In this contribution the kinetic mechanism and substrate specificity of *Escherichia coli* diacylglycerol kinase were examined. Steady state kinetic studies were carried out under mixed micellar conditions using a novel continuous coupled assay system. The kinetic data were consistent with a random equilibrium mechanism, implying that diacylglycerol kinase catalyzes direct phosphoryl transfer from MgATP to diacylglycerol. This was supported by failure to detect an enzyme-phosphate covalent intermediate and by the observation that the bisubstrate analog adenosine 5'-tetraphosphoryl-3-O-(1,2-dihexanoyl)-sn-glycerol inhibits the enzyme ($k_i \ll K_m$). While diacylglycerol kinase’s $k_{cat}/K_m$ is modest compared with the efficiency of many water-soluble enzymes, the enzyme nevertheless appears to be an evolutionarily optimized biocatalyst in the sense that its chemical reaction rate approaches the substrate diffusion-controlled limit. The in vivo rate-limiting step of DAGK’s reaction appears to be, in part, the transbilayer diffusion of diacylglycerol from the outer leaflet to the inner leaflet of the cytoplasmic membrane where DAGK’s active site is located. DAGK was observed to maintain a high nucleotide substrate specificity, with most of this specificity being expressed in the form of reductions in $k_{cat}$ for ATP analogs.

*Escherichia coli* diacylglycerol kinase (DAGK) represents a family of prokaryotic DAGKs that play an important role in biological extracts (4). DAGK is structurally distinct from other kinases. It is a homotrimer (5), has virtually no detectable sequence homology to other kinases, and lacks sequence motifs typically present in enzymes catalyzing phosphoryl transfer (6, 7). With a molecular mass of only 13 kDa, DAGK is easily the smallest known kinase. DAGK is an integral membrane protein with at least 50% of its sequence being located within the lipid bilayer (Fig. 1). The reaction catalyzed by DAGK is unique by virtue of its phase heterogeneity; ATP is water-soluble while DAG is a bilayer-associated lipid.

Kinetic studies carried out in the laboratories of R. Bell and H. Sandermann have established that DAGK can be subjected to detailed mechanistic characterization in mixed micelles (8–11). Their work has provided a phenomenological characterization of DAGK’s apparent activation by metal ions and phospholipids. The Bell laboratory also examined DAGK’s diacylglycerol substrate specificity in elegant detail (12). In this report, we present additional kinetic studies that resolve the previously unaddressed question of whether DAGK catalyzes direct phosphoryl transfer or employs an enzyme-phosphate intermediate. The new kinetic data also allow us to examine the question of whether or not DAGK is an evolutionarily optimized biocatalyst in the sense that it can catalyze its reaction near the substrate diffusion-controlled rate limit (13). In addition, DAGK’s nucleotide substrate specificity is scrutinized in this study.

**MATERIALS AND METHODS**

**Purification of DAGK**—DAGK, in which the N-terminal Met residue has been replaced with a MGHHHHHHHEL “poly(His)” sequence tag, was purified from a high level overexpressing strain of *E. coli* using nickel ion chelate chromatography as described previously (14). For the studies of this work the final purification step was elution of pure DAGK from the Ni(II)-agarose (Qiagen, Chatworth, CA) column using a 1% β-decyl maltoside (DM) solution containing 0.4 M NH₄OH. DAGK is completely stable for several hours in such a solution. The DAGK pool was then frozen in liquid nitrogen and lyophilized to remove the ammonium and to yield an easily handled DAGK-DM powder which is typically about one-third by weight DAGK. Lyophilization of DAGK in a DM solution has been found to result in little or no loss of DAGK activity upon redissolution. For kinetic studies 0.3–30 mg/ml stock DAGK solutions were made by dissolving the DAGK-DM powder in assay buffer (see below). DAGK concentration was assessed by measuring solution absorbance at 280 nm ($e_{\text{mg/mol}} = 2.1$ for DAGK). DM is the detergent of choice for the stock DAGK solutions because DAGK is completely stable for several days in DM solutions at room temperature. The DM and β-octyl glucoside used in our studies were ultrapure and were purchased from Anatrace (Maumee, OH).

**Coupled Assay System**—For routine assays and for the kinetic studies DAGK was assayed by enzymatically coupling its reaction to NADH oxidation, a process conveniently followed in a continuous mode by monitoring assay solution absorbance at 340 nm and 30 °C. Routine assays were initiated by adding DAGK to assay mixtures containing pH 6.8 buffer (60 mM PIPES, 50 mM LiCl, 0.1 mM EDTA, 0.1 mM EGTA), 60 mM β-octyl glucoside (OG), 7 mM dimyristoylphosphatidylcholine (DMPC), 1 mM phosphoenolpyruvate, 3 mM ATP, 2.6 mM sn-1,2-dihexanoyl-sn-glycerol, 20 mM MgCl₂ (acetate salt used), 0.25 mM NADH, and 20 units each of lactic dehydrogenase and pyruvate kinase (from a glycerol-containing stock). Because the critical micelle concentration of OG is ~22 mM, the micellar OG concentration is 38 mM. Thus, the two lipid components of the assay mixture, DMPC and DHG, are present at levels of about 20 and 7.5 mol %, respectively. Reactions were carried out in 1-cm, 1.5-ml quartz cuvettes in temperature-controlled sample cells within a Hewlett Packard (Palo Alto, CA) model 8452 ultraviolet/visible spectrophotometer.
visible spectrophotometer. For rate calculations the extinction coefficient of NADH at 340 nm was taken to be 6110 absorbance units/(cm·μM). Under these "standard" assay conditions, DAGK activity was normally observed to be about 22 units/mg, where 1 unit is defined as 1 μM of DHG phosphorylated per min. DHG was synthesized in our laboratory (procedure available upon request), although it is now commercially available. DMPC was purchased from Princeton Lipids (Princeton, NJ).

For steady state kinetic studies the following modifications were made to the standard assay: the PIPES concentration was somewhat higher (75 mM), the Mg²⁺ concentration was slightly higher (20 mM), DMPC was replaced by 8 mol % (relative to micellar OG) cardiolipin (beef heart mitochondrial, Avanti Polar Lipids, Alabaster, Alabama), and the ATP and DHG concentrations were variable.

Fluorescent/Thin Layer Chromatography-based DAGK Assay—Reactions involving ATP analogs were not run using the coupled assay system because of the potential complication that some analogs might be poor substrates for pyruvate kinase, such that the rate of the coupling enzyme could become partially rate-limiting. In these cases, an assay in which pyrene-tagged DAG (1-pyrenebutyryl-2-butyrl-sn-glycero, PBBG) served as the substrate for DAGK. At various time points during the reaction, aliquots were quenched, and the pyrene-tagged product phosphatidic acid was separated from PBBG using TLC. TLC plates were then subjected to ultraviolet light, and the conversion of substrate to product was quantified via densitometric analysis of the fluorescent spots on the TLC plates. This assay has been described in detail previously (14). For the studies of this report, TLC assay conditions, DAGK activity was normally possible to resolve the "major product" into two major species ("high" and "medium") products) and one minor product (lowest of the three possible species) and could be detected by TLC (5:2:3 1-butanol:acetic acid:water). The reaction was then warmed to 45 °C and allowed to proceed for 12 h, at which point a high RF (0.57) UV-absorbing spot was observed to be present with an intensity greater than that of the ATP precursor (RF near 0).

The reaction mixture was then rotary-evaporated and flash-chromatographed (5:2:3 1-butanol:acetic acid:water). The major product pool was rotary-evaporated to yield 0.26 g of a yellow oil. The product exhibited an RF of 0.22 in 5:2:1:2 1-butanol:water:acetic acid:ethyl ether and was flash-chromatographed using this solvent system. This time it was possible to resolve the "major product" into two major species ("high" and "medium" RF products) and one minor product (lowest of the three in RF terms), all with RF values near 0.22. Following drying of the pooled product by rotary evaporation, medium RF product weighed 33 mg. This oil was then dissolved by an 1-propanol/water mixture and passed over 1-ml "medium" product weighed 33 mg. This oil was then dissolved by an 1-propanol/water mixture and passed over 1-ml columns, followed by rotary evaporation to yield a white solid. The identity of this products as an ATP-DAG conjugate where adenosine is 5-linked to dihexanoylglycerol through a tetraphosphoryl linkage was supported by 1H and 31P NMR. However, fast atom bombardment mass spectroscopy carried out at the NIH Mass Spectrometry Facility at Michigan State University indicated an apparent molecular weight of 821 rather than the expected 857. Thus, it is possible that the inhibitor characterized in this study is not exactly that depicted in Fig. 1, but is instead a related bisubstrate analog. This ambiguity does not alter the interpretation of the data described under "Results."
Structure and Mechanism of Diacylglycerol Kinase

**Steady State Kinetic Models and Data Fitting—**DAGK reaction rates measured in studies where both DAG and MgATP concentrations were varied were fit by the steady state kinetic model for a two-substrate random equilibrium enzyme (15),

\[
\text{Rate} = \frac{V_{\text{max}} \cdot [\text{DAG}] \cdot [\text{ATP}]}{1 + [\text{ATP}] / K_{\text{m,ATP}} + [\text{DAG}] / K_{\text{m,DAG}} + [1/K_{\text{M}} + [\text{ATP}]] \cdot [\text{DAG}] / K_{\text{m,DAG}}} \quad \text{(Eq. 1)}
\]

where \(\alpha\) is the factor by which the \(K_m\) of MgATP and DAG are attenuated by prior association of the other substrate. Data were fit by this equation using a nonlinear least squares routine within the program MINSQ-II, with weighting of the data proportional to rate (Micromath Software, Salt Lake City, UT). Because DAGK kinetic studies were carried out in a mixed micellar medium (see above and “Results”) and involve a lipid substrate and an integral membrane protein appropriate use of Equation 1 (and Equation 2 below) requires that the \(K_m\) of the lipid substrate be expressed in mole fraction units (16). Furthermore, appropriate use of these equations is based upon the assumption that the rate of intermicellar diffusion for lipid substrates is not rate-limiting to the enzymic reaction. For DAGK in mixed micelles involving the detergent \(\beta\)-octyl glucoside and short-tailed forms of diacylglycerol such as DHG, this assumption has been carefully examined and unambiguously demonstrated to hold (9).

Reaction rates determined in the presence of the ATP-DAG bisubstrate analog were fit by a random equilibrium-derived model (15) which assumes: (i) that the analog is competitive with both substrates, (ii) that binding of the analog to the completely free enzyme is much tighter than to either binary complex or to the ternary complex (so that only the dissociation constant for the analog to the free enzyme needs to be treated), and (iii) the degree of synergism between substrates is negligible (\(\alpha = 1.0\) in Equation 1).

\[
\text{Rate} = \frac{V_{\text{max}} \cdot (\alpha \cdot [\text{DAG}] / K_{\text{m,DAG}} \cdot K_{\text{m,ATP}})}{1 + [\text{ATP}] / K_{\text{m,ATP}} + [\text{DAG}] / K_{\text{m,DAG}} + [1/K_{\text{M}} + [\text{ATP}]] \cdot [\text{DAG}] / K_{\text{m,DAG}}} \quad \text{(Eq. 2)}
\]

**RESULTS**

**Development of a Continuous Coupled Mixed Micellar Assay System for DAGK—**Because DAGK is an integral membrane protein and because one of its substrates is a lipid, previous kinetic studies of DAGK have relied upon the use of mixed micelles as a means to solubilize the enzyme and DAG. Extensive justification has previously been presented regarding the validity of using mixed micelles to mimic the lipid bilayer for detailed kinetic study of DAGK (8, 9) (also see “Materials and Methods”) and other membrane enzymes (16). A limited number of DAGK assays have also been carried out in bilayers, which suggest that DAGK’s activity in vesicles is similar to its activity in mixed micelles at similar levels of substrates (14). The studies of this report were greatly facilitated by integrating the central aspects of the mixed micellar DAGK assay system developed by Bell and his co-workers (17) with a classical kinase assay wherein substrate phosphorylation is coupled to UV absorbance-detectable NADH oxidation via the mediation of pyruvate kinase and lactate dehydrogenase (18). This new assay method is much easier than the existing assay methods (11, 17) and also provides a continuous means for monitoring the DAGK reaction. In developing this assay several observations were made. First, the measured rates for a given set of assay conditions were linear with the quantity of DAGK added to the assay, as expected. Second, the slopes of the \(A_{340} \text{ vs. time plots were linear following sample mixing until nearly all of the NADH had been consumed. Finally, provided that lactate dehydrogenase and pyruvate kinase are present at levels of \(\geq 10\) units per 1-ml assay, the measured rates are independent of the level of the coupling enzymes. The key to the success of this assay system is the apparent tolerance of lactate dehydrogenase and pyruvate kinase to high levels of octyl glucoside.

**DAGK Catalysis in the Direction of DAG Phosphorylation Can Be Kinetically Approximated as Random Equilibrium in Nature—**A very fundamental question with regard to the mechanism of any kinase is whether it catalyzes phosphoryl transfer via a direct substrate to acceptor pathway or whether it first catalyzes formation of an enzyme-phosphate intermediate followed by a second transfer of the phosphoryl from enzyme to acceptor. In general, a mechanism involving a covalent enzyme-phosphate complex is expected to be reflected by ping-pong kinetic behavior where both substrates are not bound at the same time. The direct transfer mechanism would be expected to display “random equilibrium” or “ordered” kinetic patterns, in either case involving formation of an enzyme-MgATP-DAG ternary complex. We carried out kinetics where the concentrations of both substrates were varied systematically. The data were fit by the random equilibrium kinetic model (in direct form, see “Materials and Methods”), the results of which were used to generate the double reciprocal plots shown in Fig. 3. \(V_{\text{max}}\) was determined to be 50 ± 7 units/mg, corresponding to a \(k_{\text{cat}}\) of 12 s\(^{-1}\), based on one active site per DAGK monomer (5). \(K_{\text{m,ATP}}\) was determined to be 1.2 ± 0.5 mm, and \(K_{\text{m,DAG}}\) is 5.0 ± 2.2 mm. The substrate cooperativity factor, \(\alpha\), was calculated to be 0.48 ± 0.17, indicative of a modest degree of positive cooperativity between the two substrates. Similar results were obtained if other data weighting schemes were used (see “Materials and Methods”) or if a was assumed to be 1.0. The \(\alpha \cdot K_m\) determined for MgATP and DHG of 0.58 ± 0.25 mm and 2.4 ± 1 mol % are in agreement with apparent \(K_m\) previously determined for each substrate in the presence of saturating levels of the other substrate (0.3 mm for MgATP and 2.4 mol % for DHG) (9, 12). The true \(V_{\text{max}}\) determined in the present work is a little higher than the apparent \(V_{\text{max}}\) determined previously (28 units/mg) (12).

The data appear to be reasonably well fit by the random equilibrium model and are clearly not consistent with ordered or ping-pong mechanisms, as can be qualitatively discerned in Fig. 3. This result supports the notion that either MgATP or DAG can bind to the enzyme in both the presence and absence of the other substrate and that the reaction likely proceeds through a direct MgATP to DAG transfer pathway.

**FIG. 2.** Possible structure of the DAG-ATP bisubstrate analog synthesized and characterized in this report. As explained under “Materials and Methods,” our analytical data are unclear as to whether the inhibitor characterized is actually this compound or a closely related derivative.
DAGK Is Inhibited by a Bisubstrate Analog—If DAGK catalyzes direct phosphoryl transfer from MgATP to DAG, then the binding sites for these two substrates should be proximal and oriented so that the \( \gamma \)-phosphoryl of ATP is appropriately placed for transfer to the sn-3 oxygen of DAG. In this case, as been demonstrated for a number of water-soluble kinases (19–22), a bisubstrate analog in which ATP is covalently linked to DAG through a polyphosphate bridge may be an inhibitor of the enzyme. For a number of kinases, analogs in which there are four bridging phosphodiester bonds have been observed to be better inhibitors than the true bisubstrate analogs with only three (20–22), probably because most kinase-active sites involve a spatially distal reaction pathway such that catalysis in the ternary complex occurs only upon a transient conformational change bringing the donor phosphomonoester in reactive proximity with the acceptor moiety (23, 24). The extra phosphate in the \( n = 4 \) analogs permits both active sites to be filled in the ternary “ground state” structure. Based on these considerations we synthesized a tetraphosphate-linked ATP-DAG bisubstrate analog whose structure is similar or identical to that depicted in Fig. 2 (see “Materials and Methods”).

A series of 10 rates were measured where the apparent concentration of the inhibitor was varied from 5 to 100 \( \mu \text{M} \) (0.01 to 0.26 mol %), ATP was varied from 0.16 to 0.94 mM, and DHG was varied from 0.4 to 3.1 mol %. As described under “Materials and Methods,” these data were fit by a model that assumes the inhibitor to be competitive against both substrates (as expected for bisubstrate inhibition (25, 26). A variety of different fits were attempted in which, variably, the \( K_m \) for the two substrates were either allowed to vary or were fixed to their predetermined values. Because, under the conditions of the mixed micellar assay, effectively all of the ATP-DAG bisubstrate analog will be associated with the mixed micelles (27), mole fraction units were used to express \( K_I \) in these calculations. In all cases, the \( K_I \) inevitably was determined to be in the 0.03–0.06 mol % range. For the specific fit where \( V_{\text{max}} \) and \( K_m \) were fixed to values determined in the studies of the previous section, a \( K_I \) of 0.036 ± 0.01 mol % was determined, about two orders of magnitude lower than \( K_m \). It should be noted that when the above calculations were repeated using \( molar \) units to express the inhibitor concentration, an apparent \( K_I \) was calculated to be in the 10–21 micromolar range, well below the MgATP’s \( K_m \). These results demonstrate that the tetraphosphate-linked ATP-DAG bisubstrate analog is a reasonably good inhibitor of DAGK. These inhibition results reinforce the interpretation of the steady state kinetic data that DAGK catalysis occurs via direct phosphoryl transfer from MgATP to proximally associated DAG.

Attempts to Detect a Covalent DAGK-Phosphate Intermediate—To provide final confirmation that the DAGK reaction does not involve an enzyme-phosphate intermediate, two additional experiments were carried out to attempt detection of a phosphoenzyme intermediate. First, pure DAGK was bound through its poly(His) tag to Ni(II)-agarose resin and bathed in a mixed micellar solution in which the enzyme is fully active. At least 40% of the enzyme was passed through the column in the presence of MgATP. Equivalents of the fluorescently tagged of the DAG was then assayed using the thin layer chromatography DAGK assay method (see “Materials and Methods”) and using a highly sensitive chemical assay to see if any conversion to phosphatidic acid occurred when DAG passed through the column. Within the limits of detection by these methods (which could detect an \(-1\% \) conversion of PBBG to product), no phosphatidic acid product could be detected. A control experiment in which PBBG was passed through the DAGK column in the presence of MgATP yielded \(-50\% \) conversion to phosphatidic acid. Since ATP hydrolysis by DAGK is slow (hours) on the time scale it took to run these experiments (minutes), these results provide no ev-
idence for the presence of a DAGK-phosphate intermediate that could react with PBGB in the absence of nucleotide.

In a second series of experiments, DAGK bound to the Ni(II)-resin was exposed under standard mixed micellar assay conditions to 5 mM MgATP and then washed with ATP-free solution, conditions in which an enzyme-phosphate intermediate would be expected to persist if present. The current was then extracted with 1% decyl maltoside containing 2% formic acid to release the DAGK and any associated phosphate. This resulting solution was then ashed and tested for the presence of phosphate using the method of Van Veldhoven and Mannaerts (28). Within the limits of detection (sensitive enough to detect a 1% population of phosphorylated DAGK within the total DAGK population), no phosphate could be detected. This result provides additional confirmation that the DAGK reaction does not involve a covalent intermediate.

**Nucleotide Specificity of DAGK**—The nucleotide specificity of the DAGK reaction was investigated using the TLC/fluorescent assay system to measure reaction rates at four to eight concentrations of each MgATP analog examined. Both adenosine tetraphosphate and adenosine diphosphate were tested as alternate substrates for DAGK's forward reaction. MgADP is a very poor phosphoryl donor (Table I), exhibiting a >104 reduction in $V_{\text{max}}$ relative to MgATP. On the other hand, while adenosine tetraphosphate shows a substantially reduced affinity for DAGK relative to either MgADP or MgATP, once bound it is a much better substrate than MgADP ($V_{\text{max}}$ within a factor of 500 that of MgATP).

The ribose and adenine moiety specificities of DAGK were also probed. 2’-deoxy-ATP is a moderately good substrate for DAGK ($V_{\text{max}}/K_m$ is 0.05 that of MgATP). However, while the $K_m$ of MgGTP and MgITP are within a factor of 10 to that of ATP, they exhibit decreases in $k_{\text{cat}}$ on the order of $10^{-4}$ to $10^{-5}$. Within the limits of detection, triphosphate was not a substrate at all.

### DISCUSSION

**Is DAGK an Evolutionarily Optimized Biocatalyst?**—In this work DAGK’s $k_{\text{cat}}$ and $K_m$ were accurately determined. A key criterion of the classical definition of an “evolutionarily optimized enzyme” is that $k_{\text{cat}}/K_m$ should approach the substrate diffusion rate limit for enzyme-substrate bimolecular association (13). In the case of an enzyme such as DAGK which has two substrates, the rate limit will be defined by the substrate for which the rate of diffusion-controlled bimolecular association is lower. For DAGK, DAG most likely sets the diffusion limit rather than MgATP. To understand this, we must examine DAGK's physiological role as a component of the “diglyceride cycle” (1).

Phosphatidylglycerol (PG) is a major anionic lipid of *E. coli* and of many other bacteria (29). When *E. coli* and, most likely, all other bacteria having periplasm are subjected to even moderately low ionic strength, part of their metabolic response includes high level production of membrane-derived oligosaccharides (MDO) in their periplasmic space (1, 2, 30). One step of this pathway is the transfer of PG's phosphoglycerol head group in the *outer leaflet* of the cytoplasmic membrane to the nascent MDO, generating DAG as a by-product. It has been estimated that under resting conditions there are roughly 4 million PG molecules per *E. coli* cell, but that in response to a change in osmolality from 0.15 to 0.07 osmolar about 10 million molecules of PG are consumed in MDO production (31). DAGK plays its essential metabolic role by converting the DAG produced during MDO biosynthesis to phosphatidic acid, which then reenters the primary lipid catabolic pathways of the microorganism (32). This conversion is necessary both so that PG can be rapidly replenished and to detoxify the cytoplasmic membrane of DAG, a lipid known for its ability to promote formation of nonbilayer lipid phases (see Sanders (33) and references therein). Based upon the above observations, it appears that the conditions under which natural selection has exerted pressure to optimize DAGK's catalytic efficiency are those experienced by the enzyme during high level MDO biosynthesis. Thus, it is the DAG concentration near steady state at the peak of MDO biosynthesis that is most relevant to DAGK's $K_m$. Furthermore, it is the diffusion of DAG from its site of production on the *periplasmic* face of the membrane to DAGK's active site on the *cytoplasmic* face which represents the process which an evolutionarily optimized DAGK would have to be able to keep up with. It should be noted that DAGK's level of expression does not change in response to changes in solution osmolarity (34).

In the report, we limit our discussion to the more **in vivo** substrate concentration (13). For enzymes such as DAGK, which operate in pathways where **in vivo** substrate and product concentrations vary according to environment (3, 34) and which may sometimes operate under reversible conditions and sometimes under irreversible conditions, it is not completely clear how to judge the degree of optimization of $K_m$. Thus, in the report, we limit our discussion to the more strictly chemical component of catalytic perfection, the question of whether DAGK's reaction rate can approach the diffusion-controlled limit.
In the absence of a functional DAGK, DAG accumulates in the cytoplasmic membrane of *E. coli*. When the concentration reaches a level of about 8 mol %, cells lose their ability to proliferate under conditions of low osmolarity (3). In the presence of DAGK, the DAG concentration is maintained at about 0.6 mol % under low osmolarity conditions, almost identical to DAGK's observed *K_m* for forms of DAG resembling those actually found in *E. coli* (3, 9, 12, 34).

To reach the active site of DAGK under the critical *in vivo* conditions described above, DAG must first transverse the lipid bilayer. Almost certainly, this process occurs via spontaneous DAG flip-flop. Despite extensive generation of mutants defective in almost all known components of lipid metabolism in *E. coli* (31, 32), no phenotypes that we are aware of exhibit characteristics which would be consistent with a defect in DAG bilayer transversal. Furthermore, the unimolecular rate constant for *spontaneous* DAG flip-flop in both natural membranes (i.e. erythrocytes) and in model membranes is rather rapid, roughly 50 s⁻¹ (35, 36). Based on the considerations of this and the preceding paragraph, we can estimate a second order rate constant for transbilayer movement of DAG under critical conditions of high MDO biosynthesis to be 50 s⁻¹/0.6 mol % = 83 mol %⁻¹ s⁻¹.

The rate of DAG transbilayer diffusion as estimated above almost certainly represents the diffusion rate limit for DAG-substrate bimolecular association. The second order rate constant for two-dimensional lateral DAG diffusion to the active site of the enzyme once it is on the same side of the membrane can be safely approximated to be within a factor of 100 (once a common standard state has been chosen) to that of MgATP diffusion in three dimensions through solution to reach the active site. This assertion is based upon two facts. First, the diffusion coefficients exhibited by lipids in membrane bilayers are a factor of about 10 lower than that for similarly sized molecules in aqueous solutions (37). Second, while the rate of unimolecular diffusion will be lower because of the increased viscosity of the membrane, the probability of a DAG-DAGK complex forming upon a DAG-DAGK collision will be a little higher than for MgATP because of the reduced dimensionality of the bilayer (38) and because a membrane protein and its lipid substrate will be predisposed for orientationally correct collision by bilayer topological constraints (39). The second order rate constant for MgATP diffusion to an enzyme active site is in the range of 10⁶–10⁸ s⁻¹ (40). To compare this rate constant to that for DAG bilayer transversal (80 mol %⁻¹ s⁻¹), both values must be converted to a common standard state appropriate for such a comparison, volume fraction units. Given that the relative molecular volumes of DAG and other lipids in the membrane and for ATP relative to water are roughly proportional to molecular weights, rough estimates of the diffusion rate constants are 100 vol %⁻¹ s⁻¹ for DAG and ≥2 × 10⁶ vol %⁻¹ s⁻¹ for MgATP. Even considering the very rough nature of the approximations made in the above analysis, the factor of more than 10⁶ leaves little doubt that the diffusion controlled rate limit for the DAGK reaction is defined by the rate of DAG transbilayer diffusion. This conclusion was originally hypothesized by Walsh et al. (34) based on their *in vivo* study of DAGK's role in DAG metabolism.

For a form of diacylglycerol akin to that found in the inner membrane of *E. coli*, diaclylglycerol (12), DAGK's *k_cat/K_m* catalysis can be determined to be 12 s⁻¹/0.4 mol % = 50 mol %⁻¹ s⁻¹. This value is well within an order of magnitude of the estimated diffusion rate limit of 80 mol %⁻¹ s⁻¹ and is probably the same within the uncertainty limits of our analysis. This leads to the conclusion that, to a rough approximation, DAGK satisfies a crucial criterion for being an evolutionarily optimized biocatalyst.

The fact that DAGK's *k_cat* and *k_cat/K_m* are only modest when compared with the most efficient of water-soluble kinases (41, 42) reflects the fact that the evolutionary constraints placed upon DAGK are membrane-specific: DAGK does not have to be as "good" an enzyme because its membranous metabolic pathway cannot presently deliver substrate to the active site as rapidly as in many metabolic pathways involving water-soluble substrates. This observation may shed light upon a very puzzling quality of DAGK; despite the small size of this enzyme and the complexity of the reaction it catalyzes, the microbial isoforms for which sequences are available are often highly divergent (sequence identity for some pairs is <20%). Mutagenesis studies have also shown dramatic sequential plasticity in functional *E. coli* DAGK molecules (43). Perhaps this sequence divergence reflects the fact that DAGK is not required by natural selection to be as chemically sophisticated and is therefore more tolerant of variations in sequence space than are water-soluble kinases. This leads to an interesting and experimentally testable conclusion: using mutagenesis it should be possible to dramatically "improve" DAGK's catalytic efficiency as measured under mixed micellar conditions, where transbilayer flip-flop is not a factor and the diffusion limit is much higher than in *in vivo*.

Despite its Structural Singularity, DAGK's Catalytic Mechanism Appears to Be Fairly Conventional—Like most kinases which involve non-diphosphonucleotide phosphoryl acceptors (44), DAGK catalyzes direct MgATP-to-acceptor phosphoryl transfer.

The fact that DAGK's high nucleotide specificity is exhibited primarily in the form of reductions in *k_cat* for ATP analogs supports the possibility that a conformational change occurs following the binding of one or both substrates prior to actual phosphoryl transfer. The fact that the tetraphosphate-linked bisubstrate analog was a good inhibitor also supports this possibility. If DAGK does undergo a substantial conformational change, then it is similar in this regard to other kinases catalyzing direct phosphoryl transfer (24, 45, 46). The purpose of this conformational change, as for other kinases, is probably to exclude water from the active site so that hydrolysis does not significantly compete with transfer.

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Structure and Mechanism of Diacylglycerol Kinase
