaired protein folding under well-controlled "test tube" conditions while at the same time failing to perturb protein function or stability (once properly folded). However, it is quite possible that many of the hundreds of documented disease-related membrane protein missense mutations may also represent this general class of "folding-defective-only" mutations.

Additional experiments will be required to examine why the Y16C mutation promotes misfolding early in the folding pathway. Perhaps this site is critical in forming transient tertiary or quaternary structural contacts, without which the rate of misfolding is much higher. Additional experiments are also required to address the question of why Tyr16 appears to play an important role in bilayer insertion even though it is an interfacial residue which does not transverse the membrane as part of the folding/assembly process. It is recognized that aromatic amino acids in membrane proteins tend to be clustered near the water−bilayer interface (*11*, *12*). The "helper" role of Tyr16 in the membrane insertion of DAGK may reflect a role for interfacial aromatic residues which may in some cases be shared by analogous residues in other membrane proteins. It is also interesting to note that there are a number of disease-associated aromatic-to-non-aromatic mutations involving nontranslocated sites in juxtamembrane domains of various human membrane proteins. These include the vasopressin receptor (*13*) (Y128S, Y164S, W323R, diabetes insipidus), bestrophin (*14*) (Y227N, macular dystrophy), the cystic fibrosis transmembrane regulator (*15*) (F311L), and presenilin (*16*) (Y115C, Alzheimer's disease). Future elucidation of the mechanisms by which the Y16C mutation of DAGK interferes with folding and membrane insertion may provide insight into the molecular bases for the disease phenotypes caused by related mutations in human proteins.

A final and important question raised by the results of this work is whether the impairment in folding observed for Y16C DAGK under test tube conditions is also observed under in vivo conditions of expression. It is well-established that most integral membrane proteins fold and insert in living cells with the assistance of an array of proteins dedicated to this task (*17*−*20*). Whether the mechanisms associated with this cellular machinery are able to override the intrinsic propensity of Y16C to misfold or not is an issue which is presently being experimentally pursued.

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