Escherichia coli Diacylglycerol Kinase: A Case Study in the Application of Solution NMR Methods to an Integral Membrane Protein

Olga Vinogradova, Prakash Badola, Lech Czerski, Frank D. Sönnichsen, and Charles R. Sanders, II
Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106-4970 USA

ABSTRACT Diacylglycerol kinase (DAGK) is a 13-kDa integral membrane protein that spans the lipid bilayer three times and which is active in some micellar systems. In this work DAGK was purified using metal ion chelate chromatography, and its structural properties in micelles and organic solvent mixtures studies were examined, primarily to address the question of whether the structure of DAGK can be determined using solution NMR methods. Cross-linking studies established that DAGK is homotrimeric in decyl maltoside (DM) micelles and mixed micelles. The aggregate detergent-protein molecular mass of DAGK in both octyl glucoside and DM micelles was determined to be in the range of 100–110 kDa—much larger than the sum of the molecular weights of the DAGK trimers and the protein-free micelles. In acidic organic solvent mixtures, DAGK-DM complexes were highly soluble and yielded relatively well-resolved NMR spectra. NMR and circular dichroism studies indicated that in these mixtures the enzyme adopts a kinetically trapped monomeric structure in which it irreversibly binds several detergent molecules and is primarily α-helical, but in which its tertiary structure is largely disordered. Although these results provide new information regarding the native oligomeric state of DAGK and the structural properties of complex membrane proteins in micelles and organic solvent mixtures, the results discourage the notion that the structure of DAGK can be readily determined at high resolution with solution NMR methods.

INTRODUCTION

Based on the completed total genomic sequence analyses of both prokaryotic and eukaryotic microorganisms, it can be estimated that about one-third of all proteins are membrane proteins (Proteome, 1997; Goffeau, 1995), and yet less than 0.1% of the solved structures currently present in the Brookhaven Protein Data Bank represent intact membrane proteins (Brookhaven National Laboratory, 1997). In part, this is due to well-documented difficulties encountered in preparing diffraction-grade crystals of membrane proteins for crystallography (Garavito et al., 1996). Solution NMR methods potentially offer an alternative to crystallography for structural analysis if the integral membrane protein of interest can be solubilized in rapidly tumbling detergent micelles or in an organic solvent system. However, except for a few studies of bacteriorhodopsin (Seigneuret and Kainosho, 1993; Pervusihin et al., 1994; Orekhov et al., 1992) and subunit c of the F_1F_o ATP synthase (Girvin and Fillingame, 1993, 1995), integral membrane proteins subjected to NMR analysis to date have contained only a single transmembrane segment, and have not had readily assayable functional properties (see reviews in Opella et al., 1994; Henry and Sykes, 1994). Thus, in many cases it is difficult to assess the exact degree to which a structure determined in micelles or in organic solvents resembles its functional, bilayer-associated structure. Furthermore, although the present molecular mass limitation for total structural analysis of water-soluble proteins by solution NMR methods is ~40 kDa (Sattler and Fesik, 1996), it remains unclear whether the structures of larger integral membrane proteins (>10 kDa) can be attained by these methods.

At the onset of the studies described in this paper, *Escherichia coli* diacylglycerol kinase (DAGK) appeared to be an attractive candidate for solution NMR structural analysis. DAGK is a 13-kDa integral membrane protein that catalyzes a readily assayable reaction: the phosphorylation of diacylglycerol by MgATP to yield phosphatidic acid. Although it is an integral membrane protein with three transmembrane segments (Fig. 1), DAGK has been purified and maintains a catalytically viable structural state when reconstituted into lipid vesicles, mixed micelles, or certain micellar systems (Loomis et al., 1985; Russ et al., 1988; Walsh and Bell, 1986a,b; Sanders et al., 1996). DAGK is also known to be soluble in a variety of organic solvent systems (Loomis et al., 1985; Russ et al., 1988).

In this contribution we report our efforts to probe, as systematically as possible, the potential for carrying out solution NMR structural studies on DAGK. Where results appear to be discouraging, we have sought not only to document, but also to explain the nature of the negative results. These studies required that we first determine the oligomeric state of DAGK under catalytically viable conditions, a matter of import not only to NMR structural studies, but also to enhancing our general understanding of this unique enzyme.

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Address reprint requests to Dr. Charles R. Sanders, Department of Physiology and Biophysics, Case Western Reserve School of Medicine, 2109 Adelbert Rd., Cleveland, OH 44106-4970. Tel.: 216-368-8651; Fax: 216-368-1693; E-mail: crs4@po.cwru.edu.

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MATERIALS AND METHODS

Labeling and purification of DAGK

E. coli strain WH1061, harboring the inducible plasmid pSD0004 for polyHis-tagged DAGK, was a gift from James Bowie of UCLA. The exact

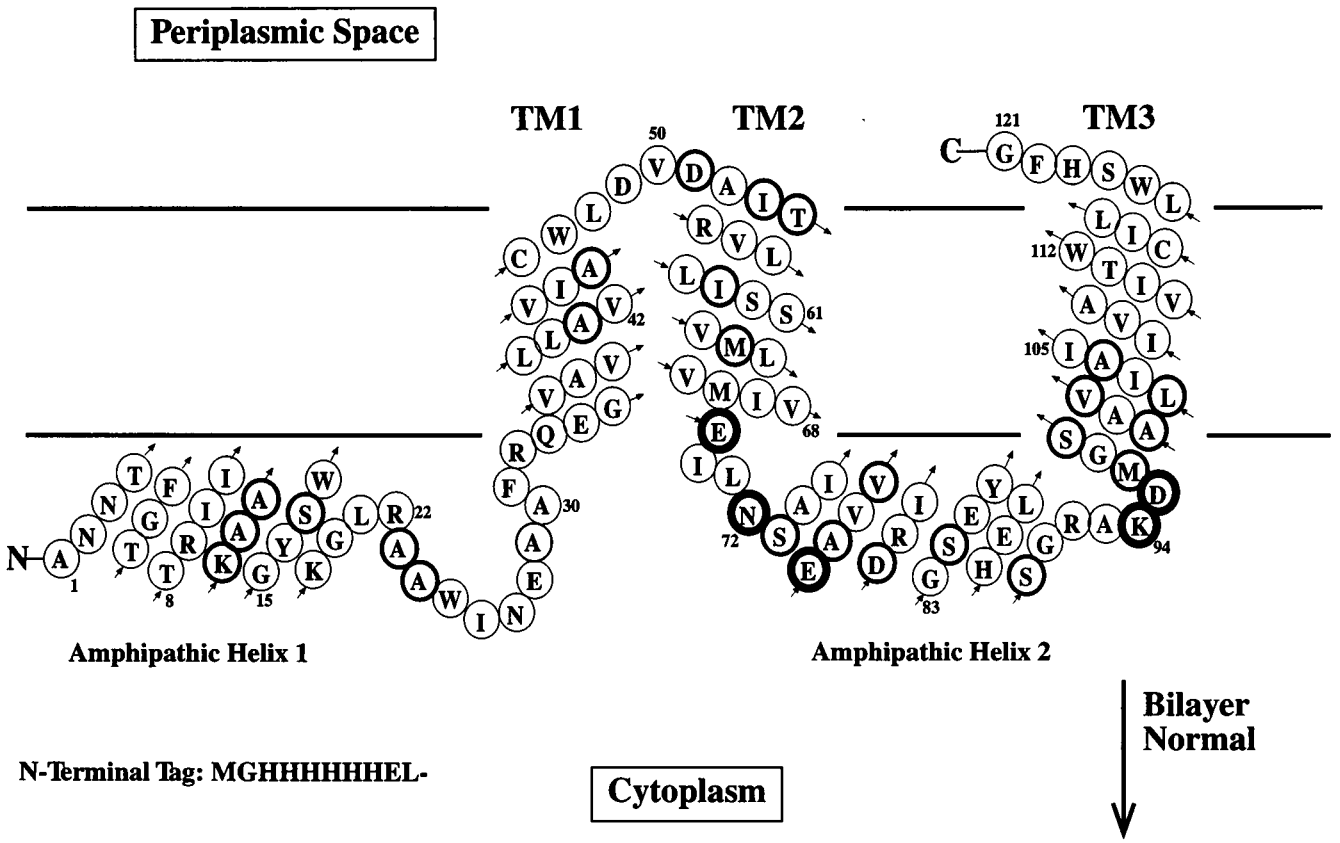

FIGURE 1 Model for the topology, secondary structure, and probable helical orientations of DAGK, based on a variety of experimental data. The topology was determined by gene fusion experiments (Smith et al., 1994). The percentage of helical content was determined by Fourier transform infrared spectroscopy (Sanders et al., 1996). The exact beginnings and ends of the helices are putative, but are reasonable based upon multiple sequence alignment and sequence analysis (Smith et al., 1994; Sanders et al., 1996). The helical orientations with respect to the bilayer normal are consistent with polarized attenuated reflection Fourier transform infrared data (Sanders et al., 1996). Both the locations of the TM segments and the approximate locations of the helices are fully consistent with multiply aligned sequence analysis of the microbial DAGK isozymes (Smith et al., 1994; Sanders, unpublished observations). The bold-circled residues are those that were identified as being functionally essential, based on the mutagenesis study of Wen et al. (1996), in which all 121 residues of DAGK were mutated. 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. This model should not be taken to imply anything about the tertiary structure of DAGK. For historical reasons, the form of DAGK used in this study is not the tagged wild type (as shown), but is a tagged double mutant (W117R,S118T) that appears to be catalytically and structurally indistinguishable from the wild-type protein.

sequence of the tag, which replaces the N-terminal Met of the 122-residue native DAGK, is MGHHHHHHEL. Cells were grown in Luria broth medium and induced with isopropyl thiogalactoside when the optical density of the cultures at 600 nm reached 1.0. When uniform ^{15}N enrichment was desired, cells were grown in minimal medium, which included $^{15}NH_4Cl$ as the sole nitrogen source. Cells were harvested, lysed, extracted with detergent, and incubated with Ni(II)-agarose resin as described previously (Sanders et al., 1996). The Ni-agarose resin to which DAGK and impurities were bound was washed with a solution containing 40 mM imidazole, 1.5% empigen detergent (Calbiochem, San Diego, CA), 50 mM phosphate, and 300 mM NaCl, pH 7.8. After this step, DAGK was the only protein remaining bound to the resin in significant quantities. Normally, the resin-DAGK was then equilibrated with 1% β-decyl maltoside (DM) in water (Anatrace, Maumee, OH) followed by elution of the enzyme with 1% decyl maltoside plus either 2% d_2-formic acid, 0.3 M imidazole (pH 7.8), or 0.5 M NH_4OH to yield >95% pure DAGK in decyl maltoside solutions. It should be pointed out that acetic acid was not a viable substitute for formic acid, probably because its pK_a is not low enough to elute the enzyme from the column. The eluted DAGK was normally 10–30 mg/ml and exhibited specific activities of 15 U/mg (see below). The DAGK solutions were then frozen in liquid N_2 and lyophilized to yield a powdered DAGK-DM mixture, which was usually about 33% DAGK by weight and which can be redissolved in the buffer of choice with little (<15%) or no loss of enzyme activity relative to the prelyophilized solution when NH_4OH was used for elution.

A majority of the samples described in this paper involve the use of DAGK prepared as described above, such that decyl maltoside was present in the final solution actually studied. Decyl maltoside was chosen as the "default" detergent because DAGK is both highly soluble and highly stable in DM micelles. In cases where another detergent was of interest, the above procedure was generally used, except that decyl maltoside was replaced by the detergent of interest in the final two steps of the purification (column equilibration and final elution). It should be pointed out that the use of sodium dodecylsulfate is incompatible with the Ni(II)-agarose resin.

DAGK was in some cases purified in a salt/lipid/detergent-free form using the following method. After the 1% DM rinse step of the standard purification method, the column was rinsed with successive 2 × 1 column volumes of 25%, 50%, and 75% 1:1 water:tetrahydrofuran (THF) in 1% DM, followed by rinsing with 10 × 1 column volumes of 1:1 water:THF and elution with 1:1 water:THF plus 2% *d*-formic acid to yield a solution of pure DAGK lacking salt, detergent, or lipid. These solutions were frozen and lyophilized to yield DAGK powder, which could be redissolved in acidic organic solvent mixtures (but not in DM micelles). An analogous procedure involving 1:7 water:hexafluoroisopropanol could also be used.

Cross-linking methods

Chemical cross-linking was carried out by incubating glutaraldehyde or Cu(II)-phenanthroline with DAGK in micelles or in mixed micelles at room temperature. Salt-free lyophilized DAGK/DM powder was typically redissolved by 25 mM DM solutions buffered with 20 mM phosphate, 150 mM NaCl, pH 7.5. The DAGK:DM molar ratio was typically about 1:1000. Glutaraldehyde (GA) was added from fresh 25% stock solutions made in the above buffer plus 1% DM. GA reactions were effectively quenched by dilution into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (see below). Disulfide-based cross-linking reactions were initiated by adding aliquots of a solution containing 30 mM phenanthroline and 10 mM $CuSO_4$ (in the same buffer as the GA stock) into DAGK solutions, followed by quenching of the reaction at the appropriate time by adding EDTA to a level of 10 mM. For SDS-PAGE analysis aliquots from both types of cross-linking reactions were usually diluted 1:5 into a loading buffer consisting of 50 mM Tris, 2–5% SDS, 0.1% bromophenol blue, and 10% glycerol. Samples were not boiled before loading because such treatment results in irreversible DAGK aggregation. SDS-PAGE was run using 20% gels according to standard protocols.

DAGK assay methods

For aqueous micellar solutions, DAGK was generally assayed by coupling the DAGK reaction to the reactions of lactic dehydrogenase and pyruvate kinase, so that the DAGK reaction could be monitored continuously by measuring the decrease in light absorbance at 340 nm due to NADH oxidation. The details of this assay system will be presented elsewhere.

For direct assays in organic solvent mixtures, the coupled assay system could not be used because of solubility/activity loss problems involving components of the coupling system. Instead, a thin-layer chromatography-based assay system was used in which DAGK activity was monitored via the conversion of pyrene-tagged diacylglycerol into phosphatidic acid. DAGK stock solutions were normally prepared by dissolving 2 mg of DAGK/DM powder (33% DAGK by weight) in the solvent mixture of interest plus just enough formic acid to ensure dissolution of the enzyme (typically 30–60 mM). Aliquots of the stock solutions were then added to small tubes containing 100 μl of solvent in which was dissolved ATP (4–20 mM), Mg^{2+} (20–40 mM), 1-pyrenebutyrl-2-butyrl-*sn*-glycerol (PBBG) (1 mM), 21 mM DM, and (sometimes) 2 mM dimyristoylphosphatidylcholine (DMPC). These mixtures were normally buffered using either 50 mM imidazole formate (pH 6.5) or 38 mM PIPES, 25 mM LiCl (pH 6.7). Reactions were allowed to run for various periods of time. Aliquots were removed and spotted on thin-layer chromatography plates, followed by chromatography to separate the fluorescently labeled phosphatidic acid (PA) product from the substrate PBBG. Details of the chromatographic methods and subsequent quantitation have been reported elsewhere (Sanders et al., 1996).

NMR methods and diffusion measurements

Lyophilized DAGK or DAGK/DM powder was dissolved in either an aqueous buffer (often containing additional detergent) or in a premixed solvent and transferred to an NMR tube. In cases where a solvent content was systematically varied (% isopropanol, for example) separate samples were prepared for each composition, rather than titrating neat solvent into a single sample. It was observed that the process of mixing neat solvents with a DAGK-containing water-solvent mixture inevitably resulted in DAGK precipitation.

NMR experiments were carried out using either a Varian UNITYPlus 600-MHz NMR spectrometer or a Bruker AC-270 instrument (for preliminary or routine screening of solution conditions). A dedicated 5-mm ^1H probe was used for the experiments at 270 MHz, and Varian 5 mm $^1H/X$ and $^1H/^{13}C/^{15}N$ indirect z axis pulsed field gradient probes were used for experiments at 600 MHz.

^1H-^{15}N heteronuclear single quantum coherence (HSQC) spectra, which yield contour peaks only for protons that are directly attached to an ^{15}N, were acquired using a gradient-enhanced HSQC pulse sequence (Kay et al., 1992). Backbone ^{15}N T_1 and T_2 relaxation times were measured using the related pulse sequence of Farrow et al. (1994). Two-dimensional nuclear Overhauser effect (NOESY) spectra were acquired using simple presaturation of the water signal. NOESY spectra ideally yield off-diagonal cross-peaks only between pairs of spatially proximal (<5 Å) protons. Two-dimensional ^{15}N half-filtered NOESY experiments were carried out by adapting a 3D ^{15}N-edited NOESY-HSQC pulse sequence (Zhang et al., 1994), so that cross-peaks are observed only if one of the spatially proximal protons in any given pair is directly attached to a ^{15}N.

Diffusion coefficients were measured using a longitudinal eddy-current delay (LED) pulse sequence as described by Altieri et al. (1995).

Circular dichroism

Circular dichroism (CD) spectra were acquired using a Jasco-600 spectrometer. Samples for far-UV studies were prepared by dissolving ~0.5 mg/ml of salt-free lyophilized DAGK-DM powder into buffer. The exact DAGK concentration (typically ~0.17 mg/ml) was determined by measuring solution absorbance at 280 nm. Quartz cuvettes with light path lengths of 1 mm (for far-UV) or 10 mm (for near-UV) were used. The parameters employed were bandwidth, 2 nm; sensitivity, 200 mdeg (far UV) or 10 mdeg (near UV); time constant, 0.125 s (far UV) or 2 s (near UV). Spectra acquired represented an average of four scans. Reference spectra, obtained with the samples containing all components except the protein, were recorded before each spectrum and subtracted from the protein spectrum during the acquisition. All spectra were recorded in units of millidegrees. The instrument was routinely calibrated with an aqueous solution of recrystallized (1*R*)-(−)-10-camphorsulfonic acid, ammonium salt at 290 nm (θ = 7910).

RESULTS

Chemical cross-linking of DAGK under micellar and mixed micellar conditions

Knowledge of the oligomeric state of catalytically viable DAGK is of crucial relevance to the potential study of DAGK by solution NMR. For example, if DAGK is a hexamer (~80 kDa), then it would be, at best, an exceptionally difficult target for NMR structural analysis unless it could be dissociated into folded monomers.

The oligomeric state of DAGK was examined when the enzyme was solubilized as a dilute component in decyl maltoside micelles or DM-cardiolipin mixed micelles. Micelles or mixed micelles were preferred to lipid vesicles to eliminate difficulties arising from the potential failure of the cross-linking agent to reach the interior compartment of vesicles. DAGK is only slightly active in neutral DM micelles but is fully active in DM/CL mixed micelles, where its specific activity is comparable to that observed for DAGK in bilayers (Sanders et al., 1996). Chemical cross-linking experiments were first carried out using glutaraldehyde (GA) as the cross-linking reagent (see review in Gaffney, 1985). Fig. 2, *A* and *C*, shows the dependence of covalent oligomerization of DAGK upon the concentration of cross-linking reagent. For both micelles and mixed micelles, the covalent trimer emerged as the dominant covalent oligomer as the concentration of GA was increased. Fig. 2, *B* and *D*, shows the dependence on the length of cross-

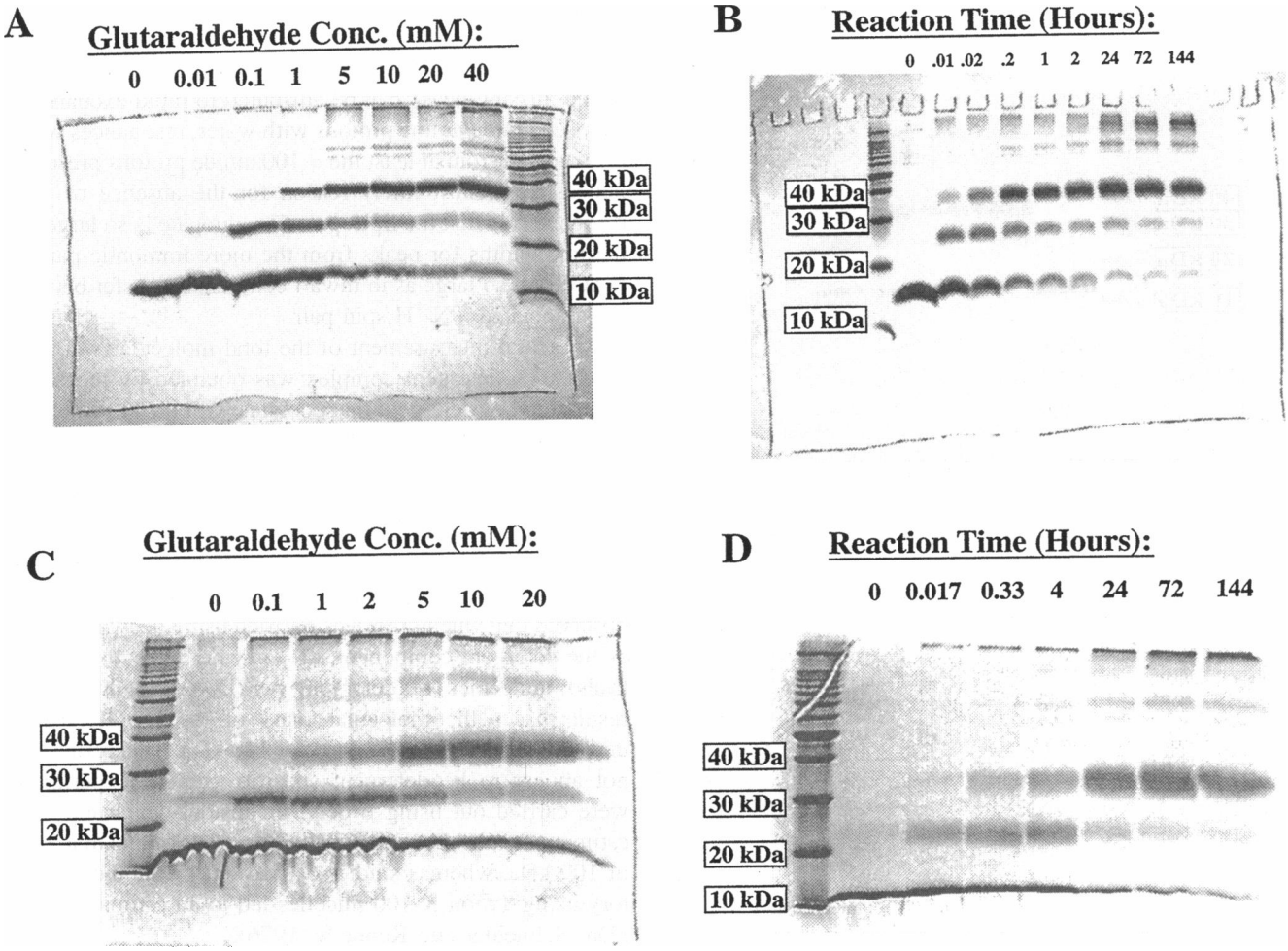

FIGURE 2 Chemical cross-linking of DAGK in micelles (*A* and *B*) and mixed micelles (*C* and *D*) at room temperature. (*A*) 25 μM DAGK in 25 mM decyl maltoside (DM) was incubated for 24 h at room temperature in the presence of the indicated concentrations of glutaraldehyde (GA). (*B*) 28 μM DAGK in 50 mM DM was incubated with 16 mM GA for various periods of time, as labeled. (*C*) 28 μM DAGK was incubated in 50 mM DM containing 10 mol% DMPC (5 mM) at various levels of GA for 5 h. (*D*) 28 μM DAGK was incubated in 50 mM DM containing 10 mol% (5 mM) DMPC and 5 mM GA for varying lengths of time.

linking reaction time at a fixed concentration of GA for micellar and mixed micellar DAGK, respectively. Again, as the cross-linking reaction time was increased, the covalent trimer emerged as the dominant covalent oligomer in both cases.

The results shown in Fig. 2 indicate that DAGK in micelles exhibits cross-linking properties very similar to those of DAGK in mixed micelles. Two additional experiments were carried out in micelles only. First, GA cross-linking was examined as the concentration of DAGK was varied with a constant concentration of detergent. As shown in Fig. 3 *A*, from 42 to 167 μM DAGK, there appeared to be little change in the distribution of observed covalent oligomers, indicating that cross-linking is occurring to a significant extent only within specific DAGK oligomers, not as a result of random collisions between DAGK monomers or between oligomers. This observation appears to break down at 334 μM DAGK, where higher oligomers appear to be pronounced relative to monomers, dimers, and trimers. Second, a chemically distinct type of cross-linking reaction was carried out by adding a reagent that promotes disulfide bond formation through a free radical-mediated mechanism (Cu(II)-phenanthroline; Careaga and Falke, 1992). DAGK contains two Cys residues, which are located near the periplasmic termini of TM1 and TM3 (Fig. 1). As shown in Fig. 3 *B*, Cu(II)-phenanthroline does indeed induce covalent oligomerization to an almost exclusively trimeric oligomeric state at high levels of the catalyst, supporting the conclusion that DAGK is a homotrimer. This result also suggests that Cys46 and Cys113 on adjacent subunits (but not on the same subunit) may be spatially proximal, providing a distance restraint useful for modeling the subunit interface. However, further work will be required to establish this because the results cannot rule out the possibility that the intersubunit disulfides form only via transient contact due to protein motions, and could actually

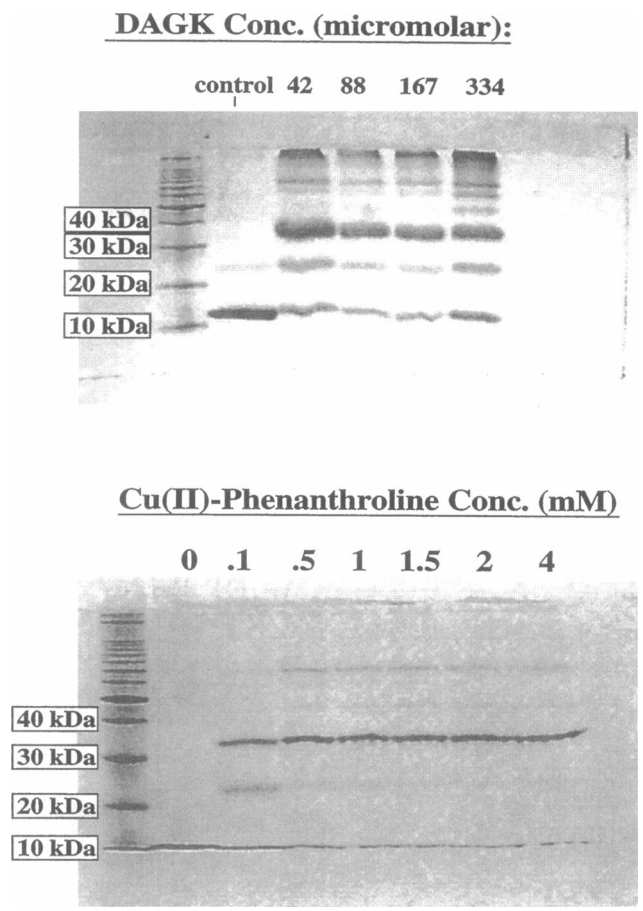

FIGURE 3 Additional cross-linking of DAGK in micelles at room temperature. (*Top*) Cross-linking of varying concentrations of DAGK in 25 mM DM in the presence of 33 mM GA for 5 h. (*Bottom*) Cross-linking of DAGK (14 μM) in 25 mM DM for 45 min in the presence of varying levels of copper(II)-phenanthroline.

have somewhat distal average positions (cf. Careaga and Falke, 1992).

Screening of micelles and organic solvent mixtures for optimal proton NMR conditions for observing DAGK

The fact that DAGK in DM micelles appears to be trimeric in both the presence and absence of added lipid suggests that the DM-DAGK complex is probably much too large for extensive structural analysis by solution NMR methods. This appears to be confirmed by the ^{1}H NMR spectra illustrated in Fig. 4, *A* and *B*, showing the aromatic/amide region of DAGK in DM micelles and DM/DMPC mixed micelles, respectively. Except for resonances from the poly-His tag, the resonances from the enzyme are extremely broad, as expected for a trimeric protein with associated detergent. The 1-D results were echoed by 2-D HSQC results (data not shown) for DAGK in neutral DM micelles in H_2O, obtained with a method for solvent suppression (Kay et al., 1992), which is designed to avoid elimination of amide resonances through saturation transfer with water. The spectrum exhibits only about 40 of the 150 possible cross-peaks from ^{15}N-attached protons. Although the some of the missing peaks can be attributed to rapid exchange of exposed amide/amine protons with water, resonances would be expected from at least the ~100 amide protons present in helices. The most likely reason for the absence of these resonances is that the lipid-protein aggregate is so large that the line widths for peaks from the more immobile parts of DAGK are so large as to thwart coherence transfer between the associated ^{15}N-^{1}H spin pair.

An actual measurement of the total molecular weight of the DAGK-detergent complex was obtained by measuring the amount of detergent that coelutes with the enzyme from the Ni(II)-agarose column in excess of the basal concentration of detergent present in the eluting buffer. This was determined by measuring the volume of the DAGK pool, measuring the DAGK content in the pool by spectrophotometric means, and then lyophilizing the pool and weighing the resulting salt-free powder. By this method it has been observed that when DAGK is purified using decyl maltoside as the detergent component, the enzyme binds 46 ± 5 (six trials) molecules of detergent per DAGK subunit. This result places the molecular mass of the DAGK trimer-detergent complex in the range of 110 kDa. This result does not appear to be detergent specific. Similar experiments were carried out using β-octyl glucoside, which led to an estimate for the DAGK-micelle aggregate molecular mass of 100 kDa, whereas studies carried out by another laboratory using Triton X-100 micelles led to an estimate of 120 kDa (Schneider and Kennedy, 1976).

As summarized in Table 1, micelles in no case yielded a well-resolved ^{1}H NMR spectrum of DAGK, despite a considerable exploration of composition space in which detergent type, detergent:DAGK ratio, salt content, pH, short chain-to-long chain detergent ratio (in binary mixtures), and temperature were varied. This result does not necessarily mean that DAGK is a trimer under all micellar conditions, because spectral broadening can arise from other factors. For example, a DAGK monomer-detergent mixed micelle might have an unexpectedly large aggregate size or the DAGK monomer might be conformationally heterogeneous. Indeed, DAGK runs as a monomer in SDS-PAGE gels and yet displays poor spectral resolution (Table 1, NMR data not shown).

The discouraging nature of the above results for DAGK in micelles led to the investigation of organic solvent mixtures as a medium in which both a transition to a monomeric state and a decrease in the detergent:DAGK ratio in the aggregate state could be induced. In these studies, lyophilized DAGK-DM powder was taken up by various solvent mixtures and screened for DAGK solubility, catalytic activity, and ^{1}H NMR spectral quality. At neutral pH DAGK maintained detectable catalytic activity, even in the presence of sometimes substantial percentages of organic solvent (Table 1, e.g., up to 75% methanol). However, the solubility of DAGK in those mixtures at neutral pH was

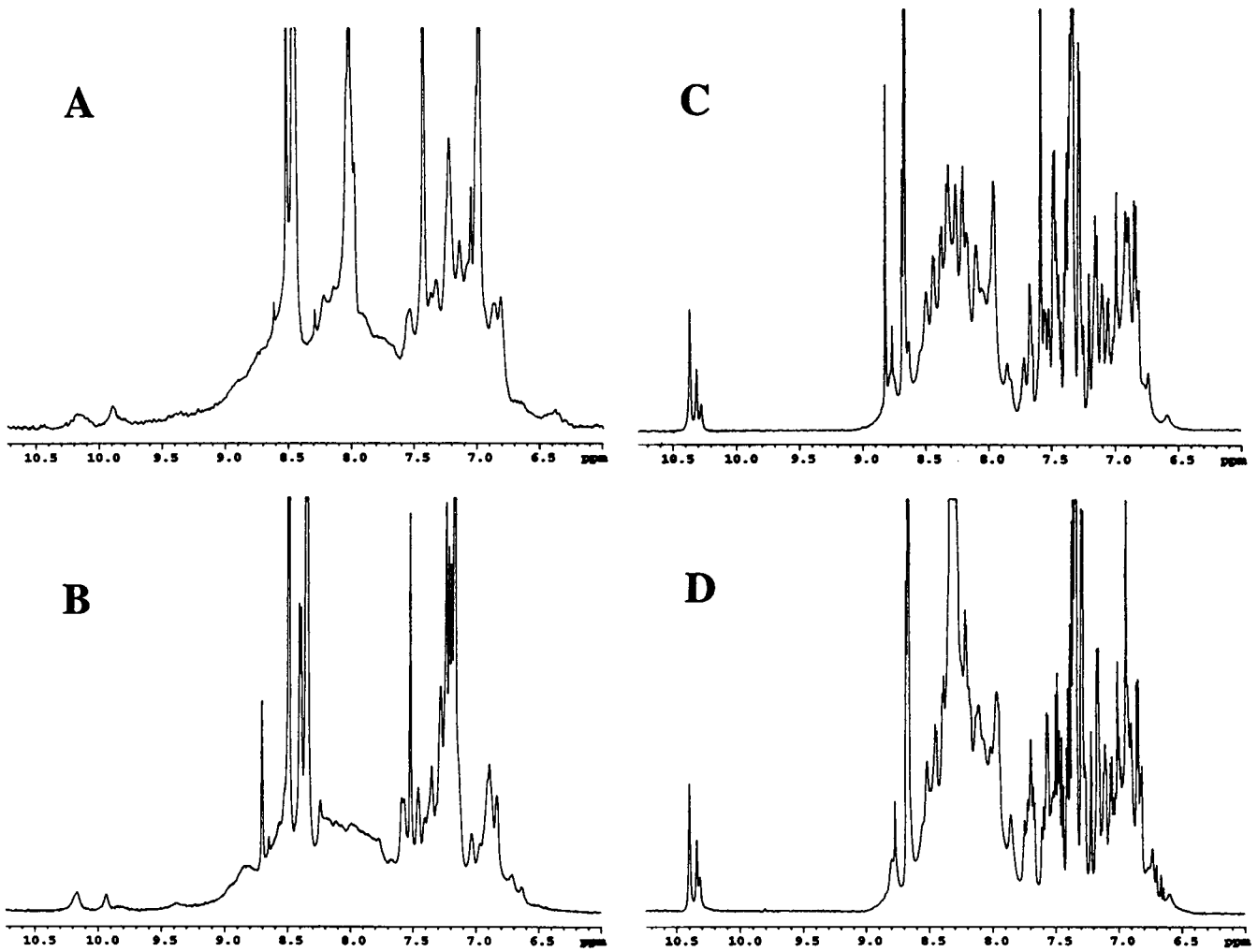

FIGURE 4 Aromatic/amide region of 600 MHz ^{1}H NMR spectrum of DAGK at 40°C. (*A*) DAGK in DM/DMPC mixed micelles: pH 7, 0.7 mM DAGK, 30 mM DM, 6 mM DMPC. (*B*) 0.7 mM DAGK in 30 mM DM micelles with no lipid; pH 7.0. (*C*) 0.6 mM DAGK plus 50 mM DM in 1:1 IPA:H_2O with 0.5% *d*-formic acid. (*D*) 0.6 mM DAGK with no lipid or detergent in 1:1 IPA:H_2O with 0.5% formic acid. The resonances chopped off at the top of each spectrum arise from either imidazole (left over from purification) or from the mobile residues on DAGK's polyhistidine purification tag. It should be pointed out that spectra very similar to *A–C* were obtained when otherwise similar samples containing much higher DM:DAGK ratios were examined.

generally very low, precluding acquisition of even simple 1-D NMR spectra.

The DAGK/DM powder was highly soluble in most organic solvent mixtures acidified by 0.5–1% formic acid. Given that the enzyme is always inactive at acidic pH, it is not surprising that the enzyme is not active in the acidic solvent systems. However, in many of these mixtures DAGK yields a proton NMR spectrum of fairly high resolution, suggestive of a substantial reduction in effective molecular mass due to trimer dissociation and/or loss of detergent binding. Among the systems listed as yielding a "high resolution" NMR spectrum in Table 1, the acidic IPA:water system was chosen for more extensive characterization. Its NMR spectrum is shown in Fig. 4 *C*; it is very similar to the spectra from a number of other acidic solvent mixtures listed in Table 1. It should be pointed out that many of the procedures used for water-IPA mixtures in the following sections were also carried out for DAGK in acidic water-tetrahydrofuran mixtures and inevitably yielded results similar to those for water-IPA.

Characterization of the detergent-associating properties of DAGK in acidic 1:1 isopropanol:water

DAGK (1 mM) in DM micelles (110 mM DM) was titrated with IPA from 0% to 75% IPA, and both DM and DAGK peaks were followed by NMR. For purposes of simplicity, only 7 of the 12 points actually acquired are represented in Fig. 5, and only the Trp indole N-H and a small portion of the DM headgroup/alkyl linkage are shown. As exemplified by the indole N-H, the relatively high resolution shown in the DAGK aromatic/amide region illustrated in Fig. 4 *C* becomes evident only at IPA levels of >30%.

Classical DM micelles do not appear to be present in 1:1 water-IPA mixtures. Spectra of aqueous DM (no DAGK)

TABLE 1 Micelles and solvent mixtures screened

Detergent or solvent mixture	pH	Temp.	Solubility*	DAGK activity#	CD:2° structure	^{1}H NMR spectrum
Aqueous detergent micelles						
Decyl maltoside + added DMPC or cardiolipin (many compositions)	6–8	25–50	High	20 U/mg	Largely helical	Very broad
Decyl maltoside + added DMPC or cardiolipin (many compositions)	2–5	25–50	High	None	ND§	NA
Decyl maltoside, 4–14%	5–8	25–50	High	0.4 U/mg	Helical	Broad
Decyl maltoside, 4–14%	2–4	25–50	High	None	Helical	Broad
4% DM + 10% sodium dodecylsulfate	7	25–60	High	None	ND	Broad
4% DM + 3.4% β-hexyl glucoside	7	25	High	ND	ND	Broad
9% β-octyl glucoside ± added phospholipid	7	25	Moderate	None in absence of lipid, 15 U/mg with lipid	ND	Broad
10% dodecyl maltoside	7	25–50	High	>1 U/mg	ND	Broad
10% dodecyl maltoside + 7% CHAPSO	7	25	High	ND	ND	Broad
9% cymal-4	7	25–50	High	ND	ND	Broad
6% empigen (with or without added DMPC)	7.8	25	High	ND	ND	Broad
10% dihexanoylphosphatidylcholine	6–8	25	High	15 U/mg with lipid	ND	Broad
8% CHAPSO (with or without added DMPC)	7.9	25–40	Moderate	>1 U/mg with lipid None in absence	ND	Broad
4% dodecyltrimethylammonium bromide	7.8	25–40	High	ND	ND	Broad
5% zwittergent 3-12	7.9	25–35	Moderate	ND	ND	Broad
5% zwittergent 3-12 + 5% zwittergent 3-8	7.9	25	Moderate	ND	ND	Broad
5% Triton X-100	7.9	30–50	Moderate	>5 U/mg with lipid	ND	Broad
9% dodecylphosphocholine	4.5	20–50	High	0.7 U/mg	ND	Broad
Organic solvent mixtures¶						
15–75% methanol in water	5.3–6.5	20	Low	Yes‖	ND	NA
15–75% methanol in water + 1% Formic acid	2.5	20–40	High	No	Helical	Medium resolution
10–25% IPA in water	6.5	20	Low	Yes	ND	NA
40–50% IPA in water	6.5	20	Low	No	ND	NA
50% IPA in water + 0.5% formic acid**	2.5	40	High	No	Helical	High resolution
12% THF in water	6.5	20	Low	Yes	ND	NA
20–50% THF in water	6.5	20	Low	No	ND	NA
50% THF in water + 0.5% formic acid**	2.5	40	High	No	No	High resolution
6% hexafluoroisopropanol in water	6.5	20	High	Yes	ND	Broad
12–80% HFIP in water	4–6.5	20–40	High	No	Helical	Med. to high resolution
6% trifluoroethanol in water	6.5	20	ND	Yes	ND	NA
12–100% TFE in water	6.5	20	ND	No	ND	High (taken at 100%, only)
Chloroform:methanol:water 66:33:4##	6.5	20	Low	No	ND	NA
Chloroform:methanol:water 66:33:4 + 1% formic acid	2.5	40	High	No	ND	High resolution
1:1 benzene:ethanol, 25–90% in water##	6.5	20	Low	No	ND	NA
Benzene:methanol:water 5:5:1 + 0.5% formic acid	2.5	40	High	No	ND	Medium resolution
Butanol:methanol:water 2:1:1 + 1% formic acid##	2.5	40	High	No	ND	High resolution
Dimethylsulfoxide:water 1:1 with 1% acetic acid	3.5	40	High	ND	ND	Broad

*"High" indicates that DAGK was observed to be soluble in the >5 mg/ml range, with no aggregation normally being observed under any circumstances in the medium of relevance. "Moderate" indicates that some aggregation could sometimes be observed in the form of visible precipitate or suspension, but that the solubility was at least high enough to allow facile acquisition of a 1-D ^{1}H NMR spectrum. "Low" refers to solubility at a level too low for NMR to be carried out.

#One unit of DAGK activity equals 1 μmol of DAG converted to PA per minute. Fully active DAGK normally exhibits a specific activity of about 15 units/mg in the coupled assay system (see Methods) containing mixed micelles composed of β-octylglucoside and DMPC. DAGK was not directly assayed in many of the micellar mixtures listed in this table. In the case of the organic solvent mixtures, we report in this table simply whether any reaction could be detected within 48 h. At the typical DAGK concentrations used and given the sensitivity of detection associated with this assay, the reaction could be detected if the DAGK specific activity was ca. $\geq 1 \times 10^{-6}$ of the normal activity of DAGK in octyl glucoside-DMPC mixed micelles (15 U/mg).

§ND, not determined; NA, not acquired.

¶Unless otherwise noted, all of these mixtures also contained decyl maltoside (typically at a level of ~60 mM in the NMR experiments and 20 mM in the kinetic assays. As described in the Methods section, purified DAGK has considerable DM associated unless specific effort is made to remove it.

‖Even in 60% methanol and in the absence of DMPC (as "lipid activator"), reaction could be detected.

**NMR spectra were also taken at lower percentages of THF and IPA and at lower temperatures. Spectral resolution gradually decreases as either variable is gradually lowered.

##Assays were difficult to carry out at neutral pH because attempts to increase the water content resulted in phase separation.

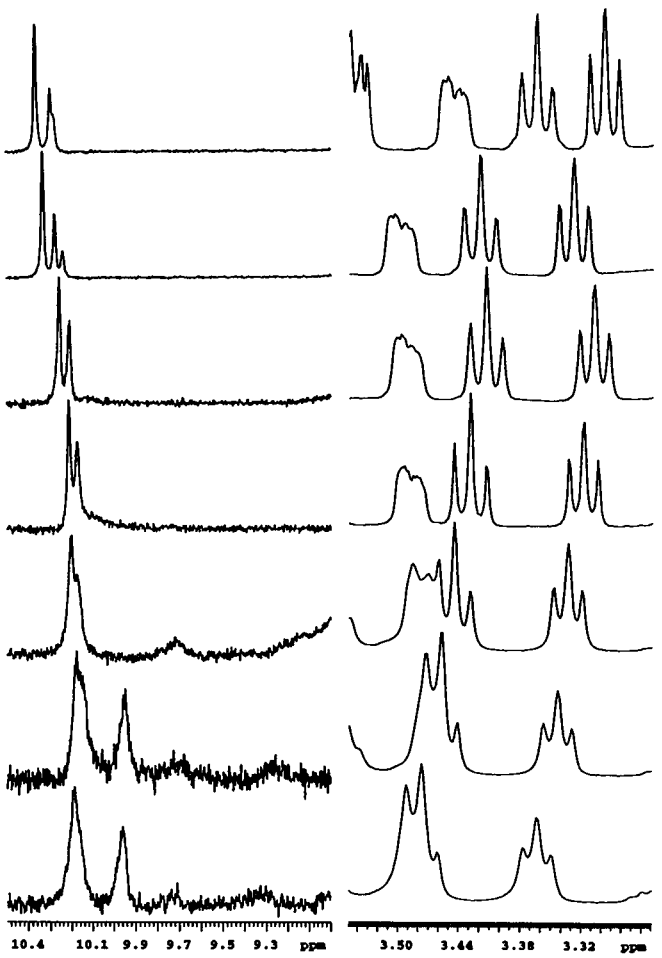

FIGURE 5 Selected regions of the 1-D ^{1}H NMR spectra of 0.7 mM DAGK plus 27 mM decyl maltoside in acidic H_2O during titration by isopropyl alcohol at 40°C. Spectra are labeled with the percentage of IPA present. (*Left*) Trp indole N-H signals from DAGK. (*Right*) peaks from the headgroup/linker region of DM.

were acquired at concentrations both well above and well below the critical micelle concentration and exhibit distinctive differences in the maltoside headgroup resonances (data not shown). Using these free monomer and micellar spectral fingerprints and examining the region of the DM spectrum shown in Fig. 5, it was possible to ascertain that by ~25% IPA, the DM micelles are dispersed. The additional shifts of the detergent peaks between 50 and 75% most likely result from bulk solvent effects.

While micelles are not present, some of the DM present in the acidic 1:1 water-IPA sample remains associated with DAGK. The 1-D ^{1}H NMR spectrum of detergent-free DAGK (Fig. 4 *D*; see Methods) was compared with that from the standard DM-containing mixture (Fig. 4 *C*). There are significant differences (cf. 6.5–7.0 ppm), implying that DM persists in associating with DAGK. This interpretation was confirmed by measuring the translational diffusion constant for DM in acidic water-IPA in both the presence and absence of 1 mM DAGK (see NMR Methods). It was observed that the diffusion constant for the detergent in these samples was considerably higher in the absence of DAGK ($210 \times 10^{-7} \pm 80 \times 10^{-7}$ cm^2/s) than in the presence of the enzyme ($33 \times 10^{-7} \pm 5 \times 10^{-7}$ cm^2/s). This indicates that a fraction of the DM present in the DAGK-containing sample is in a relatively slow diffusion environment, presumably bound to DAGK. Relaxation rate measurements (see next paragraph) led to the observation that the global correlation time for DAGK is smaller in the DM-free sample (9.5 ± 0.5 ns) than in the detergent-containing sample (11.5 ± 0.9 ns), as expected. Because correlation times are roughly proportional to molecular mass (assuming similar molecular shapes), the observed 12% increase in correlation time for DAGK when DM is present suggests that there are several detergent molecules bound to each DAGK.

The complexation of DM with DAGK appears to be irreversible: when the detergent-free sample represented by Fig. 4 *D* was titrated with DM, the spectrum of the enzyme did not change significantly (no reversion to Fig. 4 *C*). This result indicates that when DAGK-DM powder is dissolved by acidic water-IPA, an irreversible detergent-protein complex is present. This does not imply that there is no exchange between bound and free detergent populations. Rather, this result suggests that the DAGK-DM complex represents a kinetically "trapped" aggregate, which to some extent reflects components of the original structure of the DAGK-DM complex in water before lyophilization and redissolution in acidic water-IPA.

Characterization of the oligomeric state of DAGK in acidic 1:1 isopropanol-water

The effective molecular mass of DAGK in the DAGK/DM complex in acidic water-IPA was investigated by measuring ^{15}N T_1 and T_2 relaxation times for some of the DAGK backbone amides (see Methods). From these relaxation times a correlation time (τ_c) was calculated, assuming that only a single motion (overall molecular tumbling) affects the relaxation rates. For the narrowest amide peaks (which most likely arise from the polyHis tag), correlation times calculated from T_1 did not generally agree with those calculated from T_2, indicating that the assumption of only a single relevant motion does not apply. On the other hand, when the broadest resolvable peaks were examined or when the entire amide region was integrated, T_1 and T_2 led to the same correlation time (below), indicating this measurement is a valid indicator of overall DAGK tumbling. A globular 14.5-kDa protein would be expected to yield a correlation time of 3.6 ns, based on the Stokes-Einstein equation. However, experimental correlation times for globular proteins in water are generally in the neighborhood of 2× the predicted τ_c (Marshall, 1978; Sanders et al., 1989), such that a monomeric DAGK is expected to exhibit a τ_c of about 7 ns. DAGK exhibited a τ_c of 11.5 ± 0.9 in acidic water-IPA, a result consistent with DAGK being predominately a monomer with some associated DM in that solvent system. We

next pursued a closer examination of the nature of the structural state adopted by monomeric DAGK when complexed with DM in acidic 1:1 water-IPA.

Environment dependence of DAGK's secondary structure

FTIR studies indicated that at least 90 of DAGK's native 121 residues reside within α-helices in the bilayer-associated enzyme (Sanders et al., 1996). In this study DAGK's secondary structure under selected mixed micellar, micellar, and organic solvent conditions was examined using far-UV CD spectroscopy, as summarized in Table 1 and illustrated in Fig. 6 *A*. The CD data can be qualitatively interpreted to indicate that DAGK is a largely α-helical protein under the various conditions tested. Although it would appear that there may be some variation in the exact percentage of helix from case to case, under no conditions is a major transition to a nonhelical secondary structure evident. The persistence of DAGK's helical secondary structure probably extends over most of the compositions represented in Table 1, based on the similarity of the NMR spectra from many of those samples to spectra from samples for which CD data were actually acquired. Unpublished results from another laboratory (J. Bowie, personal communication) suggest that DAGK is also highly helical in both urea and SDS solutions. A more quantitative analysis of the CD data of Fig. 6 *A* is not presented because quantitative interpretation of CD data from membrane proteins is not straightforward (Park et al., 1992; Fasman, 1995).

Examination of the general nature of the tertiary structural state adopted by DAGK in acidic 1:1 water-IPA

The observation that DAGK is monomeric in acidic organic solvent mixtures and yet maintains detergent-binding capacity led to the question of whether DAGK maintains any native-like tertiary structure. This issue was investigated using NMR and near-UV CD spectroscopy.

The 1-D proton NMR spectrum of DAGK in acidic 1:1 water-IPA shows less spectral dispersion in the amide/aromatic (Fig. 4 *C*) and upfield-shifted aliphatic (not shown) spectral regions than do spectra of the enzyme in micelles. For example, in DM micelles (Fig. 4 *B*), at least two of the five Trp ring N-H protons are shifted well upfield from their random coil chemical shift positions ($\sim$10.2 ppm) and are very broad, whereas in water-IPA all four indole N-H resonances appear near 10.2. Several broad peaks are clearly visible in the $<$0 ppm region of DAGK's spectrum in DM micelles that are completely missing in the water-IPA spectrum (not shown). These results suggest that the degree of defined tertiary structure maintained by DAGK in water-IPA is less than in aqueous detergent micelles. The amide/aromatic region of the ^{1}H NMR spectrum of DAGK in water-IPA shows a relatively low degree of chemical shift dispersion (Fig. 4 *C*), an observation not surprising for a largely helical protein. However, the amide resonances tend to be very broad, whereas many of the aromatic resonances are quite sharp. These observations are characteristic of proteins that exist in nonrandom but partially disordered conformational states (Alexandrescu et al., 1993). This conclusion is further supported by a ^{1}H-^{15}N HSQC spectrum acquired in which about 60 of DAGK's amide/amine peaks can be resolved (Fig. 7 *A*)—more than expected for a random coil protein, but less than for a well-folded 13-kDa monomer. Because the method used for water peak suppression in the HSQC experiment is designed to avoid amide resonance saturation via saturation transfer with water (Kay et al., 1992), the missing peaks are most likely absent as a result of conformational heterogeneity and/or intermediate time scale conformational exchange

Attempts were made to determine the extent of residual tertiary structure for DAGK in acidic water-IPA. A 2-D NOESY spectrum was acquired (Fig. 7 *B*), which shows a considerable number of aromatic/amide-aliphatic NOEs. However, most of the cross-peaks appear to arise from DAGK-detergent cross-relaxation rather than from intraprotein NOEs. One fairly clear example is provided by the four indole N-H, which resonate near 10 ppm. These protons exhibit only intraresidue aromatic-aromatic NOEs. Furthermore, most of their NOEs, to 0–5 ppm species, appear to involve detergent protons. These results, along with additional ^{15}N half-filtered NOESY and 1-D NOE difference experiments (not shown), support the notion that DAGK complexed with DM in acidic 1:1 water-IPA maintains only a modest degree of tertiary structure.

Near-UV CD spectra can also be used to provide a qualitative measurement of the degree of tertiary structure present in a protein (Strickland, 1974), based on the magnitude of dichroism observed in the 250–300-nm region. Spectra are shown for DAGK in DM-DMPC mixed micelles, DM micelles, and acidic water-IPA (with DM present) in Fig. 6 *B*. The minimal near-UV dichroism exhibited by DAGK in the mixture, along with the NOESY data, strongly supports the conclusion that the degree of stable tertiary structure present for the enzyme in the organic solvent mixtures is modest.

DISCUSSION

Metal ion chelate chromatography is ideally suited for preparation of membrane protein NMR samples

In this paper we have demonstrated that metal ion chelate chromatography can be uniquely adapted to preparing polyhistidine-tagged membrane proteins for NMR in either of two distinct purification modes. First, once the stage in the purification process was reached where only DAGK remained bound to the resin, it was possible to rapidly and efficiently reequilibrate the resin/enzyme with the detergent of choice for NMR, followed by elution. This capability to

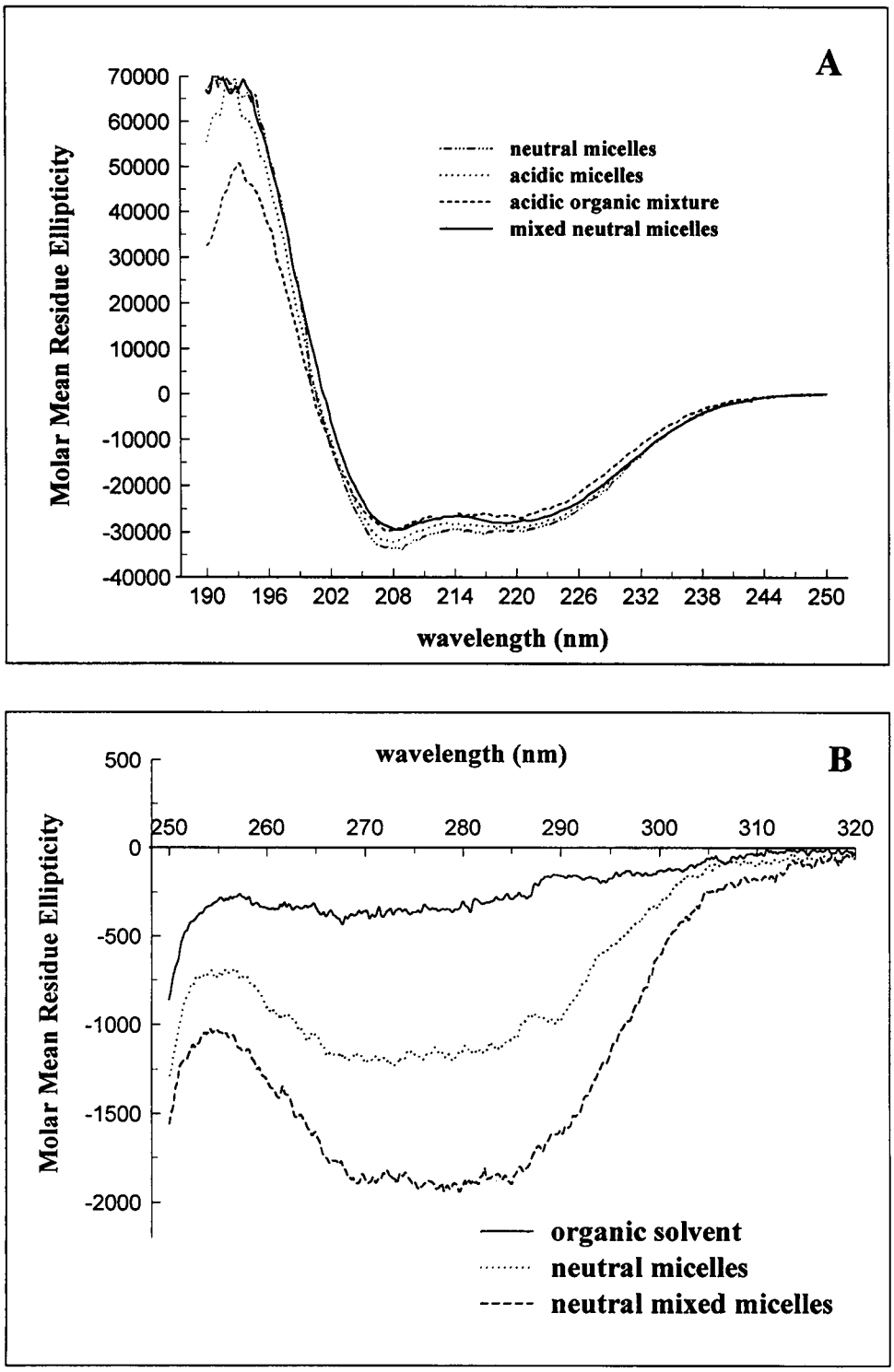

FIGURE 6 Circular dichroism of DAGK in various solutions at 25°C. (*A*) Far-UV CD spectra; (*B*) near-UV CD spectra. The acidic organic solvent mixture was 1:1 isopropanol-water plus 0.5% formic acid. The acidic micelles were composed of 21 mM decyl maltoside at neutral or low pH (acidified with 0.5% formic acid). The mixed micelles contained 4 mM DMPC in the sample represented by the far-UV spectrum and 1 mM cardiolipin in the sample represented by the near-UV spectrum. Additional details can be found in the Methods section.

carry out a low volume exchange of detergent types immediately before purification is attractive because it allows one to switch from an inexpensive detergent that is not optimal for NMR (used in the extraction and column washing stages, which require a large amount of detergent) to a more expensive detergent at the critical elution step. Because

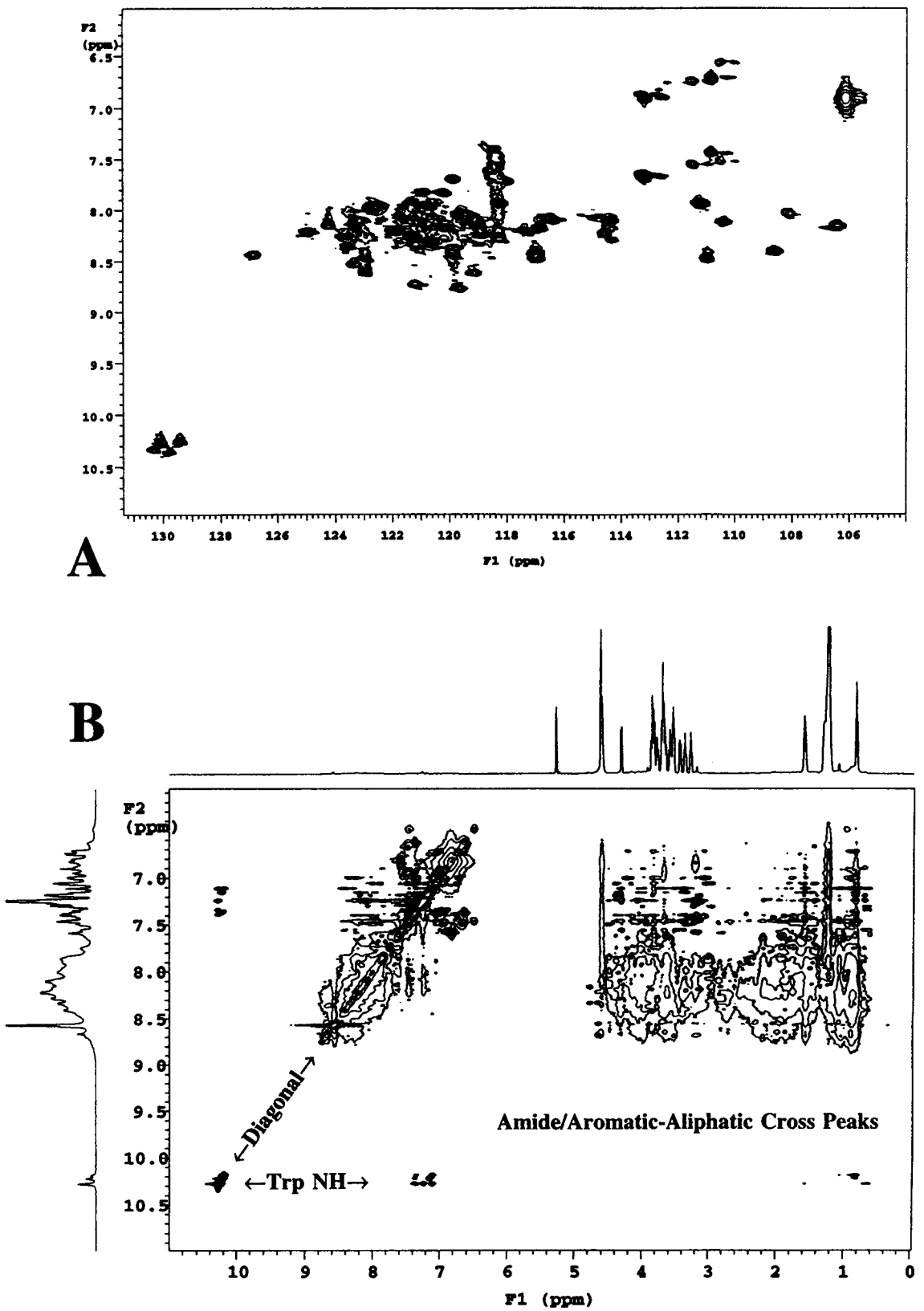

FIGURE 7 600 MHz ^{1}H-^{15}N HSQC (*A*) and NOESY (*B*) of 0.9 mM DAGK plus 150 mM decyl maltoside dissolved by 1:1 isopropanol:water (10% D_2O) plus 0.5% *d*-formic acid at 40°C. For the HSQC experiment 512 t1 points of 64 scans each were taken, and DAGK was uniformly ^{15}N-labeled. For the NOESY spectrum, 300 points of 128 scans each were acquired, the protein was unlabeled, and the water resonance was presaturated. The f1 projection of the NOESY spectrum shows the decyl maltoside peaks, which dominate the 1-D spectrum (the DAGK aromatics can just barely be seen). The f2 projection shows a close-up of the DAGK aromatic/amide region.

deuterated detergents are often used in NMR studies to suppress background detergent proton resonances and sometimes cost in excess of $1000/g (e.g., d_{38}-dodecylphosphocholine), this is an attractive option.

A second chelate chromatographic purification mode, demonstrated for the first time in this paper, involves re-equilibration of the pure protein on-resin with a salt-free organic solvent mixture, followed by elution in that same mixture. This procedure allows complete removal of detergent and lipid from the membrane protein and solubilization in that form. In addition to its utility in preparing samples for NMR, another area to which this method may be particularly well suited is in the preparation of membrane protein samples for mass spectroscopy. Both electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)-based mass spectroscopic techniques have been generally difficult to implement on membrane proteins, in large part because of interference by associated lipid/detergent/salt (Zaluzec et al., 1995; Hufnagel et al., 1996).

General environment dependence of the structure of DAGK

The studies of this paper established that catalytically functional DAGK in micelles and mixed micelles is a trimer. It is not surprising that DAGK is an oligomer. The presence of amphipathic helix 2 between TM2 and TM3 (see Fig. 1) makes it almost impossible to derive a tertiary structural model for a monomeric DAGK in which all three TM helices are packed with one another (regardless of the orientation of amphipathic helix 2). This issue is resolved for a DAGK trimer because a variety of models can be derived (Sanders et al., unpublished observations) in which TM3 of one subunit contacts TM helices in adjacent subunits, and in which all nine TM helices pack to form a continuous membrane domain. It is, perhaps, notable that DAGK is a trimer, because protein trimers appear to be relatively rare compared to dimers and tetramers (Jones and Thornton, 1996), and because we are unaware of any other kinase that is a trimer. The cross-linking results also indicated that whereas the presence of phospholipid in micelles is required for the full catalytic activation of DAGK (Walsh and Bell, 1986a,b), lipid is not required for the formation of DAGK's oligomeric state.

The results of this study established that solubilization of DAGK in acidic isopropanol-water results in a loss of its trimeric oligomeric state and much of its tertiary structure. Whereas detergent micelles are not sustained in these mixtures, DAGK persists in binding several molecules of detergent. Based on the fact that these observations were made for the enzyme in a number of distinct solvent systems (e.g., both IPA-H_2O and THF-H_2O; see Table 1), it seems probable that DAGK monomerization, DAGK unfolding, and micelle dissolution are highly cooperative. The loss of DAGK's quaternary structure and much of its tertiary structure in the presence of isopropanol did not result in a substantial loss of its helical secondary structure.

DAGK was observed to bind multiple DM in an irreversible fashion when DAGK/DM powder is dissolved in acidic water-IPA. Because this complex represents a kinetically trapped state, having its origins in solvent free DM-DAGK micelles, it likely reflects a particular avidity and possible specificity for of DAGK monomers for lipid-like molecules. Irreversibility also suggests a certain degree of stable tertiary structure, indicative of a certain "core" conformation for part of the DAGK molecule, which can persist even in the absence of micelles or bilayers. However, the results suggested that even this tertiary structural core is probably conformationally heterogeneous.

The behavior of DAGK in organic solvent mixtures appears to be similar to that of bacteriorhodopsin (BR) (27 kDa, seven transmembrane helices). In extensive NMR studies of BR and derived fragments in acidic organic solvents, Arseniev and co-workers have shown that although secondary structure is generally maintained for BR's transmembrane helices, and although there is evidence for residual tertiary structure, it appears to be disordered, seriously hindering structural analysis by NMR. The DAGK and BR results are in clear contrast to the case of subunit c of the F_1F_o ATP synthase (8.5 kDa), which does maintain a defined conformation involving observable contacts between its two transmembrane segments in certain organic solvent mixtures (Girvin and Fillingame, 1993, 1995).

Aggregate molecular weights of protein micelle complexes

When planning studies of a membrane protein in micelles, it is generally desirable to estimate in advance the effective molecular mass of the detergent-protein micellar complex to ensure that the size of the complex will be within the molecular mass limitations for NMR analysis. In making this estimate, it is tempting to simply sum the molecular mass of the protein of interest and the molecular mass of protein-free micelles of the detergent. By this method and based on the known aggregation number for micellar β-OG (84; Neugebauer, 1988), we would have expected an aggregate molecular mass for trimeric DAGK in β-OG micelles of 39 + 25 = 63 kDa. In fact, as described in the Results, the actual molecular mass was determined to be close to 100 kDa, indicating that the effective aggregation number of β-OG in the mixed protein-detergent micelles increased by a factor of nearly 3 relative to simple β-OG micelles.

The above observation is not unexpected. The amount of detergent that will be present in a mixed detergent-protein micelle is largely dependent upon the hydrophobic surface area of the protein, which must be stabilized by interaction with the nonpolar chains of the detergents (Moller and le Maire, 1993). Thus molecular weights of protein-detergent complexes may be very difficult to predict. These observations suggest that the experimental determination of the

actual aggregate molecular mass of a given membrane protein with the detergent or detergents of interest before NMR analysis may save time and the expense of preparing isotopically labeled NMR samples, only to find that the effective molecular mass of the complex is prohibitively large.

Implications for future structural study of DAGK

Although DAGK appears to maintain some residual tertiary structure in organic solvent mixtures, determination of the details of this structure would require considerable effort, and it is unclear whether the information ultimately gleaned would be worth the investment. DAGK's structure in some micellar systems and many mixed micellar systems is probably native-like, based on its catalytic viability. However, the results of this study indicate that even for a short-chained detergent such as β-OG, the molecular mass of the DAGK-detergent mixed micelles is in the neighborhood of 100 kDa. This is well beyond the present nominal molecular weight limit of about 40 kDa for structural analysis by NMR. Nevertheless, because only 121 residues would have to be assigned, the possibility cannot be completely ruled out that one might be able to carry out productive studies on uniformly $^{15}N/^{13}C$-labeled DAGK which is also randomly fractionally deuterated to enhance line widths and eliminate spin diffusion pathways (Sattler and Fesik, 1996). More attractive immediate routes for structural analysis would appear to be the emerging methods of solid-state NMR and disulfide mapping, both of which are well suited to the study of membrane proteins in bilayers.

We thank James Bowie of UCLA for providing us with wild-type and mutant DAGK-overexpressing strains of *E. coli* used in this work, and for much helpful discussion.

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