Biochemistry

 Volume 40, Number 17 May 1, 2001

Accelerated Publications

Conformationally Specific Misfolding of an Integral Membrane Protein†

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Received December 21, 2000; Revised Manuscript Received March 15, 2001

ABSTRACT: Membrane protein misfolding is related to the etiology of many diseases, but is poorly understood, particularly from a structural standpoint. This study focuses upon misfolding of a mutant form of diacylglycerol kinase (s-DAGK), a 40 kDa homotrimeric protein having nine transmembrane segments. Preparations of s-DAGK sometimes contain a kinetically trapped misfolded population, as evidenced by lower-than-expected enzyme activity (with no accompanying change in substrate K_m) and by the appearance of a second band in electrophoresis gels. Misfolding of s-DAGK may take place during cellular overexpression, but can also be reproduced using the purified enzyme. TROSY NMR spectra of s-DAGK as a 100 kDa complex with detergent micelles exhibit a single additional set of resonances from the misfolded form, indicating a single misfolded conformational state. The relative intensities of these extra resonances correlate with the percent reduction in enzyme activity below the maximum observed for fully folded s-DAGK. Misfolded s-DAGK exhibits a modest difference in its far-UV CD spectrum compared to the folded enzyme, consistent with a small degree of variance in secondary structural content between the two forms. However, differences in NMR chemical shift dispersion and temperature-dependent line widths exhibited by folded and misfolded s-DAGK support the notion that they represent very different structural states. Cross-linking experiments indicate that both the correctly folded enzyme and the kinetically trapped misfolded form are homotrimers. This work appears to represent the first documentation of conformationally specific misfolding of an integral membrane protein.

Protein misfolding is a phenomenon of considerable importance to human health and disease. It is now recognized that eukaryotic cells have elaborate machinery for recognizing and correcting or disposing of nascent misfolded proteins (*1*−*6*). It is also known that many different disease states are associated with the consequences of protein misfolding (*7*−*13*). Many proteins for which misfolding is disease-related are integral membrane proteins (*1*, *14*). However, only limited data are available regarding the molecular mechanisms which lead to the misfolding of membrane proteins, and little structural information is available which provides even general insight into the nature of their misfolded states (*1*, *15*). For example, it is not known whether, in general, proteins misfold to adopt specific misfolded conformations or whether misfolding generally leads to a heterogeneous mixture of aberrant conformations. Membrane proteins represent particularly difficult targets for detailed folding and misfolding studies because of the well-known difficulties associated with the practical handling and experimental structural biology of this class of proteins (*16*−*19*).

Microbial diacylglycerol kinase (DAGK)[1] represents a suitable model system for studying membrane protein folding and misfolding under well-defined conditions. This enzyme functions as a homotrimer of 13 kDa subunits, each of which

† The support of this work was provided through NIH Grants RO1 GM47485 and R21 GM59071.

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10.1021/bi0029090 CCC: $20.00
Published on Web 04/05/2001

has three transmembrane helices (*20*−*22*). DAGK catalyzes the formation of phosphatidic acid from diacylglycerol (DAG + ATP → PA + ADP). DAGK can be purified in milligram quantities and can be functionally reconstituted into a variety of model membrane systems (*21*, *25*). A large mutant library is available for the enzyme (*24*, *25*), and it is known that preparations of most of these mutants tend to be plagued by the presence of kinetically trapped misfolded DAGK populations (*25*). Empirical methods have been developed for correcting misfolded protein in such samples (*25*).

On the basis of chemical cross-linking results from previous work, different DAGK mutants appear to misfold to adopt structurally distinct forms, with different misfolded mutants generally exhibiting discrete oligomeric states rather than forming high-molecular mass aggregates (*25*). However, little more is known regarding the nature of the misfolded forms of the enzyme. In this contribution, we demonstrate the direct detection of a misfolded DAGK mutant using NMR spectroscopy and observe that, for this mutant, misfolding is a conformationally specific process.

MATERIALS AND METHODS

Production, Purification, and Assay of [U-^{2}H,^{15}N]s-DAGK. The pSD005 plasmid containing an inducible synthetic gene for the I53C/I70L/V107D triple mutant of *Escherichia coli* diacylglycerol kinase (s-DAGK) was obtained from J. Bowie of the University of California, Los Angeles (UCLA) (*26*). This synthetic gene also encodes an N-terminal MGHHHHHHEL purification tag in place of the Met1 residue of the native sequence. *E. coli* BL21 was transformed with the pSD005 plasmid and cultured in minimal medium (recipe given in Protocol 1 of ref *27*, supplemented with extra nutrients and vitamins as described in Protocol 2 of that same reference). All cultures were incubated with rotary shaking at 37 °C. First, a 50 mL H_2O culture was grown to an OD_{600} of 0.5. Next, 0.5 mL of this culture was used to inoculate 50 mL of medium containing 70% D_2O, which was then grown to an OD_{600} of 0.5. Then, 0.5 mL of this culture was then used to inoculate 50 mL of medium containing 99% D_2O and $^{15}NH_4Cl$. Once this culture reached an OD_{600} of 0.5, 5 mL was used to inoculate 500 mL of medium containing 99% D_2O. When the OD_{600} of this culture reached 1.0 (∼20 h), expression was induced with isopropyl thiogalactoside and allowed to proceed for 8 h before the cells were harvested. DAGK was then purified by metal ion chelate chromatography using procedures described elsewhere (*25*). Yields varied from 10 to 30 mg of pure s-DAGK from 1 L of D_2O/$^{15}NH_4Cl$ culture. Final chelate column elution solutions contained 0.5% DPC (Anatrace Inc., Maumee, OH) and 250 mM imidazole (pH 7.8). After elution, the pH of the purified DAGK solution was adjusted to 6.5 with acetic acid and EDTA was added to a concentration of 1 mM.

Catalytic activity of s-DAGK was determined in decyl maltoside/cardiolipin mixed micelles using a coupled spectrophotometric assay at 30 °C in which dihexanoylglycerol served as the phosphate acceptor (*28*). The activity of fully active unlabeled s-DAGK under standard conditions is ∼120 units/mg, where 1 unit of activity equals 1 μmol of DAG converted to PA per minute. The activity of the fully active *perdeuterated* s-DAGK is slightly lower (∼90 units/mg). The "% maximal" activities reported in the work are relative to the values of 120 or 90 units/mg, depending upon whether the protein is perdeuterated or not. Steady-state kinetic measurements of $K_{m,MgATP}$ and $V_{max,app}$ were conducted under standard assay conditions in which dihexanoylglycerol was present at a saturating level (8 mol %), free Mg(II) was maintained at a concentration of 10 mM, and the level of MgATP was varied.

NMR Sample Preparation. For NMR experiments, DAGK in solution containing 250 mM imidazole, 1 mM EDTA, and 0.5% DPC (pH 6.5) was concentrated 10−20-fold by ultrafiltration using a Centricon centrifugal filter cartridge (Millipore, Bedford, MA) with a 50 kDa molecular mass cutoff. By this operation, both DAGK *and* the detergent DPC were concentrated (since DPC has a low critical micelle concentration). Samples were then transferred to a 5 mm NMR tube.

Chemical Cross-Linking of s-DAGK. To assess the oligomeric state of DAGK, it was cross-linked with glutaraldehyde. Reactions were performed in 200 μL solutions containing 0.35 mg/mL DAGK, 0.5% DPC, 250 mM imidazole, and 1 mM EDTA (pH 6.5). Cross-linking was initiated by adding GA to a final concentration of 16 mM, and the reaction was allowed to proceed for 24 h at room temperature with vigorous shaking. Samples were then subjected to SDS−PAGE analysis.

NMR Methods. Experiments were performed on an INOVA 600 spectrometer (Varian, Palo Alto, CA). ^{1}H−^{15}N TROSY spectra were acquired using the pulse sequence described in ref *29*. Ninety-six increments were collected in the t_1 dimension, with 32−256 transients per increment and 1.2 s delays between transients. Data were processed using VNMR software (Varian). A square sine bell weighting function $\{\sin^2[(t-\tau)\pi/2\lambda]\}$ was applied in the time domain, with the constants τ and λ being 0.024 and 0.016 for the proton dimension and 0.046 and 0.032 for the ^{15}N dimension, respectively. Data in the t_1 dimension were zero-filled to 512 points.

Circular Dichroism Spectroscopy. CD spectra were measured using a Jasco 810 spectropolarimeter. s-DAGK samples that were used were derived from NMR samples by desalting using a Biogel P-60 column (Bio-Rad, Hercules, CA) equilibrated and eluted with 10 mM sodium phosphate and 0.5% DPC (pH 6.5). Measurements were taken at 30 °C with samples having a path length of 1 mm and an s-DAGK concentration of ∼0.3 mg/mL. Presented data are the average of three scans with a scanning rate of 0.2 nm/s. Baseline correction was performed using a blank containing no protein.

RESULTS

s-DAGK Can Undergo Misfolding but Remains Homotrimeric. Many different DAGK mutants are known to misfold (*25*). However, the studies of this paper focus upon the triple mutant form (I53C/I70L/V107D) of DAGK (s-

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; CD, circular dichroism; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DPC, dodecylphosphocholine; GA, glutaraldehyde; NMR, nuclear magnetic resonance; PA, phosphatidic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TROSY, transverse relaxation-optimized spectroscopy; UV, ultraviolet.

DAGK) engineered by the lab of J. Bowie. The original motivation for conducting NMR studies of this particular mutant is that, when correctly folded, it has a much higher thermal stability than the wild-type protein (*26*) and may therefore be especially suitable for long NMR experiments at elevated temperatures. We have overexpressed and purified s-DAGK many times (>20). s-DAGK does not exhibit as great a tendency to misfold as many other DAGK mutants, but preparations sometimes yield s-DAGK with lower-than-normal specific activity. This is suggestive of the presence of an inactive misfolded protein population, despite the fact that *identical* cell culture, protein purification, and enzyme assay procedures were employed. Attempts to induce misfolding during cell lysis and protein purification by relaxing good lab practice were not successful,[2] suggesting that misfolding may occur at the level of cellular expression. It is perhaps significant in this regard that the purification yield of DAGK from the batches of cells which yielded MF-A and MF-B (see below) were in each case about 10 mg/L of culture, while for the cells yielding sample FO (below), the yield was 30 mg/L even though all three cultures were grown and induced by the exact same procedure.

Two of the s-DAGK samples were subjected to additional examination for this paper. Sample "MF-A" exhibited a specific activity which was about 25% lower than that observed for maximally active samples, suggesting a 25% misfolded population. A second sample, "MF-B", exhibited a 50% reduced activity, suggestive of a 50% misfolded population. These samples were prepared several months apart in time and were derived from different *E. coli* cultures.

Support for the interpretation that the reduced catalytic activities observed in samples such as MF-A and MF-B indicate coexisting populations of fully active and fully inactive s-DAGK was provided by the results of a simple steady-state kinetic experiment. Apparent V_{max} and $K_{m,MgATP}$ values were determined both for a fully active s-DAGK "sample FO" and for sample MF-B (50% active). As illustrated in Figure 1, the apparent V_{max} for these samples differed by a factor of 2 (103 ± 10 units/mg for FO and 48 ± 6 units/mg for MF-B). However, the apparent $K_{m,MgATP}$ values for both samples were the same within experimental error (~0.45 mM), consistent with the notion that the folded population is kinetically identical in both samples FO and MF-B, with the misfolded population being completely inactive.[3]

Direct Detection of a Misfolded Population in TROSY NMR Spectra of s-DAGK. The NMR spectra presented in this paper were acquired using the TROSY pulse sequence, which takes advantage of relaxation pathway interference effects occurring at high magnetic fields to potentially yield spectra that have a higher resolution than is possible using conventional heteronuclear correlation sequences (*30*, *31*).

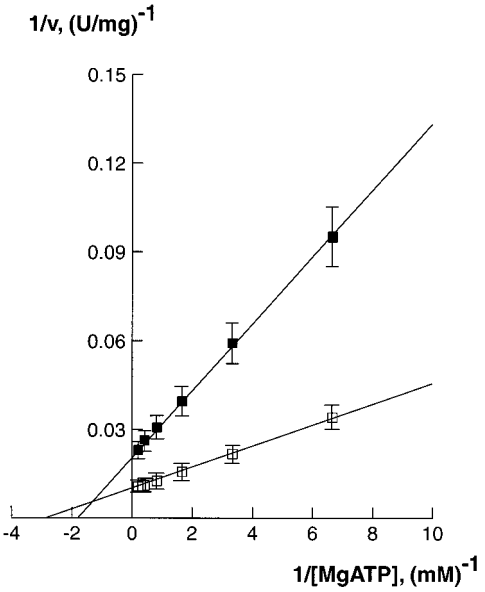

FIGURE 1: Steady-state kinetic results for s-DAGK at 30 °C. Data were fit to the Michaelis−Menten model as direct rate vs concentration using nonlinear least-squares analysis. The results are presented here in double-reciprocal form for convenience of viewing. Empty squares represent 100% folded sample FO. Closed squares represent sample MF-B, believed to be ~50% misfolded. Additional experimental details can be found in Materials and Methods.

Optimizing sample conditions so that interference effects can be maximally exploited for amide protons requires perdeuteration of all nonexchangeable protons. Samples MF-A and FO were biosynthetically perdeuterated for this study, followed by purification into DPC micelles and back exchange of amide deuterons for protons by prolonged (at least 1 week) incubation of samples in H_2O at room temperature. That back exchange of amide deuterons was complete under the conditions of the NMR experiments was demonstrated by comparing the spectrum of ^{15}N-labeled DAGK which was not perdeuterated or exposed to D_2O (data not shown) to the spectra presented in this study; there are no peaks in the spectrum from fully protonated DAGK that were not observed in the spectrum from perdeuterated and back exchanged DAGK.

Two-dimensional TROSY spectra ideally exhibit a single peak for each nitrogen-attached proton in a uniformly ^{15}N-labeled protein (*30*, *31*). The 600 MHz TROSY spectrum from a 100% correctly folded sample FO is illustrated in Figure 2A. This spectrum has been reproduced for other 100% active samples prepared at different times and at protein concentrations ranging from 0.5 to 3.5 mM. The amide region of Figure 2A exhibits about 100 of the expected 130 resonances from amide protons, with all five Trp indole protons clearly visible in the 9.5−10.5 ppm range. Missing resonances are the result of cancellation of antiphase magnetization for very broad peaks (broadened through any of several possible mechanisms) or may simply be poorly resolved.[4] The TROSY spectrum of sample MF-A (Figure

[2] For example, s-DAGK purified from one-half of a single batch of cells which had been "carefully" lysed by enzyme/detergent treatment at 4 °C yielded the same (high) activity as enzyme purified from the same batch of cells which were lysed by a similar procedure, but at room temperature and including extended stir-bar agitation and high-power sonication steps.

[3] It should be noted that while $K_{m,DAG}$ was not determined for these samples, the Michaelis constants for DAGK's lipid and nucleotide substrates are known to be coupled (28) such that a change in $K_{m,DAG}$ without a change in $K_{m,MgATP}$ is highly unlikely.

[4] While we did not have access to an 800 MHz magnet for most of the NMR studies of this paper, we have remotely acquired an 800 MHz TROSY spectrum of an s-DAGK sample similar to FO (not shown). One hundred twenty of 130 expected amide peaks can be clearly observed in that spectrum, indicating that most of the "missing" peaks in Figure 2A are not absent due to exchange broadening.

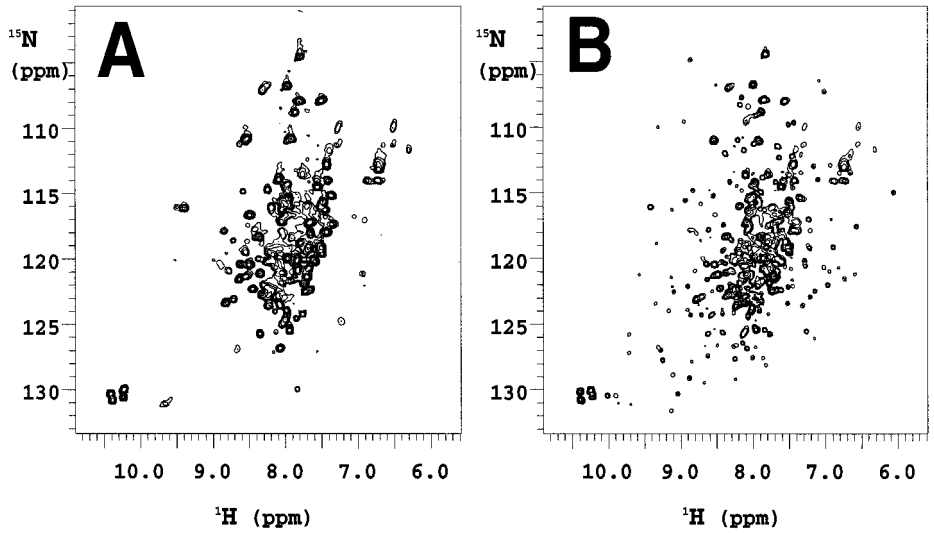

FIGURE 2: ^{1}H−^{15}N TROSY spectra of [U-^{2}H,^{15}N]s-DAGK in DPC micelles at 45 °C. Panel A is for sample FO which contains little or no misfolded protein. Panel B is for sample MF-A which contains a 25% population of misfolded protein. Both samples contained 0.25 M imidazole, 1 mM EDTA, and 10% D_2O at pH 6.5. Sample FO contained 3.5 mM s-DAGK and 10% dodecylphosphocholine (0.32 M), while sample MF-A contained 0.5 mM s-DAGK and 5% dodecylphosphocholine (0.16 M). Thirty-two transients per t_1 point were acquired for sample FO, while 256 transients per t_1 point were acquired for sample MF-A. Additional spectroscopic details are given in Materials and Methods.

2B) exhibits the same resonances that appear in the spectrum of FO *plus* an additional set of resonances which are reduced in intensity relative to the others, as expected for a set of resonances reflecting the ~25% misfolded s-DAGK population in this sample. Approximately 120 additional resonances are observed, indicating a single conformational state for the misfolded protein. This spectrum was reproduced when the TROSY experiment was executed 19 days later on the same sample. We also acquired a TROSY spectrum of sample MF-B which was very similar to that of MF-A,[5] except that the putative "misfolded form" peaks are similar in intensity to the peaks arising from the folded population, as expected on the basis of the ~50% misfolded population in this sample. The relative intensities of the misfolded form peaks with respect to *themselves* were the same in spectra from both MF-A and MF-B, as expected on the basis of the above interpretation.

The extra set of peaks observed for samples MF-A and MF-B cannot result from aggregation due to an overly low detergent:protein ratio, as has been documented for at least one membrane protein (*32*). The detergent:protein ratio is actually lower in sample FO than in sample MF-A by a factor of 6, while in sample MF-B, the ratio is very similar to that in FO. Indeed, we have previously shown that DAGK's NMR spectrum is insensitive to the DAGK:detergent ratio over a wide range (*21*). Misfolding of s-DAGK and the appearance of TROSY peaks representing the misfolded form are not the results of sample perdeuteration, as evidenced by observation of both reduced activities and misfolded form TROSY peaks from some s-DAGK samples which were not perdeuterated (data not shown).

One final potential artifactual source of the misfolded form TROSY peaks is the presence of a second protein impurity or the presence of a proteolytically clipped form of DAGK. These possibilities were ruled out by dividing a single batch of pure enzyme bound to Ni(II)−agarose resin into two portions and processing each by different procedures. The first half of the s-DAGK−resin sample was eluted with DPC and prepared for NMR spectroscopy by the usual method. This "control" sample exhibited a near-100% maximal activity. The other half of enzyme−resin sample was equilibrated with detergent and lipid-free 8 M guanidine hydrochloride and then eluted in 8 M guanidine-HCl with 0.5% formic acid. The denaturant-eluted s-DAGK was then dialyzed for 4 h against 0.5% formic acid to remove the denaturant and then passed through a Biogel P-60 desalting column which was equilibrated with 0.5% DPC (pH 7.8). The resulting s-DAGK/DPC mixture was then concentrated to yield an NMR sample having the exact same composition as the first sample. This "denatured/renatured" sample exhibited a lower catalytic activity than the control, suggesting that the deliberately flawed unfolding/refolding procedure involving guanidine hydrochloride resulted in some misfolding. The TROSY spectrum of the control sample was very similar to that of sample FO (Figure 2A), while the spectrum of the denatured/renatured sample was similar to that of sample MF-A (Figure 2B), exhibiting the extra set of peaks representing the misfolded form. Because both samples were derived from the same batch of purified DAGK, differences in the catalytic activity and TROSY spectrum for the two samples cannot reflect differences in sample purity or partial proteolysis.

Characterization of the Misfolded Form of s-DAGK. Because of its high stability, folded s-DAGK retains its homotrimeric oligomeric state even under conditions of SDS−PAGE, unlike wild-type DAGK or most other mutants (*26*). The results from high-resolution SDS−PAGE and silver staining of very small aliquots of samples FO and MF-B are illustrated in Figure 3A. Sample FO yielded only a single band, characteristic of the homotrimer. MF-B (50% active) yielded two barely resolved bands of about equal intensity, consistent with both folded and misfolded forms being

[5] Minor differences in the TROSY spectra from MF-A and MF-B reflect the facts that, for historical reasons, sample MF-B had a higher pH (7.8) than MF-A (6.5) and also included a small amount of lipid along with the DPC micelles used to solubilize s-DAGK.

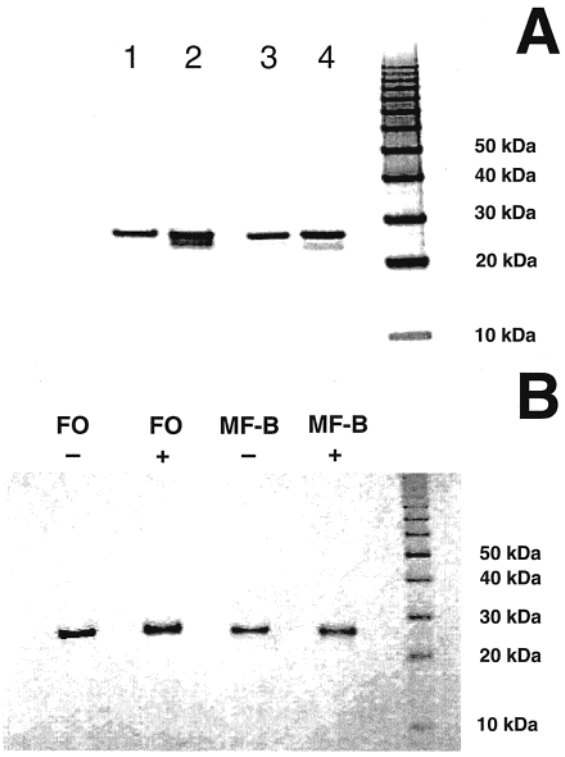

FIGURE 3: (A) Silver-stained SDS−PAGE gel results for (1) sample FO, (2) sample MF-B (50% active), (3) control s-DAGK processed by the usual DPC-based method from one-half of a batch of the enzyme/Ni(II)−agarose sample, and (4) s-DAGK prepared from the second half the same batch of the enzyme/Ni(II)−agarose sample by a deliberately flawed denaturation/renaturation procedure. (B) Coomassie-stained SDS−PAGE gel following glutaraldehyde cross-linking of a sample of 100%-folded s-DAGK (FO) and a sample believed to contain a 50% population of misfolded protein (MF-B): (−) samples which were not cross-linked and (+) samples which were cross-linked.

homotrimers, but migrating slightly differently because they have different conformations. Also illustrated in Figure 3A are the results for the pair of s-DAGK samples described immediately above which were derived by different methods from a single batch of pure s-DAGK. The presence of a ~15% misfolded population is clearly observed in the lane representing the sample subjected to the deliberately flawed denaturation/renaturation procedure, consistent with TROSY and activity results for this sample. The control sample which exhibited near-100% activity showed only a barely detectable band representing a small population of misfolded protein which was not clearly detectable above the "noise" of either the TROSY spectrum or the precision of activity measurements.

Chemical cross-linking was employed to verify that both folded and misfolded forms of s-DAGK have the same oligomeric state under NMR sample conditions. s-DAGK from samples FO and MF-B was subjected to random cross-linking using glutaraldehyde to covalently trap oligomers and then subjected to SDS−PAGE. GA cross-linking has been extensively employed in studies of DAGK's oligomeric state (*21*, *25*), where it has been documented that the cross-linked trimer migrates between 25 and 30 kDa relative to molecular mass standards (*25*). Cross-linking was monitored by SDS−PAGE under conditions suitable for Coomassie staining, where the folded and misfolded bands are not resolved. As shown in Figure 3B, only the trimeric oligomeric state is

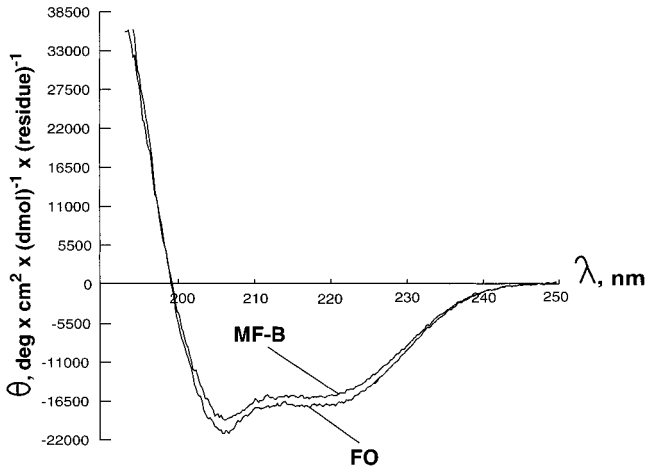

FIGURE 4: Far-UV CD spectra of sample FO and sample MF-B under identical conditions at 30 °C in DPC micelles. Additional experimental details are presented in Materials and Methods.

observed for samples FO and MF-A in both the absence and presence of cross-linking. There was also no loss of total observed protein due to the formation of high-molecular mass aggregates which would not migrate into the gels. This confirms that the population of misfolded DAGK in sample MF-A retains its native-like oligomeric state under NMR sample conditions.

Far-UV circular dichroism (CD) spectra of s-DAGK from a fully folded sample were acquired and compared to spectra from sample MF-B (containing a ~50% misfolded population). The far-UV spectra were similar but not identical (Figure 4), consistent with a modest change in secondary structural content for the misfolded form of s-DAGK. Attempts to calculate the secondary structural differences in the two samples by fitting these spectra did not lead to results which were judged to be reliable based on variations in results when data were fit by different algorithms and taking experimental uncertainty into account. However, the difference between the two spectra of Figure 4 is consistent with a modest (perhaps 10%) change in secondary structural content in s-DAGK upon misfolding.

A final result which points to differences between the misfolded and folded forms of s-DAGK is illuminated by comparison of the peaks representing these populations in TROSY spectra acquired at 35 °C rather than 45 °C. The folded form peaks exhibit considerable line broadening at the reduced temperature, while the peaks representing the misfolded form do not (data not shown). These data indicate substantial temperature-sensitive differences between the folded and misfolded forms in terms of either conformational motions or overall tumbling rates.

DISCUSSION

Use of TROSY NMR To Detect Misfolded s-DAGK. The TROSY experiment exploits relaxation interference effects which occur at very high magnetic fields to yield NMR spectra exhibiting enhanced resolution compared to those from traditional heteronuclear correlation experiments (*30*, *31*). This has enabled spectral assignment of relatively large biomolecules and their complexes, as exemplified by recent reports of completed main chain spectral assignments for water soluble oligomers having aggregate masses of 110 and

67 kDa (*33*, *34*). Moreover, the structure of a 17 kDa integral membrane porin as part of a 60 kDa micellar complex has recently been determined using TROSY-based methods (*35*). Even though the experiments involving s-DAGK were carried out at a field (600 MHz proton frequency) which is too low to fully exploit relaxation interference effects (*31*), the spectra of both folded and misfolded s-DAGK are of remarkable quality. The effective molecular mass of s-DAGK in the samples of this work was ~100 kDa: 40 kDa from the protein homotrimer and the remainder from the associated detergent, DPC. The observation that at least 70% of the amide resonances from the correctly folded s-DAGK[4] and ~90% of the resonances from the misfolded protein can be detected is extremely encouraging, indicating that extensive resonance assignment for both forms is feasible, particularly if future experiments are carried out at a higher field where relaxation interference effects will be maximally activated and spectral dispersion will be higher. A key advantage of the use of NMR to detect the misfolded protein population as compared to the use of activity measurement or other spectroscopic techniques such as FT-IR or CD is that NMR is able to shed light upon whether the misfolded protein population is structurally homogeneous, as for s-DAGK, or whether it represents a variety of different trapped conformations.

Origin and Characteristics of Misfolded s-DAGK. While the results do not completely rule out the possibility that imperfect reproduction of cell lysis/protein purification methods results in the misfolded population of s-DAGK observed in some samples, the available evidence suggests that misfolding may occur at the stage of cellular expression in the *E. coli* host. Why this occurs in some cultures and not in others grown under the same conditions is not clear, but may reflect events related to the adaptation of host cell cultures to the selective pressure of high-level protein expression. It would be premature to speculate regarding what the specific nature of such stochastic "events" might be. However, it must also be pointed out that we were able to generate a misfolded s-DAGK population using a deliberately flawed denaturation/renaturation procedure. This misfolded form exhibited the same NMR spectrum and electrophoretic behavior as the putatively in vivo-misfolded s-DAGK. This indicates that the pathway leading to the specific misfolded conformation documented in this work is accessible to s-DAGK in the absence of native membrane and other protein factors; the "information" required for misfolding is encoded solely by s-DAGK's sequence.

Unlike the misfolded forms of many other DAGK mutants (25), misfolded s-DAGK was seen to remain homotrimeric. The fact that a single set of well-defined resonances arose from the misfolded protein strongly suggests that it adopted a single conformational state which is separated by a kinetic barrier from the folded state. The misfolded protein does not populate a series of structurally distinct trapped structures. For s-DAGK, the misfolding process evidently has *conformational specificity.*

The TROSY peaks arising from misfolded s-DAGK (Figure 2B) are striking in that they exhibit a much higher chemical shift dispersion, particularly in the proton dimension, than the resonances arising from the folded form, which is known to be highly helical (*20*). Indeed, chemical shift differences are so great that in most cases it is not possible to correlate peaks from sites in the misfolded form with the peaks from the corresponding sites in the folded form. While amide proton and ^{15}N chemical shifts are not very reliable predictors of protein structure (*36*, *37*), the dramatically enhanced dispersion of these resonances from the misfolded form suggests a significant difference in conformation from the folded form. This was reflected by a slight difference in electrophoretic mobility relative to the folded form (Figure 3A) and also by a modest variation in the far-UV CD spectra from a 100% folded sample compared to that from a 50:50 mixture of folded and unfolded s-DAGK (Figure 4). This latter observation is consistent with some degree of change in secondary structural content. Another apparent difference between the folded and misfolded forms of s-DAGK is reflected by differences in the temperature dependence of the TROSY line widths of resonances representing the two forms. This observation is consistent with either a significant difference in internal protein dynamics between the folded and misfolded forms or differences in overall protein tumbling, as might occur if the two forms interacted with the host DPC micelles in very different manners. However, despite all of the differences described above, the folded and misfolded forms were both observed to be homotrimers.

Relationship of Misfolding by the s-DAGK Mutant to Misfolding by Other Forms of DAGK. DAGK is a protein for which both the wild-type protein and a host of mutants exhibit a high propensity to misfold to generate kinetically trapped states (*25*). The s-DAGK mutant of this study differs from the wild-type protein and most other mutants by its high stability, once correctly folded (*26*). Misfolded s-DAGK also differs from most other misfolded forms of DAGK in at least two regards. First, misfolded s-DAGK retains the native-like oligomeric state, unlike most other misfolded forms (*25*) which tend to exhibit specific oligomeric states, but are usually not homotrimers. This is interesting since two out of three mutations which confer high stability to s-DAGK occur at positions within the second transmembrane segment of DAGK, the segment believed to form the central axis of 3-fold symmetry within the DAGK trimer (*38*). This suggests that misfolding of s-DAGK may occur at a relatively late stage of protein assembly, perhaps representing a post-trimerization excursion off of the folding pathway.

Misfolded s-DAGK is also different from most other forms of DAGK in its resistance to refolding. We have previously demonstrated that application of a procedure known as "reconstitutive refolding" to most misfolded forms of DAGK results in conversion of the misfolded protein into a correctly folded form in high yield (*25*). Application of this procedure to s-DAGK was found to be ineffective, perhaps because the misfolded form of this "super stable" mutant is also much more stable than most other forms of DAGK. This is consistent with the observed persistence of the misfolded form of s-DAGK following long NMR experiments at 45 °C and with the observed retention of its distinct homotrimeric state even in SDS. Perhaps part of the reason proteins such as DAGK do not evolve high thermodynamic and kinetic stability is because the same mutations which result in high stability for the correctly folded form often confer extremely high stability upon accessible misfolded forms, making them harder to "correct" or dispose of.

Is Misfolding of s-DAGK Relevant to Disease-Related Membrane Protein Misfolding? Conformationally specific misfolding of water soluble proteins has been documented at atomic resolution (*39*, *40*) and appears to be a central theme of "conformational diseases" such as amyloid disorders (*3*, *7*−*11*, *41*−*43*). The work presented here appears to represent the first biophysical documentation of conformationally specific misfolding by an integral membrane protein. There are compelling physical chemical reasons to suppose that misfolding of integral membrane proteins may be very common and may occur through pathways and interactions which are in some cases distinct from those of water soluble proteins (review in ref *1*). In particular, the entrapment of non-native hydrogen bonding patterns within the hydrocarbon phase of membranes would appear to offer a potent mechanism for locking in aberrant membrane protein conformations (*44*−*48*). Given such considerations, it is not surprising both that there is a complex cell biological machinery dedicated to recognizing and dealing with misfolded membrane proteins in the secretory pathway (*2*−*6*) and that there are a number of diseases known to be associated with membrane protein misfolding (*1*). These include cystic fibrosis (*14*), Charcot-Marie-Tooth disease (*49*), and common forms of diabetes (*50*), blindness (*51*, *52*), and deafness (*53*). In these cases, pathogenesis appears to be most often derived from the loss of protein function rather than from toxicity of the misfolded protein. Do eukaryotic integral membrane proteins which misfold in the endoplasmic reticulum generally undergo similar conformationally specific misfolding? If so, are there common structural themes for misfolded membrane proteins which cellular quality control machinery has evolved to recognize as aberrant? These questions await future experimental address but may be piqued by the results of this paper.

ACKNOWLEDGMENT

We thank James Bowie and his lab at UCLA for providing the s-DAGK mutant used in this work and Bonnie Gorzelle of Case Western Reserve University for both technical assistance and proofreading.

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BI0029090