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Prokaryotic Diacylglycerol Kinase and Undecaprenol Kinase

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Abstract

Prokaryotic diacylglycerol kinase (DAGK) and undecaprenol kinase (UDPK) are the lone members of a family of multispan membrane enzymes that are very small, lack relationships to any other family of proteins—including water soluble kinases, and that exhibit an unusual structure and active site architecture. *Escherichia coli* DAGK plays an important role in recycling diacylglycerol produced as a byproduct of biosynthesis of molecules located in the periplasmic space. UDPK seems to play an analogous role in Gram-positive bacteria, where its importance is evident by the fact that UDPK is essential for biofilm formation by the oral pathogen *Streptococcus mutans*. DAGK has also long served as a model system for studies of membrane protein biocatalysis, folding, stability, and structure. This review explores our current understanding of the microbial physiology, enzymology, structural biology, and folding of the prokaryotic diacylglycerol kinase family, which is based on over 40 years of studies.

Keywords

Diacylglycerol kinase; Undecaprenol kinase; *dgkA* gene; Membrane protein structure; Membrane protein folding

Introduction

Members of the prokaryotic diacylglycerol kinase (DAGK) and undecaprenol kinase (UDPK) family are encoded by the *dgkA* gene and are among the smallest known kinases (usually < 140 residues) yet have a complex structure, forming domain-swapped homotrimers in which each monomer subunit has three transmembrane segments. These enzymes catalyze formation of phosphatidic acid (PA) and undecaprenyl phosphate, respectively. UDPK and DAGK do not share significant homology with other proteins and do not recognizably contain a P-loop or any other structural or functional motif that is common among water soluble kinases. Members of this family are unrelated to the eukaryotic diacylglycerol kinases and their prokaryotic homologs (encoded by the *dgkB* gene), which usually are water soluble. DAGK and UDPK are located in the plasma membrane with their active sites facing the cytosol. Their lipid substrates, *sn*-1,2-diacylglycerol and undecaprenol share an unusual function as covalent transmembrane carriers of hydrophilic molecules from the inner to the outer leaflet of the plasma membrane for use in biosynthesis of molecules located in the cell wall or periplasm.

DAGK is small, has a readily assayable function, and was cloned, overexpressed, and purified relatively early. However, it also exhibits complexity in structure, enzyme mechanism, and folding behavior. The fact that it is experimentally tractable but complex has led to focus on *E. coli* DAGK as a model system for studies of membrane protein structure, catalysis, and folding.

Proteomics and Microbial Physiology

Function of *E. coli* DAGK

Diacylglycerol kinase (DAGK) activity was first documented in *E. coli* by Pieringer and Kunnes in 1965 (69). Soon after, Chang and Kennedy showed that DAGK is not usually the major source of phosphatidic acid (PA) central to the pathway of *de novo* glycerophospholipid biosynthesis (18). Instead, this pool of PA is usually generated by the sequential acylation of 3-phosphoglycerol (110). In 1978 Raetz and Newman mapped the location of the *dgkA* gene that encodes DAGK and went on to use a combination of genetic and biochemical experiments to show that a major function of DAGK in *E. coli* is to participate in the membrane-derived oligosaccharide (MDO) cycle (73,74). The MDO path was originally discovered by Eugene Kennedy and co-workers (41). Large quantities of MDOs are synthesized in the periplasm of *E. coli* under certain conditions of environmental stress such as low osmolarity medium. Part of the MDO biosynthetic pathway involves transferring phosphoglycerol from phosphatidylglycerol (PG) in the outer leaflet of the plasma membrane (PM) to decorate the nascent MDOs. The diacylglycerol that is generated via this process is phosphorylated by DAGK after it transverse the PM to reach the inner leaflet (Figure 1). DAGK thereby serves to convert a lipid (DAG) that is potentially membrane-disruptive because of its preference for non-bilayer lipid phases into non-toxic PA, a central intermediate of glycerophospholipid biosynthesis in bacteria. DAGK thereby provides the basis for lipid recycling to restore the PG consumed in MDO biosynthesis.

In the early 1980's Raetz and co-workers recognized there is at least one other major source of DAG in *E. coli* besides the MDO cycle (79), indicating the presence of at least one other major function for DAGK catalysis in this organism. At about that same time Bell, Modrich, and Raetz sequenced the *dgkA* gene and its neighboring DNA (51) and established that transcription of this gene can be enhanced by *trans*-acting mutations in a far-removed locus, which was designated *dgkR* (75,102). The *dgkA* gene was also found (51) to be in close proximity to the *plsB* gene that encodes the 3-phosphoglycerol acyltransferase that initiates the major phospholipid biosynthetic pathway of *E. coli* (which includes PA as a major intermediate). An elegant recent study by Bouveret and co-workers has offered a satisfying explanation for the various relationships implied above (99). The *dgkA* and *plsB* genes are regulated in a reciprocal manner. The heat stress-induced factor σ_E activates *plsB* transcription while down-regulating *dgkA*. *plsB* transcription is also subject to control by a second promoter that reduces basal transcription under conditions of the stringent response, which had previously been shown to up-regulate *dgkA* transcription (24). *dgkA* transcription was also found to be up-regulated by two-component signaling originating in the gene product of *basR*, which it turns out is the long-unidentified *dgkR*. The *basRS* two-component signaling system helps to regulate the transcription of genes involved in lipopolysaccharide (LPS) biosynthesis, and includes a gene encoding an enzyme that transfers phosphoethanolamine from the outer leaflet of the PM to lipid A— an LPS biosynthetic intermediate—a reaction that generates diacylglycerol as its byproduct. The recycling to phospholipid of DAG produced during conditions of LPS biosynthesis therefore appears to be a second major function of *E. coli* DAGK.

Identification of Undecaprenol Kinase in Gram-positive Bacteria

In both Gram-negative and Gram-positive bacteria C₅₅-undecaprenyl pyrophosphate serves as a covalent scaffold on which peptidoglycan components are assembled in the cytoplasm of bacteria in preparation for eventual “flip-flop” from the inner leaflet to the outer leaflet of the PM, where the peptidoglycan unit is then transferred from its undecaprenol scaffold to molecules such as mature peptidoglycans and teichoic acids located in the periplasm, outer membrane, and/or cell wall. The byproduct of these reactions is production of undecaprenyl

pyrophosphate, which is subjected to phosphatase cleavage and recycled back to the inner membrane to generate undecaprenyl phosphate for re-use (Figure 1). The *de novo* biosynthetic pathway to undecaprenyl phosphate does not include free undecaprenol as an intermediate (37), meaning that UDPK activity does not participate in this pathway. Gram-negative *E. coli* does not have detectable quantities of free undecaprenol (6) and may lack an undecaprenol kinase. *E. coli* DAGK exhibits very low activity as an undecaprenol kinase (36,53). Moreover, an enzyme present in *E. coli* and long thought to be an undecaprenol kinase, *bacA*, was recently shown to be an undecaprenyl pyrophosphatase (25).

In contrast to *E. coli*, Gram-positive bacteria have a significant pool of free undecaprenol, which represents a major form of undecaprenoids found in these microorganisms (6,13,35). The source of free undecaprenol is not well understood. Perhaps it is generated by phosphatase/pyrophosphatase cleavage of undecaprenyl phosphate and/or pyrophosphate (see (9)) or perhaps it is directly generated as the byproduct of one or more yet-to-be-discovered biosynthetic pathways. In any case it has been known for many years that Gram-positive bacteria contain a UDPK activity (27–29,38,81). Indeed, Sandermann and Strominger purified UDPK from *Staphylococcus aureus* in 1971, showed that it is an extremely hydrophobic protein, and proposed a monomeric molecular weight of 17,000—remarkably small and DAGK-like. However, the protein was never sequenced. In the early 1990s Kuramitsu and his colleagues became interested in the putative *dgkA* gene product from another Gram-positive organism, *Streptococcus mutans*. The *dgkA*-encoded protein was found by that group to be essential in conferring acid-resistance to *S. mutans*, of particular importance during dental cavity formation by this oral pathogen (106). This led to them to purify the 15.4 kDa *dgkA*-encoded enzyme and then reconstitute its activity. This enzyme was found (53), and later confirmed (34), to have high activity as an undecaprenol kinase, but much lower activity as a diacylglycerol kinase. Rock and co-workers recently confirmed that the *dgkA* gene of another Gram-positive organism, *Bacillus subtilis*, is also a UDPK (36). These considerations indicate that the small and hydrophobic *S. aureus* UDPK originally purified in 1971 was almost certainly the *dgkA* gene product, now known to have a molecular weight of 13 kDa.

We are not aware of any bacterium that contains two *dgkA*-like genes. In the case of *E. coli* this is not surprising since there appears to be no need for UDPK activity because this microorganism does not contain detectable levels of undecaprenol. In the case of Gram-positive organisms there are two possible (and non-exclusive) explanations for how the needs of both DAGK and UDPK activities can be met. One possibility is that the *dgkA*-encoded UDPK retains sufficient diacylglycerol kinase activity to serve a bifunctional role. The available data regarding the substrate specificity of UDPK is not very quantitative and so this possibility cannot yet be ruled out. However, a second and much more likely explanation is provided by a post-genomic realization that many bacteria, including Gram-positives, contain homologs to the water soluble eukaryotic diacylglycerol kinases. Rock and colleagues recently demonstrated that *B. subtilis* encodes a functional DAGK protein homologous to eukaryotic DAGK, with the coding gene being dubbed *dgkB* (36). They determined the structure of this water soluble enzyme (60) and showed that it provides the critical DAGK activity required for recycling diacylglycerol as a by-product of lipoteichoic acid production in Gram-positive organisms (43,54,94).

The *dgkA* gene that encodes UDPK in Gram-positive bacteria such as *Streptococcus mutans* is often part of a three gene operon (19) in those organisms that includes a small GTPase, Sgp (5,85). Sgp is homologous to the *E. coli* and *Streptococcus pneumoniae* Era GTPase (see reviews in (16,17,20)). These G proteins are known to directly bind ribosomal RNA and somehow regulate ribosomal biogenesis and/or function (33,86,95). Era has also been shown to reversibly bind to membranes in response to physiological stress, which in the case of the

homologous Sgp was found to include acidic culture conditions (5,33,52). Association of Era with membranes versus association with RNA appears to be competitive (33). It should be added that the third gene of the operon, *ygfG*, encodes a protein homologous to *E. coli* YbeY, which is also thought to be involved in regulating ribosomes (77,78) and is structurally similar to certain eukaryotic metalloproteases (107). These observations suggest that UDPK plays a role in a stress-induced pathway that modulates ribosome function. Whether the formation of undecaprenyl phosphate by UDPK is for the purpose of signaling related to this pathway or whether phosphorylation of undecaprenol is for biosynthetic purposes is not yet known.

Regardless of the exact physiological functions of UDPK, it clearly is an important enzyme, at least in *Streptococcus mutans*. It is required by this organism for growth at low pH (106), for biosynthesis of certain lantibiotics (19), and for biofilm formation (109). It is also a virulence factor for the formation of smooth surface dental caries (91) and has been proposed as a possible anti-cariogenic drug target (92). UDPK is also essential for sporulation of *Bacillus subtilis* (3).

DAGK in Other Microorganisms and in Plants

Genes homologous to *dgkA* are widely distributed in the eubacterial domain. Distribution is not ubiquitous, however. For example, mycobacteria do not have a *dgkA* homolog although they do have homologs to the water soluble eukaryotic DAGK. The *dgkA* gene is rarely found in *Archaea* and *Eukaryota*, although a handful of organisms contain the gene.

While *dgkA*-like genes are usually predicted to code for proteins with <150 amino acids, the *dgkA*-homologous gene of the Gram-positive genus *Clostridium* is an interesting exception. The open reading frame of this gene in various *Clostridia* is predicted to encode for a ca. 230 residue protein, the N-terminus of which appears to encode a conventional DAGK or UDPK, while the C-terminus is homologous to members of the type 2 phosphatidic acid phosphatase/bacitracin permease family. The actual function of the members of this latter family was recently determined to be as undecaprenyl pyrophosphate phosphatases (8). This raises the intriguing question of whether the *Clostridium dgkA* gene encodes a bifunctional enzyme that can catalyze undecaprenyl phosphate formation both by phosphorylating undecaprenol and by cleaving the terminal the phosphoryl group from undecaprenyl pyrophosphate. The three *dgkA*-like genes found in *Eukaryota* arise in the *Viridiplantae* and *Rhizaria* clades; specifically the California poplar tree (also black cottonwood or *Populus trichocarpa*), the castor oil plant (*Ricinus communis*), and a freshwater amoeba (*Paulinella chromatophora*). The function of the protein encoded by the rare eukaryotic gene that is homologous to prokaryotic *dgkA* is currently uncharacterized and unknown. Given its paucity in eukaryotes it seems possible that this gene, when present, is an evolutionary vestige. All three eukaryotic species in which it is found are photosynthetic organisms that are believed to represent a product of endosymbiosis with cyanobacteria (40,65,108). Given that cyanobacteria are Gram-negative and usually have *dgkA* genes, we hypothesize that the eukaryotic *dgkA* gene product would share function with cyanobacteria.

In conclusion, the *dgkA* gene has been shown to encode diacylglycerol kinases (DAGKs) and undecaprenol kinases (UDPKs). While these proteins have been investigated for more than 40 years, important functional results are still being uncovered.

Structure of DAGK

Solution NMR was used to determine the backbone DAGK structure in 2009, as illustrated in Figure 2 (97). When published, this structure represented a benchmark of sorts for NMR-based structural biology given that DAGK is a 40 kDa homotrimer comprised of nine

transmembrane helices and forms a 100 kDa complex with the dodecylphosphocholine (DPC) micelles used as model membranes in the structural studies. Determination of the DAGK structure required use of a variety of structural restraints including paramagnetic relaxation enhancement (PRE)-derived long range distances, residual dipolar coupling (RDC)-based orientational restraints, and distance restraints garnered from biochemical disulfide mapping. DAGK is active in DPC micelles with a high $V_{\max}$ but exhibits much higher substrate K_m than under more native-like model membrane conditions.

Much of the NMR structural effort was informed by orthogonal biochemical and biophysical studies. For example, generating a cysteine-less DAGK mutant suitable for making single-cysteine mutants relied on previous work in which stability-enhanced mutant forms of DAGK were developed (111). Also, results from extensive cysteine scanning mutagenesis studies enabled rational choices of sites for cysteine replacement as the basis for introducing spin labels for PRE distance measurements (97). Similarly, earlier studies probing the topology of DAGK were useful in designing disulfide-mapping experiments (66,67,93).

The structure of *E. coli* DAGK is distinct from other kinases, including water soluble DAGKs. DAGK is a trimer with an intricate fold. The three-fold structural symmetry is built around a left-handed parallel three-helix bundle formed by the second transmembrane helix (TM2) from each of the three subunits (97). This structural core is central to three overlapping four-helix bundles. Each four-helix bundle elaborately consists of helices from all three subunits (Figure 2D). A hallmark of the DAGK structure is that TM3 is domain-swapped and interacts only with TM1 and TM2 from an adjacent subunit, not from the same subunit (Figure 2C). The interface between each 4-helix bundle is a membrane submerged cavity that resembles a classical portico (Figure 2A). Each portico is bound by TM1 and TM3 (pillars) and is capped by the loop connecting TM2 and TM3 (the cornice). The portico contains a majority of DAGK's most highly conserved residues.

The active site was mapped to the portico by NMR-based titrations that monitored chemical shift perturbation as a function of increased substrate concentration (Figure 3A). The active site was also characterized by exhaustive cysteine scanning mutagenesis studies in which both catalytic activity and structural integrity (foldability) of each single-cysteine mutant was evaluated (22,30,97). Low activity mutants were characterized to establish whether inactivity reflects active site perturbation only or whether the mutation also results in misfolding of the enzyme (Figure 3). Information on location of active site residues gleaned from the NMR titrations and cysteine scanning mutagenesis was found to closely correlate with the degree of residue conservation observed among homologs. All indications point to key residues on the cytosolic side of TM2 and TM3 and to a lesser extent on TM1. The structure and functional studies also illuminate the conclusion of previous mutagenesis that DAGK's active site must lie at the interface between subunits, involving TM1 and TM3 from one subunit and TM2 from an adjacent subunit (47,68).

The portico provides the basis for DAGK's lipid substrate specificity (Figure 2). The cornice formed by the TM2-TM3 loop sterically occludes the binding of lipids with headgroups larger than the phosphate moiety of DAGK's reaction product PA. At the same time the relatively large gap between TM2 and TM3 explains DAGK's known promiscuity with respect to variations in substrate lipid acyl chain composition (103). It is also likely that the TM2-TM3 loop is responsible for protecting the active site from unwanted water penetration which could lead to catalytically unproductive ATP hydrolysis. Note that unlike many protein loops, the TM2-TM3 cornice loop has a highly conserved length and a moderately conserved sequence, suggesting its importance in function.

DAGK's intricate fold sheds light on its high thermodynamic and kinetic stability. It has long been recognized that correctly-folded *E. coli* DAGK is extremely stable, yet the protein is also susceptible to mutation-induced misfolding (see Folding section below). The domain-swapped fold and the extensive inter-subunit interactions in DAGK explain its stability. Paradoxically, the same structural properties that impart stability appear also to be associated with a folding *pathway* along which there are ample opportunities for off-pathway misfolding or for kinetic entrapment of folding intermediates. Another surprising feature from the structure and results of cysteine-scanning mutagenesis is that the residues most important in catalysis and the residues most important for folding are close in space, clustering around the portico-based active site (Figure 3). Intimate structural linkage between the determinants of folding with the determinants for catalysis suggests that it may be unusually difficult for evolution to remodel DAGK's active site—tinkering with the active site has significant repercussions on protein folding. This may help to explain why DAGK is an evolutionary orphan—a protein that has neither orthologs nor paralogs beyond UDPK.

Despite the wealth of insight provided by the current DAGK structure, there are inherent limitations. The first is that the structure is backbone only; that is, the side-chain conformations are not explicitly experimentally restrained. The lack of side-chain restraints is a general weakness for NMR structures of multispan helical membrane proteins (7,42,97,112), with notable exceptions (26). Recently, promising computational methods have been developed to deal with the lack of side-chain constraints for challenging NMR structures (76). Nonetheless, the NMR field is ripe for the development of improved methods for determining side chain conformations in membrane proteins.

Another limitation to the current structure is that while the general location of phosphoryl transfer has been determined based on NMR titrations involving ATP and phosphatidic acid, the location of the ATP site is ill-defined, as is the conformation of the bound nucleotide. Part of the problem is that part of the ATP site may be associated with the N-terminus. The N-terminus of DAGK is comprised of two short amphipathic helices and was seen to be dynamic in the DPC micelles used in the structural studies. It is unclear whether these dynamics reflect the native state of DAGK or whether they are the consequences of destabilization of the protein by the DPC micelles used as the medium in structural studies. Sequence alignment and mutagenesis results point to a handful of residues in the N-terminus that are very important for DAGK folding and function. Resolving the ATP binding site, the role of dynamics in DAGK function, and the nature of the conformation of its N-terminus remain important matters for future studies.

Caffrey and coworkers recently showed that functional DAGK can be incorporated into the lipidic cubic mesophase, a class of model membranes that has emerged as a crystallization milieu for a number of challenging membrane proteins(49). Based on spectacular recent success of *in meso* crystallization it is anticipated that a high-resolution x-ray crystal structure of DAGK should follow. On another technical front the Glaubitz lab has recently initiated studies of DAGK in lipid bilayers using solid-state NMR, illustrating that another orthogonal method is positioned to provide structural and mechanistic insight (57). In addition, Becker and coworkers have recently published the total chemical synthesis of DAGK, resulting in functional enzyme(45). Access to a robust synthetic scheme should allow for residue-specific manipulation beyond what is readily feasible using biological methods, possibly providing the basis for new structural and mechanistic insight.

Enzymology of DAGK

E. coli DAGK and *S. aureus* UDPK were among the first integral membrane enzymes to be subjected to detailed enzyme kinetic studies. The general conditions used for most subsequent studies were established by Pieringer and Kunnes in their 1965 paper (69). In that work a detergent with the unappealing trade name of Cutscum was used to solubilize DAGK and also to make it possible to co-dissolve both DAGK and synthetic long chain DAG in the same mixed micelles. Under these conditions and in the presence of ATP, lipids from its native membranes, and a divalent cation (preferably Mg^{2+}), DAGK was found to catalyze phosphorylation of *sn*-1,2-diacylglycerol using the MgATP as the phosphoryl donor. While Cutscum was soon abandoned in favor of other detergents, most kinetic studies of DAGK over the past 40 years have been carried out using mixed detergent-lipid micelles as the model membranes. Pioneering early kinetic studies carried out during the 1970s and 80s relied on the use of Triton X-100/lipid mixed micelles (10–12,80,87,88), but often employed DAGK stock solutions for activity measurements that had first been purified into organic solvent mixtures, conditions that it is now appreciated would have unfolded the protein (98). The low specific activities observed for DAGK in such studies (<1 U/mg) almost certainly reflect low efficiency of refolding of the solvent-purified enzyme when diluted into the aqueous mixed micelles in which activities were measured. Nevertheless, much was learned from these studies, as summarized below. After cloning, overexpressing, and initially purifying DAGK in organic solvent mixtures (55). Bell and co-workers side-stepped the organic solvent problem by conducting subsequent mixed micellar kinetics studies using reactions that were initiated by the addition of aliquots of isolated *E. coli* membranes into which DAGK had been overexpressed (100,101,103). Octylglucoside/phospholipid mixed micelles were used in that work, conditions in which DAGK exhibits a V_{max} of 28 U/mg in the presence of optimal lipid activators. These early studies established that *E. coli* DAGK exhibits high activity towards numerous forms of *sn*-1,2-diacylglycerol as long as it has 4 or more carbons on each of its acyl chains and the chains are hydrophobic enough to ensure membrane association (103). From this and previous work it was seen that DAGK also exhibits modest ability to phosphorylate 1-monocylglycerols and ceramide, but not undecaprenol (10,87,88,103). Full activation of DAGK requires the presence of phospholipids or lipid analogs in the micelles (12,80,88,100,101). Cardiolipin is a particularly effective activator, exhibiting a K_{act} of 2.3 mol% in octylglucoside micelles (100). This can be compared to the K_{act} for dioleoylphosphatidylglycerol (6.3 mol%), dioleoylphosphatidylcholine (10.4 mol%), and sodium hexadecylsulfate (9.8 mol%), all of which increase V_{max} to approximately the same level (100). Early studies also revealed that in addition to a preference for Mg(II) as the divalent metal ion complexed to ATP, DAGK appears to have an additional requirement for activation by free Mg(II) (100,101). DAGK exhibits a strong preference for ATP as the phosphate donor over other nucleotides (4,88).

The overexpression of *E. coli* DAGK in hexa-His tagged form by the Bowie lab in the mid-1990s (46) enabled the facile purification of DAGK into either detergent micelles or into a range of recently-developed model membrane systems such as bicelles or amphipols. This work was accompanied by a switch from use of octylglucoside as the detergent used in activity assays (4) to decylmaltoside (DM) (22,30,84). A convenient spectrophotometrically-based assay system was also developed by adapting the classic pyruvate kinase/lactic dehydrogenase reaction coupling system long used in studies of water soluble kinases in which rate-limiting ADP production by DAGK is coupled to NADH oxidation (22,30,84). The maximal activity of DAGK in mixed DM/phospholipid micelles at 30°C is ca. 110 U/mg, which is roughly the same as the activity of this enzyme when reconstituted into lipid vesicles of optimal composition (see next paragraph). Evidently, fully optimized mixed micelles are as proficient at supporting native function as *bona fide* membranes. Similar activity is observed for DAGK in CHAPSO-DMPC or CHAPSO-DPPC bicelles (22).

Indeed, high activity is also seen for DAGK in lyso-myristoyl-phosphatidylcholine detergent micelles in the absence of any lipid additive (44), in lipid- and detergent-free amphipathic polymers known as amphipols (31), and in the lipidic cubic mesophase formed by monoolein (49). These results point to a great degree of non-specificity in terms of which features of the native membrane must be faithfully mimicked by model membranes in order for DAGK to be fully activated as a catalyst.

Recent kinetic studies have shown that DAGK is more active when reconstituted into vesicles composed solely of phosphatidylcholine (PC) than in vesicles composed of other common phospholipids, than in PC vesicles containing 20 mol% of anionic phospholipids (including cardiolipin), or in vesicles with lipid compositions mimicking the plasma membrane of *E. coli* (70). It is also more active when both acyl chains of PC contain a single *cis* double bond (71). For the symmetric monounsaturated PC series from C14 to C24, DAGK exhibits the highest $V_{\max}/K_m$ in di(C18:1)-PC (71). When cholesterol is added to di(C14:1)-PC to a level of 50 mol%, DAGK's activity was seen to double, reaching about 70% of its fully optimal activity (71). The fact that neither cholesterol nor PC is found in the membranes of *E. coli* emphasizes the degree to which DAGK is promiscuous in terms of its preferences for the composition of its surrounding membrane. The recent development of time-resolved solid state NMR methods for monitoring DAGK's reaction kinetics offers a powerful set of capabilities for future mechanistic studies of this enzyme in lipid vesicles (96). These methods were used to show that in lipid vesicles the rate of DAGK-catalyzed ATPase activity can be surprisingly high, at least in the presence of certain forms of DAG. Moreover, that study documented inhibition of DAGK by vanadate and also that ATP γ S can serve as a phosphoryl donor in place of ATP.

Binding studies indicated that a single DAGK trimer can bind up to three molecules of nucleotide substrate (47). Steady-state kinetic studies of DAGK in mixed micelles indicated no homocooperativity in the association of either ATP or DAG as substrates with the enzyme (4). However, binding by one substrate does result in a modest enhancement in the binding of the other substrate, suggestive of a modest degree of heteroallostery (4). DAGK exhibits a random equilibrium kinetic mechanism, indicative of lack of order to binding that leads to a requisite ternary DAGK-DAG-MgATP complex at a given active site before phosphoryl transfer takes place. This observation, combined with the failure to detect a covalent phosphoenzyme intermediate, strongly supports a direct ATP to DAG phosphoryl transfer pathway.

$k_{\text{cat}}/K_{m,\text{ATP}}$ for DAGK is on the order of $10^4 \text{ M}^{-1}\text{sec}^{-1}$ (4) which reflects only modest catalytic efficiency compared to some water soluble kinases. However, under physiological conditions binding of MgATP to DAGK is unlikely to contribute to the rate-limiting step. Instead, it is the flip-flop of diacylglycerol from its site of production on the outer leaflet of the PM to the inner leaflet that likely limits the rate of the DAGK reaction *in vivo*. While it cannot be ruled out that a transporter or permease accelerates the rate of DAG flip-flop across the membrane, because DAG is so hydrophobic its rate of spontaneous unassisted flip-flop across the membrane is rather rapid, having been measured to be ca. 50 sec^{-1} in lipid vesicles (2,32). This is strikingly similar to DAGK's k_{cat} of roughly 26 sec^{-1} . Moreover, DAGK's K_m for DAG as substrate appears to be on the same order as the concentration of DAG in the *E. coli* PM under conditions in which the MDO cycle is active (4). Based on these observations it has been argued that DAGK is able to catalyze phosphorylation of DAG under physiological conditions at a rate that is on the same order as the rate by which DAG can diffuse to its active site in the inner leaflet of the PM. It follows then that while DAGK is not a very rapid enzyme when compared to many evolutionarily optimized water soluble enzymes, it nevertheless appears to satisfy Knowles' classic definition of a "perfect enzyme" (1) in the sense that its chemical step is able to keep up with the rate of

substrate diffusion (i.e., DAG) to the active site. Just as the imperative of being an integral membrane kinase has led to a unique structural architecture for DAGK, the unusual nature of substrate transbilayer diffusion associated with its reaction has imposed unique mechanistic restraints.

The Gram-positive UDPK was subjected to a burst of studies from 1971–1981 that established its strong preference for undecaprenol and closely related isoprenoid alcohols as substrates. As for DAGK, these studies also showed that many different lipids can serve as “lipid activators” for the UDPK reaction under mixed micellar conditions (27–29,38,39,81,82). Now that the UDPK has been identified to be the *dgkA* gene product in Gram-positive bacteria the stage is set for a revival of compare and contrast studies of UDPK vs. DAGK.

Folding and Misfolding of DAGK

Both *E. coli* DAGK and Gram-positive UDPK have been shown to be phenomenally stable in native membranes, being resistant to irreversible activation upon incubation for a few minutes at 100°C (69,80,82). Even in detergent micelles, it can be quite stable: in decylmaltoside micelles at 70°C and pH 6.5 the $t_{1/2}$ for irreversible inactivation is on the order of a number of hours (44,50). Such stability is reflected by a general tolerance by wild type DAGK to mutations. Bowie and coworkers showed that DAGK remained ca. 50% active even when residues 35–48 in its first transmembrane domain were simultaneously mutated to alanine or to a mixture of alanines and leucines (113). The same lab also exploited an *E. coli* conditional phenotype assay for DAGK function *in vivo* that is based on a *dgkA* knockout strain (74) as the basis for a random mutagenesis study of DAGK’s tolerance to mutations (104). It was revealed that only 31% of DAGK’s residues failed to be tolerant of nonconservative mutations and only 8 of these were absolutely conserved. More than 400 mutants were found to maintain high enough folding efficiency, stability, and catalytic function to enable the DAGK(-) strain to grow under conditions in which it would otherwise be non-viable. Later, Lau and Bowie also developed a method for quantitating the thermodynamic stability of purified DAGK under micellar conditions (46). Folded DAGK in decylmaltoside micelles was titrated with sodium dodecylsulfate (SDS), which serves as a denaturing agent. Based on quantitating the SDS-dependence of unfolding it was concluded that the transmembrane domain of DAGK has an impressive folding stability of –16 kCal/mol, while even the cytosolic domain has respectable stability, favored by –6 kCal/mol.

The data summarized above lead to an impression that DAGK is a highly robust membrane protein. However, it has long been known that DAGK is much less stable in detergent micelles than in native membranes (80,88). For example, in octylglucoside micelles at 55°C, wild type DAGK is inactivated with a $t_{1/2}$ of only 6 minutes, with inactivation believed to be associated with dissociation of the functional homotrimers, possibly leading to aggregation of monomers (114). Moreover, initial studies of a library of single-Cysteine mutants (46) revealed that many of these mutants are prone to misfold to form inactive kinetically-trapped aberrant structures, which usually are non-trimeric (30)Footnote 1. Misfolding of DAGK can usually be avoided or corrected using a procedure referred to as “reconstitutive

Footnote 1: Soon after this library was prepared it was realized that in addition to containing the two Cys-to-Ala mutations required to remove all wild type Cys residues, the parent “Cysless” mutant contained—by mistake—two additional mutations, W117R and S118T—near the end of the 3rd transmembrane segment (46). Therefore, each single-Cys mutant form of DAGK in this library is four mutations away from wild type. However, because the Cysless DAGK exhibited the same catalytic properties as true wild type enzyme, there appeared to be no reason to re-generate an entirely new library. It is, however, now clear that the 4 mutations that separate the original “Cysless” mutant from wild type DAGK do significantly reduce the stability of this enzyme. Most studies published to date have relied on use this original Cysless mutant, although some more recent studies have generated single-Cys mutants starting from a cysteine-less mutant in which the two extra mutations in the 3rd transmembrane segment were corrected back to wild type residues.

refolding” wherein a detergent is used to solubilize the protein followed by mixing with lipid-containing mixed micelles and subsequent removal of the detergent using dialysis (30). DAGK refolds during the final stages of detergent removal and vesicle formation. This procedure makes it possible to obtain the properly folded single-Cys mutant forms of DAGK that were used for kinetic studies to determine the identities of active site residues (see Figure 3C), as well as for Cys side-chain modifications to enable ^{19}F NMR studies of the N-terminal domain (66), for site-specific thiol modification rate measurements (21), for site-directed amide exchange rate measurements (23), and to carry out disulfide mapping of DAGK’s oligomeric interface (61,97).

The availability of a large library of single-Cys DAGK mutants with varying degrees of propensity to misfold afforded an opportunity to examine the biophysical basis for misfolding of a membrane protein. This represents a matter of some importance as many studies during the past 20 years have established that misassembly of integral membrane proteins underlies many genetic disorders and is also a common contributing factor to complex (multi-factorial) diseases, as reviewed in (83). A folding assay for purified DAGK was therefore developed as shown in Figure 4 (62). DAGK can be purified into lipid/detergent-free urea or guanidinium solutions, conditions in which the protein is partially (urea) or highly (guanidinium) unfolded. When small aliquots of these solutions are added to mixed micelles or to solutions containing pre-formed lipid vesicles a fraction of the DAGK spontaneously inserts into the model membrane and folds correctly, a process that can be followed with time by monitoring the appearance of DAGK’s catalytic activity. From these folding assays both the overall rate of the insertion/folding process and the folding efficiency can be measured. For example, wild type DAGK from an acidic urea stock solutions inserts and folds into POPC vesicles with a $t_{1/2}$ of ca. 370 seconds and a folding efficiency of 26%, whereas insertion/folding is much more rapid into mixed micelles ($t_{1/2}$ = 16 sec) and the folding efficiency is higher (46%) (62). It is acknowledged that this folding assay may imperfectly mimic the folding of nascent DAGK under native conditions in *E. coli* where it remains unclear whether the enzyme folds in a spontaneous manner (as in this assay) or whether it takes one of the protein-mediated energy-consuming pathways typically taken by integral membrane proteins of this organism to assemble in the membrane. However, in light of the tremendous success of two decades of thermodynamic studies of the stability of purified membrane proteins in model membranes (15,58,72,105) it was reasoned that useful insight would be gleaned from exploring the factors that determine purified DAGK’s folding kinetics and efficiency.

Studies of a series of over 20 mutants revealed that that the overall rate of folding and insertion of DAGK into lipid vesicles correlates well with folding efficiency: mutants that insert and/or folding slowly are more likely to become trapped in misfolded states (59,62–64). Most mutations appear to lower folding efficiency by disfavoring the pathways leading to the folded state rather than by actively favoring misfolding (59). It was observed that there is a generally strong correlation between protein stability (both kinetic and thermodynamic, which seem to be well-correlated for DAGK) and folding efficiency (59,64). By far the most common mutations that results in significantly reduced folding efficiency are mutations that destabilize DAGK (59,64). A second class of misfolding-promoting mutations appears to be rare: amino acid changes that have little effect on the stability of DAGK but that reduce the folding rate and efficiency. Such mutations impact the energetics of the folding pathways without perturbing the energy difference between the unfolded and folded states. One mutation that falls into this category, Tyr16Cys, appears to result in impaired ability of DAGK to insert into lipid bilayers (63). Given that Tyr16 is located in an amphipathic helix that does not traverse the membrane, this suggests that the Tyr side chain somehow transiently promotes membrane insertion of DAGK’s transmembrane domain. A third and also rare class of mutation is represented by I110W

(59). This mutation, located at a site deep within the 3rd transmembrane segment, was seen to be more stable than the wild type protein when folded and yet inserts and folds with very low efficiency. In light of the fact that the I110Y mutant form is also more stable than the wild type protein and yet continues to fold efficiently, the promotion of misfolding by Trp at site 110 appears to be very specifically related to the indole ring and may occur via a mechanism that is independent of how this mutation enhances the stability of the folded form.

In addition to the set of >20 mutants that were characterized using folding/insertion assays and stability measurements as described above, a final (4th) set of misfolding-prone DAGK mutants has also been documented (97). These do not seem to be highly destabilized, but are aggregation-prone and tend to express very poorly in *E. coli*. Remarkably, these mutations are all located in or near the active site of DAGK (see Figure 3). 7 out of 121 single-Cys DAGK mutants fall into this category, suggesting it is the 2nd most common class of misfolding, after destabilization-promoted misfolding. It is also the class of misfolding that *E. coli* apparently finds to be the most difficult to manage, as reflected by low expression levels.

Documentation of at least 4 classes of misfolding-promoting mutations in DAGK raises interesting questions about the nature of membrane protein folding quality control in various organisms and in the endoplasmic reticulum of higher organism. Which of these classes of misfolding is cellular protein folding quality control able to recognize? Of these, which can quality control most easily coax into the correctly folded form using chaperones? Which are preferentially targeted for degradation? Which classes of mutants tend to escape the surveillance of quality control to form misfolded protein that is not disposed of? These questions remain to be addressed in a general manner, but are clearly of great relevance to the many human diseases that are associated with membrane protein misassembly.

Booth and co-workers extended the relatively crude folding studies summarized above to incorporate sophisticated rapid kinetic measurements to elucidate the detailed folding and misfolding pathways taken by wild type DAGK to reach its folded state in vesicles starting from diluted aliquots of acidic urea solutions. These pathways appear to be complex, with the insertion/folding pathway including 4 kinetically discernable steps (56). Misfolding was found to occur prior to association with vesicles and was proposed to lead to aggregated DAGK (56). Later work suggests that while aggregates may result from misfolding, the committed step to irreversible misfolding takes place at the level of monomer (59). It was also seen that the rate and efficiency of DAGK's association with DOPC bilayers on the productive folding pathway can be modulated by varying the lipid composition, with folding rate and efficiency being enhanced by added anionic DOPG or reduced by lyso-OPC (89). The latter effect was proposed to be related to the lowering of membrane curvature stress and/or the increased lateral pressure in the head group region induced by inclusion of the ice cream cone-shaped lyso-OPC in the bilayer.

A final area in which studies of DAGK folding and stability have made an important contribution is in the engineering of enhanced stability into membrane proteins. Soon after generating the original set of single-Cysteine DAGK mutants Bowie and co-workers found that about 10% of the purified mutants exhibited higher stability in detergent micelles than the wild type protein (47). They then discovered that combining multiple mutations into a single DAGK mutant results in partial additivity of the stabilization enhancements observed for the parent single-site mutant forms (111). This led to a quadruple-mutant form of DAGK that exhibits a functional half-life at 80°C in octylglucoside micelles of 35 minutes, which compares to <<1 minute for the wild type protein. The original DAGK-based observation that stabilizing mutations are not uncommon in membrane proteins has been confirmed for

other membrane proteins (14), a fact that is now being powerfully exploited to optimize the stabilities of membrane proteins to make them more amenable to study and/or to enhance biotechnological applications (48,90).

Conclusions

Much is now known about the structure, catalysis, and folding of DAGK and UDPK. Some of what has been learned appears to be broadly applicable to other membrane proteins. However, many important questions remain, which include: What is the physiological role of the UDPK reaction in Gram-positive bacteria? Is this enzyme of any relevance as a potential antimicrobial drug target? What are the structures of DAGK's N-terminus and of the DAGK-substrate complexes? How does the structure of UDPK differ from that of its DAGK homolog? By what pathway does nascent DAGK integrate into the membrane and fold *in vivo*? Three generations of scientists have toiled to attain our current state of knowledge. It is to be hoped that a fourth will find the motivation and the resources to continue the pursuit.

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Mini-Glossary Terms

Diacylglycerol	A lipid consisting of two fatty acid chains esterified to glycerol leaving a neutral and small hydroxyl head group. This is the primary substrate of DAGK
Membrane-derived oligosaccharide cycle	A biosynthetic pathway whereby branched and substituted β -glucans are synthesized in the periplasmic space, with certain precursors being generated on cytoplasmic side of the inner membrane and then flipfopped across the membrane to the periplasm while attached to a covalent carrier—the lipid diacylglycerol
Undecaprenoids	A group of polyisoprenoids that are constituted from 11 isoprene units, resulting in a 55 carbon molecule. Undecaprenoids are particularly important in cell wall biosynthesis and have an assortment of head groups including; hydroxyl (undecaprenol), phosphoryl (undecaprenyl phosphate), or pyrophosphoryl-head group (undecaprenyl pyrophosphate)
Lipopolysaccharide	A molecule composed of lipids and polysaccharides that is the main constituent of the Gram-negative bacterial outer cell membrane. Lipopolysaccharides are generally highly immunogenic

Acronyms

DAGK	diacylglycerol kinase
UDPK	undecaprenol kinase
UDPr	Undecaprenol

PA	Phosphatidic acid
MDO	membrane-derived oligosaccharide
PG	phosphatidylglycerol
PM	plasma membrane
DAG	diacylglycerol
TM	transmembrane
LPS	lipopolysaccharide

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Summary Points

The *dgkA* gene encodes small multispan membrane proteins that function as diacylglycerol kinase in Gram-negative organisms and undecaprenol kinase in many Gram positive organisms.

E. coli diacylglycerol kinase (DAGK) phosphorylates diacylglycerol produced as a byproduct of both membrane-derived oligosaccharide and lipopolysaccharide biosynthetic pathways.

Undecaprenol kinase (UDPK) phosphorylates undecaprenol, which is abundant in Gram positive microorganisms, an activity that is critical for biofilm formation by the oral pathogen, *Streptococcus mutans*.

Diacylglycerol kinase catalyzes direct ATP-to-diacylglycerol phosphoryl transfer via a random equilibrium kinetic mechanism at a modest rate that nevertheless roughly matches the rate at which diacylglycerol can flip-flop across the bacterial membrane from its periplasmic site of production to diffuse to the cytosolically-disposed DAGK active site.

The structure of homotrimeric DAGK was determined using NMR spectroscopy and contains three portico-like active sites located at the subunit-subunit interfaces, the architecture of which explains the lipid substrate specificity of the enzyme.

DAGK subunits are domain-swapped in the homotrimer, a fact that likely explains both the high stability of DAGK (once folded) and its propensity to misfold.

DAGK represents a rare example of a membrane protein for which extensive studies of both folding and misfolding have been conducted, work that has revealed several distinct classes of misassembly, the most common of which is misfolding that is linked to mutation-induced destabilization of the protein.

From analysis of a complete library of single-cysteine mutants it is clear that the most critical residues of DAGK for both folding and catalysis are located in or immediately adjacent to the active site, indicating entanglement of the primary determinants for folding with the catalytic site, a relationship that may explain why evolution has not remodeled DAGK and UDPK for more diverse functions.

Figure 1. Physiological role of diacylglycerol kinase (**A**) in the membrane-derived oligosaccharide (MDO) cycle of Gram-negative bacteria and the purported role of undecaprenol kinase (**B**) in cell wall biosynthesis in Gram-positive bacteria. DAG, PA, PG, and MDO in (**A**) indicate diacylglycerol, phosphatidic acid, phosphatidylglycerol, and membrane-derived oligosaccharide respectively. In (**B**) UDPr and UDPrP indicate undecaprenol and undecaprenol phosphate respectively.

Figure 2.

NMR-determined backbone structure of diacylglycerol kinase. In all cases the N-terminus (ca. 20 residues) of the protein is not depicted because it was structurally under-restrained, even though it appears to include active residues. **(A)** Ribbon diagram of the DAGK structure viewed from the membrane plane to highlight a single portico-like active site and the enzyme's most highly conserved residues (minus those located on the structurally unresolved N-terminus). Side chain conformations were not experimentally-restrained. **(B)** Close-up of the DAGK portico with the same highly conserved residues as depicted in **(A)** labeled. **(C)** Cytosolic view of the DAGK structure illustrating key active site residues (in black stick representation) and the partially membrane-submerged portico that houses the lipid substrate. **(D)** Topological organization of DAGK. The clipping planes have been reduced to simplify the subunit helical orientation illustrating the domain-swapped structure, the central TM2 three-helix bundle, and one of the four-helix bundle (highlighted by a dashed square). This figure is adapted from (97) and reprinted with permission.

Figure 3.

(A) and (B) summarize active site-mapping from NMR-detected substrate titrations. (C) and (D) indicate crucial residues in DAGK catalysis and/or folding as determined based on characterization of an extensive library of single-cysteine DAGK mutants. The residues that are crucial to catalysis (in yellow) are co-localized with the folding determinants (in pink). (E) and (F) illustrate the residues mostly highly conserved across *dgkA* orthologs. Note that surface representations of the structure only highlight a single DAGK active site. (A–D) are reprinted with permission (97).

Figure 4. Schematic of the DAGK folding assay (62). Purified DAGK is partially unfolded in detergent/lipid-free urea or guanidinium solutions, with small aliquots being added to a buffer containing vesicles or mixed micelles plus DAGK's substrates. The spontaneous insertion and folding of a fraction of the DAGK present in the added aliquot results in the appearance of DAGK catalytic activity that is coupled to spectrophotometrically-detected NADH oxidation. Both the rate of folding/insertion and the folding efficiency are determined using this assay.