Lipid cubic phase as a membrane mimetic for integral membrane protein enzymes

Dianfan Li and Martin Caffrey[1]

Schools of Medicine and Biochemistry and Immunology, Trinity College, Dublin, Ireland

Edited by Christopher Miller, Brandeis University, Waltham, MA, and approved April 7, 2011 (received for review February 4, 2011)

The lipidic cubic mesophase has been used to crystallize important membrane proteins for high-resolution structure determination. To date, however, no integral membrane enzymes have yielded to this method, the *in meso*. For a crystal structure to be meaningful the target protein must be functional. Using the *in meso* method with a membrane enzyme requires that the protein is active in the mesophase that grows crystals. Because the cubic phase is sticky and viscous and is bicontinuous topologically, quantitatively assessing enzyme activity *in meso* is a challenge. Here, we describe a procedure for characterizing the catalytic properties of the integral membrane enzyme, diacylglycerol kinase, reconstituted into the bilayer of the lipidic cubic phase. The kinase activity of this elusive crystallographic target was monitored spectrophotometrically using a coupled assay in a high-throughput, 96-well plate format. *In meso*, the enzyme exhibits classic Michaelis–Menten kinetics and works with a range of lipid substrates. The fact that the enzyme and its lipid substrate and product remain confined to the porous mesophase while its water-soluble substrate and product are free to partition into the aqueous bathing solution suggests a general and convenient approach for characterizing membrane enzymes that function with lipids in a membrane-like environment. The distinctive rheology of the cubic phase means that a procedural step to physically separate substrate from product is not needed. Because of its open, bicontinuous nature, the cubic phase offers the added benefit that the protein is accessible for assay from both sides of the membrane.

coupled enzyme assay | DgkA | glycerophospholipid | phosphatidylglycerol phosphate synthase | PgsA

The lipidic cubic phase has been used to grow crystals of membrane proteins for high-resolution structure determination. This approach has had spectacular successes of late with the publication of structures for G-protein coupled receptors (1–7). The method has also been used with a variety of other membrane protein and peptide types that include photo-sensitive proteins like the assorted bacterial rhodopsins, a light harvesting complex and the photosynthetic reaction centers, a transporter, a channel, and an adhesin (8–15). Conspicuous by their absence on this list are the integral membrane enzyme class of proteins.

We have an interest in the structure-function relationships that pertain to membrane enzymes involved in glycerolipid metabolism. Our immediate focus is on the diacylglycerol kinase, DgkA, in *Escherichia coli* responsible for the ATP-dependent conversion of diacylglycerol to phosphatidic acid for use in membrane-derived oligosaccharide synthesis (16). A solution NMR structure of this small, hydrophobic trimer was published recently (17), and a high-resolution crystal structure is needed now to complement and extend the solution work. Efforts at crystallizing DgkA by conventional means have produced crystals but none of structure quality (18). The lipidic cubic mesophase (*in meso*) method is now being examined as an alternative approach that may yield the requisite crystal structure given the success it has had with other small, hydrophobic integral membrane proteins and peptides.

In support of such *in meso* crystallization trials it is important to demonstrate that the enzyme is functionally active in the cubic mesophase, in and from which crystals must grow. The cubic phase can be viewed as consisting of a single, highly curved, continuous lipid bilayer that permeates 3D space the midplane of which sits on a periodic minimal surface. The lipid membrane is bathed on either side by the aqueous component of the mesophase, which takes the form of continuous, branching networks of channels. Assaying for enzymatic activity in such a "bicontinuous" medium is not straightforward and is the focus of this work.

To demonstrate and to characterize the activity of an integral membrane enzyme *in meso* presents two major challenges. To begin with, the cubic phase is extremely viscous and sticky, having the rheology of a thick toothpaste. Accordingly, handling the mesophase is not trivial. However, these viscosity and stickiness features have been used to advantage in setting up and performing *in meso* assays, as described.

The second challenge arises because the mesophase is a porous, liquid crystalline medium in which the enzyme must be assayed. It can be viewed as a molecular sponge where the fabric of the sponge is a lipid bilayer and the holes in the sponge are filled with aqueous medium. Thus, like the common household sponge, water-soluble materials can diffuse into and out of the holes or aqueous channels that permeate the mesophase and that are continuous with the bulk medium. The water-soluble DgkA substrate, ATP, and product, ADP, must, respectively, diffuse into and out of the mesophase in the course of the assay to support kinase activity and for detection. The latter is done in the current application using a coupled enzyme system (19–21) that includes pyruvate kinase (PK), lactate dehydrogenase (LD), phosphoenolpyruvate (PEP), and nicotinamide adenine dinucleotide (NADH). Thus, ADP production as a result of DgkA activity leads to a decrease in NADH concentration in the bathing aqueous solution that can be monitored conveniently by following the change in dinucleotide absorption at 340 nm. In contrast, the DgkA enzyme as well as its lipid substrate and product are confined to the lipid bilayer of the mesophase and are free to move within the plane of that continuous membrane.

DgkA is a promiscuous enzyme. It can use a variety of lipid and surfactant alcohols as substrates for γ-phosphoryl group transfer from ATP (22–25). It has been shown to work with monoolein (22), which is also the lipid most commonly used to create the hosting mesophase for crystallogenesis. In the current investigation, therefore, monoolein serves double duty, as substrate and hosting mesophase lipid.

Here, we show that the lipidic cubic phase is a suitable and a convenient system in which to reconstitute and to assay an integral membrane enzyme. Specifically, DgkA has been shown to reconstitute into the bilayer of the cubic phase prepared with monoolein in a functionally active form. The kinetic properties of the enzyme *in meso* have been characterized, and the kinase has

Author contributions: D.L. and M.C. designed research; D.L. performed research; D.L. and M.C. analyzed data; and D.L. and M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: martin.caffrey@tcd.ie.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1101815108/-/DCSupplemental.

BIOCHEMISTRY

been shown to work with a range of lipid substrates. The system and approach described here have general applicability as demonstrated in measurements made with the phosphatidylglycerol phosphate synthesizing enzyme, PgsA.

Results

Reconstitution. DgkA is a small, hydrophobic integral membrane protein (Fig. 1, *Inset*). The expectation therefore is that upon mixing with the lipid, monoolein, under conditions that produce the cubic phase the protein will reconstitute into the bilayer of the mesophase. This was demonstrated convincingly by fluorescence quenching. Thus, the intrinsic tryptophan fluorescence of DgkA was monitored as monoolein was replaced, mole for mole, by the quenching lipid, bromo-MAG. Bromo-MAG is a monoacylglycerol with a bromine atom on carbon atoms 9 and 10 of its stearoyl chain. Its phase behavior is very similar to that of monoolein, and the two are entirely miscible. The quenching curve (Fig. 1) is analogous to that observed with other integral membrane proteins known to reconstitute *in meso* (13, 15, 26) and is convincing evidence that DgkA similarly reconstitutes into the bilayer of the cubic phase.

Transport in the Mesophase. It was proposed to monitor DgkA activity using a coupled enzyme system. Thus, the reconstituted enzyme must have access to both of its substrates, monoolein and ATP, during the course of the assay. Monoolein is immediately available in the bilayer of the cubic phase as hosting lipid. In contrast, ATP is provided in the bathing solution. The nucleotide must therefore diffuse into the aqueous channels and be available to the enzyme, which is distributed throughout the mesophase. At the same time, the product, ADP, must diffuse from its site of production through the aqueous channels of the cubic phase out and into the bathing solution. There it is available for reaction with the PK and LD enzyme couple that leads to a measurable drop in A_{340} reflecting consumption of NADH.

Before proceeding with the assay it was important to demonstrate that the cubic phase is porous and can support the diffusion of small, soluble organics under assay conditions. This was done by preparing the cubic phase with a buffer containing ADP. The mesophase, whose aqueous channels were thus preloaded with nucleotide, was placed in the well of a 96-well plate and covered with buffer solution mimicking assay conditions. Release of ADP from the cubic phase was conveniently monitored by following the rise in absorbance of the bathing solution at 260 nm. ADP floods out of the bolus with approximately 98% of the nucleotide released within 10 min (Fig. 2). This is expected behavior and clearly demonstrates that transport of the type needed for a successful assay of DgkA reconstituted *in meso* should be possible.

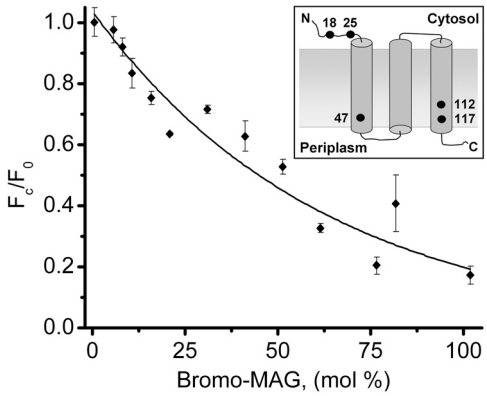


Fig. 1. Fluorescence quenching of DgkA reconstituted in the lipidic cubic mesophase. Quenching of intrinsic tryptophan fluorescence was effected by replacing monoolein, mole for mole, with bromo-MAG. Intrinsic fluorescence (F_c) was normalized to the unquenched fluorescence value (F_o) recorded in the absence of bromo-MAG. The inset shows a model of DgkA in the membrane with tryptophans identified by dots and sequence position.

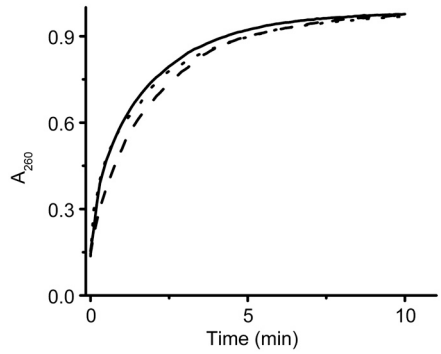


Fig. 2. ADP release from the cubic phase. The mesophase was prepared by homogenizing monoolein with 8.8 mM ADP in 10 mM HEPES pH 7.5. Five microliters of the cubic phase was placed in a 96-well plate and the bolus was overlain with 0.2 mL 10 mM HEPES pH 7.5. A_{260} of the bathing solution was monitored as a function of time in a microplate reader at 30 °C with shaking. Release profiles are shown for triplicate samples. To facilitate absorbance measurements the polystyrene base of the microplate, which strongly absorbs at 260 nm, was replaced with the more transparent Crystal Clear Sealing Film (Hampton).

Kinase Activity. Monoolein as Substrate, LPA as Product. DgkA can use a variety of lipid substrates including monoacylglycerols. In the current study, monoolein creates the bilayer fabric of the cubic phase and the question of most immediate interest was, Can monoolein serve double duty—as a hosting, cubic phase-forming lipid and as a substrate for DgkA? The thin layer chromatographic (TLC) data (Fig. 3) clearly show that the enzyme is indeed active *in meso* and that lyso-phosphatidic acid (LPA) is a product of the DgkA reaction. Thus, monoolein can function both as a substrate and as a hosting lipid.

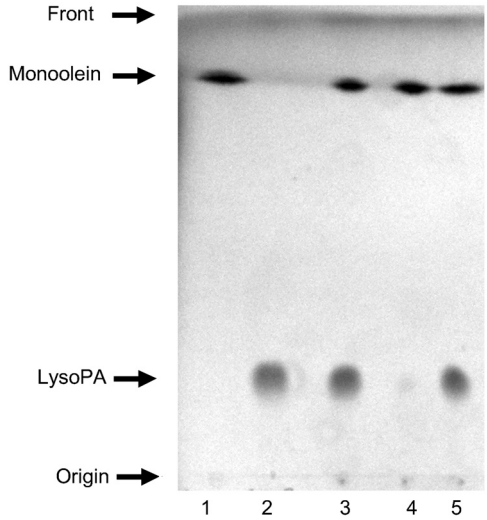


Fig. 3. Thin layer chromatographic analysis of the lipid substrate for and product of DgkA action on monoolein in the cubic mesophase. Lane 1, monoolein standard. Lane 2, LPA standard. Lane 3, monoolein and LPA standards. Lane 4, reaction mix, no DgkA. Lane 5, reaction mix, with DgkA. The reaction was allowed to proceed overnight at 30 °C. The data in Lanes 4 and 5 clearly show DgkA-dependent conversion of monoolein to LPA (Lane 5). The control measurement in Lane 4 illustrates that conditions prevailing during assay support a low level of nonenzymatic phosporylation of monoolein by ATP, which is not surprising.

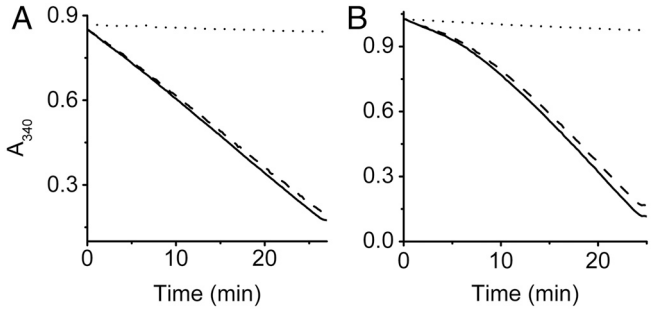


Fig. 4. Progress of the DgkA reaction *in surfo* and *in meso* monitored using a coupled enzyme assay. In *A*, the reaction was carried out in a micellar dispersion (*in surfo*) with DHG as substrate and cardiolipin as activator (Assay Mix 1, see *SI Text*). In *B*, the reaction was conducted with the enzyme reconstituted into the cubic mesophase formed by monoolein, which also serves as lipid substrate, and Assay Mix 2 (see *SI Text*). The progress curve recorded with enzyme (solid line) is corrected (dashed line) for background changes in A_{340} recorded in the absence of enzyme (dotted line). Initial and final A_{340} values reflect, respectively, the concentration of NADH in the reaction mix at the start of the reaction and when all NADH has been consumed.

Kinase Activity Characterization. Before initiating kinetic characterization *in meso* control measurements of DgkA activity were carried out with the protein dispersed in a surfactant solution—the *in surfo* form of the protein. The substrate employed was dihexanoylglycerol (DHG), and measurements were made in the presence of cardiolipin, a DgkA activator (27). A typical progress curve recorded at 30 °C is shown in Fig. 4*A*. The corresponding specific activity is 120 U/mg protein, as expected (20). The *in surfo* assay was repeated with monoolein in place of DHG as substrate. With and without activating cardiolipin the specific activities were 37.4 ± 0.3 and 26.9 ± 0.3 U/mg, respectively. The cardiolipin and monoolein concentrations at which the assays were performed were 2.9 and 25.5 mol%, respectively.

Having shown that DgkA behaves *in surfo* as expected it was necessary to determine if it could be assayed conveniently in a 96-well format *in meso* with monoolein acting both as substrate and hosting lipid. Accordingly, the protein was reconstituted into the cubic phase, the mesophase was added to the well of a 96-well plate along with the assay solution (Assay Mix 2, see *Methods*), and the A_{340} of the bathing solution was monitored as a measure of kinase activity. The progress curve (Fig. 4*B*) begins with an initial lag followed by a region where the change in A_{340} becomes linear with time. The latter, linear region was used to calculate an operational specific activity of 16.5 U/mg under standard conditions (see *SI Text*). This result shows that the activity of the reconstituted kinase can indeed be monitored conveniently in a coupled assay system *in meso*.

Operational K_m and V_{max} *in Meso*. DgkA has two substrates, Mg^{2+}-ATP and diacylglycerol. As noted, monoolein also functions as a substrate. But because monoolein serves as the hosting lipid *in meso* its concentration cannot be sensibly varied to quantify the catalytic characteristics of the enzyme, K_m and V_{max}, with respect to this substrate. Presumably, monoolein saturates the enzyme under assay conditions *in meso*. However, the concentration of Mg^{2+}-ATP can be adjusted at will, and how this affected enzymatic activity *in meso* is illustrated in Fig. 5*A*. Classic Michaelis–Menten saturation behavior was observed with K_m and V_{max} values of 3.5 ± 0.3 mM Mg^{2+}-ATP and 14.4 ± 0.4 U/mg, respectively. Corresponding values of 1.2 ± 0.5 mM Mg^{2+}-ATP and 50 ± 7 U/mg, respectively, have been reported for DgkA *in surfo* with DHG as lipid substrate and cardiolipin as activator (20). These data support the view that DgkA reconstituted into the bilayer of the lipidic cubic phase functions as a classic, well behaved enzyme.

Magnesium Optimization. The kinase activity of DgkA *in meso* responds to Mg^{2+} concentration (Fig. 5*B*) in a way that is similar to its behavior *in surfo* with diolein as substrate (27). Based on these data our standard *in meso* assay mix (Assay Mix 2) includes magnesium acetate at 55 mM.

Enzyme Loading Effects. The measured rate of kinase activity depended on the degree to which the mesophase was loaded with enzyme (Fig. 5*C* and Fig. S1). Rate increased linearly with loading initially as expected. The result indicates therefore that DgkA functions *in meso* as a well behaved catalyst. At higher concentrations, the rate dropped but became linear with loading again in the range of 4 to 16 mg protein/mL. This change in measured rate with concentration and with loading of the mesophase will be discussed.

Alternative Lipid Substrates. As noted, DgkA can use a variety of lipid substrates. With a view to producing crystals of the enzyme by the *in meso* method there is the possibility that one or more of these may preferentially stabilize the protein and, in so doing, facilitate the growth of structure-grade crystals. Accordingly, in this study we examined different lipids that might act as a substrate for the enzyme under *in meso* conditions. The survey was limited to those lipids that form the cubic phase in a single lipid system.

The lipids included in this survey and their chemical structures and the recorded reaction rates under standard conditions are listed in Table S1. Several *cis*-monounsaturated monoacylglycerols (MAGs) were examined that differed in acyl chain length and position of the double bond along the chain. Activity remained relatively constant for MAGs with acyl chain lengths in the range from 16 to 18 carbons. However, when chain length was reduced to 14 carbons a significant drop in activity followed. Adding

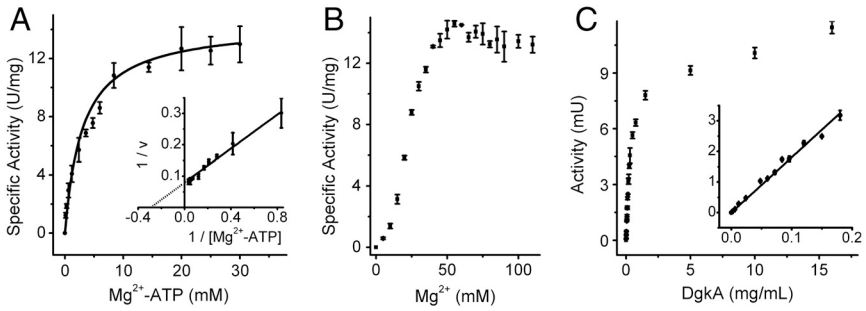


Fig. 5. Enzymatic activity of DgkA reconstituted into the cubic mesophase of monoolein and its dependence on substrate, activator and protein concentration. (*A*) A linear plot of DgkA saturation with respect to Mg^{2+}-ATP concentration where the inset shows the Lineweaver–Burk representation for K_m and V_{max} determination (GraphPad Prism 5). (*B*) Specific activity of DgkA is shown as a function of total Mg^{2+} concentration. In this experiment, Mg^{2+} was added as magnesium acetate and ATP as the sodium salt. (*C*) Dependence of DgkA activity on enzyme loading in a fixed volume of the cubic phase.

BIOCHEMISTRY

bromine to the chain, as in bromo-MAG, had a profound effect on activity, reducing it by three- to fourfold compared to the reference lipid, monoolein. It is of note that monoolein and bromo-MAG both have chains 18 carbon atoms long. Myverol is a food-grade lipid containing mono-, di-, and tri-acylglycerols, glycerol, and free fatty acids. The predominant component is monoolein (28). It was not unexpected therefore that the activity of DgkA in Myverol (17.6 ± 1.2 U/mg) is similar to that observed in monoolein.

The two other lipids included in this study resemble, but are not *bone fide*, MAGs. The first, PAL, is an amide lipid. It consists of a multiply branched 20 carbon phytanoyl chain linked to a glycerol analogue where one of the terminal $-CH_2OH$ moieties is replaced by an amino group. It supported a very reasonable kinase activity *in meso* analogous to the rate observed with 7.7 MAG. The final lipid in the study was phytantriol, which is a 20 carbon, multiply branched phytane with hydroxyl groups attached to three sequential carbons at one end of the molecule. The enzyme shows very little activity with this cubic phase forming lipid.

Discussion

Reconstitution *in Meso*. The most important outcome of this work is the demonstration that DgkA reconstitutes into the bilayer of the cubic phase and that it is enzymatically active therein. The fact that the protein contains five tryptophans distributed throughout its sequence (Fig. 1, *Inset*) made it relatively easy to show by fluorescence quenching that the protein resides in the bilayer following standard reconstitution procedures. Reconstituting the protein into pure bromo-MAG resulted in the quenching of approximately 80% of its intrinsic fluorescence (Fig. 1). The model that emerges from the recent solution NMR structure study places three of DgkA's five tryptophans (Trp47, Trp112, Trp117) in the transmembrane region and thus susceptible to quenching. The fourth and fifth tryptophans (Trp18, Trp25) are expected to reside at the membrane–aqueous interface, although these are located in the N terminus of the protein (residues 1–25) that was deemed mobile and less well defined conformationaly in the NMR study. If indeed these telltale residues are at the interface then they are likely to succumb, at least partially, to quenching consistent with the data in Fig. 1.

Kinase Activity *in Meso*. That DgkA was enzymatically active *in meso* was convincingly demonstrated directly by TLC (Fig. 3). Our next task was to determine if a high-throughput coupled assay system could be used with DgkA reconstituted into a sticky and viscous mesophase. The progress curve in Fig. 4*B* shows clearly this to be the case. The progress curve includes an initial lag phase followed by a region where rate is linear with time. A lag phase is expected. Enzymatic activity resides in protein distributed throughout a porous bolus that is accessed by one of its substrates, ATP, primarily by way of aqueous channels permeating the mesophase. Thus, ATP must diffuse from the bulk bathing solution into and throughout the aqueous channels to fuel the distributed kinase. At the same time, the water-soluble product, ADP, has to diffuse in these same permeating aqueous channels out of the bolus and into the bulk medium. There it can engage with the components of the coupled assay system that leads to a reduction in A_{340} reflecting kinase activity. Both of these processes take time, and together they account for the initial lag.

Following on from the lag is a region where A_{340} changes linearly with time, reflecting the establishment of a steady state. It is this linear region that was used to calculate an operational specific activity for the enzyme. The value recorded under standard conditions (see *SI Text*) was 14.4–16.5 U/mg. The specific activity recorded *in surfo*, with monoolein as substrate, was 26.9 U/mg. It is important to appreciate that the *in surfo* and *in meso* systems differ in many respects and similar specific activities are not necessarily expected.

To our knowledge, this is a unique demonstration of enzymatic activity and turnover for an integral membrane protein reconstituted *in meso*. Perhaps it is not surprising to find that this enzyme functions in the cubic mesophase in light of the fact that other membrane proteins, similarly disposed, have been shown to be biologically active. Thus, for example, the *in meso* form of the vitamin B_{12} transporter, BtuB, was shown to bind its substrate, cobalamin, with nanomolar affinity, as does the native protein in the outer membrane of *E. coli* (13). And OpcA, an adhesin from *Neisseria*, bound sialic acid *in meso* with an affinity similar to that recorded for the protein *in surfo* (15). Together, these and other data in the literature (29), support the view that the lipid bilayer of the cubic phase is a reasonable membrane mimetic. It also highlights the fact that the cubic phase is a generally useful system with which to study membrane proteins and that, despite its sticky and viscous nature, it is a tractable and versatile nanoporous biomaterial. In the current application, it works nicely in a multiwell format and is therefore well suited to economical, parallel, and high-throughput screening of the type that should benefit future crystallization and structure-function determination studies.

The K_m and V_{max} values for Mg^{2+}-ATP reported here are operational in the sense that they reflect the particulars of the system under investigation. Thus, for example, the cubic phase is arranged in the assay well under standard conditions as a set of string-like boluses. The cubic phase that constitutes the bolus is an open and porous material into and out of which diffusion of water-soluble molecules and ions occurs. The aqueous channels permeating the mesophase are molecularly narrow with a diameter of 5–10 nm (30). Diffusion is therefore limited in rate but nonetheless significant as demonstrated in the release study (Fig. 2). It is clear, therefore, that the concentration of added ATP in the bathing solution is always going to be an upper limit to the nucleotide concentration experienced by the enzyme *in meso*. At time zero, those enzymes at the bolus surface will experience bulk concentration while DgkA molecules within the bolus have no ATP in their immediate vicinity. At steady state, a similar situation prevails at the surface of the bolus as at time zero. However, now enzymes in the bolus are operating under conditions where ATP concentration falls off from the surface toward the core of the bolus. Thus, the activity measured reflects the activity of enzymes operating over a range of ATP concentrations the maximum of which is that of the initial bulk bathing solution. Consequently, the operational K_m value reported (3.5 mM) is an upper limit; the actual, intrinsic value is lower. For comparison, the corresponding values reported in the literature for DgkA in liposomes and *in surfo* are 0.2–1.8 mM (31) and 1.2 mM (20), respectively.

By the same reasoning, the activities reported for DgkA *in meso* must be considered operational values. Enzyme molecules at the surface of the bolus will produce ADP directly into the bulk bathing solution for immediate detection as a reduction in A_{340} by the coupled assay method. These then will exhibit and give rise to maximum measured activity. In contrast, enzymes within the bolus will operate at a lower concentration of ATP than prevails at the surface exhibiting lower activity. At the same time, the ADP produced must diffuse through and out of the bolus into the bathing solution for detection by the coupled assay system. Thus, the measured rate will reflect this movement of product from site of production and will be slower the closer is the enzyme to the cylindrical core of the bolus. The operational rate recorded for the bolus therefore is always going to be less than the true rate. The operational V_{max} recorded *in meso* under standard conditions was 14.4 U/mg. For comparison, the V_{max} value reported for DgkA *in surfo* with monoolein as substrate was 26.9 U/mg.

Despite the rationalization just provided it can be argued that the reduced V_{max} *in meso* is attributable to unfolded and inactive enzyme. In the absence of a convenient, direct binding method to quantify active protein *in meso* we have approached the issue indirectly. A mutationally thermostabilized form of DgkA, CLLD (32), was shown to exhibit the same relative kinase activity *in surfo* and *in meso* as did wild type (Table S2). Given the extraordinary stability of CLLD, this finding corroborates the notion that the lower measured rates recorded *in meso* are a reflection of full enzyme activity in this membrane mimetic and not an indication of unfolded protein. Our ability to grow crystals of DgkA *in meso* (*vide infra*, Fig. S2) is consistent with this view.

Despite all of the above real but understandable complications with assaying DgkA *in meso* progress curves enter a phase where the measured rate is linear with time reflecting the establishment of a steady state under standard assay conditions. Further, when the rate in this linear region was monitored as a function of protein concentration in the mesophase and thus, in the assay solution, it too was linear with loading at lower protein concentrations (Fig. 5*C*). This is an important finding because it means that under standard assay conditions operational rates can be used as a reliable measure of the concentration of active protein and substrate.

At the higher protein loading the recorded kinase rate fell off (Fig. 5*C*). Reasons for this are likely to include a diffusion rate of water-soluble substrate into and product out of the mesophase that cannot cope with the elevated demands of the protein to access substrate and for product to diffuse away. It may also have to do with the changing characteristics of the mesophase that come about as the concentration of decyl maltoside (DM) detergent, brought along by the protein, rises. This will tend toward a lamellar and away from the cubic mesophase at higher surfactant levels (33, 34). As noted, the bulk lamellar phase is not compatible with the diffusion of water-soluble substrate and product of the type needed to support activity that can be monitored by coupled enzyme assay. For reference, a loading of 10 mg DgkA/mL corresponds to a DM concentration of approximately 0.1 M *in meso*, which is still well below the level needed to trigger a transition *in meso* (33, 34). Parenthetically, 10 mg/mL is within the concentration range used for *in meso* crystallization trials. Importantly, the protein loading study demonstrates that the enzyme is catalytically active under such conditions.

Toward *in Meso* Crystallogenesis and Structure-Function Studies. The primary aim of this study was to determine if the integral membrane kinase, DgkA, was catalytically active (and assayable) upon incorporation into the lipidic cubic phase. Clearly, it is. The stage is therefore set to proceed with crystallization trials with a view to generating structure-grade crystals of functional DgkA by the *in meso* method for which diffraction quality crystals are already in hand (Fig. S2). The kinase has been shown to phosphorylate a variety of lipid substrates several of which should prove useful in future crystallographic studies aimed at deciphering the mode of action of the kinase and the origins of its substrate selectivity.

In this study, the lipidic cubic phase that incorporates an integral membrane kinase has been shown to be compatible with a microplate-based coupled assay of catalytic activity for convenient and high-throughput screening. The sticky and viscous nature of the cubic phase facilitated such measurements. The fact that the enzyme and its lipid substrate and product remain confined to the porous mesophase while the other water-soluble substrate and product are free to partition into the aqueous bathing solution suggests a general and convenient approach for characterizing integral membrane enzymes that function with lipids in a membrane-like environment. In support of this, the activity of a phosphatidylglycerol phosphate (PGP) synthesizing enzyme, PgsA, has been successfully assayed *in meso* (Figs. S3 and S4). Here, the lipid substrate, cytidine diphosphate-diacylglycerol (CDP-DAG), and product, PGP, remain in the host cubic phase; their water-soluble counterparts, glycerol 3-phosphate and cytidine monophosphate, respectively, move freely in and out of the PgsA laden bolus facilitating direct spectrophotometric assay. The distinctive rheology of the cubic phase means that a separate step to physically separate substrate from product is not always needed. Accordingly, if one of the water-soluble substrates or products can be measured spectrophotometrically the need for labeling, with radioisotopes for example, and for cumbersome lipid extraction (35) is dispensed with and activity can be monitored directly and continuously. Because of its open, bicontinuous nature, the cubic phase offers the advantage that the protein is accessible for assay from both sides of the membrane. By extension, the cubic mesophase system described should lend itself to the simultaneous reconstitution, functional characterization, and exploitation of integral membrane enzymes that catalyze sequential reactions in pathways of lipid metabolism.

Methods

Detailed procedures and additional results can be found in *SI Text*.

Molecular Biology, Protein Production, and Purification. The *dgk*A gene was synthesised (Genscript) based on the sequence of *dgk*A in *E. coli* K12 and was cloned into pTrcHisB (Invitrogen) using *Nco*I and *Eco*RI. The amino acid sequence of the expressed protein is the same as that reported for pSD0005 (32) where the N-terminal methionine is replaced by a hexa-His tag-containing peptide (MGHHHHHHEL). The CLLD mutant gene was generated as for its wild-type counterpart. DgkA and CLLD production and purification were done following published procedures (36) with minor modifications (see *SI Text*).

Reconstitution *in Meso*. DgkA was reconstituted into the bilayer of the cubic phase following a standard protocol (8). The diluted protein solution was homogenized with monoolein in a coupled syringe device (37) at room temperature (RT, 19–24 °C) using two volumes of protein solution for every three of monoolein. For spectroscopy, where transparent samples were needed, a slightly less hydrated phase was used; the corresponding volume ratio was 29:71. Control samples that lacked enzyme were prepared with 1 % (w/v) DM in buffer in place of protein solution.

Kinase Activity Measurements. A coupled assay system was used to monitor DgkA activity. Thus, the consumption of ATP in the phosphorylation of monoolein was coupled through the sequential action of PK and LD to the oxidation of NADH monitored by a change in A_{340} (19) in a 96-well plate format (20).

The kinase activity of DgkA reconstituted into the cubic phase was assayed under standard conditions as follows. The *in meso* assay mix (Assay Mix 2) contained 20 mM ATP, 0.1 mM EDTA, 0.1 mM EGTA, 55 mM magnesium acetate, 1 mM PEP, 0.2 mM DTT, 50 mM LiCl, 0.4 mM NADH, 20 U/mL of PK and LDH, and 75 mM PIPES pH 6.9. The protein loaded mesophase, prepared by homogenizing 3 volumes of monoolein with 2 volumes of DgkA solution at 66 μg/mL as above, was transferred to a 50-μL syringe (Hamilton) attached to a 50-step dispenser (Hamilton) and fitted with a 22-gauge needle. Five microliters of the mesophase was dispensed in 1-μL rod-shaped boluses along the sidewall of a well in a 96-well plate (Nunc) and out of the light path of the plate reader. Because the mesophase is sticky it remains in place on the sidewall during the assay. To start the reaction 0.2 mL Assay Mix 2 was added to each well, which was enough to cover the bolus. Immediately, the plate was placed in the microplate reader at 30 °C and shaken for 5 s. A_{340} readings were recorded every 15 s for 45 min with shaking for 2 s between each reading. DgkA-free blanks were run with each trial using mesophase prepared with buffer. Background rates, corresponding to nonenzymatic hydrolysis of ATP and to NADH photobleaching, were subtracted from those recorded in the presence of DgkA. Assays were done in triplicate. Average values in units (1 U = 1 μmol substrate converted to product per min) ± standard deviation are reported.

Spectrophotometry. Absorbance and fluorescence measurements on the protein *in surfo* and *in meso* were carried out as previously described (13, 15, 26) with minor modifications (see *SI Text*).

BIOCHEMISTRY

Thin Layer Chromatography. TLC was used to verify that LPA is a product of DgkA action on monoolein. Thus, 5 μL cubic phase with and without DgkA was incubated overnight in a 1.5-mL Eppendorf tube at 30 °C bathed in 0.2 mL of Assay Mix 2. Mesophase was harvested by centrifuging at 20,000 g for 10 min at 25 °C and used for lipid extraction (see *SI Text*). Three microliters of the extracted lipid and standard solutions in chloroform were spotted on silica gel TLC plates and developed in chloroform : methanol : acetone : aceticacid : water (10 : 2 : 4 : 2 : 1 by vol.). After staining with phosphomolybdic acid the plate was placed on a hot plate at 150 °C to develop.

ACKNOWLEDGMENTS. We thank M. Aherne and C. Doherty for technical assistance and A. Dukkipati and C. R. Sanders for helpful discussion. Supported by Science Foundation Ireland (07/IN.1/B1836), FP7 COST Action CM0902, and the National Institutes of Health (GM75915, P50GM073210, U54GM094599).

1. Rosenbaum DM, et al. (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318:1266–1273.
2. Cherezov V, et al. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258–1265.
3. Jaakola VP, et al. (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322:1211–1217.
4. Wu B, et al. (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330:1066–1071.
5. Chien EY, et al. (2010) Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* 330:1091–1095.
6. Rasmussen SG, et al. (2011) Structure of a nanobody-stabilized active state of the beta (2) adrenoceptor. *Nature* 469:175–180.
7. Rosenbaum DM, et al. (2011) Structure and function of an irreversible agonist-beta(2) adrenoceptor complex. *Nature* 469:236–240.
8. Caffrey M, Cherezov V (2009) Crystallizing membrane proteins using lipidic mesophases. *Nat Protoc* 4:706–731.
9. Caffrey M (2009) Crystallizing membrane proteins for structure determination: Use of lipidic mesophases. *Annu Rev Biophys* 38:29–51.
10. Landau EM, Rosenbusch JP (1996) Lipidic cubic phases: A novel concept for the crystallization of membrane proteins. *Proc Natl Acad Sci USA* 93:14532–14535.
11. Cherezov V, Clogston J, Papiz MZ, Caffrey M (2006) Room to move: Crystallizing membrane proteins in swollen lipidic mesophases. *J Mol Biol* 357:1605–1618.
12. Katona G, Andreasson U, Landau EM, Andreasson LE, Neutze R (2003) Lipidic cubic phase crystal structure of the photosynthetic reaction centre from Rhodobacter sphaeroides at 2.35 A resolution. *J Mol Biol* 331:681–692.
13. Cherezov V, et al. (2006) In meso structure of the cobalamin transporter, BtuB, at 1.95 A resolution. *J Mol Biol* 364:716–734.
14. Hofer N, Aragao D, Caffrey M (2010) Crystallizing transmembrane peptides in lipidic mesophases. *Biophys J* 99:L23–25.
15. Cherezov V, et al. (2008) In meso crystal structure and docking simulations suggest an alternative proteoglycan binding site in the OpcA outer membrane adhesin. *Proteins* 71:24–34.
16. Raetz CR, Newman KF (1979) Diglyceride kinase mutants of *Escherichia coli*: Inner membrane association of 1,2-diglyceride and its relation to synthesis of membrane-derived oligosaccharides. *J Bacteriol* 137:860–868.
17. Van Horn WD, et al. (2009) Solution nuclear magnetic resonance structure of membrane-integral diacylglycerol kinase. *Science* 324:1726–1729.
18. Lorch M, et al. (2005) How to prepare membrane proteins for solid-state NMR: A case study on the alpha-helical integral membrane protein diacylglycerol kinase from E. coli. *Chembiochem* 6:1693–1700.
19. Tanzer ML, Gilvarg C (1959) Creatine and creatine kinase measurement. *J Biol Chem* 234:3201–3204.
20. Badola P, Sanders CR (1997) *Escherichia coli* diacylglycerol kinase is an evolutionarily optimized membrane enzyme and catalyzes direct phosphoryl transfer. *J Biol Chem* 272:24176–24182.
21. Czerski L, Sanders CR (2000) Functionality of a membrane protein in bicelles. *Anal Biochem* 284:327–333.
22. Bohnenberger E, Sandermann H, Jr (1982) Diglyceride kinase from *Escherichia coli*. Modulation of enzyme activity by glycosphingolipids. *Biochim Biophys Acta* 685:44–50.
23. Schneider EG, Kennedy EP (1973) Phosphorylation of ceramide by diglyceride kinase preparations from *Escherichia coli*. *J Biol Chem* 248:3739–3741.
24. Schneider EG, Kennedy EP (1976) Partial purification and properties of diglyceride kinase from *Escherichia coli*. *Biochim Biophys Acta* 441:201–212.
25. Walsh JP, Fahrner L, Bell RM (1990) sn-1,2-diacylglycerol kinase of *Escherichia coli*. Diacylglycerol analogues define specificity and mechanism. *J Biol Chem* 265:4374–4381.
26. Liu W, Caffrey M (2005) Gramicidin structure and disposition in highly curved membranes. *J Struct Biol* 150:23–40.
27. Walsh JP, Bell RM (1986) sn-1,2-Diacylglycerol kinase of *Escherichia coli*. Mixed micellar analysis of the phospholipid cofactor requirement and divalent cation dependence. *J Biol Chem* 261:6239–6247.
28. Clogston J, Rathman J, Tomasko D, Walker H, Caffrey M (2000) Phase behavior of a monoacylglycerol: (myverol 18–99 K)/water system. *Chem Phys Lipids* 107:191–220.
29. Hochkoeppler A, et al. (1995) Photochemistry of a photosynthetic reaction center immobilized in lipidic cubic phases. *Biotechnol Bioeng* 46:93–98.
30. Qiu H, Caffrey M (2000) The phase diagram of the monoolein/water system: Metastability and equilibrium aspects. *Biomaterials* 21:223–234.
31. Pilot JD, East JM, Lee AG (2001) Effects of bilayer thickness on the activity of diacylglycerol kinase of *Escherichia coli*. *Biochemistry* 40:8188–8195.
32. Zhou Y, Bowie JU (2000) Building a thermostable membrane protein. *J Biol Chem* 275:6975–6979.
33. Misquitta Y, Caffrey M (2003) Detergents destabilize the cubic phase of monoolein: Implications for membrane protein crystallization. *Biophys J* 85:3084–3096.
34. Ai X, Caffrey M (2000) Membrane protein crystallization in lipidic mesophases: Detergent effects. *Biophys J* 79:394–405.
35. Dowhan W (1992) Phosphatidylglycerophosphate synthase from *Escherichia coli*. *Methods Enzymol* 209:313–321.
36. Sanders CR, et al. (1996) *Escherichia coli* diacylglycerol kinase is an alpha-helical polytopic membrane protein and can spontaneously insert into preformed lipid vesicles. *Biochemistry* 35:8610–8618.
37. Cheng A, Hummel B, Qiu H, Caffrey M (1998) A simple mechanical mixer for small viscous lipid-containing samples. *Chem Phys Lipids* 95:11–21.

Supporting Information

Li and Caffrey 10.1073/pnas.1101815108

Experimental Procedures

1. Reagents. Monoolein (9.9 MAG) (M239-JY27-T, M239-O24-S, M239-F15-U, M239-M27-U) and 9.7 MAG (M219-JY22-N) were purchased from Nu-Chek Prep. 7.7 and 7.11 MAG were synthesized and purified in-house by Dr. J. Lee and Mr. J. Lyons following established procedures (1, 2). 1,2-dihexanoyl-sn-glycerol [C6:0]] (DHG, Cat. No. D-8531/D-6662; lot No. 33H84584), pyruvate kinase (Cat. No. P9136, Lot 010M1228), L-lactate dehydrogenase (61309, lot 1439120 43609087), NADH (N4505, lot 029K5151), ADP (Cat. A2754, lot 078K7001), ATP (Cat. No. A7699, lot 087K7001), Empigen BB (Cat 45165 lot 1384611 33408313), DL-dithiothreitol (DTT, 43815, lot 1333018 63007006), magnesium acetate (Cat M0631, lot 069K03391), tris (2-carboxyethyl) phosphine hydrochloride (TCEP, C4706, lot 030M1523), phosphoenol pyruvic acid (PEP, 860077, lot 055K37992), imidazole (Cat. I0250, lot 068K5303), DNAse I, (Cat. DN2, lot 056K7680), lysozyme (Cat. L6876, lot 094K1454), Tris (Cat. T8,760-2, lot 1133AH-437), lithium chloride (Cat. 62486, lot 134700814107043), lithium hydroxide (Cat. 402974, lot MKBC0399), HEPES (Cat. H3375, lot 038K5429), EDTA (E5134, lot 087K0049), EGTA (Cat 03777, lot 0001426960), butylated hydroxytoluene (BHT, Cat. W21,840-5-K, lot 15796PH-478), phenylmethanesulfonyl fluoride (PMSF, 78830, lot 1419691 31509037), chloroform (650496, lot 09690HJ), hydrochloric acid (Cat. 258148, lot. 1403487 23808281), and phosphomolybdic acid (Cat. No. 31,927-9, lot 50281-448), glycerol phosphate disodium salt hydrate (Cat. G6501, lot 029k0189) were obtained from Sigma. Acetone (A/0606/7, lot 095939), methanol (M/4056/PB17, lot 1074898), and sodium chloride (BP358-10, lot 060875) were from Fisher Scientific. PIPES (lots M10550899 and 20373) was purchased from Molekula Ltd. Magnesium chloride (Cat. 1.05833.0250, lot A410133) was obtained from Merck (Merck Biosciences). 1,1′,2,2′-tetraoleoyl-cardiolipin (cardiolipin, CL, Cat. 710335P, lot. 181CA-44), 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphate (LPA, Cat. 857130, Lot. 181LPA-62), 1-(9,10-dibromooctadecanoyl)-rac-glycerol (18:0 dibromo monoglyceride, bromo-MAG, Cat. 790777, lot 180BRMG-15), 1,2-dioleoyl-sn-glycero-3-(cytidine diphosphate) (Cat. 87052P, lot 181CDPDG-24) were from Avanti Polar Lipids, Inc. n-Decyl-β-D-maltopyranoside (DM, Cat. D322LA, lot 120455), n-dodecyl-β-D-maltopyranoside (DDM, D310, D310S, lots 127193, 126671) were from Anatrace. Phytantriol (lot PTL-03423) was obtained from Kuraray Co. Ltd. Ni-NTA resin (Cat. 1018142, lot 136231239) was from Qiagen. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Cat. MB1008, lot A21345), glycerol (Cat. G1345, lot A21344), and ampicillin (Cat A0104, lot 20614) were purchased from Melford Labs. 96-well plates (Cat. 265301, lot. 655700; Cat. 265302, lot. 658021) were purchased from Nunc. *Nco*I (Cat. R0193) and *EcoR*I (R0101) were from New England Biolabs.

2. Molecular Biology, Protein Production, and Purification. The *dgk*A wild-type gene and a *dgk*A gene construct encoding for a thermostable mutant (CLLD) (3) were synthesised (Genscript). The wild-type gene has the same DNA sequence as *dgk*A in *E. coli* K12. CLLD was modified to incorporate four missense mutations, as described (3). Both genes were cloned into pTrcHisB (Cat. V360-20, Invitrogen) using *Nco*I and *EcoR*I. The amino acid sequence of the expressed DgkA and CLLD are the same as that reported for pSD0005 and pSD0005-CLLD, respectively, by Bowie (3), where the N-terminal methionine is replaced by a hexa-His tag-containing peptide (MGHHHHHHEL) to facilitate purification.

The *pgs*A gene from *Pseudomonas aeruginosa* PAO1 was amplified by PCR using primer pairs 5′-GGAATTCCATATGAATATCCCCAATCTGCTC-3′ and 5′-CGGGATCCTTTCTCTTTCGGGGTGGTCG-3′ incorporating *Nde*I and *BamH*I restriction enzyme sites (underlined). The PCR product was cut with *Nde*I and *BamH*I and ligated into the pWaldo-d vector (4) treated with the same enzymes.

The production of DgkA and its purification, primarily from inclusion bodies, were done following published procedures with minor changes (5). Briefly, BL21 cells carrying the pTrcHisB-DgkA plasmid and grown in *Luria-Bertani* broth were induced at an absorbance at 600 nm of 0.6 for 3 h at 37 °C with 1 mM IPTG. Biomass was harvested by centrifugation at 5,000 g for 15 min at 4 °C. The cell pellet from 1 L of culture was suspended in 0.1 L of lysis buffer (0.2 mM TCEP, 0.3 M NaCl, 0.2 mg/mL lysozyme, 50 μg/mL DNAase, 10 μM BHT, 1 mM PMSF (toxic), 0.2 mM EDTA, 5 mM $MgCl_2$, 75 mM Tris/HCl pH 7.8) and left with gentle stirring at 25 °C for 30 min. The suspension was cooled on ice and cells were broken using a probe sonicator at 4 °C for 10 min with a 0.3 s on - 0.7 s off duty cycle and a power setting of 30% (Model HD2200, Probe KE76. Bandelin). The zwitterionic detergent, Empigen BB, was added to the cell lysate at 3% (w/v) to solublize DgkA from inclusion bodies and membrane fragments. All subsequent steps were carried out at 4 °C unless otherwise noted. After 30 min of mixing at 10 rpm on a Stuart SB3 rotator (Bibby Scientific Ltd.), the solublized protein was separated from cell debris and insoluble material by centrifuging the detergent-treated lysate at 9,000 g for 10 min. To the supernatant was added 4 mL Ni-NTA resin preequilibrated with 1.5% (w/v) Empigen BB in *Buffer A* (0.3 M NaCl, 0.2 mM TCEP, 40 mM HEPES pH 7.5). After 30 min incubation in the SB3 rotator, the resin was placed in a gravity flow column (Cat. 732-1010, BioRad). The resin was washed with 50 mL 10 μM BHT, 3% (w/v) Empigen BB in Buffer A, followed by a 50 mL wash with 40 mM imidazole and 1.5% (w/v) Empigen BB in Buffer A. The Empigen BB detergent was exchanged to DM by washing the column with 60 mL of 0.25% (w/v) DM in *Buffer B* (50 mM LiCl, 0.2 mM TCEP, 20 mM HEPES pH 7.5). Protein was eluted with 0.25 M imidazole/HCl pH 7.5 and 0.5% (w/v) DM in *Buffer C* (1 mM TCEP, 50 mM LiCl, 10 mM HEPES pH 7.5) and concentrated to 16 mg/mL using a concentrator (Amicon Ultracel-50 membrane, Cat. UFC905008, Millipore). In the process, imidazole was reduced to less than 20 mM by diluting the retentate in the concentrator twofold with 0.25% (w/v) DM in Buffer C and repeating the concentration/dilution cycle four times. The concentrated protein solution was immediately divided into 10-μL aliquots, flash frozen in liquid nitrogen and stored at −80 °C. Protein concentration was determined by measuring absorbance at 280 nm ($\epsilon_{1\,\mathrm{mg/ml}} = 2.1$) (6) in a NanoDrop 1000 spectrometer (Thermo Fisher Scientific Inc.). Protein purity was estimated at >95% based on SDS-polyacrylamide gel electrophoresis. Typically, the yield per litre of culture was 23 mg DgkA. The CLLD mutant was produced and purified exactly as described above for DgkA.

PgsA was produced in C43 (DE3) cells carrying the pWaldo-PgsA plasmid in *Luria-Bertani* broth. Cells were induced at 25 °C with 0.4 mM IPTG at an absorbance at 600 nm of 0.6. Biomass was harvested 16 h postinduction by centrifugation at 5,000 g for 15 min at 4 °C. The cell pellet from 6 L of culture was suspended in 0.2 L of lysis buffer (0.2 mM TCEP, 0.3 M NaCl, 0.2 mg/mL

lysozyme, 50 μg/mL DNAase, 1 mM PMSF (toxic), 0.2 mM EDTA, 20 mM $MgCl_2$, 75 mM Tris/HCl pH 8.0) and left with gentle stirring at 25 °C for 30 min. The suspension was cooled on ice and cells were broken using a probe sonicator at 4 °C for 10 min with a 0.3 s on - 0.7 s off duty cycle and a power setting of 30% (Model HD2200, Probe KE76. Bandelin). The membrane fraction was pelleted by centrifugation at 100,000 g and 4 °C for 1 h. The pellet was resuspended in *Buffer D* (1 M NaCl, 0.2 mM TCEP, 50 mM Tris/HCl pH 8.0) and gently stirred at 25 °C for 30 min. The suspension was centrifuged at 100,000 g and 4 °C for 1 h and the pellet was suspended in 200 mL of *Buffer E* (0.3 M NaCl, 0.2 mM TCEP, 50 mM Tris/HCl pH 8.0). To the suspension was added 4 g of DDM (corresponding to 2% (w/v) DDM) and solubilization of the membranes was allowed to continue overnight (approximately 13 h) at 4 °C with gentle stirring. After centrifugation at 100,000 g and 4 °C for 1 h, the supernatant containing solublized PgsA was diluted four times by adding 600 mL of Buffer E slowly with stirring. To this was added 10 mL Ni-NTA resin preequilibrated with *Column Buffer* (0.3 M NaCl, 0.2 mM TCEP, 0.03% (w/v) DDM, 50 mM Tris/HCl pH 8.0). After overnight incubation at 4 °C with gentle stirring the resin was placed in a gravity flow column (Cat. 732-1010, BioRad). The resin was washed with 50 mL of Column Buffer followed by 50 mL of Column Buffer containing 50 mM imidazole. Protein was eluted with 0.25 M imidazole in Column Buffer.

3. Reconstitution *in Meso*. DgkA was reconstituted into the bilayer of the cubic phase following a standard protocol (7). The stock protein solution at 16 mg/mL as above was first diluted with 1% (w/v) DM in *Buffer C*. Most measurements were done using cubic phase prepared with a 242-fold diluted protein solution at 66 μg/mL. Others, that included the assay of DgkA activity in phytantriol and absorbance and fluorescence spectroscopy used protein solutions at 1 and 5 mg/mL, respectively. The diluted protein solution was homogenized with monoolein in a coupled syringe device (8) at room temperature (19–24 °C) using two volumes of protein solution for every three volumes of monoolein. For spectroscopic measurements, where optically transparent samples were needed, a slightly less hydrated cubic phase was used; the corresponding volume ratio was 29:71. For Myverol, phytantriol, and 7.7 MAG which have slightly different phase behaviours compared to monoolein, the ratio used was 25:75, 28:72, and 50:50, respectively. Control samples that lacked enzyme were prepared with 1% (w/v) DM in Buffer C in place of protein solution. Delivering small volumes of the viscous and sticky mesophase reproducibly was greatly facilitated by using a repeat dispenser, as described (9).

4. Kinase Activity Measurements. Throughout this study a coupled assay system was used to monitor DgkA activity. Thus, the consumption of ATP in the phosphorylation of monoolein was coupled through the sequential action of PK and LD to the oxidation of NADH. The latter has a strong absorbance at 340 nm (A_{340}) and the change in A_{340} with time was used to monitor kinase activity (10). The Sanders' group has implemented this coupled assay of DgkA in a 96-well format (6). We have adapted this approach to quantify the activity of DgkA reconstituted *in meso*.

5. Activity Assay *in Surfo*. The kinase activity of detergent solubilised DgkA was monitored under standard *in surfo* conditions in 96-well plates as follows. The *in surfo* assay mix (*Assay Mix 1*) contained 0.66 mM cardiolipin, 0.95 mM DHG, 1% (w/v) (21 mM) DM, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM PEP, 3 mM ATP, 15 mM magnesium acetate, 50 mM LiCl, 0.2 mM DTT, 0.3 mM NADH, 20 U/mL PK and LDH, 75 mM PIPES pH 6.9. Thus, Assay Mix 1 has 2.9, 4.2 and 92.9 mol% cardiolipin, DHG and DM, respectively. To eliminate any ADP in the system and for thermal equilibration the reaction mixture was incubated at 30 °C for 10 min prior to assay. The kinase reaction was initiated by adding 0.2 mL Assay Mix 1 to 2 μL DgkA solution prepared as a 2,400-fold dilution (6.6 μg protein/mL) of stock, purified protein in 1% (w/v) DM in Buffer C. Immediately, the plate was placed in a SpectraMax M2e plate reader (Molecular Devices) prewarmed to 30 °C. A_{340} was recorded every 15 s, typically for 30 min. DgkA-free blanks were run with each trial using 1% (w/v) DM in Buffer C in place of protein solution. Background rates, corresponding to nonenzymatic hydrolysis of ATP and NADH photobleaching, were subtracted from those recorded in the presence of DgkA.

6. Activity Assay *in Meso*. *6.1. DgkA*. The kinase activity of DgkA reconstituted into the lipidic cubic phase was assayed under standard conditions in 96-well plates as follows. The *in meso* assay mix (Assay Mix 2) contained 20 mM ATP, 0.1 mM EDTA, 0.1 mM EGTA, 55 mM magnesium acetate, 1 mM PEP, 0.2 mM DTT, 50 mM LiCl, 0.4 mM NADH, 20 U/mL of PK and LDH, and 75 mM PIPES pH 6.9. To eliminate residual ADP and to equilibrate temperature the assay mix was incubated at 30 °C for 10 min immediately after preparation and just prior to assay. The protein loaded mesophase, prepared by homogenizing 3 volumes of monoolein with 2 volumes of DgkA solution at 66 μg/mL as above under Reconstitution *In Meso*, was transferred to a 50-μL syringe (Cat. 80930, Hamilton) attached to a 50-step repeating dispenser (Cat. PB600-1, Hamilton) and fitted with a 22-gauge, flat-tipped, 19.1 mm-long needle. Five microliters of the mesophase was dispensed in 1-μL rod-shaped boluses along the sidewall of a well in a 96-well plate (Cat. 265302, Nunc, *Roskilde*, Denmark) and out of the light path of the plate reader. For samples prepared with 7.7 MAG and with Myverol, 4 and 8 μL of cubic phase were used respectively, to have the same amount DgkA (132 ng) in the assay. Because the mesophase is naturally sticky and viscous it remains in place on the sidewall of the well during the course of the assay. To start the reaction 0.2 mL Assay Mix 2 was added to each well which was enough to completely cover the mesophase bolus. Immediately, the plate was placed in the micro-plate reader at 30 °C and shaken (autoshaker function setting-on) for 5 s. A_{340} readings were recorded every 15 s typically for 45 min with shaking for 2 s between each reading. DgkA-free blanks were run with each trial using mesophase prepared with Buffer C. Background rates, corresponding to nonenzymatic hydrolysis of ATP and to NADH photobleaching, were subtracted from those recorded in the presence of DgkA. Activity assays were done in triplicate requiring 15 μL mesophase per activity assay datum. Values reported are the average ± standard deviation of triplicate measurements.

The effect of holding constant the total DgkA concentration in the assay well but dispersing the fixed amount of enzyme in different, total volumes of mesophase, was examined. The results show that over a wide mesophase volume range from 0.5 to 12.5 μL the measured rate remained relatively constant (Fig. S1). Note that a mesophase volume of 5 μL is used under standard assay conditions. Interestingly, at the lower volumes examined down to 200 nL the measured rate fell off dramatically.

***6.2. PgsA*.** To monitor PgsA activity *in meso* the enzyme was reconstituted into the cubic phase as described for DgkA except that the hosting monoolein mesophase was predoped with substrate CDP-DAG. The concentration of the PgsA solution used for mesophase preparation was 0.35 mg/mL. Ten microliters of the PgsA-laden mesophase was dispensed in 1-μL rod-shaped boluses along the sidewall of a well in a 96-well plate (Cat. 265302, Nunc) and out of the light path of the plate reader, as described for DgkA. The base of the plate was replaced with ClearSeal Film (HR4-521, Hampton Research) to allow absorbance at 272 nm (A_{272}) of the bathing solution to be recorded.

To start the reaction 0.2 mL of prewarmed (37 °C) PgsA Assay Mix (16 mM glycerol-3 phosphate, 0.1 mM EDTA, 0.2 mM TCEP, 0.1 M $MgCl_2$, 50 mM Tris/HCl) was added to each well, which was enough to completely cover the mesophase bolus. Immediately, the plate was placed in the micro-plate reader at 37 °C and shaken (autoshaker function setting-on) for 5 s. A_{272} readings were recorded every 30 s typically for 100 min with shaking for 2 s between each reading.

7. Spectrophotometric Measurements. Absorbance and fluorescence measurements on the protein *in surfo* and *in meso* were carried out as previously described (11–13) with the following modifications. The mesophase was prepared by mixing to homogeneity lipid and protein solution in the volume ratio of 71/29 as outlined above under Reconstitution *in Meso*. Most studies were done with the lipid, monoolein. For fluorescence quenching measurements mixtures of monoolein and bromo-MAG were used. All spectra were recorded in cuvettes (0.1 mm pathlength) consisting of a front and a back quartz window. To load, 2 μL of mesophase was placed on one of the windows in the region to be interrogated by the light beam and immediately a sandwich of optically clear mesophase was made by covering it with the second window. Measurements were made using the micro-plate reader (medium sensitivity and normal reading setting) as above and a micro-plate as a cuvette holder. Thus, the base of a well in the 96-well plate (Cat. 265301, Lot. 655700, Nunc) was removed, the cuvette was taped in its place, and then positioned in the plate reader for data collection at 25 °C. Fluorescence emission spectra were recorded from 325 nm to 360 nm in 1 nm steps with an excitation wavelength of 295 nm. Since sample absorbance at all wavelengths used was below 0.1 inner filter effects are not significant and a correction for same was not applied. Control measurements were run in which buffer was used in place of the DgkA solution for mesophase preparation. In all cases, fluorescence values recorded for protein containing samples were corrected by subtracting those recorded with protein-free blanks. *In surfo* measurements were made following this protocol with the exception that the cuvette was loaded with 2 μL DgkA solution at 1.45 mg/mL in place of the lipidic mesophase.

Mesophase samples containing different mole percentages of quenching lipid for fluorescence quenching studies were prepared using monoolein and bromo-MAG, as described (3).

8. Thin Layer Chromatography (TLC). TLC was used to verify that lyso-phosphatidic acid (LPA) is the phorphorylated product of DgkA activity when reconstituted into the lipidic cubic phase prepared with monoolein. For this purpose, 5 μL cubic phase with and without DgkA was incubated overnight in a 1.5-mL Eppendorf tube at 30 °C bathed in 0.2 mL of Assay Mix 2. Mesophase was harvested by centrifuging the tubes at 20,000 g for 10 min at 25 °C. Interestingly, the mesophase with DgkA added formed a pellet while the protein-free, control mesophase floated on the aqueous solution. This behavior likely reflects the presence of reconstituted DgkA and enzymatic conversion of monoolein to LPA, both of which will alter mesophase density. Excess aqueous solution was removed and the mesophase was washed three times with 1 mL 50 mM LiCl in 10 mM HEPES pH 7.5. Excess buffer was removed and the mesophase was solubilized by vortexing with 0.8 mL of a chloroform : water (1 : 1 by vol.) solution. Following centrifugation at 20,000 g for 10 min at 25 °C the upper aqueous phase was removed and discarded. The chloroform solution containing the extracted lipid was centrifuged again at 20,000 g for 10 min at RT and diluted 1 : 10 in chloroform for direct use in TLC analysis.

TLC plates (8-cm silica gel plate, Cat. No. 1.05554.0001, Lot No. HX068423. Merck) were prerun in chloroform : methanol : acetone : aceticacid : water (10 : 2 : 4 : 2 : 1 by vol.), dried at 50 °C for 20 min and cooled to RT. Three microliters of the extracted and standard lipid solutions in chloroform were spotted at the origin, solvent was removed by drying at 22 °C for 15 min under a stream of nitrogen gas and the plates were developed in chloroform : methanol : acetone : aceticacid : water (10 : 2 : 4 : 2 : 1 by vol.) at 22 °C. After drying on a heat block at 40 °C under nitrogen for 15 min and staining with 20% (w/v) phosphomolybdic acid in ethanol the plate was placed on a hot plate at 150 °C for stain development. A digital image of the chromatogram was recorded using a FluoroChem SP Imaging System (Cell Biosciences). Lipid standards that included monoolein and lyso-phosphatidic acid were prepared for TLC as 0.5 mg/mL solutions in chloroform.

1. Coleman BE, et al. (2004) Modular approach to the synthesis of unsaturated 1-monoacyl glycerols. *Synlett* 2004:1339–1342.
2. Caffrey M, Lyons J, Smyth T, Hart DJ (2009) *Membrane Protein Crystallization (Current Topics in Membranes)* ed Larry D (Academic Press, Maryland Heights, MO), pp 83–108.
3. Zhou Y, Bowie JU (2000) Building a thermostable membrane protein. *J Biol Chem* 275:6975–6979.
4. Drew D, Lerch M, Kunji E, Slotboom DJ, de Gier JW (2006) Optimization of membrane protein overexpression and purification using GFP fusions. *Nat Methods* 3:303–313.
5. Sanders CR, et al. (1996) *Escherichia coli* diacylglycerol kinase is an alpha-helical polytopic membrane protein and can spontaneously insert into preformed lipid vesicles. *Biochemistry* 35:8610–8618.
6. Badola P, Sanders CR (1997) *Escherichia coli* diacylglycerol kinase is an evolutionarily optimized membrane enzyme and catalyzes direct phosphoryl transfer. *J Biol Chem* 272:24176–24182.
7. Caffrey M, Porter C (2010) Crystallizing membrane proteins for structure determination using lipidic mesophases. *J Vis Exp* e1712.
8. Cheng A, Hummel B, Qiu H, Caffrey M (1998) A simple mechanical mixer for small viscous lipid-containing samples. *Chem Phys Lipids* 95:11–21.
9. Caffrey M, Cherezov V (2009) Crystallizing membrane proteins using lipidic mesophases. *Nat Protoc* 4:706–731.
10. Tanzer ML, Gilvarg C (1959) Creatine and creatine kinase measurement. *J Biol Chem* 234:3201–3204.
11. Cherezov V, et al. (2008) *In meso* crystal structure and docking simulations suggest an alternative proteoglycan binding site in the OpcA outer membrane adhesin. *Proteins* 71:24–34.
12. Cherezov V, et al. (2006) *In meso* structure of the cobalamin transporter, BtuB, at 1.95 A resolution. *J Mol Biol* 364:716–734.
13. Liu W, Caffrey M (2005) Gramicidin structure and disposition in highly curved membranes. *J Struct Biol* 150:23–40.

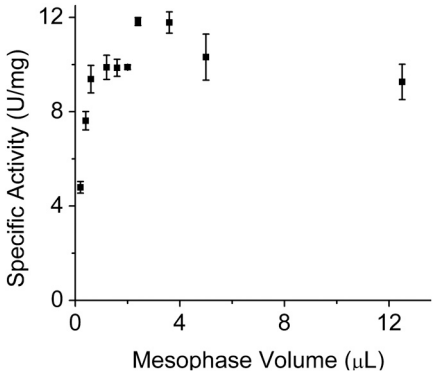


Fig. S1. Kinase activity of DgkA reconstituted into the cubic mesophase of monoolein and its dependence on mesophase volume. The total amount of DgkA used for all volumes tested is 12 μg. Mesophase was dispensed in boluses of 200 nL. Thus, in the smallest volume tested (200 nL) all of the DgkA was reconstituted into a single 200 nL bolus. For the largest volume tested (12.5 μL), the same amount of DgkA was distributed among sixty-two 200 nL boluses.

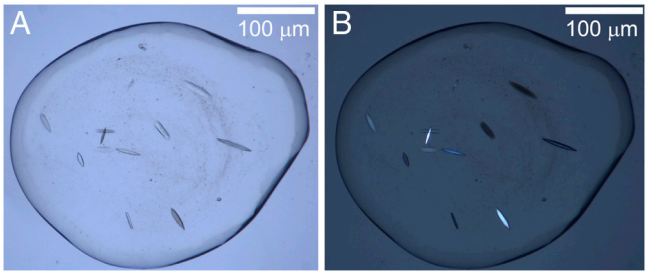


Fig. S2. Crystals of DgkA growing in the lipidic mesophase formed by monoolein viewed with normal (*A*) and polarized light (*B*). Trials were conducted as described (6, 11) using 12 mg DgkA/mL. The precipitant included sodium chloride, potassium nitrate and sodium citrate pH 5.6.

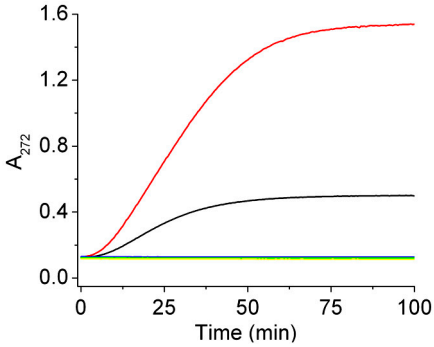


Fig. S3. Progress of the PgsA reaction *in meso* monitored by the increase in absorbance at 272 nm due to release of the product, CMP, from the mesophase into the bathing aqueous solution. The cubic phase was prepared with 0 (green), 0.08 (black), or 0.34 mol% CDP-DAG (red) in monoolein. The assay was performed as detailed in Section 6. Control measurements performed in the absence of PgsA (yellow) or glycerol 3-phosphate (blue) show no measurable activity as observed with the enzyme-free control.

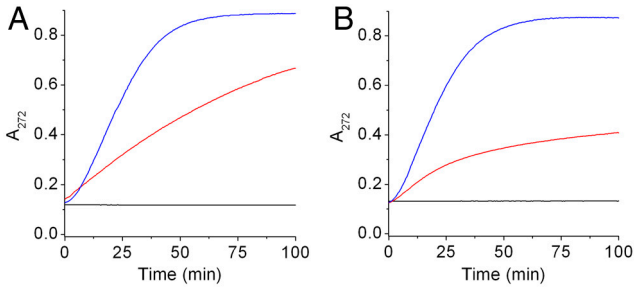


Fig. S4. Dependence of PgsA enzymatic activity *in meso* on substrate, glycerol 3-phosphate (*A*), and activator, $MgCl_2$, concentration (*B*). The concentrations of glycerol 3-phosphate used in (*A*) were 0 mM (black), 1 mM (red), and 16 mM (blue). The concentrations of $MgCl_2$ used in *B* were 0 mM (black), 3 mM (red), and 100 mM (blue). Progress curves were recorded as described in the legend to Fig. S3 at a CDP-DAG concentration of 0.17 mol% in monoolein.

Table S1. DgkA kinase activity *in meso* using different lipids to form the hosting cubic mesophase and to act as subtrate

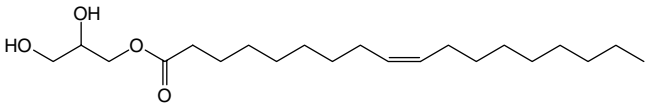
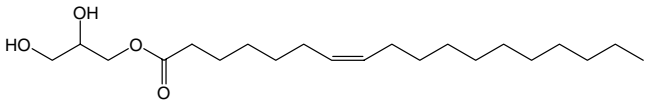
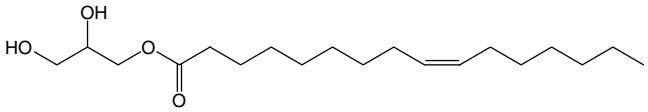
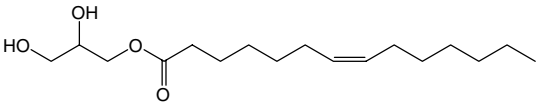
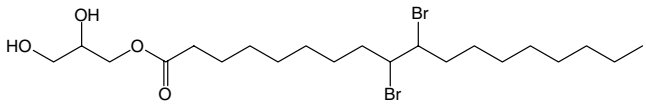
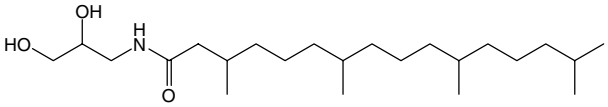
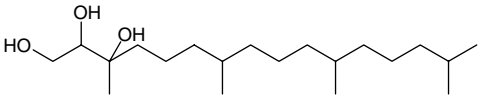
Lipid	Chemical Structure	Specific Activity (U/mg)
9.9 MAG Monoolein		16.4
7.11 MAG		16.1
9.7 MAG		14.2
7.7 MAG		9.8
Bromo-MAG		4.4
PAL		8.6
Phytantriol		0.7

Table S2. Kinase activity of DgkA and its stable mutant, CLLD, under *in meso* and *in surfo* conditions

Condition	Kinase activity (Units/mg)*	
	Wild Type	CLLD
In surfo, Assay Mix 1	109.0 ± 1.6	127.6 ± 2.4
In meso, Assay Mix 2[†]	16.5 ± 1.2 (15%)	16.8 ± 1.3 (13%)

*Assays for wild type and CLLD kinases were performed in parallel as described under *Methods*. Standard deviations represent the average of three separate reconstitutions each assayed in triplicate.

[†]The percentages refer to the rate recorded *in meso* compared to that recorded *in surfo*.