Articles

Effects of Bilayer Thickness on the Activity of Diacylglycerol Kinase of *Escherichia coli*†

Jeffrey D. Pilot, J. Malcolm East, and Anthony G. Lee*

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton SO16 7PX, U.K.

Received February 15, 2001; Revised Manuscript Received May 14, 2001

ABSTRACT: We have developed a procedure for the reconstitution of *Escherichia coli* diacylglycerol kinase (DGK) into phospholipid bilayers containing diacylglycerol substrate. When DGK is reconstituted into a series of phosphatidylcholines containing monounsaturated fatty acyl chains, activity against dihexanoylglycerol (DHG) as a substrate was found to be markedly dependent on the fatty acyl chain length with the highest activity in dioleoylphosphatidylcholine [di(C18:1)PC] and a lower activity in bilayers with shorter or longer fatty acyl chains. Low activities in the short chain phospholipid dimyristoleoylphosphatidylcholine [di(C14:1)PC] followed from an increase in the K_m value for DHG and ATP, with no effect on v_{max}. In contrast, in the long chain lipid dierucoylphosphatidylcholine [di(C24:1)PC], the low activity followed from a decrease in v_{max} with no effect on K_m. In mixtures of two phosphatidylcholines with different chain lengths, the activity corresponded to that expected for the average chain length of the mixture. Cholesterol increased the activity in di(C14:1)PC but slightly decreased it in di(C18:1)PC or di(C24:1)PC, effects that could follow from changes in bilayer thickness caused by cholesterol.

The lipid component of the biological membrane provides more than a simple permeability barrier and support for membrane proteins. These additional roles often require the presence of lipids with particular structures and physical properties, helping to explain the many different chemically distinct species of lipid found in an average membrane. In part, the complexity of the lipid composition follows from the wide variety of fatty acyl chains contained in the lipids (*1*). The length of the fatty acyl chains is a major determinant of the thickness of the hydrophobic core of the membrane. In turn, the thickness of the hydrophobic core can be expected to match the thickness of the transmembrane domains of the intrinsic membrane proteins because the cost of exposing either the fatty acyl chains or hydrophobic amino acids to water is high (*2*).

The variety of lipid fatty acyl chain lengths can lead to a nonhomogeneous distribution of lipids within a single membrane. For example, the sphingolipids in the plasma membranes of mammalian cells are enriched in long chain (C24) fatty acids that have a high gel to liquid crystalline phase transition temperature (*3*). Thus, in mixtures of sphingolipids and glycerophospholipids, the sphingolipids tend to separate out as solid domains of the gel phase lipid (*4*). Association of sphingolipids with cholesterol can lead to the formation of lipid *rafts* that have been suggested to carry membrane proteins to their appropriate destinations within a cell (*4*).

Correct matching of the hydrophobic region of a membrane protein to the hydrophobic core of the lipid bilayer is likely to be important for membrane protein function. Most membrane proteins span the membrane in the form of one or more hydrophobic α-helices. Studies with model peptides have suggested that the extent to which lipid fatty acyl chains can stretch or compress to match the hydrophobic length of the peptide is rather limited (*5*). This means that short hydrophobic α-helices are excluded from thick lipid bilayers but that long hydrophobic α-helices can incorporate into thin bilayers by simply tilting to decrease their effective length across the bilayer (*5*−*8*). This tilting could result in a change in activity for the protein, particularly if the transmembrane region of the protein is important for function. For example, the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum contains 10 transmembrane α-helices tilted ∼10° with respect to the bilayer normal (*9*), and bilayer thickness has been shown to be important for the function of the ATPase (*10*). ATPase activity is highest in bilayers of dioleoylphosphatidylcholine [di(C18:1)PC],[1] with lower activities in bilayers of shorter or longer chain phospholipids (*10*−*13*). Although

† We thank the MRC for a studentship (to J.D.P.).

* To whom correspondence should be addressed. Phone: 44 (0) 2380 594331. Fax: 44 (0) 2380 594459. E-mail: agl@soton.ac.uk.

[1] Abbreviations: DGK, diacylglycerol kinase; di(C14:1)PC, dimyristoleoylphosphatidylcholine; di(C16:0)PC, dipalmitoylphosphatidylcholine; di(C16:1)PC, dipalmitoleoylphosphatidylcholine; di(C18:1)PC, dioleoylphosphatidylcholine; di(C20:1)PC, dieicosenoylphosphatidylcholine; di(C22:1)PC, dierucoylphosphatidylcholine; di(C24:1)PC, dinervonylphosphatidylcholine; di(C18:1)PA, dioleoylphosphatidic acid; di(C18:1)PS, dioleoylphosphatidylserine; di(C18:1)PE, dioleoylphosphatidylethanolamine; DM, *n*-decyl *β*-D-maltopyranoside; OG, octyl *β*-D-glucopyranoside; DOG, 1,2-dioleoylglycerol; DHG, 1,2-dihexanoylglycerol.

10.1021/bi0103258 CCC: $20.00
Published on Web 06/20/2001

changing the bilayer thickness leads to changes in the state of aggregation of the Ca^{2+}-ATPase (*14*), this has been shown not to be the explanation for the effects on activity (*15*). Rather, it is thought that changes in helix packing lead to the observed changes in the rates of phosphorylation and dephosphorylation, in the stoichiometry of Ca^{2+} binding, and in the equilibrium between the two major conformation states of the ATPase, E1 and E2 (*10*).

A disadvantage of the Ca^{2+}-ATPase for studies of lipid−protein interactions is that the mechanism of the ATPase is relatively complex, involving at least one phosphorylated intermediate, with many reaction steps contributing to the observed overall rate (*16*). We have therefore decided to study a kinetically simple intrinsic membrane protein, diacylglycerol kinase (DGK) of *Escherichia coli.* DGK carries out the reaction

$$\text{diacylglycerol} + \text{ATP} \rightleftharpoons \text{phosphatidic acid} + \text{ADP}$$

With a molecular mass of just 13 kDa, DGK is the smallest known kinase. DGK of *E. coli* has been cloned and overexpressed as a poly(His)-tagged protein (*17*). Sanders et al. (*18*) have shown that the activity of the poly(His)-tagged enzyme is very similar to that of the native enzyme and thus that the poly(His) tag does not need to be cleaved from the enzyme in studies of enzyme structure or function; the poly(His) tag has been shown to be mobile in NMR spectroscopy, suggesting that it does not interact with the rest of the protein (*19*).

DGK is predicted to contain three transmembrane α-helices with two amphipathic helices on the cytoplasmic side of the membrane (*20*). It is trimeric (*19*) with three active sites per trimer, the active sites being at the subunit−subunit interfaces with residues from different subunits making up the active site (*21*). The kinetics of DGK are consistent with the formation of a MgATP−DAG−enzyme ternary complex, suggesting a direct phosphoryl transfer mechanism for DGK, rather than a mechanism involving the formation of a phosphorylated enzyme intermediate (*22*). Activity shows Michaelis−Menten kinetics with respect to both MgATP and diacylglycerol (*23*), and ATP and diacylglycerol show random order binding, either substrate being able to bind in the absence of the other (*22*).

The activity of DGK has usually been determined in the presence of detergent micelles, necessary for solubilizing the hydrophobic diacylglycerol substrate (*17*, *23*, *24*). The activity of DGK in micelles of a detergent such as octyl β-D-glucopyranoside (OG) in the absence of phospholipid is very low when the substrate is a short chain diacylglycerol such as dioctanoylglycerol (*24*). Addition of phospholipids leads to increased activity, cardiolipin being the most efficient phospholipid in restoring activity (*24*). Higher activities are seen with dioleoylglycerol (DOG) as the substrate, but the situation is complex since long chain diacylglycerols can act as both the substrate and activating lipid (*24*). Walsh et al. (*25*) have shown that, in the presence of activating phospholipids, the lower activities observed for short chain diacylglycerols follow from higher K_m values for the diacylglycerol with v_{max} values being independent of chain length.

In this paper, we establish a reconstitution protocol for DGK, allowing us to measure enzyme activity in a lipid bilayer. We show that, in the bilayer system, the activity of DGK is sensitive to bilayer thickness.

MATERIALS AND METHODS

Materials and General Procedures. Dimyristoleoylphosphatidylcholine [di(C14:1)PC], dipalmitoleoylphosphatidylcholine [di(C16:1)PC], dioleoylphosphatidylcholine [di(C18:1)PC], dieicosenoylphosphatidylcholine [di(C20:1)PC], dierucoylphosphatidylcholine [di(C22:1)PC], dinervonylphosphatidylcholine [di(C24:1)PC], dioleoylphosphatidic acid [di(C18:1)PA], dioleoylphosphatidylserine [di(C18:1)PS], dioleoylphosphatidylethanolamine [di(C18:1)PE], and cardiolipin were obtained from Avanti Polar Lipids. 1,2-Dioleoylglycerol (DOG) and 1,2-dihexanoylglycerol (DHG) were obtained from Lipid Products and Sigma, respectively. Potassium cholate was purified by dissolving equimolar quantities of cholic acid and potassium hydroxide in methanol, followed by precipitation with excess diethyl ether.

A plasmid expressing His-tagged DGK was generously provided by J. Bowie of the University of California (Los Angeles, CA). The plasmid was expressed in *E. coli* strain WH1061 in which the chromosomal DGK gene was disrupted (*17*). DGK was purified as described by Lau and Bowie (*17*). In brief, cells were treated with 3% (w/v) OG, and the solubilized material was adsorbed onto Ni−NTA−agarose resin (Qiagen). Following washing and packing into a column, the detergent was changed to 0.5% *n*-decyl β-D-maltopyranoside (DM) and the protein was eluted with buffer [50 mM sodium phosphate and 0.3 M NaCl (pH 7.5)] containing 0.5% DM and 0.25 M imidazole. The eluted protein gave a single band on SDS−polyacrylamide gel electrophoresis and was flash-frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were estimated either using an extinction coefficient of 25 200 M^{-1} cm^{-1} at 280 nm or using a bicinchoninic acid protein assay.

Reconstitution of DGK. Purified DGK was reconstituted into lipid bilayers by mixing lipid and DGK in cholate followed by dilution to decrease the concentration of cholate below its critical micelle concentration, using a protocol established previously for the Ca^{2+}-ATPase (*27*). Phospholipid (8 μmol) and the required concentration of diacylglycerol (usually 2 μmol of DHG) were dried from a chloroform solution onto the walls of a thin glass vial. Buffer [400 μL; 60 mM Pipes (pH 6.9)] containing 28 mM cholate was added, and the sample was sonicated to clarity in a bath sonicator (Ultrawave). DGK (22 μg) was then added and the suspension left at room temperature for 15 min, followed by incubation on ice until use. Twenty microliters of the sample was then diluted into 1 mL of the assay buffer described below, representing a 50-fold dilution.

Gradient centrifugation was used to characterize the reconstituted preparation.

Di(C18:1)PC (20 μmol) was mixed with [^{3}H]dipalmitoylphosphatidylcholine (0.2 nmol) in chloroform and dried onto the walls of a glass vial. The mixture was resuspended in 0.8 mL of buffer [10 mM Pipes (pH 7.1) and 100 mM K_2SO_4] containing 28 mM cholate. The sample was sonicated to clarity in a sonication bath (Ultrawave). DGK (150 μg) was added and the mixture left at room temperature for 15 min. The sample was then dialyzed at 4 °C against two lots of 500 mL of buffer [10 mM Pipes (pH 7.1) and 100 mM

Table 1: DGK Activities in Micelles of OG

phospholipid	activity against DOG [μmol min^{-1} (mg of protein)$^{-1}$][a]	activity against DHG [μmol min^{-1} (mg of protein)$^{-1}$][a]
di(C18:1)PC	63.3	1.5
di(C14:0)PC	58.3	–
di(C22:1)PC	66.2	–
di(C18:1)PE	65.1	1.8
di(C18:1)PS	67.1	1.2
di(C18:1)PA	70.3	6.8
cardiolipin	72.6	24.2
none added	65.3	1.0

[a] Activities were measured in 1.5% (w/v) OG at 25 °C and pH 6.9 with 5 mM ATP and 15 mM Mg^{2+}. Concentrations of phospholipid and DAG were 2.4 and 5.1 mol %, respectively, expressed as the mol fraction of the mixture of OG, phospholipid, and DAG.

K_2SO_4] for a total of 5 h. Samples of dialysate (0.4 mL) were then loaded onto sucrose gradients containing the following solutions of sucrose (w/w) in 10 mM Pipes (pH 7.1) and 100 mM K_2SO_4: 2.5, 5.0, 10.0, 15.0, 20.0, and 30.0%; the 30% sucrose solution also contained 0.05% (w/v) Triton X-100. Samples were spun at 80000*g* for 18 h at 4 °C, and then 1 mL fractions were collected from the gradients and analyzed for lipid and protein by liquid scintillation counting and protein assay (modified Lowry assay from Sigma), respectively.

Assay of DGK Activity. DGK activity was measured using a coupled enzyme assay based on that developed for studies of the Ca^{2+}-ATPase (*26*). The production of ADP by the action of DGK on ATP was coupled to the oxidation of NADH through pyruvate kinase and lactate dehydrogenase. The assay medium consisted of buffer [60 mM Pipes (pH 6.9)] containing phosphoenolpyruvate (2 mM), NADH (0.2 mM), ATP (5 mM), Mg^{2+} (20 mM), pyruvate kinase (18 units), and lactate dehydrogenase (22 units). The mixture was incubated at 25 °C for 10 min to ensure that any residual ADP in the ATP sample was consumed. For studies in which DGK activity was measured in micelles of OG, the assay medium also contained OG (1.2%, w/v) and the required concentrations of diacylglycerol and phospholipid. The assay was initiated by addition of DGK (4 μg) to 1 mL of the assay medium. For studies of DGK reconstituted into phospholipid bilayers, diacylglycerol at the required concentration was included with the phospholipid at the reconstitution stage as described above, and the reaction was initiated by addition of DGK (1.5 μg) dissolved in cholate with phospholipid and DHG to 1 mL of the assay medium. The oxidation of NADH was monitored by the decrease in absorbance at 340 nm.

RESULTS

*Assay in Detergent Micelles.*The activity of DGK against DHG in OG micelles is very low in the absence of added phospholipid (Table 1). Addition of di(C18:1)PC, di(C18:1)PE, or di(C18:1)PS had little effect on activity; however, addition of di(C18:1)PA led to a small increase in activity, and addition of cardiolipin led to a large increase in activity. In contrast, higher activities are seen with DOG as substrate, but addition of phospholipids had little effect on the measured activity (Table 1). This is consistent with published reports that long chain diacylglycerols can act both as a substrate and as an activating lipid (*24*).

Table 2: Effects of Dilution on DGK Activity[a]

dilution factor	final concentration (μM)		activity [μmol min^{-1} (mg of protein)$^{-1}$]
	di(C18:1)PC	DHG	
20	900	225	58.5
33	540	135	59.3
50	360	90	59.8
200	90	22	60.3

[a] DGK (22 μg) was incubated with di(C18:1)PC (8 μmol) and DHG (2 μmol) in cholate, then the mixture diluted by the given factor into 1 mL of assay medium, and the activity determined.

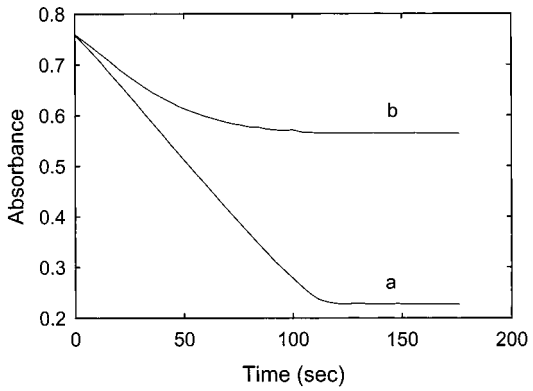

FIGURE 1: Kinetics of ATP hydrolysis for reconstituted DGK. ATP hydrolysis was coupled to the oxidation of NADH and followed by the decrease in absorbance at 340 nm. DGK was reconstituted at a di(C18:1)PC:DGK molar ratio of 4000:1 into bilayers of di(C18:1)PC containing a mole fraction of DHG of 20 (a) or 7.5% (b). The concentration of DGK was 5 μg/mL.

Reconstitution of DGK into Lipid Bilayers. The first step in the reconstitution of DGK involved mixing DGK in 0.5% DM with a phospholipid/DHG mixture, normally containing 20 mol % DHG, solubilized in 28 mM cholate to give a molar ratio of total lipid (phospholipid and DHG) to DGK of 6000:1. This ratio was chosen to ensure the appropriate DHG:DGK ratio, as described below. The mixture was incubated for 15 min at room temperature, and was stored on ice until use. The reconstitution mixture was diluted 50-fold into buffer, decreasing the concentration of cholate below its critical micelle concentration [10 mM (*28*)], reforming membrane fragments. Dilution factors between 20- and 200-fold were found to be equally effective (Table 2).

An important requirement for the success of the reconstitution procedure is that DGK has to be reconstituted into a bilayer already containing the diacylglycerol substrate. Further, the amount of diacylglycerol in the bilayer has to be sufficient for steady state kinetics to be measurable over a time period of several minutes. Figure 1 shows data for ATP hydrolysis recorded as a function of time for DGK reconstituted into bilayers of di(C18:1)PC containing DHG at 20 and 7.5 mol % DHG, at a di(C18:1)PC:DGK molar ratio of 4000:1. For bilayers containing 20 mol % DHG, linear kinetics are observed for 100 s (curve a), whereas for bilayers containing 7.5 mol % DHG, kinetics are linear for only ~30 s.

The value for v_{max} with DHG as a substrate in bilayers of di(C18:1)PC is ~75 IU/mg, as described below (see Figure 3). This is comparable to the activity with DOG as a substrate observed in OG micelles (Table 1). Walsh et al. (*25*) have shown that changing the chain length of the diacylglycerol substrate affects only K_m and not v_{max}. The similar v_{max} values

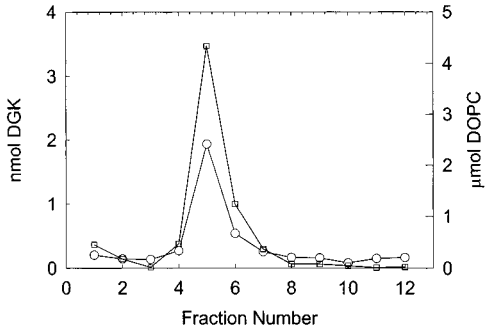

FIGURE 2: Sucrose density gradient analysis of the reconstituted DGK. A sample of DGK reconstituted with di(C18:1)PC at a lipid:protein molar ratio of 1750:1 was separated on a discontinuous sucrose gradient from 30 to 2.5% sucrose. Fractions (1 mL) were taken and analyzed for lipid (□; right-hand axis) and DGK (○; left-hand axis).

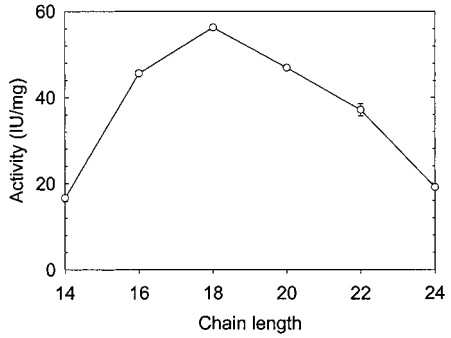

FIGURE 3: Effect of phosphatidylcholine fatty acyl chain length on DGK activity. DGK was reconstituted with phosphatidylcholines with two monounsaturated fatty acyl chains of the given length, all being in the liquid crystalline phase. Activities were measured at 25 °C with 5 mM MgATP and 20 mol % DHG.

for DHG in reconstituted bilayers and for DOG in micelles therefore confirm that the cholate dilution procedure forms unsealed membrane fragments, as observed when the Ca^{2+}-ATPase was reconstituted in this way (*27*). If sealed vesicles had been formed with a random distribution of DGK across the bilayer, a maximum activity of 50% of v_{max} would have been observed because lipid bilayers are impermeable to ATP. DGK was also reconstituted using OG as a detergent instead of cholate. In this case, activities observed following dilution into buffer were half those observed in detergent micelles, suggesting the formation of sealed vesicles (data not shown).

The observed maximum decrease in NADH adsorption agreed closely with the expected decrease if all the diacylglycerol in the membrane had been converted to phosphatidic acid. For example, total conversion of the 20 mol % DHG in Figure 1 would lead to the production of 98 nmol of ADP and so to a decrease in NADH absorption of 0.61, in good agreement with the measured value of 0.53. This shows that DGK mixes properly with diacylglycerol and phospholipid on reconstitution; a mixed system in which significant amounts of membrane lack DGK does not form.

The dilution experiments described in detail in Table 2 confirm that the majority of the added DHG partitions into the phospholipid bilayer. The partition coefficient K_p for DHG between the lipid bilayer and the aqueous phase is defined as

$$K_p = [\mathrm{DHG}]_L/[\mathrm{DHG}]_w \quad (1)$$

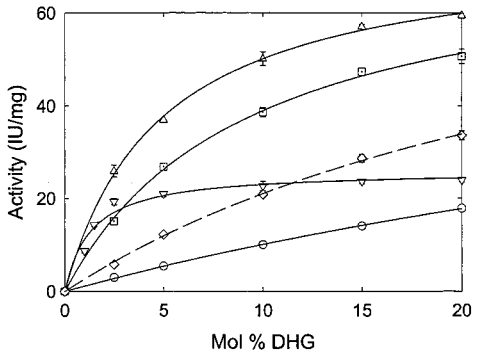

FIGURE 4: Effects of phosphatidylcholine fatty acyl chain length on DGK activity as a function of DHG concentration. DGK was reconstituted with phosphatidylcholines of the given chain length, at a constant total lipid:DGK molar ratio of 6000:1 and the given mole fraction of DHG. Activities were measured at 25 °C with 5 mM MgATP. Phospholipids were as follows: (○) di(C14:1)PC, (□) di(C16:1)PC, (△) di(C18:1)PC, and (▽) di(C24:1)PC. The solid lines show fits to the Michaelis−Menten equation with the values for K_m and v_{max} plotted in Figure 5A. Also shown is the activity for DGK reconstituted into a di(C14:1)PC:cholesterol molar ratio of 1:0.5 (◇).

where $[\mathrm{DHG}]_L$ and $[\mathrm{DHG}]_w$ are the concentrations of DHG in the phospholipid bilayer and aqueous phase, respectively, in units of moles per liter. This can be written more conveniently as

$$K_p = \frac{(n_{DHG})_L}{(n_{DHG})_w \beta [L]} \quad (2)$$

where $(n_{DHG})_L$ and $(n_{DHG})_w$ are the numbers of moles of DHG in the phospholipid and aqueous phases, respectively, [L] is the concentration of phospholipid (moles per liter of the aqueous medium), and β is the volume of a mole of lipid in liters per mole. The fraction of added DHG that is bound to the phospholipid bilayer is thus given by

$$\text{fraction bound} = \frac{(n_{DHG})_L}{(n_{DHG})_L + (n_{DHG})_w} = \frac{1}{1 + 1/K\beta[L]} \quad (3)$$

If $K\beta[L] \gg 1$, then essentially all the added DHG will be bound to the bilayer. However, for smaller values of $K\beta[L]$, the added DHG will be only partly bound, the fraction bound decreasing with decreasing concentrations of phospholipid. For example, if 50% of the added DHG is bound at one particular phospholipid concentration, at a 10-fold lower concentration of phospholipid the fraction of DHG that is bound will be only 5%. As described later, DGK activity is dependent on the concentration of DHG (see Figure 4), and a large change in the bound concentration of DHG would result in a significant change in measured activity. As shown in Table 2, a 10-fold change in the concentration of phospholipid at a fixed molar ratio of DHG to phospholipid had no effect on the measured activity of DGK, consistent with all the added DHG being membrane-bound.

The state of the added DHG was also investigated by measuring DGK activity after DGK reconstituted with di(C18:1)PC was mixed with di(C18:1)PC reconstituted with 20 mol % DHG. Di(C18:1)PC (720 μM) containing 20 mol % DHG was mixed with di(C18:1)PC (360 μM) containing DGK at a phospholipid:protein molar ratio of 6000:1, giving a mixture with an overall DHG content of 14.3 mol %. The

Table 3: Effects of Reconstitution Are Reversible[a]

lipid	time for reconstitution	activity in phospholipid against DHG [μmol min^{-1} (mg of protein)$^{-1}$]	activity in OG micelles against DOG [μmol min^{-1} (mg of protein)$^{-1}$]
di(C14:1)PC	5 min	21.1	53
	3 h	20.6	52.4
di(C18:1)PC	5 min	63.3	56.7
	1 h	63.0	58.1

[a] DGK was incubated in cholate with a 20 mol % mixture of DHG and the given phospholipid for the given period of time. A sample was then reconstituted by dilution and the activity determined. A second sample was diluted into an assay cuvette containing OG and DOG and the activity determined.

final DGK activity of the mixture was 28 μmol min^{-1} (mg of protein)$^{-1}$, compared to an activity of 57 μmol min^{-1} (mg of protein)$^{-1}$ when DGK was reconstituted into di(C18:1)-PC containing 15 mol % DHG. These experiments suggest that although DHG is able to partition from the initial protein-free membranes into the membranes containing DGK, the rate of partitioning is slow enough to lead to a reduced rate of reaction.

The reconstituted DGK was also characterized by centrifugation on a discontinuous sucrose gradient (Figure 2). When DGK and lipid were run separately on the gradient, all the DGK was found at the bottom of the gradient and all the lipid at the top. In contrast, when DGK was mixed with di(C18:1)PC in cholate followed by dialysis to remove the detergent, DGK was found at the 10% sucrose interface, together with the lipid (Figure 2). The lipid:protein molar ratio for the DGK-containing fraction was 2250:1, compared to 1750:1 in the original mixture. This molar ratio gives an average density for the sample of 1.033, corresponding to a density between that of a 6% sucrose solution (1.0219) and a 10% sucrose solution (1.0381), agreeing with the location of the sample at the interface between the 6 and 10% sucrose solutions (Figure 2). The experiment shows that reconstitution has resulted in a homogeneous sample.

Effects of Phospholipids on DGK Activity Are Reversible. Reconstitution of DGK into bilayers of di(C14:1)PC gave a lower activity against DHG than for DGK reconstituted into bilayers of di(C18:1)PC (Table 3). To demonstrate that the effect of di(C14:1)PC was fully reversible, a sample of the reconstituted DGK was added to micelles of OG containing DOG, conditions under which DOG acts as the activating lipid so giving full activity (Table 1). As shown in Table 3, when added to micelles of OG, DGK reconstituted into di-(C14:1)PC or into di(C18:1)PC gave the same activity, equal to that for the un-reconstituted enzyme. We conclude that effects of reconstitution are reversible.

Reconstitution using the cholate procedure is complete within 5 min. If DGK is mixed with di(C14:1)PC in cholate and samples are taken after 5 min or 3 h, the same activity is recorded (Table 3). DGK is stable in bilayers of di(C14:1)PC for at least 3 h (data not shown).

Effect of Phospholipid Chain Length on DGK Activity. DGK was reconstituted into bilayers of phosphatidylcholines containing monounsaturated fatty acyl chains of lengths between 14 and 24 carbons. Phase transition temperatures for all these lipids are <25 °C (*29*) so that all the lipids are in the liquid crystalline phase at the assay temperature. DGK

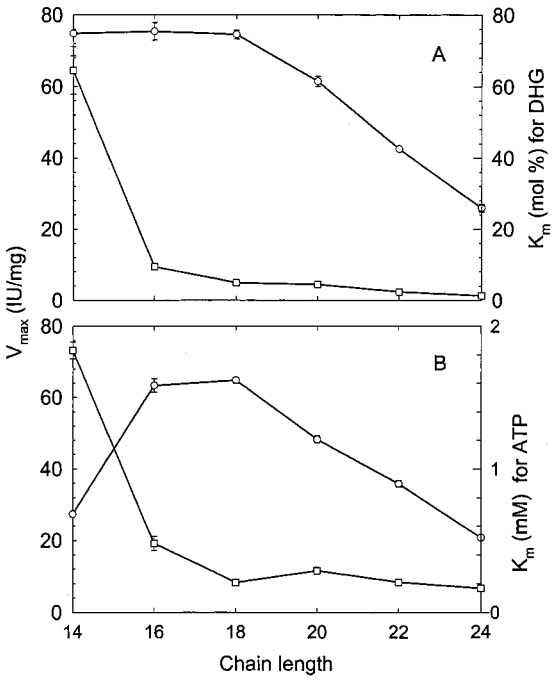

FIGURE 5: Effects of phosphatidylcholine chain length on K_m and v_{max} values for DHG (A) and ATP (B). K_m (□) and v_{max} (○) values obtained from fits of rates vs the mole fraction of DHG at a fixed ATP concentration of 5 mM (A) or from fits of rates vs ATP concentration at a fixed DHG concentration of 20 mol % (B) are plotted vs chain length.

activity was assayed at 5 mM MgATP and 20 mol % DHG. The highest activity was observed in di(C18:1)PC with lower activities for phosphatidylcholines with shorter or longer chains (Figure 3). Activities were measured as a function of DHG concentration, at a MgATP concentration of 5 mM (Figure 4). The data fit well to a simple Michaelis−Menten scheme with the K_m and v_{max} values plotted in Figure 5A. As shown, in di(C14:1)PC the K_m value for DHG is high but the value for v_{max} is the same as in di(C18:1)PC, whereas in long chain phosphatidylcholines, K_m values for DHG are comparable to those in di(C18:1)PC but values for v_{max} are low. Activities as a function of ATP concentration at a fixed DHG concentration of 20 mol % also fit well to Michaelis−Menten kinetics (Figure 6) with the K_m and v_{max} values plotted in Figure 5B. The K_m value for ATP is higher in di(C14:1)PC than in di(C18:1)PC but is little changed on increasing the phosphatidylcholine chain length from 16 to 24 carbons. The low value for v_{max} seen in di(C14:1)PC in Figure 5B follows because activity was assayed at 20 mol % DHG and the K_m value for DHG is high in di(C14:1)PC (Figure 5A). Thus, the inverted-U shape profile for activity as a function of chain length shown in Figure 3 for activity measured with 20 mol % DHG and 5 mM ATP follows largely from a high K_m value for DHG in di(C14:1)PC and a low value for v_{max} in long chain phosphatidylcholines.

Effects of mixtures of phosphatidylcholines are shown in Figure 7. Addition of increasing proportions of di(C14:1)-PC or di(C24:1)PC to DGK reconstituted in di(C18:1)PC leads to a gradual decrease in activity (Figure 7). However, addition of di(C24:1)PC to DGK reconstituted in di(C14:1)PC results in an initial increase in activity so that at a mole fraction of di(C24:1)PC of 0.2 activity is almost equal to that in di(C18:1)PC. Further increases in the mole fraction

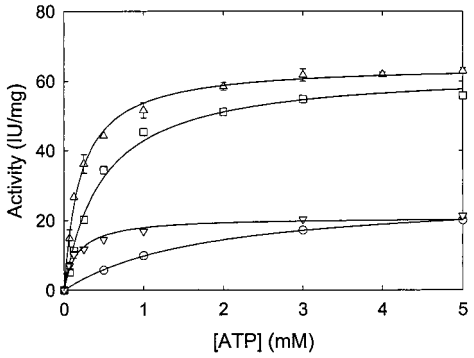

FIGURE 6: Effects of phosphatidylcholine fatty acyl chain length on DGK activity as a function of ATP concentration. DGK was reconstituted with phosphatidylcholines of the given chain length, at a constant total lipid:DGK molar ratio of 6000:1 and 20 mol % DHG. Activities were measured at 25 °C as a function of ATP concentration. Phospholipids were as follows: (○) di(C14:1)PC, (□) di(C16:1)PC, (△) di(C18:1)PC, and (▽) di(C24:1)PC. The solid lines show fits to the Michaelis−Menten equation with the values for K_m and v_{max} plotted in Figure 5B.

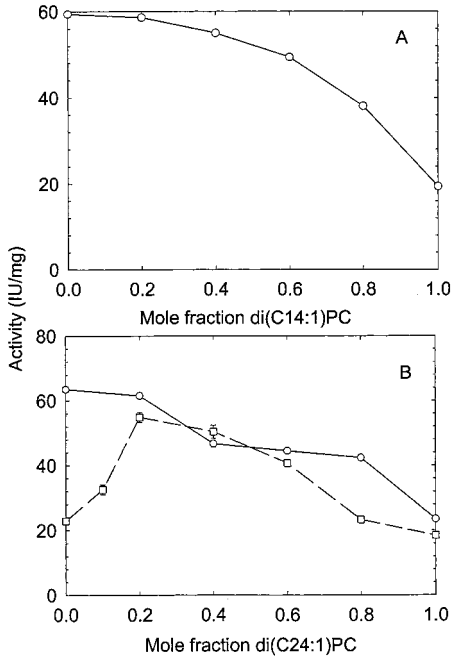

FIGURE 7: Effect of mixtures of phosphatidylcholines on DGK activity. DGK was reconstituted with mixtures of (A) di(C18:1)-PC and di(C14:1)PC and (B) di(C18:1)PC and di(C24:1)PC (○) or di(C14:1)PC and di(C24:1)PC (□). Activities were measured at 25 °C with 5 mM MgATP and 20 mol % DHG.

of di(C24:1)PC up to 0.6 lead to only a small decrease in activity, but activity decreases significantly beyond this (Figure 7). These results suggest that the thickness of the bilayer is an important parameter determining enzyme activity.

Effects of Cholesterol. The presence of cholesterol has marked effects on enzyme activity (Figure 8). In di(C14:1)-PC, addition of cholesterol to a cholesterol:di(C14:1)PC molar ratio of 0.5:1 led to a 2.5-fold increase in activity, with higher concentrations of cholesterol resulting in slightly lower activities. In contrast, addition of cholesterol to DGK in di(C18:1)PC or di(C24:1)PC led to a 40 or 75% decrease in activity, respectively, at a cholesterol:phospholipid molar ratio of 1:1. The effect of cholesterol on DGK in di(C14:1)PC is an effect on the K_m for DHG rather than an effect

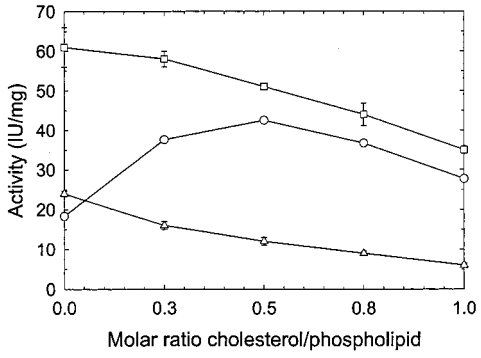

FIGURE 8: Effects of cholesterol on the activity of DGK. DGK was reconstituted into bilayers of phospholipid and cholesterol at the given cholesterol:phospholipid molar ratios. Phospholipids were as follows: (○) di(C14:1)PC, (□) di(C18:1)PC, and (△) di(C24:1)PC. Activities were measured at 25 °C with 5 mM MgATP and 20 mol % DHG.

on v_{max}; at a 0.5:1 cholesterol:di(C14:1)PC molar ratio, a plot of activity against the mol fraction of DHG fits to a K_m of 31.9 ± 3.6 mol % with a v_{max} of 87.8 ± 6.7 IU/mg, compared to values of 64.5 ± 6.7 mol % and 74.9 ± 6.3 IU/mg for K_m and v_{max}, respectively, in the absence of cholesterol (Figure 4).

DISCUSSION

Many physical features of a lipid bilayer have been suggested to be important in determining the activity of a membrane protein embedded within the bilayer, including bilayer fluidity, bilayer thickness, the free volume available within the bilayer, the charge on the bilayer surface, and any bilayer "frustration" arising from the presence within the bilayer of lipids that prefer nonbilayer structures (*30*). In addition to these nonspecific effects, specific phospholipids may bind strongly to a small number of sites on a membrane protein, acting as cofactors, the classic example being provided by cardiolipin, essential for the activity of many proteins important in bioenergetics (*31*).

The simplicity of both the structure and the kinetics of DGK make DGK an ideal protein with which to define the important features of the surrounding lipid bilayer. We have established a protocol by which the DGK can be reconstituted into lipid bilayers containing DHG as a substrate. With 20 mol % DHG in the lipid bilayer, linear steady state kinetics can be recorded for up to 100 s when the DHG:DGK molar ratio is 800:1 (Figure 1). The normal substrate for DGK is a diacylglycerol with fatty acyl chains about 18 carbons in length. These DAGS will be fully membrane bound, and thus, the binding site for diacylglycerol on DGK is likely to be accessible from the lipid bilayer. An important parameter in any kinetic analysis of DHG as a substrate for DGK will therefore be the proportion of the total DHG that is bound to the membrane. If DHG partitions between water and the lipid bilayer with a significant fraction of the DHG remaining in the aqueous phase, then decreasing the lipid concentration in the sample will decrease the proportion of DHG bound to the bilayer, according to eq 3. Varying the concentration of phospholipid was, in fact, found to have no effect on DGK activity against DHG (Table 2) so that, under the conditions of these experiments, the bulk of the DHG must be membrane-bound.

Long chain diacylglycerols have complex effects on the properties of phospholipid bilayers with the formation of high-melting temperature complexes between the phospholipids and the diacylglycerol, with the complex often being immiscible with the uncomplexed phospholipid (*32*). However, 25 mol % DHG has been shown to be miscible with dipalmitoylphosphatidylcholine in the liquid crystalline phase with no evidence for phase separation of the type seen with long chain diacylglycerols (*33*). The presence of 25 mol % DHG had only a small effect on the order parameter profile for the phospholipid fatty acyl chains, and the effect of DHG has been likened to that of benzyl alcohol which binds at the lipid−water interface, hydrogen bonding to the phospholipid phosphate groups with the benzyl group penetrating a small way into the hydrocarbon core of the bilayer (*33*, *34*). NMR studies are consistent with a similar location for the diacylglycerols with the OH group of the diacylglycerol located close to the lipid−water interface (*35*). The effects of a short chain diacylglycerol binding at the lipid−water interface on the physical properties of a phospholipid bilayer would be expected to be independent of the chain length of the phospholipid.

Using the reconstitution protocol, we have shown that bilayer thickness has a marked effect on the activity of DGK with a maximum activity at a chain length of 18 carbons (Figure 3). DGK is predicted to contain three transmembrane α-helices (*20*). Wen et al. (*36*) have suggested that transmembrane α-helix M1 is relatively short, containing just 14 residues, running from Glu-34 to Asp-49; M2 running from Asp-51 to Glu-69 contains 17 residues, and M3, from Met-96 to Trp-117, contains 22 residues. However, if it is assumed that M1 starts at Glu-28 rather than Glu-34, the length of M1 becomes 21 residues. Cross-linking experiments have suggested that M2 is at the trimer interface of DGK (*37*), suggesting that M1 and M3 will be exposed to lipid. If helices M1 and M3 contain 21−22 residues, the length of the helices will be ∼32 Å, assuming an ideal α-helix with a helix translation of 1.5 Å per residue. The hydrophobic thickness of a bilayer of di(C18:1)PC is ∼29.8 Å (*38*, *39*). The best hydrophobic match for DGK will therefore be with a bilayer of di(C18:1)PC, consistent with the observation that the highest activity is observed in a bilayer of this lipid (Figure 3).

The low activity observed in di(C14:1)PC measured at 20 mol % DHG and 5 mM ATP (Figure 3) follows from an increase in the K_m value for DHG (Figure 5); the K_m value for DHG increases from 4.9 ± 0.7 mol % in di(C18:1)PC to 64.5 ± 6.7 mol % in di(C14:1)PC, whereas v_{max} in di(C18:1)PC (74.5 ± 1.2 IU/mg) is the same as in di(C14:1)PC (74.9 ± 6.3 IU/mg). The K_m value for ATP also increases from 0.21 ± 0.01 mM in di(C18:1)PC to 1.83 ± 0.06 mM in di(C14:1)PC. The observation that the low activity in di(C14:1)PC follows from changes in K_m values and not from changes in v_{max} shows that any changes in the dynamic properties of the lipid bilayer (such as changes in fluidity or viscosity) are unimportant since changes in a dynamic property of the system cannot result in changes in an equilibrium property such as a K_m value (*40*). Since DGK catalyses direct phosphoryl transfer from ATP to diacylglycerol, it is likely that ATP binds to DGK with its γ-phosphate close to the OH group of the diacylglycerol (*22*). The binding sites for ATP have been located at the subunit−subunit interfaces in the DGK trimer (*21*), suggesting that the diacylglycerol binding site could also be located at the subunit−subunit interfaces. The observed increases in K_m for both ATP and DHG in di(C14:1)PC therefore suggest that packing at the subunit−subunit interface has changed as the transmembrane α-helices tilt to match the thin bilayer.

Effects of long chain phosphatidylcholines are very different from those of short chain phosphatidylcholines. For phosphatidylcholines with chain lengths of greater than 18 carbons, the observed decreases in activity with increasing chain length do not follow from any changes in K_m for ATP or DHG but, rather, from a decrease in v_{max} (Figure 5). Such a change might occur, for example, if the binding sites for ATP and diacylglycerol on DGK became misaligned so that the rate of phosphoryl transfer is decreased.

Phosphatidylcholines in the liquid crystalline phase show close to ideal mixing even when there is a marked difference in chain length for the component phospholipids (*41*). The lipid environment experienced by DGK reconstituted into a mixture of two phosphatidylcholines with different chain lengths will therefore correspond to an averaged environment, unless DGK shows markedly stronger binding to one of the phosphatidylcholine species. In fact, effects of mixtures of phosphatidylcholines with different chain lengths are consistent with the idea that an important feature of the bilayer is its average thickness. For example, addition of di(C14:1)PC or di(C24:1)PC to a bilayer of di(C18:1)PC will result in a decrease or increase, respectively, in average bilayer thickness, resulting in a decrease in activity, as observed (Figure 5). Particularly striking is the observation that suitable mixtures of di(C14:1)PC and di(C24:1)PC support high activities even though each lipid supports only a low activity on its own (Figure 5). In these mixtures, the average chain length determines the activity. For example, the average chain length for a 0.8:0.2 mixture of di(C14:1)PC and di(C24:1)PC is 16 carbons, a chain length giving close to maximum activity (Figure 3), consistent with the high activity observed in the mixture. At a di(C14:1)PC:di(C24:1)PC molar ratio of 0.4:0.6, the average chain length has become 20 carbons, and in di(C20:1)PC, activities are still close to that seen in di(C18:1)PC (Figure 3). However, further increases in the proportion of di(C24:1)PC lead to average chain lengths corresponding to regions of the chain length dependency curve where low activities are observed (Figure 3), explaining the decrease in activity observed in mixtures containing a mole fraction for di(C24:1)PC of more than 0.6. Similar observations have been made for the Ca^{2+}-ATPase, although for the Ca^{2+}-ATPase activity changes more steeply with changes in lipid composition, suggesting that effects of chain length on the Ca^{2+}-ATPase are more cooperative than those on DGK (*13*).

Addition of cholesterol to DGK in di(C14:1)PC roughly doubles activity, whereas addition of cholesterol to DGK in di(C18:1)PC or di(C24:1)PC causes a slight decrease in activity (Figure 8). It has been estimated from the NMR order parameter data that, in the liquid crystalline phase, the effective length of the fatty acyl chains of di(C14:0)PC and di(C18:0)PC increases by 2.1−2.5 Å on incorporation of 50 mol % cholesterol (*42*). The bilayer hydrophobic thickness d is related to chain length n by (*38*

$$d = 1.75(n - 1)$$

, *39*) The effect of 50 mol % cholesterol is therefore to increase the effective chain length by the equivalent of ~1−1.5 carbons, consistent with the observed increase in activity seen on addition of cholesterol to DGK in di(C14:1)PC to give an activity somewhat lower than that observed in di(C16:1)PC (Figure 3). An increase in effective chain length on addition of cholesterol would be expected to reduce activity in the longer chain phosphatidylcholines, as observed experimentally (Figure 8). Also consistent with the proposal that cholesterol increases the activity of DGK in di(C14:1)PC through an effect on bilayer thickness is the observation that cholesterol decreases the K_m value for DHG in di(C14:1)PC with no significant effect on v_{max} (Figure 4).

Cholesterol and other sterols have also been shown to increase the activity of the Ca^{2+}-ATPase in di(C14:1)PC but with no effect on the activity in di(C24:1)PC (*13*). The activity in di(C14:1)PC at a 1:1 sterol:phospholipid molar ratio was equal to that in the optimum phospholipid, di(C18:1)PC; this is significantly different from the observations made here with DGK, where activities in mixtures of di(C14:1)PC and cholesterol were always lower than those in di(C18:1)PC (Figure 8). This suggests that effects of cholesterol on the Ca^{2+}-ATPase could be more complex than those on DGK, and indeed, it has been suggested that the effects of cholesterol on the Ca^{2+}-ATPase could follow from direct binding to the ATPase (*13*, *43*).

In conclusion, *E. coli* DGK shows its highest activity in di(C18:1)PC, the lipid which most closely matches the expected length of the DGK transmembrane α-helices. These can be compared with the effects of phospholipid chain length on the function of *E. coli* melibose permease where the highest rates of transport were seen in di(C16:1)PC (*44*). Since membrane proteins seem to have little ability to select between lipids on the basis of chain length (*10*), it appears that either DGK or the melibose permease (or both) must be functioning in the *E. coli* membrane at lower than maximal rates.

ACKNOWLEDGMENT

We thank Professor James Bowie for the generous gift of the clone for DGK and Elizabeth Clark for help with some of the experiments.

REFERENCES

1. Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C. (1973) *Form and function of phospholipids*, Elsevier, Amsterdam.
2. Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, John Wiley, New York.
3. Koynova, R., and Caffrey, M. (1995) *Biochim. Biophys. Acta 1255*, 213−236.
4. Simons, K., and Ikonen, E. (1997) *Nature 387*, 569−572.
5. Webb, R. J., East, J. M., Sharma, R. P., and Lee, A. G. (1998) *Biochemistry 37*, 673−679.
6. Mall, S., Broadbridge, R., Sharma, R. P., Lee, A. G., and East, J. M. (2000) *Biochemistry 39*, 2071−2078.
7. Ren, J. H., Lew, S., Wang, J. Y., and London, E. (1999) *Biochemistry 38*, 5905−5912.
8. Ren, J. H., Lew, S., Wang, Z. W., and London, E. (1997) *Biochemistry 36*, 10213−10220.
9. Toyoshima, C., Nakasako, M., Nomura, H., and Ogawa, H. (2000) *Nature 405*, 647−655.
10. Lee, A. G. (1998) *Biochim. Biophys. Acta 1376*, 381−390.
11. Caffrey, M., and Feigenson, G. W. (1981) *Biochemistry 20*, 1949−1961.
12. Johannsson, A., Keightley, C. A., Smith, G. A., Richards, C. D., Hesketh, T. R., and Metcalfe, J. C. (1981) *J. Biol. Chem. 256*, 1643−1650.
13. Starling, A. P., East, J. M., and Lee, A. G. (1993) *Biochemistry 32*, 1593−1600.
14. Cornea, R. L., and Thomas, D. D. (1994) *Biochemistry 33*, 2912−2920.
15. Starling, A. P., East, J. M., and Lee, A. G. (1995) *Biochem. J. 308*, 343−346.
16. de Meis, L. (1981) *The Sarcoplasmic Reticulum*, John Wiley, New York.
17. Lau, F. W., and Bowie, J. U. (1997) *Biochemistry 36*, 5884−5892.
18. Sanders, C. R., Czerski, L., Vinogradova, O., Badola, P., Song, D., and Smith, S. O. (1996) *Biochemistry 35*, 8610−8618.
19. Vinogradova, O., Badola, P., Czerski, L., Sonnichsen, F. D., and Sanders, C. R. (1997) *Biophys. J. 72*, 2688−2701.
20. Smith, R. L., O'Toole, J. F., Maguire, M. E., and Sanders, C. R. (1994) *J. Bacteriol. 176*, 5459−5465.
21. Lau, F. W., Chen, X., and Bowie, J. U. (1999) *Biochemistry 38*, 5521−5527.
22. Badola, P., and Sanders, C. R. (1997) *J. Biol. Chem. 272*, 24176−24182.
23. Walsh, J. P., and Bell, R. M. (1986) *J. Biol. Chem. 261*, 6239−6247.
24. Walsh, J. P., and Bell, R. M. (1986) *J. Biol. Chem. 261*, 15062−15069.
25. Walsh, J. P., Fahrner, L., and Bell, R. M. (1990) *J. Biol. Chem. 265*, 4374−4381.
26. Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 622−626.
27. Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974) *Biochemistry 13*, 5501−5507.
28. Lee, A. G. (2000) in *Membrane Transport* (Baldwin, S. A., Ed.) pp 21−46, Oxford University Press, Oxford, U.K.
29. Koynova, R., and Caffrey, M. (1998) *Biochim. Biophys. Acta 1376*, 91−145.
30. Brown, M. F. (1997) *Curr. Top. Membr. 44*, 285−356.
31. McAuley, K. E., Fyfe, P. K., Ridge, J. P., Isaacs, N. W., Cogdell, R. J., and Jones, M. R. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 14706−14711.
32. Boden, N., Jones, S. A., and Sixl, F. (1991) *Biochemistry 30*, 2146−2155.
33. De Boeck, H., and Zidovetzki, R. (1992) *Biochemistry 31*, 623−630.
34. Hamilton, J. A., Bhamidipati, S. P., Kodali, D. R., and Small, D. M. (1991) *J. Biol. Chem. 266*, 1177−1186.
35. Heimburg, T., Wurz, U., and Marsh, D. (1992) *Biophys. J. 63*, 1369−1370.
36. Wen, J. A., Chen, X., and Bowie, J. U. (1996) *Nat. Struct. Biol. 3*, 141−148.
37. Nagy, J. K., Lau, F. W., Bowie, J. U., and Sanders, C. R. (2000) *Biochemistry 39*, 4154−4164.
38. Lewis, B. A., and Engelman, D. M. (1983) *J. Mol. Biol. 166*, 211−217.
39. Sperotto, M. M., and Mouritsen, O. G. (1988) *Eur. Biophys. J. 16*, 1−10.
40. Lee, A. G., and East, J. M. (1993) in *Protein−Lipid Interactions* (Watts, A., Ed.) pp 259−299, Elsevier, Amsterdam.
41. Lee, A. G. (1978) *Biochim. Biophys. Acta 507*, 433−444.
42. Sankaram, M. B., and Thompson, T. E. (1990) *Biochemistry 29*, 10676−10684.
43. Ding, J., Starling, A. P., East, J. M., and Lee, A. G. (1994) *Biochemistry 33*, 4974−4979.
44. Dumas, F., Tocanne, J. F., Leblanc, G., and Lebrun, M. C. (2000) *Biochemistry 39*, 4846−4854.

BI0103258