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NMR Assignments for a Helical 40 kDa Membrane Protein

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The observation of the TROSY phenomenon and the introduction of related NMR pulse sequences by Wüthrich and co-workers[1] enables the detailed NMR characterization of relatively large biomolecules and complexes. This development has been especially welcome in the field of integral membrane protein (MP) structural biology. Using TROSY-based NMR methods, several labs have recently completed the structures of 15−20 kDa monomeric β-barrel outer membrane proteins as part of much larger micellar complexes.[2] For MPs with multiple transmembrane (TM) helices, the only examples of completed NMR structures are represented by classic studies of glycophorin A and subunit c of the F_1,F_o-ATPase, both of which have two transmembrane segments.[3] Preliminary reports of completed assignments for micellar MPs with two to three transmembrane segments have also appeared.[4] Resonance assignments have also been made for polytopic membrane proteins solubilized in organic solvent mixtures, but in these cases retention of stable native tertiary structure is doubtful.[5] Here we report NMR assignment of the backbone resonances for a functional polytopic membrane protein with nine TM segments in detergent micelles.

Escherichia coli diacylglycerol kinase (DAGK) is a 40 kDa homotrimeric membrane protein composed of 121 residue subunits, each of which has three transmembrane segments. DAGK catalyzes the phosphorylation of the lipid diacylglycerol by MgATP to form phosphatidic acid and MgADP and has served as a model system for MP biocatalysis, stability, folding, and misfolding.[6] Biochemical studies have suggested that the second transmembrane segment of each subunit forms a bundle representing the threefold axis of symmetry of the homotrimer[7] and that DAGK's three active sites lie at the interfaces between subunits.[8] Here, we describe studies involving functionally similar wild-type (wt-DAGK) and superstable mutant[9] (s-DAGK) forms.

All NMR experiments were carried out on DAGK which is solubilized using dodecylphosphocholine (DPC) micelles.[10] Wt- and s-DAGK retain their homotrimeric oligomeric state in DPC and are catalytically functional (V_{max} is similar to that under ideal membrane conditions, but K_m is significantly elevated for both MgATP and diacylglycerol). By four different methods, the aggregate molecular weight for the protein micellar complexes was determined to be in the 90−110 kDa range.[10]

The sequential backbone resonance assignments of uniformly ^{2}H,^{13}C,^{15}N-labeled DAGK proteins were carried out at 45 °C using TROSY-class triple-resonance experiments.[11] The use of TROSY and very high magnetic fields (750−800 MHz proton frequency) were found to be essential to avoid widespread loss of signals due to large line widths. It was also found that 100% perdeuteration of DAGK was essential for assigning many resonances. The N-terminus of the ultrastable s-DAGK was almost completely assigned, as noted previously.[12] However, it was found that a cluster

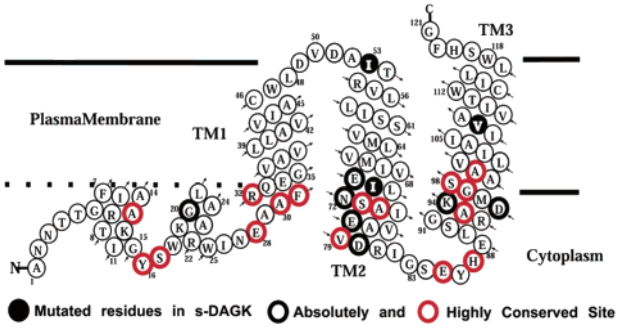

Figure 1. Secondary structure and membrane topology of DAGK based on the present work and on previous results.[12] The exact location of the linker between TM2 and TM3 is not unambiguous based on the data.

of about 20 residues located in transmembrane segments 2 and 3 were exceedingly resistant to back-exchange because of the high stability. Despite many months of effort, we were unable to develop a successful unfolding/refolding protocol to force back-exchange. Moreover, the resonances for these residues could not be accessed by using samples subjected to <100% perdeuteration. In the case of wt-DAGK, an unfold/back-exchange/refold protocol was developed that allowed amide protons to be detected from these exchange-resistant sites.[10] The disadvantage of working with wt-DAGK is that, unlike s-DAGK, the protein degrades over a period of days at 45 °C. Thus, samples of wt-DAGK had to be monitored during the course of a lengthy series of NMR experiments to ensure that samples were discarded at the point where signals from the denatured protein became problematic. While differing in stability, wt- and s-DAGK yielded very similar TROSY spectra (see Table of Contents Graphic), suggesting little difference in overall structure.

Sequential NMR spin system connectivities were established using [^{15}N−^{1}H]−TROSY−HNCA and [^{15}N−^{1}H]−TROSY−HNCACB experiments at 750 and 800 MHz, which provided intraresidual and sequential cross-peaks of C^{α} and C^{α}/C^{β}, respectively. Ambiguities were resolved by [^{15}N−^{1}H]−TROSY−HN(CO)CA and [^{15}N−^{1}H]−TROSY−HN(CO)CACB, which provide only sequential cross-peaks. For s-DAGK, 4D HNCACO and 4D HNCOCA experiments[13] were also used to confirm assignments. Approximately 80% (98 of 121 residues) and 90% (108 of 121) of backbone resonance assignments were completed for s-DAGK and wt-DAGK, respectively. The lower percentage achieved for s-DAGK reflects incomplete amide D→H back-exchange, as described above.

The analysis of ^{13}C secondary chemical shifts of wt- and s-DAGK indicates that they have the same secondary structural elements, mainly α-helices.[14] The NMR results confirm that DAGK's three transmembrane segments are helical, while also indicating that segments 2 and 3 are considerably longer than previously thought[15a] (Figure 1).

The amide exchange-resistant residues in s-DAGK are located in the second and third transmembrane helices of s-DAGK. All

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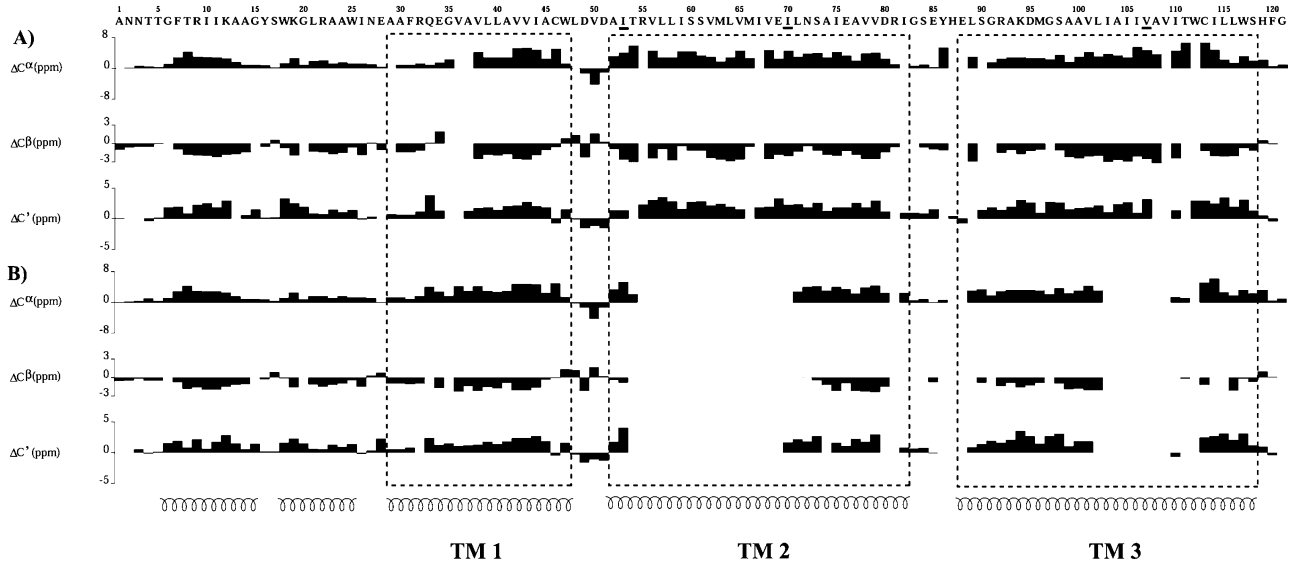

Figure 2. ^{13}C secondary chemical shifts, secondary structure elements, and transmembrane (TM) segments[12,15] (hatched boxes) of (A) wt-DAGK and (B) s-DAGK. On the basis of positive chemical shifts of $^{13}C^{\alpha}$ and $^{13}C'$ and the negative chemical shifts of $^{13}C^{\beta}$, the α-helical structure elements are identified, as displayed at the bottom of this figure.

three residues that are mutated (I53C, I70L, V107D) to convert the wild-type enzyme into s-DAGK[9] are located within or immediately adjacent to this region.

The completion of the majority of NMR resonance assignments for wt-DAGK represents an important step toward structural determination using NMR methods. Supporting the feasibility of future progress, we have recently been able to marginally align DAGK in DPC micelles using stretched[16] polyacrylamide gels (unpublished). Moreover, $^{1}H/^{13}C$ HMQC spectra of DAGK, which has been Leu/Ile/Val methyl-protonated in an otherwise perdeuterated background,[17] exhibit well-resolved spectra in which 90% of the expected resonances are observed (unpublished). In addition to being a milestone in the process of structural determination for DAGK, the results of the present work suggest that helical membrane proteins having molecular weights and numbers of transmembrane segments similar to DAGK may also now be regarded as feasible targets for NMR resonance assignments. This would include monomeric G protein-coupled receptors, although as 40 kDa monomers they would be expected to be significantly more difficult to assign than the homotrimeric DAGK.

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Supporting Information Available: Methods, NMR spectra, and tabulated assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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