Reconstitutive Refolding of Diacylglycerol Kinase, an Integral Membrane Protein†

Bonnie M. Gorzelle,‡ Joanna K. Nagy,‡ Kirill Oxenoid,‡ Willis L. Lonzer,‡ David S. Cafiso,§ and Charles R. Sanders*,‡

Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4970, Department of Chemistry, University of Virginia, Charlotte, Virginia 22901

Received June 8, 1999; Revised Manuscript Received October 4, 1999

ABSTRACT: While the formation of kinetically trapped misfolded structural states by membrane proteins is related to a number of diseases, relatively few studies of misfolded membrane proteins in their purified state have been carried out and few methods for refolding such proteins have been reported. In this paper, misfolding of the trimeric integral membrane protein diacylglycerol kinase (DAGK) is documented and a method for refolding the protein is presented; 65 single-cysteine mutants of DAGK were examined. A majority were found to have lower-than-expected activities when purified into micellar solutions, with additional losses in activity often being observed following membrane reconstitution. A variety of evidence indicates that the low activities observed for most of these mutants results from kinetically based misfolding of the protein, with misfolding often being manifested by the formation of aberrant oligomeric states. A method referred to as "reconstitutive refolding" for correcting misfolded DAGK is presented. This method is based upon reconstituting DAGK into multilamellar POPC vesicles by dialyzing the detergent dodecylphosphocholine out of mixed micellar mixtures. For 55 of the 65 mutants tested, there was a gain of DAGK activity during reconstitutive refolding. In 33 of these cases, the gain in activity was greater than 2-fold. The refolding results for cysteine replacement mutants at DAGK sites known to be highly conserved provide teleological insight into whether sites are conserved, because they are critical for catalysis, for maintenance of the proper folding pathway, or for some other reason.

Our understanding of the folding of integral membrane proteins is at a much earlier stage of development than in the case of water soluble proteins. Studies of membrane protein folding to date have typically focused on assembly and stability under equilibrium conditions (e.g., *1*−*4*) or upon elucidating the complex molecular machinery believed to be associated with the proper assembly and insertion of membrane proteins in vivo (e.g., *5*, *6*). Less work has been done to characterize misfolding of membrane proteins into kinetically trapped states, even though this phenomenon is related to a variety of diseases (*7*−*13*). In this contribution, we document the misfolding of one particular integral membrane protein. Furthermore, a simple and reliable method for refolding this protein is presented.

Microbial diacylglycerol kinase (DAGK) is a membrane protein found in the cytoplasmic membrane of many bacteria, where it is part of the membrane-derived oligosaccharide cycle and the lipoteichoic acid biosynthesis pathway (*14*, *15*). DAGK is a homotrimeric protein composed of ca. 120 residue subunits. As illustrated in Figure 1 each subunit has three α-helical transmembrane segments (*16*, *17*). Despite its very small size and the complexity of the reaction it catalyzes, both the results from random mutagenesis studies of the *E. coli* isozyme (*18*), and the results from multiple sequence alignment of the available microbial sequences (*17*) have suggested that DAGK is functionally tolerant of amino acid substitutions at most positions. However, the functional measurements made in the random mutagenesis studies involved assays conducted at the whole cell level under

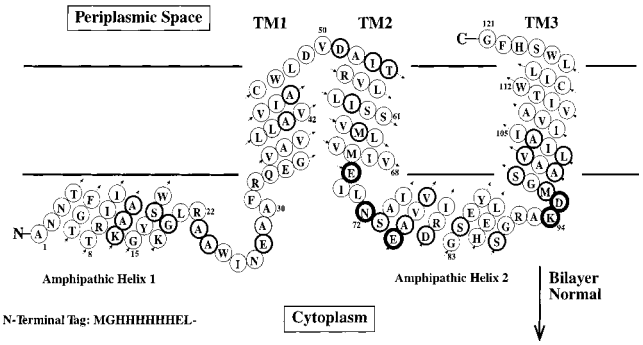

FIGURE 1: Model for DAGK's topology, secondary structure, and probable helical orientations based upon a variety of experimental data (*16*, *17*, *19*). The bold-circled residues are those that were judged to be functionally essential based either upon high conservation among natural isozymes or upon results of the extensive mutagenesis study of Wen et al. (*18*). The five residues in extra-bold are those that have been observed to be absolutely conserved both in the mutagenesis study and in all available native microbial DAGK sequences. For historical reasons described in ref 1, the form of DAGK used in this study is not the tagged wild-type (as shown) but is a mutant (W117R,S118T) that appears to be very similar to the wild-type protein.

† This paper is dedicated to the memory of Professor Matthew Petersheim of Seton Hall University, who prematurely departed from this vale of tears in October of 1998. Support for this work was provided by the U.S. National Institutes of Health (RO1 GM47485 and R44 DK49911) and through an American Heart Established Investigatorship to CRS (94001540).

* Corresponding author: Telephone: 216-368-8651. Fax: 216-368-1693. E-mail: crs4@po.cwru.edu.

‡ Case Western Reserve University.

§ University of Virginia.

10.1021/bi991292n CCC: $18.00
Published on Web 11/17/1999

conditions where DAGK was highly overexpressed. Some of the mutants that were observed to exhibit high units/cell DAGK activities in the original mutagenesis studies were later purified and found (*16*) to yield surprisingly low specific activities (units/mg of pure protein). These apparently contrasting results are consistent with the notion that there may be a significant fraction of misfolded DAGK within the total populations of many overexpressed DAGK mutants.

In this paper, we examine a set of purified single-cysteine DAGK mutants and observe that many of these mutants also exhibit low specific activities. As is always the case in mutagenesis-based structure−function studies of proteins, it is not possible, a priori, to know whether observed loss of functionality reflects purely functional roles (i.e., catalytic) for the mutated residues, or whether reduced function reflects global structural perturbations. Resolving these issues for membrane protein mutants is generally much more difficult than for water soluble proteins because of the difficulty in obtaining meaningful structural data for proteins of the former class. Here, we document that for a majority of purified DAGK mutants, activity loss is most often due to misfolding of the protein. This problem has a very negative impact upon kinetic, biophysical, and structural studies that are presently being carried out in several labs on DAGK. A solution to this problem is also presented in this paper.

MATERIALS AND METHODS

Overexpression and Purification of DAGK. DAGK mutant overexpressing strains of *E. coli* were provided by the laboratory of James Bowie of UCLA. These strains were prepared, cultured, and harvested as described elsewhere (*1*). Both wild-type DAGK and mutants were engineered to include an N-terminal polyhistidine purification tag.

The method normally used to purify DAGK and its mutants has been described elsewhere (*16*, *19*, *20*), so that only a brief summary need be presented here. Cells were lysed and then extracted with the detergent Empigen (dodecyl-*N*,*N*-dimethylglycine, Calbiochem, San Diego, CA). Cell extracts were incubated with Ni(II)-agarose resin (Qiagen, Valencia, CA). The resin was then step-eluted by (*1*) washing all non-DAGK protein from the resin with a buffer containing 40 mM imidazole and 1.5% Empigen, (*2*) reequilibrating the resin with a buffer containing 1% β-decylmaltoside (DM,[1] Anatrace, Maumee, OH) as the sole detergent component, and (*3*) eluting DAGK with 1% DM plus 0.3 M imidazole. A reducing agent (TCEP, mercaptoethanol, or DTT) was normally present at all stages of purification. Pure DAGK concentrations were quantitated by measuring light absorbance of solutions at 280 nm ($\epsilon_{280,0.1\%} = 1.8$).

[1] Abbreviations: BOG, β-octylglucoside; CD, circular dichroism; DAGK, diacylglycerol kinase; DBG, dibutyrylglycerol; DM, decylmaltoside; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DPC, dodecylphosphocholine; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GA, glutaraldehyde; HEPES, *N*-(2-hydroxyethylpiperazine)-*N*′-(2-ethanesulfonic acid); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NMR, nuclear magnetic resonance; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCEP, tris(2-carboxyethyl)phosphine; WT, wild-type.

Previous accounts of DAGK purification involving the DAGK overexpression system used in this study (*1*, *16*, *19*, *20*), did not address the question of where the highly overexpressed protein is found within *E. coli* cells. We carried out subcellular fractionation of cells harboring both wild-type DAGK and a single-cysteine mutant (C116, see Results). The vast majority of the overexpressed DAGK in both cases was found to be associated with the pellet, which ensues from low speed centrifugation ($<30000g$) following cell lysis (but prior to detergent extraction). This indicates that most of the overexpressed DAGK goes into inclusion bodies or into nonclassical membrane assemblies (cf., *21*−*24*) of unusually high density. Empigen was effective at completely solubilizing this post-lysis pellet.

Some purifications in this study were also carried out in which β-octylglucoside (Anatrace) was used in place of Empigen at early stages of purification, as described elsewhere (*1*). We observed that BOG was not as good a solubilizing agent as empigen, being able to solubilize only a fraction of the post-lysis pellets. Thus, final yields of pure DAGK obtained following extraction using Empigen were generally higher than when BOG was used at the extraction step.

Vesicular and Mixed Micellar Assays for DAGK. Purified DAGK can be assayed in mixed micellar solutions, as has been the case in prior kinetic characterizations of the enzyme (*20*, *25*, *26*). The mixed micellar method used in this study involves coupling the DAGK reaction to the reactions of pyruvate kinase and lactic dehydrogenase so that DAGK's reaction progress can be conveniently followed by monitoring the coupled oxidation of NADH at 340 nm (see ref 20 for details).

For directly assaying DAGK in unlysed bilayered lipid vesicles, the standard assay method was adapted for this study by eliminating all detergent from the assay mix and using a water soluble form of diacylglycerol:dibutyrlglycerol (DBG, ref *26*), which can partition into lipid vesicles containing DAGK. Details of this assay method will be provided elsewhere.

Refolding of Micellar DAGK Using Urea. DAGK was purified into DM micelles as described above and then diluted 1:10 into a "refolding solution" (4 M urea, 1% Triton X-100, 200 mM imidazole, 0.25 mM TCEP, pH 8) where it was incubated for 1−24 h at room temperature or 37 °C. Aliquots were then diluted back into the mixed micellar assay solution (above) to complete the refolding process and activities were measured and compared to the activities measured immediately following purification. The optimal composition for the folding solution given above was determined following systematic screening of solution pH (the 2−11 range was tested), detergent type (Empigen, BOG, DM, DPC, cholate, Triton X-100 and dodecyl sulfate were tested), presence of a lipid additive (cardiolipin and dodecylsulfonate were tested, but neither was found to enhance refolding), and urea concentration (the 0−8 M range was tested).

Attempts to refold DAGK bound to the Ni(II)-agarose resin were also made by incubating the pure enzyme on-resin with the urea refolding solution (above), followed by reequilibration of the DAGK/resin with 1% DM, and elution of the protein from the resin in DM plus 0.3 M imidazole

(the standard elution procedure). A similar refolding procedure was previously forwarded by Rogl et al. (*21*).

Bilayer Reconstitution of DAGK. In all of the membrane reconstitutions undertaken in this study, a purified micellar solution of DAGK was mixed with a POPC-detergent mixed micellar stock solution such that the moles DAGK monomer per mole POPC ratio is 1:120. These solutions were transferred to dialysis tubing (Spectra-Por 1.1 molecular weight cutoff = 8000, Spectrum, Houston, TX) and dialyzed extensively against a detergent/lipid-free buffer. Typically, each solution was subjected to 3−4 changes of a >1000X volume of buffer, for 10−30 h each.

When DAGK was reconstituted using the "glycoside-based method" the purified enzyme in DM solution was mixed with a solution containing 300 mM BOG (Anatrace, Maumee, OH), 75 mM POPC, 50 mM HEPES, 300 mM NaCl, and 1 mM EDTA, pH 8.0. DAGK mixtures were dialyzed as summarized above at room temperature or 4 °C vs multiple changes of a buffer containing 10 mM imidazole, 0.1 mM DTT, and 0.5 mM EDTA, pH 7.8. Results varied little when dialysis was against this low salt buffer rather than against an otherwise identical buffer containing 300 mM NaCl.

When DAGK was reconstituted using the DPC-based "reconstitutive refolding" method, the enzyme was purified by replacing DM for DPC in the final two steps of the standard purification process described earlier. Because DAGK in DPC micelles is prone to activity loss due to freeze−thaw, newly purified samples were immediately subjected to reconstitution rather than being frozen and stored. DPC−POPC stock solutions were prepared by mixing DPC (Anatrace, Maumee, OH) and POPC (Avanti, Alabaster, Alabama) in quantities such that the final solution contains 200 mM DPC and 50 mM POPC in a buffer containing 50 mM HEPES, 300 mM NaCl, and 1 mM EDTA, pH 8.0. To completely dissolve the POPC, it was necessary to subject the DPC−POPC stock mixtures to repeated freeze−thaw cycles using liquid nitrogen to freeze and mild bath sonication during thawing. DPC−POPC-DAGK mixtures were dialyzed as summarized above at room temperature vs multiple changes of a buffer containing 10 mM imidazole, 0.2 mM DTT, and 0.5 mM EDTA, pH 7.8. Results were slightly more favorable when dialysis was against this low salt buffer rather than against an otherwise identical buffer also containing 300 mM NaCl. It should also be noted that the success of the DPC-based method in promoting DAGK refolding is dependent upon the use of an appropriate grade of dialysis tubing. We found Spectra-Por 1.1 tubing to be satisfactory, but not Spectra-Por 1 or Pierce Chemical Co. (Rockford, Illinois) "Snakeskin" brand tubing. The membranes for these brands of tubing appear to be thicker than Spectra-Por 1.1 (while having similar molecular weight cutoffs) and the process of detergent removal was observed to be much slower, suggesting that too-slow detergent removal results in inefficient refolding.

DPC-derived POPC-DAGK vesicles can be repetitively freeze−thawed following preparation without a loss in DAGK activity.

Chemical Cross-Linking of DAGK. A 5−25 μM sample of DAGK in micelles or lipid vesicles was incubated at room temperature with 16 mM glutaraldehyde in buffer containing 2% DM, 20 mM phosphate, 50 mM NaCl, 1 mM EDTA, 0.5 mM TCEP, pH 7.5. For experiments in which DAGK was to be cross-linked within lipid vesicles, detergent was excluded from the buffer. Reaction tubes were rotary-agitated at 300 rpm for 21 h under argon at room temperature. Reactions were terminated by diluting into SDS-PAGE loading buffer (25 mM Tris-HCl, 40% glycerol, 8% SDS, 0.01% bromophenol blue). Samples were loaded directly onto Novex (San Diego, CA) 10−20% Tris-Glycine or 4−12% Bis-Tris gels and subjected to PAGE according to standard procedures.

Electron Microscopy of DAGK−POPC Aggregates. Samples were prepared for electron microscopy by spreading a small volume of reconstituted DAGK vesicle samples directly onto Formvar- and carbon-coated 200 mesh copper grids. After approximately 1 min, the excess sample was removed and the grids were washed with distilled water. The excess solution was removed and the sample was stained with 1% uranyl acetate and subjected to electron microscopy.

RESULTS

Many Purified DAGK Mutants Exhibited Problems With Misfolding. The laboratory of James Bowie recently generated a library of about 100 single-cysteine DAGK mutants (unpublished). To accomplish this, a cysteine-less DAGK mutant was first prepared by mutating the two Cys present in wild type DAGK to alanine. The Cys-less mutant is fully active and very stable, as in the case of the wild-type enzyme. A single cysteine was then introduced into the Cys-less mutant, with the sequential location of the single cysteine residue being serially varied from mutant to mutant. When 93 of these mutants were purified and assayed, it was observed that 31 of the single-cysteine mutants tested showed activities which were <20% that of wild-type DAGK and that 63 mutants exhibited ≤50% activity. Such widespread loss of activity was not expected based on the high degree of sequential divergence observed among the microbial DAGK isozymes (*17*), the results of mutagenesis studies (*18*, *28*), and activities measured by the lab of James Bowie (unpublished) for some of these same mutants. On the basis of these considerations and because it seems improbable that all of the residues mutated in the reduced-activity mutants are directly involved in catalysis, we carried out a series of tests for structural perturbations using a subset of mutants.

In Table 1, DAGK specific activities are reported for a cross-section subset of 28 mutants, which were purified using a method involving the use of the detergent Empigen to extract the enzyme from the lysed cells and decyl maltoside in subsequent column purification steps. Each mutant was purified multiple times, typically from multiple original cell cultures. As summarized in column 2 of the table, DAGK activity was often highly variable from batch to batch. Routine SDS-PAGE revealed little variation in protein purity from batch to batch, suggesting that batch to batch irreproducibility of activity reflects varying amounts of an improperly folded form of the protein in each batch. Consistent with this observation are results obtained when a number of the mutants were purified using a different detergent at the initial lysis step (BOG) instead of Empigen. The specific activity of mutants purified following BOG extraction (data not shown) were sometimes observed to be very different from those following Empigen extraction, even when identical batches of cells were used for the two methods and even

Table 1

mutant	range of activities observed following purification (no refolding)[a,b]	activity of batch used for cross-linking	GA cross-linking pattern[c]	activities before and after urea incubation[d]	activities before and after DPC-based reconstitution[e]
WT[f]	100	100	normal	100 → 96	90 ± 5 → 100 ± 3
R9C	0.6−1.0	0.2	normal	0.5 → 0.7	0.1 ± 0.08 → 0.5 ± 0.1
I11C	8−50	36	normal	ND[h]	30 → 82
I26C	36−50	3	normal	ND	45 ± 9 → 58 ± 33
R32C	0−0.5	0.5	M,d,t,tt	0.0 → 0.0	0.4 → 0.6
Q33C	3−16	13	normal	6 → 10	12 ± 5 → 26 ± 11
V38C	25−77	84	normal	ND	36 → 94
L48C	3−6	4	M,d,t,TT	ND	2 ± 1 → 64 ± 15
A52C	11−70	ND	M,D,t,TT	ND	8 ± 7 → 64 ± 9
I53C	40−120	55	normal	ND	83 ± 12 → 100 ± 10
R55C	2−120	18	M,D,t,tt	ND	11 ± 7 → 65 ± 24
L58C	0−15	0.4	M,D,t,tt	0.3 → 3	1 → 83
I59C	1−15	0.9	M,d,t	ND	2 ± 1 → 78 ± 15
V62C	10−80	75	M,d,t,TT	10 → 93	37 → 110
M63C	5−100	73	normal	ND	26 ± 9 → 61 ± 5
V65C	0−15	39	M,D,t,tt	13 → 35	9 → 99
I67C	1−10	2	M,d,t,tt,p	2 → 50	9 ± 1 → 84 ± 13
A74C	3−30	9	M,d,t,tt	ND	3 → 55
I75C	0−5	0.0	M,d,t	0 → 0	2 ± 1 → 30 ± 6
G97C	0−1	0.0	M,d	0.6 → 0.7	0.3 → 2
V101C	0.5−10	3	M,d,t	0.0 → 2	22 ± 4 → 51 ± 7
I103C	10−80	15	normal	ND	14 ± 2 → 73 ± 8
A104C	1−10	7	M,d	4 → 34	7 ± 3 → 79 ± 10
V107C	70	63	normal	ND	31 → 53
I110C	4−32	6	M,D,t,TT	ND	6 → 115
T111C	2−15	15	M,d,tt	9 → 110	2 ± 1 → 73 ± 11
C113C	100	120	normal	ND	52 → 96
G121C	5−25	25	M,d,t	ND	64 ± 3 → 83 ± 5

[a] All activities reported in this table were measured using the mixed micellar assay and are reported as % activity relative to wild-type enzyme. The absolute specific activity of WT is ca. 110 units/mg. "100" means activity is same as WT. [b] When a range of values is reported in this column, this indicates the maximum degree of variation observed when this mutant was purified multiple times from multiple batches of cells. [c] Results from glutaraldehyde cross-linking of nonrefolded DAGK in DM under a standard set of conditions (see Results) as followed by SDS−PAGE. M,D,T,TT,P stands for monomer, dimer, trimer, tetramer, pentamer; capitals vs small letters are used to indicate relative SDS−PAGE band intensities (high and low, respectively). "Normal" means primary conversion to trimer with smaller amounts of monomer and dimer present and no tetramer: by this notation "normal" would be m,d,T. [d] In addition to the mutants described in this table, additional single-Cys mutants were also tested: for mutants 23, 31, 57, 80, 86, 115, and 116 greater than 2X increases in activity were observed following urea incubation. Mutants for positions 17, 19, 21, 25, 28, 69, 73, 76, 81, 85, 89, 93, 97, and 98 exhibited < 2X increases in activity. [e] Activities measured after taking vesicular DAGK and redissolving it back into DM micelles followed by mixed micellar assay. Standard deviations are given for cases where the reconstitution procedure was carried out more than once for the mutant in question. Results for other mutants are given in Figure 2. [f] Wild-type results are from wild-type, Cysless, or the C46 DAGK mutant. These three forms of DAGK have been observed to behave extremely similarly both structurally and kinetically in a variety of studies and so results from these three forms of the protein are lumped together. [g] ND: not determined.

though the final solution compositions and DAGK purity were the same (see Methods). While DAGK activities following BOG-based purification were often higher than activities following Empigen-based purification, this was not always the case. Screening carried out in our lab to find detergents for extraction and final purification steps that led to DAGK preparations of consistent activities were not successful.

Samples purified into DM micelles by the standard method were also incubated overnight at 37 °C. For nine of the mutants listed in Table 1, there was a >15% gain in activity following incubation, consistent with spontaneous refolding of misfolded protein.

Finally, it should be pointed out that misfolded DAGK does not appear to represent the results of nonspecific aggregation. Only a few of the 93 DAGK mutants that were purified and cross-linked (below) showed evidence for such aggregation in the form of visible light scattering or very high molecular weight oligomers following cross-linking (see later section of Results).

Refolding of Micellar DAGK Can Sometimes Be Accomplished Using Urea. Rogl et al. (*21*) have reported that some integral membrane proteins can be refolded while bound to a metal ion chelate affinity resin by incubating suspensions of the resin with urea-containing detergent solutions. While we were unable to adapt their on-resin procedure to DAGK with much success, an in-solution adaptation proved more effective. The best conditions were found to involve incubation of DAGK in Triton X-100 and 4 M urea for several hours. About 30 single-cysteine DAGK mutants were subjected to urea exposure using this method and then diluted back into a urea-free DM solution and assayed. As summarized in the fifth column and footnote e of Table 1, about half of the mutants tested exhibited substantial increases in activity following incubation, indicative of refolding. As will be seen, most of the mutants for which a gain in activity was *not* observed following the urea treatment were, in fact, misfolded. This indicates that the urea procedure does not work for all mutants: it was effective for about half of the mutants tested.

Additional DAGK Misfolding Can Occur During Bilayer Reconstitution. In addition to misfolding during or prior to DAGK purification, misfolding can also be induced by the process of reconstituting the purified enzyme into lipid

bilayers using the published methods. These procedures (*16*) call for mixing pure DAGK in DM micelles with DM/POPC or BOG/POPC mixed micellar solutions, followed by removal of the detergent component(s) by dialysis and formation of DAGK−POPC vesicles. This general approach is referred to in this paper as the "glycoside-based" method. When wild-type DAGK and single-cysteine mutant numbers 26, 53, 58, 74, 75, 104, 113, and 121 were reconstituted using this method and then assayed in mixed micelles, no gains in catalytic activity were observed following reconstitution and in 7 out of 9 cases (including wild-type) there were >15% losses in activity. Variation of the glycoside-based method by varying dialysis pH (*5*−*9*), salt content (10−300 mM), or lipid type (*E. coli* lipids, DOPC, POPC/POPG mixtures, DOPC/DOPG mixtures, DPPC, and DMPC) did not lead to a method which reliably maintained the original activity following reconstitution. Moreover, in no case did the reconstitution process lead to an increase in activity. The glycoside-based reconstitution method is not ideal, even for generally well-behaved forms of DAGK.

Reconstitutive Refolding of DAGK. Given the failure of the glycoside-based reconstitution method to reliably maintain DAGK activity, an alternate set of reconstitution methods were screened. For these tests, the final steps of DAGK purification were altered. Instead of equilibrating and eluting the Ni(II)-agarose/DAGK column with decylmaltoside solutions, DM was replaced in these steps by other detergents. Accordingly, mutant I75C DAGK was purified using Triton X-100, CHAPSO, DM, BOG, β-dodecylmaltoside, or dodecylphosphocholine as the detergent in the final two steps. To each of the purified micellar DAGK solutions was then added a mixed micellar solution containing the same detergent used for purification and POPC (typically 200 mM detergent and 50 mM POPC). The DAGK:POPC molar ratio was 1:120. The single detergent component of each mixture was then removed by dialysis for 86 h (including 5 changes of dialysis solution) at room temperature. To ensure that residual detergent was not present in the final vesicular samples, we then treated each post-dialysis solution with excess Bio-Beads (SM-2, Bio-Rad, Hercules, CA) for 3 h at room-temperature according to well established procedures (*29*, *30*). Vesicular solutions were subjected to both micellar and vesicular assays before and after Bio-Bead treatment, with results being qualitatively the same before and after treatment. In each case, except for dodecylphosphocholine (DPC), the micellar and vesicular activities of the I75C mutant remained below 2 U/mg following reconstitution. However, in the case of DPC the post-reconstitution micellar and vesicular activities were 16 and 18 U/mg, respectively. This is in contrast to the pre-reconstitution micellar activity of <1 U/mg observed for DPC and all of the detergent types investigated. This result indicates that events occur that lead to refolding when DPC is dialyzed out of solutions containing I75C DAGK and POPC. This result led to the investigation of whether the DPC-based reconstitution method could be applied with success to other DAGK mutants.

Figure 2 illustrates the activities of DAGK mutants before and after reconstitution by the DPC/POPC-based procedure. These measurements were made using the mixed micellar assay system following redissolution of the DAGK−POPC vesicles into DM micelles. As illustrated by Figure 2, activities for 55 out of 65 mutants increased significantly

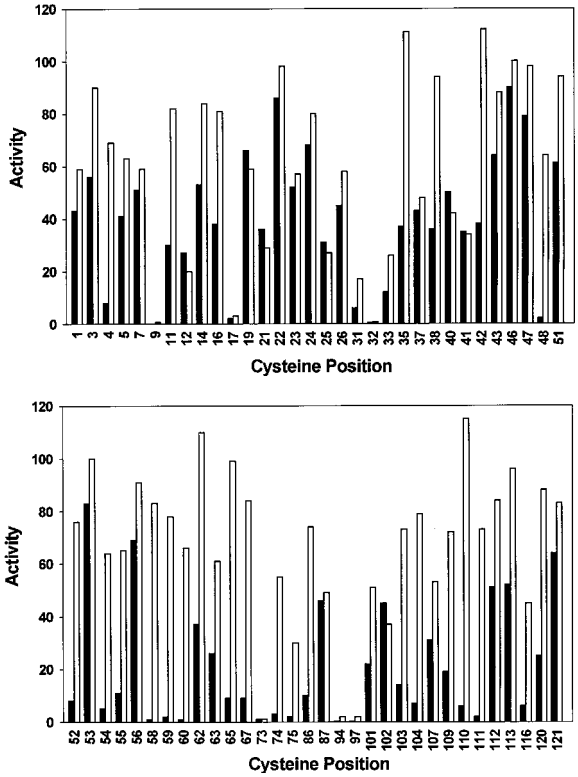

FIGURE 2: Catalytic activities for single-cysteine DAGK mutants in mixed micelles measured before (closed bars) and after (open bars) DPC-based reconstitutive refolding into POPC vesicles. Activities are reported as a percentage of the fully active wild-type DAGK activity.

following DPC-based reconstitution. Indeed, for 33 mutants the gain in activity was greater than 2-fold. Direct in-vesicle activities were also measured after reconstitution and these (not shown) generally correlated very well with the post-reconstitution micellar activities. We refer to the DPC/POPC-based method for simultaneously reconstituting and refolding DAGK as "reconstitutive refolding".

A number of the mutants that exhibited substantial refolding when reconstituted from DPC/POPC mixtures were also reconstituted by a procedure in which DPC was used but where POPC was replaced by DPPC. In these cases, refolding was not observed. Refolding was also not observed when DAGK was purified in DM followed by addition of DPC/POPC and detergent removal: it was critical that DPC be the only detergent component present when detergent removal was commenced.

Refolding of DAGK During DPC-Based Reconstitution Occurs At A Late Stage in Detergent Removal. Two mutants (C11 and C110) were subjected to DPC/POPC reconstitutive refolding, with aliquots being removed from the dialysis tubing and assayed at various stages during the reconstitution process to determine when refolding takes place. There was no observed gain in activity for either mutant at any of several points taken up through 20 h of dialysis involving two changes of solution. At this 20 h point, detergent removal had progressed to the stage where the lipid−protein aggregates were becoming large enough to scatter light. After an additional 30 h of dialysis involving two additional changes of buffer solution, the now smoothly opaque DAGK vesicular solutions were again assayed. At this final step, the activities of the two mutants had increased by factors of

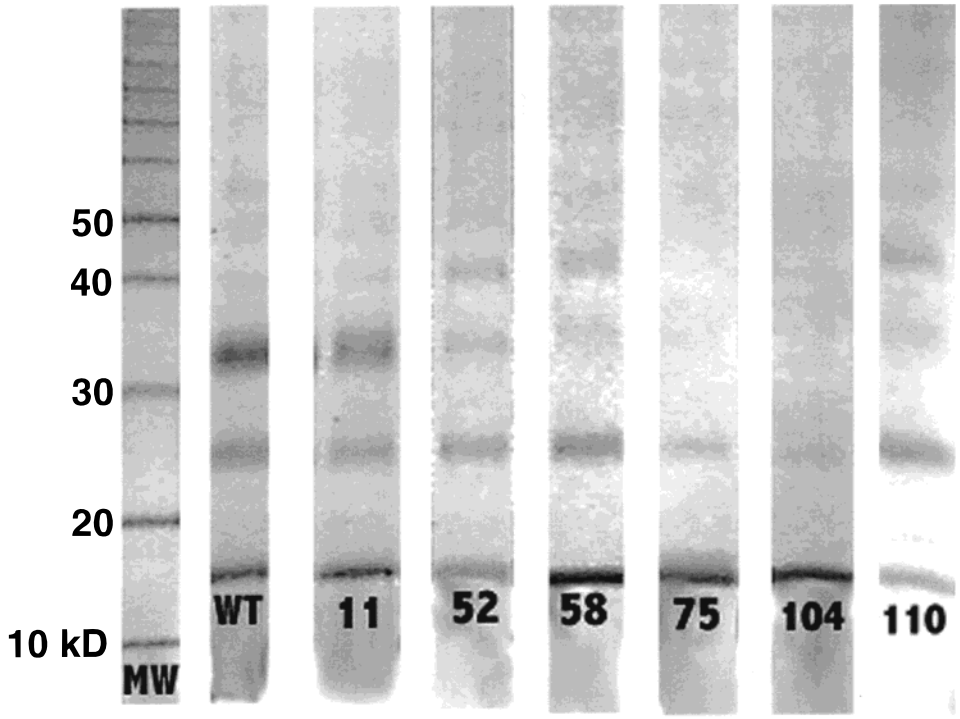

FIGURE 3: SDS-PAGE of glutaraldehyde cross-linked micellar DAGK single-cysteine mutants. Lanes are labeled according to the location of the single cysteine in the represented mutant. DAGK was incubated with 16 mM glutaraldehyde in 1% DM buffer at room temperature for 21 h with shaking. Reactions were then combined with SDS sample buffer and loaded onto Novex gradient 10−20% Tris-Glycine gels.

2.7 (C11) and 51 (C110). This result indicates that refolding did not take place at an even pace throughout the detergent removal process. Instead, all of the refolding occurred in a late stage of reconstitution, after the formation of lipid−protein-detergent aggregates large enough to scatter light.

Chemical Cross-Linking of Properly and Improperly Folded DAGK. Several techniques were examined as possible routes to more direct structural insight into the folding states of the purified mutants. Near-UV CD spectroscopy yields spectra that are sensitive to protein tertiary structure. However, micellar DAGK yields only a very weak near-UV CD signal (*19*), to the extent that we found it impossible to reliably reproduce spectra. Solution ^{1}H NMR spectra of micellar DAGK were also not illuminating because of the extremely broad resonances from most sites in the protein (cf. ref *19*). The most revealing method tested was chemical cross-linking using glutaraldehyde (GA). Fully active wild-type DAGK yields a reproducible SDS−PAGE pattern after running GA cross-linking in DM micelles (Figure 5 and reference *19*): a band which represents the cross-linked trimer predominates, while slightly less monomer and significantly less dimer persist.[2]

GA cross-linking results for the other mutants purified by the Empigen method are summarized in column 4 of Table 1, with representative SDS-PAGE data being illustrated in Figure 3. A majority of the mutants showed significant perturbations in their cross-linking patterns from that of wild-type. Cases are observed in which the monomeric form was found to predominate, in which monomer and dimer were present about equally, and in which tetrameric forms predominated. In most of the abnormal cases, cross-linking was repeated at a 20-fold lower DAGK concentration (while maintaining a constant detergent concentration) in order to establish whether the unusual cross-linking patterns observed could be the result of cross-linking between DAGK oligomers. In most cases, the dilute cross-linking results were similar to the results in more concentrated solutions, indicating that the abnormal cross-linking patterns reflect changes in the distribution of discrete DAGK oligomers rather than in perturbations in the tendency of discrete oligomers to aggregate.

DAGK activities are listed in column 3 of Table 1 for the batches of enzyme used in the cross-linking studies. It can be seen that normal cross-linking patterns do not always correlate with high enzyme activity. However, aberrant cross-linking patterns are generally excellent predictors for low DAGK activity.

Cross-linking was carried out on a number of micellar samples representing DAGK that had first been subjected to reconstitution by the DPC-based method or by the glycoside-based procedure. As illustrated in Figure 4, the DPC method led to DAGK samples exhibiting micellar cross-linking patterns on SDS-PAGE gels that better approximated correctly folded wild-type control patterns than corresponding samples, which had been reconstituted by the glycoside method. Similar results were also obtained when mutants known to be refolded by the micellar urea procedure were cross-linked before and after refolding (not shown).

DAGK mutants were also directly cross-linked in vesicles formed by both the DPC-based procedure and in bilayers formed by the glycoside-based procedure. Properly folded

[2] It should be noted that by using very long GA cross-linking times or using very high GA concentrations (*19*), it is possible to obtain SDS-PAGE that exhibit almost complete conversion to trimer (little monomer or dimer left over). We have chosen "standard" GA reaction conditions for the purpose of this work with shorter reaction times (for reasons of convenience) and lower GA concentrations (to avoid possible artifacts associated with very high concentrations of an organic solute).

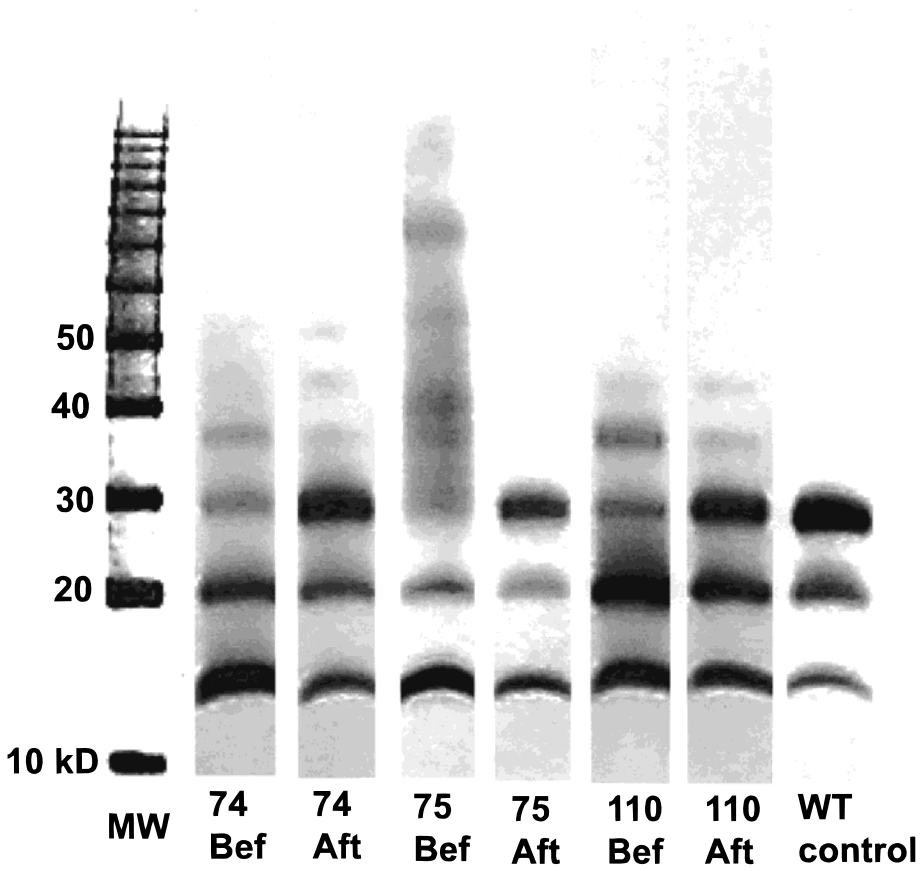

FIGURE 4: SDS-PAGE of glutaraldehyde cross-linked DAGK single-cysteine mutants in micelles before and after DPC−POPC reconstitution. Both forms were incubated with 16 mM glutaraldehyde in 1% DM buffer at room temperature for 21 h with shaking. The numbers labeling each lane give the position of the single cysteine present in the represented mutant. The gels used were Novex gradient 4−12% Bis-Tris gels (different from the gel type used for Figure 3). Silver staining was used.

(DPC-derived) samples yielded SDS-PAGE patterns (Figure 5) following cross-linking that were similar to results from micellar samples: the major species is trimeric. The fact that lesser amounts of hexamer and higher oligomers of trimers are also observed is not unexpected, given the relatively high DAGK content in the vesicles (weight DAGK:weight lipid is ca. 1:6). Samples reconstituted by the glycoside-based procedure and observed to be misfolded based on low activities clearly failed to assemble into trimers as shown by SDS-PAGE results of Figure 5. In some of the misfolded cases (C58, C65, and C74), an unusual pattern was observed where most of the protein was observed to run near the gel tracking dye. This suggests that reconstitution of these proteins by the glycoside-based method led to the formation of lipid−protein aggregates that are not completely disrupted by SDS and run anomalously on the PAGE gels.

Characterization of the POPC-DAGK Aggregates Formed During Reconstitutive Refolding. When DPC is dialyzed out of POPC-DAGK mixtures, the solutions take on a milky appearance characteristic of the formation of lipid vesicles large enough to scatter visible light. Confirmation of vesicles was sought by electron microscopy. Shown in Figure 6, A and B are electron micrographs of a DAGK vesicle preparation at two different vesicle dilutions. The more concentrated sample clearly shows that the sample contains vesicles having a heterogeneous distribution of sizes where the smallest vesicles are on the order of 300 Å and the largest vesicles exceed 2000 Å in diameter. Excluding the few large vesicles in this micrograph, the average vesicle diameter was found to be 520 ± 190 Å. In the more dilute case, the sample appears more homogeneous probably because there are fewer vesicles viewed in the sample. The vesicle sizes in this image are smaller with an average diameter of 440 ± 90 Å. In both micrographs, there appears to be either membrane and/or protein material that is nonvesicular. The exact nature of the nonvesicular material is not clear. Phosphorus-31 NMR spectra of these solutions yield a single axially symmetric powder pattern that is characteristic (*31*) of liquid crystalline lipid bilayers (data not shown) and inconsistent with the presence of a significant nonbilayer lipid population. NMR analysis also indicated that the concentration of free DPC present in DAGK−POPC samples following extensive dialysis is <0.3 mM, well below DPC's critical micelle concentration.

DAGK is associated with the vesicles. The supernatant above the lipid pellet following ultracentrifugation of DPC-derived POPC-DAGK vesicles does not contain significant amounts DAGK. Moreover, the fact that DAGK exhibits high catalytic activity when directly assayed in vesicular solutions indicates that the enzyme is freely accessed by the added diacylglycerol which partitions into the lipid bilayers present.

DISCUSSION

Misfolding of Diacylglycerol Kinase. The results of this study document widespread problems with misfolding of

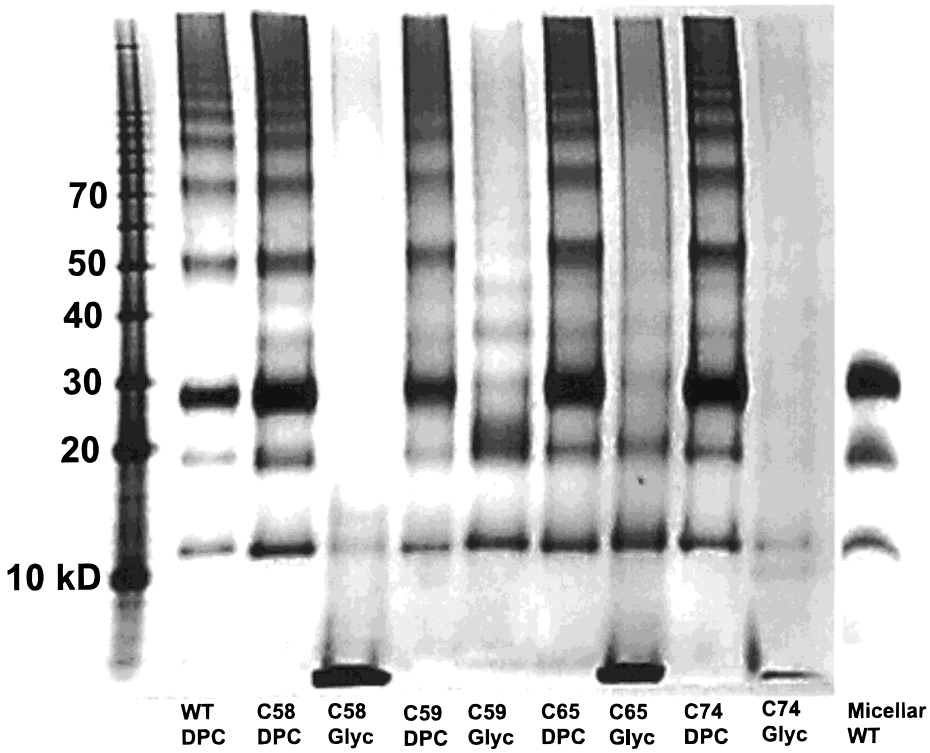

FIGURE 5: SDS-PAGE of glutaraldehyde cross-linked DAGK mutants in POPC vesicles generated using either the DPC-based (reconstitutive refolding) or the glycoside-based procedure. Vesicular DAGK was then incubated with 16 mM glutaraldehyde (in detergent-free buffer) for 21 h at room temperature with shaking. The position of the single cysteine present in each mutant represented is given below each lane. The gels used were Novex gradient 4−12% Bis-Tris gels (different from the gel type used for Figure 3). Silver staining was used.

DAGK mutants at stages of both micellar purification and membrane reconstitution. In the latter case, it was clear that the actual process of reconstitution could induce misfolding. In the case of micellar purification, the results were inconclusive as to whether DAGK was misfolded in the process of cellular overexpression into inclusion body-like aggregates (see Methods) and/or whether the purification process itself caused misfolding. In any case, misfolding does not appear to be the result of nonspecific aggregation. Misfolded forms of DAGK represent kinetically trapped structural states as opposed to thermodynamically preferred species. Whether there is a correlation between the tendency to misfold and the thermodynamic stability of the correctly folded structure remains to be seen.

The cross-linking results suggest that the structures of the misfolded mutants in micelles are heterogeneous from mutant to mutant. Some dysfunctional DAGK mutants that were ultimately shown to be refoldable yielded normal cross-linking patterns in micelles, even prior to refolding. This suggests that these mutants assembled into trimers but had aberrant subunit-subunit contacts and/or tertiary conformations. Other DAGK mutants exhibit little oligomerization at all, while others showed a preference for forming dimers or even tetramers. This does not necessarily indicate that the overall pathway for DAGK misfolding differs from mutant to mutant. Various misfolded forms may represent proteins that have headed down the same folding pathway but have made wrong turns at different points into different cul-de-sacs.

Misfolding/refolding results for cysteine replacement mutants can be correlated with what is known (see Figure 1) about the degree of sequence conservation of the mutated sites from multiple alignment of available prokaryotic DAGK sequences and from random mutagenesis studies (*17, 18*). One set of conserved site mutants show low activities both before and after refolding (e.g., Cys replacements at positions 17, 73, and 95). These results support the notion that these sites represent catalytically important residues. Other cysteine-replacement mutants for conserved positions exhibited low activities before refolding and high activity following refolding (e.g., positions 54, 59, and 104). These sites are probably not catalytically essential. Instead, our results suggest that these sites may be conserved because of their importance in DAGK's folding pathway. This is a hypothesis that may warrant additional experimental testing. Finally, there are a number of conserved sites for which Cys mutants show reasonably high specific activities (>20% of wild-type) both before and after refolding. For some of these positions (e.g., 14, 23, 24, 41, 53, 63, and 102), mutation to cysteine represents a fairly conservative mutation and so these results are uninformative. However, in two cases (K12C and D51C) the relatively high activity of the mutants before and after mutation suggest suggests that these sites are required for some role other than catalytic or folding efficiency. Such roles could include contributing to in vivo protein stability or interactions with other (non-DAGK) proteins, such as might occur during the process of biosynthesis and membrane insertion.

Reconstitutive Refolding of DAGK. Previous reports of refolding of purified membrane proteins have typically involved denaturation using urea or certain detergents followed by transfer of the protein back into a nondenaturing micelle or lipid phase (cf. refs *3, 32*−*35*). Two methods for refolding DAGK in micelles were documented but found to be inadequate for most applications. A few misfolded forms of micellar DAGK were observed to undergo a significant

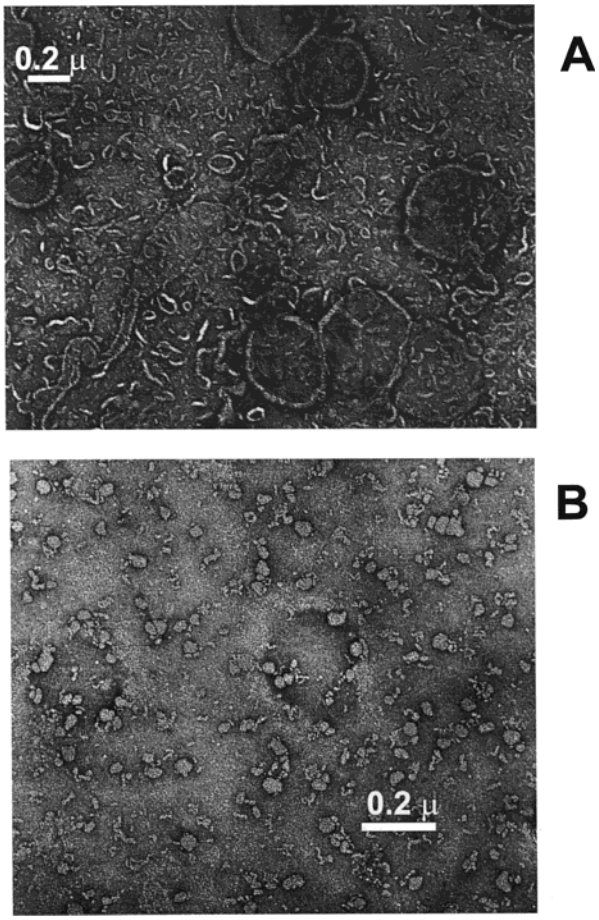

FIGURE 6: Transmission electron micrographs of an unfixed "reconstitutively refolded" DAGK/POPC sample. The DAGK/POPC sample was spread onto Formvar- and carbon-coated grids and stained with uranyl acetate. The N3C single cysteine DAGK mutant was used and the sample had been freeze−thawed once. In A, the samples were spread at a DAGK concentration of 1.5 mg/mL. In B, the samples were diluted 10:1 in distilled water before spreading. Although a few large vesicles are seen in A, the majority of the vesicles are small, having vesicle diameters of 520 ± 190 and 440 ± 90 Å in A and B, respectively.

degree of spontaneous refolding upon simple incubation in micelles, indicating that for these forms the height of the energy barrier between misfolded and folded (equilibrium) states is low. An even higher fraction of misfolded mutants could be refolded by micellar incubation in the presence of 4 M urea. The fact that urea was not effective for about half of the mutants may reflect differences in energy barriers between different misfolded forms and the properly folded protein: urea may lower refolding barriers for all mutants by the same absolute quantity, which in some cases is sufficient to permit refolding, while in others it is not. Alternately, differences in responsiveness to the urea treatment may reflect mutant-specific variation in the nature of the pathways between states such that urea's reduction of energy barriers may be very different from mutant to mutant.

This work shows that misfolded DAGK can best be refolded using a straightforward procedure involving purification of DAGK in DPC followed by addition of DPC/POPC mixed micelles and removal of DPC by dialysis. DAGK mutants in DPC-derived vesicles typically exhibit both high in-vesicle activities and high mixed micellar activities when the vesicles containing DAGK were redissolved. Other detergents tested could not be substituted for DPC. Also, refolding did not occur when POPC was replaced with another lipid (DPPC). Refolding occurs at a very late stage in the reconstitution process, during the final transition to DPC−POPC aggregates large enough to scatter light. Misfolding due to reconstitution is avoided by this method and misfolding present at the stage of micellar purification is largely corrected. About 90% of the mutants tested exhibited some degree of refolding during reconstitutive refolding, with about half showing greater than 2-fold gains in catalytic activity. However, it must be acknowledged that reconstitutive refolding may not be *absolutely* effective. Using the DPC-based method, 5 of 65 mutants exhibited modest losses (<25%) of activity. Also, as reflected by the sizes of the standard deviations in the post-refolding activities given in the last column of Table 1, there are some mutants which, when refolded from batch to batch exhibit different final specific activities. For these cases, the observed efficiency appears to be a function of the degree of misfolding under pre-reconstitution conditions and of the detailed variables of the reconstitution process (such as the rate of detergent removal). Despite these caveats, the DPC-based method is superior to other refolding or reconstitution methods we tested.

It is difficult to say exactly why the DPC-reconstitution procedure is so effective for DAGK when very similar procedures involving more commonly used detergents failed. Detergents were tested and observed to be ineffective, which had critical micelle concentrations greater than DPC (BOG, CHAPSO) about the same as DPC (DM), and much lower than DPC (Triton and DDM). There may be properties associated with the intermediate DPC−POPC aggregates that appear during the process of detergent removal that are particularly favorable for DAGK refolding and membrane insertion. Alternately, there may be unique qualities associated with DAGK-DPC interactions which facilitate proper folding and insertion into bilayers. We are not aware of any previous reconstitution procedure involving the use of DPC. Moreover, while lipid-free DPC micelles have been well-characterized and are frequently used in NMR studies of membrane proteins (see refs *36*−*38*) virtually no information exists in the literature regarding the size, structure−morphology, or dynamics of lipid-DPC aggregates. Such basic information will ultimately be required to understand why results obtained using this detergent were so uniquely and generally positive.

CONCLUSIONS

We have documented severe problems involving misfolding of single-cysteine DAGK mutants and have presented a new and extremely effective method for refolding such mutants. This method represents a very welcome development in the study of DAGK, because structural biophysical studies involving purified single-cysteine mutants of the enzyme have until now been plagued by irreproducibility, which probably stems from the misfolding problems documented in this study. For example, results we have obtained from disulfide mapping studies involving about 75 DAGK mutants that were not subjected to refolding are very different from the results for the same set of mutants following reconstitutive refolding (Nagy and Sanders, unpublished).

Relatively few membrane proteins have been overexpressed at the level of tens of milligrams per liter of culture

(as for DAGK), and for even fewer of these have large mutant libraries been prepared and purified (for notable examples where misfolding does not seem to be a problem, see refs *38*−*40*). Thus, it is difficult to say with certainty just how widespread the sort of misfolding problems we have documented will turn out to be. However, the association of membrane protein misfolding with a number of disease states (*7*−*13*) indicates that misfolding is a very important problem, regardless of how common it is. It is also difficult to say whether the reconstitutive refolding procedure worked out for DAGK in this paper will prove to be generally effective for other membrane proteins as a way to avoid reconstitution-induced misfolding or to correct misfolding that may already be present. In any case, it is hoped that the results of this paper are striking enough to motivate efforts to apply the DPC/POPC-based reconstitution method to other membrane proteins and to elucidate why this method proved so effective for DAGK.

ACKNOWLEDGMENT

We thank James Bowie and members of his laboratory at UCLA (Yufeng Zhou, Francis Lau, and Sehat Nauli) for their generosity in providing us with the DAGK overexpression systems used in this study. Finally, we thank Drs. Mel Keyes and Don Gray of Anatrace, Inc. for providing us with samples of DPC, which were used in the preliminary studies leading to this work.

REFERENCES

1. Lau, F. W., and Bowie, J. U. (1997) *Biochemistry 36*, 5884−5892.
2. Booth, P. J., and Curran, A. R. (1999) *Curr. Opin. Struct. Biol. 9*, 115−121.
3. Fleming, K. G., Ackerman, A. L., and Engelman, D. M. (1997) *J. Mol. Biol. 272*, 266−275.
4. He, M. M., and Kaback, H. R. (1998) *Mol. Membr. Biol. 15*, 15−20.
5. Fekkes, P., and Driessen, A. J. M. (1999) *Microbiol. Mol. Biol. Rev. 63*, 161−173.
6. De Gier, J.-W., Scotti, P. A., Saaf, A., Valent, Q. A., Kuhn, A., Luirink, J., and von Heijne, G. (1998) *Proc. Nat. Acad. Sci. U.S.A. 95*, 14646−14651.
7. Jung, M., Sommer, I., Schachner, M., and Nave, K. A. (1996) *J. Neurosci. 16*, 7920−7929.
8. Ridge, K. D., Lu, Z., Liu, X., and Khorana, H. G. (1995) *Biochemistry 34*, 3261−3267.
9. Seibert, F. S., Loo, T. W., Clarke, D. M., and Riordan, J. R. (1997) *J. Bioenerg. Biomembr. 29*, 429−442.
10. Goldberg, A. F., Loewen, C. J., and Molday, R. S. (1998) *Biochemistry 37*, 680−685.
11. Sisoida, S. S., and Price, D. L. (1995) *FASEB J. 9*, 366−370.
12. Thomas, P. J., Qu, B. H., and Pedersen, P. L. (1995) *Trends Biochem. Sci. 20*, 456−459.
13. Stewart, C., Bailey, J., and Manoil, C. (1998) *J. Biol. Chem. 273*, 28078−28084.
14. Kennedy, E. P. (1996) In *E. coli* and *S. typhimurium*: Cellular and Molecular Biology; Vol. I, 2nd ed., (Neidhardt, F. C., Ed.) pp 1064−1071 ASM Press, Washington.
15. Fischer, W. (1994) In Bacterial Cell Wall (Ghuysen, J.-M., Hakenbeck, R., Eds.) pp 199−215 Elsevier, New York.
16. Sanders, C. R., Czerski, L., Vinogradova, O., Badola, P., Song, D., and Smith, S. O. (1996) *Biochemistry 35*, 8610−8618.
17. Smith, R. L., O'Toole, J. F., Maguire, M. E., and Sanders, C. R. (1994) *J. Bacteriol. 176*, 5459−5465.
18. Wen, J., Chen, X., and Bowie, J. U. (1996) *Nat. Struct. Biol. 3*, 141−147.
19. Vinogradova, O., Badola, P., Czerski, L., Soennichsen, F. D., and Sanders, C. R. (1997) *Biophys. J. 72*, 2688−2701.
20. Badola, P., and Sanders, C. R. (1997) *J. Biol. Chem. 272*, 24176−14182.
21. Rogl, H., Kosemund, K., Kuhlbrandt, W., and Collinson, I. (1998) *FEBS Lett. 432*, 21−26.
22. von Meyenberg, K., Jorgensen, B. B., and van Deurs, B. (1984) *EMBO J. 3*, 1791−1797.
23. Wilkison, W. O., Walsh, J. P., Corless, J. M., and Bell, R. M. (1986) *J. Biol. Chem. 261*, 9951−9958.
24. Weiner, J. H., Lemire, B. D. Elmes, M. L., Bradley, R. D., and Scraba, D. G. (1984) *J. Bacteriol. 158*, 590−596.
25. Walsh, J. P., Fahrner, L., and Bell, R. M. (1990) *J. Biol. Chem. 265*, 4374−4381.
26. Walsh, J. P., and Bell, R. M. (1992) *Methods Enzymol. 209*, 153−163.
27. Zhou, C., and Roberts, M. F. (1997) *Biochim. Biophys. Acta 1348*, 273−286.
28. Zhou, Y., Wen, J., and Bowie, J. U. (1997) *Nat. Struct. Biol. 4*, 986−990.
29. Rigaud, J.-L., Levy, D., and Mosser, G. (1998) *Eur. Biophys. J. 27*, 305−319.
30. Levy, D., Bluzat, A., Seigneuret, M., and Rigaud, J.-L. (1990) *Biochim. Biophys. Acta 1025*, 179−190.
31. Watts, A., and Spooner, P. J. R. (1991) *Chem. Phys. Lip. 57*, 195−211.
32. Hobe, S., Prytulla, S., Kuhlbrandt, W., and Paulsen, J. (1994) *EMBO J. 13*, 3423−3429.
33. Grisshammer, R., and Tate, C. G. (1995) *Q. Rev. Biophys. 28*, 315−422.
34. Popot, J.-L., Gerchman, S.-E., and Engelman, D. M. (1987) *J. Mol. Biol. 198*, 655−676.
35. Beswick, V., Guerois, R, Corder-Ochsenbein, F., Coic, Y.-M., Huynh-Dinh, Tostain, J., Noel, J.-P., Sanson, A., and Neumann, J.-M. (1998) *Eur. Biophys. J. 28*, 48−58.
36. Kallick, D. A., Tessmer, M. R., Watts, C. R., and Li, C.-Y. (1995) *J. Magn. Reson. B109*, 60−65.
37. Vinogradova, O., Soennichsen, F., and Sanders, C. R. (1998) *J. Biomol. NMR 4*, 381−386.
38. Kaback, H. R., and Wu, J. (1997) *Q. Rev. Biophys. 30*, 333−364.
39. Frillinghos, S., Sahin-Toth, M., Wu, J., and Kaback, H. R. (1998) *FASEB J. 12*, 1281−1299.
40. Perozo, E., Cortes, D. M., and Cuello, L. G. (1998) *Nat. Struct. Biol. 5*, 459−469.

BI991292N