

Cell-free expression and *in meso* crystallisation of an integral membrane kinase for structure determination

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Abstract Membrane proteins are key elements in cell physiology and drug targeting, but getting a high-resolution structure by crystallographic means is still enormously challenging. Novel strategies are in big demand to facilitate the structure determination process that will ultimately hasten the day when sequence information alone can provide a three-dimensional model. Cell-free or *in vitro* expression enables rapid access to large quantities of high-quality membrane proteins suitable for an array of applications. Despite its impressive efficiency, to date only two membrane proteins produced by the *in vitro* approach have yielded crystal structures. Here, we have analysed synergies of cell-free expression and crystallisation in lipid mesophases for generating an X-ray structure of the integral membrane enzyme diacylglycerol kinase to 2.28-Å resolution. The quality of cellular and cell-free-expressed kinase samples has been evaluated systematically by comparing (1) spectroscopic properties, (2) purity and oligomer formation, (3) lipid content and (4) functionality. DgkA is the first membrane enzyme crystallised based on cell-free

expression. The study provides a basic standard for the crystallisation of cell-free-expressed membrane proteins and the methods detailed here should prove generally useful and contribute to accelerating the pace at which membrane protein structures are solved.

Keywords Cell-free · Crystallisation · *In meso* · Lipid cubic phase · Macromolecular crystallography · Membrane protein structure

Abbreviations

ADP	Adenosine diphosphate
ARII	<i>Acetabularia acetabulum</i> rhodopsin II
ATP	Adenosine triphosphate
C ₁₂ E ₈	<i>n</i> -octaethylene glycol monododecyl ether
C ₈ E ₄	<i>n</i> -octyl tetraethylene glycol monoether
CL	Cardiolipin
CTP	Cytidine triphosphate
CV	Column volume
DgkA	Diacylglycerol kinase
DDM	<i>n</i> -dodecyl-β-D-maltopyranoside
DM	<i>n</i> -decyl-β-D-maltopyranoside
DNA	Deoxyribonucleic acid
DTT	DL-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
hVDAC1	Human voltage-dependent anion channel 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton

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Atomic coordinates and structure factors for cell-free-expressed Δ7 DgkA are deposited in the Protein Data Bank under accession code 4D2E.

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LCP	Lipid cubic phase
LDAO	Lauryldimethylamine <i>N</i> -oxide
MAG	Monoacylglycerol
MPD	2-methyl-2,4-pentanediol
MWCO	Molecular weight cut off
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEG	Polyethyleneglycol
PEP	Phosphenolpyruvic acid
PK	Pyruvate kinase
PG	Phosphatidylglycerol
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PMSF	Phenylmethanesulfonyl fluoride or phenylmethylsulfonyl fluoride
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
SEC	Size-exclusion chromatography
TCEP	tris (2-carboxyethyl) phosphine hydrochloride
TLC	Thin-layer chromatography
tRNA	Transfer ribonucleic acid
UTP	Uridine triphosphate
YPTG	Yeast phosphate tryptone glucose

Introduction

Membrane proteins have been referred to as cellular gatekeepers regulating what enters and leaves the cell and its subcellular organelles. Gatekeeping is just one of a myriad of functions ascribed to membrane proteins which account for close to a quarter of the genetic blueprint. One of the most powerful approaches to understanding how membrane proteins operate at a molecular level relies on structure, ideally at atomic resolution obtained by the method of macromolecular crystallography. With structure comes insight into mode of action and interaction with the prospect of exploiting such relationships. Structure-inspired drug discovery is one application of relevance given the preponderance of marketed drugs that target membrane proteins.

Atomic resolution structures of integral membrane proteins and their complexes are in high demand. Alas, the number of unique solved structures is small. This is in stark contrast to the situation that prevails with soluble proteins where many thousands of structures are available. Part of the reason for the shortfall has to do with the challenging nature of membrane proteins where issues arise at almost every step along the way from gene to structure. The introduction of new materials and methods, along with a growing understanding of the properties of membrane proteins and the lipid environment in which they reside, has made the task of pursuing crystal structures somewhat

less daunting. However, challenges remain and any new approach that accelerates, is less costly and/or simplifies the process of generating high-resolution structures will be welcomed by the community.

Cell-free expression is a method with considerable promise in the membrane protein field [1]. It is easy to perform, milligram quantities of protein can be produced overnight, and costs are reasonable. To date, the cell-free method has been used to express integral membrane proteins, three of which have been crystallised but only two have yielded crystal structures [2, 3]. So while it is proven, and indeed kits for *in vitro* expression are commercially available, the method cannot be considered routine. Intrigued by what the method has to offer with regard to quality protein for structure work, we evaluated its applicability with the integral membrane enzyme, diacylglycerol kinase (DgkA).

DgkA, a 42-kDa α -helical trimer in the plasma membrane of *Escherichia coli*, is responsible for the Mg^{2+} -ATP-dependent conversion of diacylglycerol to phosphatidic acid. It plays a role in the synthesis of membrane-derived oligosaccharide and lipopolysaccharide, key components of the cellular envelope [4–6]. DgkA was an attractive target for the current study because it can be expressed *in vitro* [7]. Further, the corresponding protein produced *in vivo* had been crystallised in lipid mesophases by the ‘*in meso*’ method and a structure was reported recently [8]. Accordingly, we set out to generate a structure with cell-free DgkA and *in meso*-grown crystals. The study was performed using a fully functional, thermostabilized DgkA mutant ($\Delta 7$ DgkA) that had previously provided a 2.05-Å resolution structure by the *in meso* method. Here, we compare systematically the biophysical and functional properties of DgkA samples obtained by cell-free and cell-based expression. The materials and methods used should prove useful for generating structures, with insight into function, of additional integral membrane protein targets many of which are important to human health and disease.

Materials and methods

Materials

L-Lactate dehydrogenase (LDH, Cat. 61309, lots 1439120, 436090870), nicotinamide adenine dinucleotide (NADH, Cat. N4505, lot 029K5151), adenosine diphosphate (ADP, Cat. A2754, lot 078K7001), adenosine triphosphate (ATP, Cat. A26209, lot 028K7009), Empigen BB (Cat. 45165, lot 1384611, 33408313), DL-dithiothreitol (DTT, Cat. 43815, lot 1333018), magnesium acetate (Cat. M0631, lot 069K03391), tris (2-carboxyethyl) phosphine hydrochloride (TCEP, Cat. C4706, lot 030M1523), phosphoenolpyruvic acid (for kinase assays) (PEP, Cat. 860077,

lot 055K37992), imidazole (Cat. I0250, lot 068K5303), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Cat. H3375, lot 038K5429), chloroform (Cat. 650496, lot 09690HJ), hydrochloric acid (Cat. 258148, lot. 1403487, 23808281), PageRuler unstained protein ladder 10–200 kDa (Cat. 26614, lot 011M6232), acetyl phosphate lithium (AcP, Cat. A0262, lots 057K5317, BCBC2020 V), cytidine triphosphate (CTP, Cat. 30320, lot BCBC5048 V), sodium hydroxide (Cat. S8045, lot SZBC1290 V), uridine triphosphate (UTP, Cat. 94370, lot BCBC5487), guanosine triphosphate (GTP, Cat. G8877, lot 070M5150), potassium phosphate monobasic (Cat. P0662, lot SLBB3276 V), glucose (Cat. G7528, lot BCBH8869 V), potassium phosphate dibasic (Cat. 04248, lot SZBB2660 V), perchloric acid (Cat. 311421, lot SHBB3741), streptomycin sulphate (Cat. S9137, lot 067K00632), Q Sepharose (Cat. Q1126, lot 109K1022), 1-cm pathlength quartz cuvettes (Cat. Z276693), ammonium molybdate (Cat. 277908, lot MKBC7021), lithium chloride (Cat. 62486, lot 134700814107043), lithium hydroxide (Cat. 402974, lot MKBC0399), ethylenediaminetetraacetic acid (EDTA, E5134, lot 087K0049), ethyleneglycoltetraacetic acid (EGTA, Cat. 03777, lot 0001426960), polyethyleneglycol 8,000 (PEG 8,000, Cat. P5413, lot 068K00511), potassium acetate (Cat. P1190, lot 109K1540), magnesium acetate (Cat. M5661, lot 099K0142), folic acid (Cat. F7878, lot 039K1476), dialysis membrane MWCO 14 kDa (Cat. D9777, lot 3110), acetic acid (Cat. A6283, lot 06822HH), 2-propanol (Cat. 59310, lot BCBB0276), hydrochloric acid (Cat. 43,557-0, lot 577025-109) and phosphomolybdic acid (Cat. 31,927-9, lot STB003319) were purchased from Sigma. L-Scorvic acid (Cat. 103035G, lot H795722), methanol (Cat. M/4056/PB17, lot 1074898), chloroform (Cat. C/4966/17, lot 1134650) and sodium chloride (Cat. BP358-10, lot 060875) were obtained from Fisher Scientific. *n*-Decyl- β -D-maltopyranoside (DM, Cat. D322, lot 120455) was purchased from Affymetrix. Bacto tryptone (Cat. 211705, lot 2171101) and yeast extract (Cat. 212750, lot 2158097) were from BD Biosciences. Ni-NTA resin (Cat. 1018142, lot 139276771), PCR Purification Kit (Cat. 28104, lot 139274612) and Plasmid Midiprep Kit (Cat. 12143, lots 136269207, 136253838) were obtained from Qiagen. Piperazine-1,4-bis(2-ethanesulfonic acid (PIPES, Cat. M10550899, lot 20373) was from Molekula Ltd. Glycerol (Cat. G1345, lot A21344), isopropyl β -D-1-thiogalactopyranoside (IPTG, Cat. MB1008, lot D23592) and ampicillin (Cat. A0104, lot 20614) were purchased from Melford Laboratories Ltd. 96-well plates (Cat. 265301, lot. 655700) were from Nunc. Monoolein (9.9 MAG, Cat. M239, lot F15-U) was obtained from Nu-Chek Prep. Pyruvate kinase (PK, Cat. 10109045001, lot 11706920), complete protease inhibitor tablets (Cat. 04693132001, lot 12777700), phosphoenolpyruvic

acid (for cell-free reactions) (PEP, Cat. 10108294001, lot 13516325 and 13516328) and *E. coli* MRE600 tRNA (Cat. 10109541001, lot 13298423) were purchased from Roche Applied Science. Protein gels (Cat. 84712, lot 100413217), GelCode Blue Safe Protein Stain (Cat. 24596, lot MK162127), Slide-A-Lyzer cassettes MWCO 10 kDa (Cat. 66380, lot 10001065) and SuperSignal West HisProbe Kit (Cat. 15168, lot NK179117) were from Thermo Scientific. The iBlot dry blotting system (Cat. IB1001UK) and iBlot transfer stack mini nitrocellulose (Cat. IB3010-02, lot NM31102-02) were obtained from Invitrogen. Clontech In-Fusion HD Cloning Kit (Cat. 639648, lot 1202447A) was purchased from Takara. Amicon ultracel 50 K concentrators (Cat. UFC805024, lot R2EA14395) were from Millipore. 3-mm pathlength quartz cuvettes (Cat. 101.015-QS) were purchased from Hellma. 0.1-mm quartz cuvettes (Cat. 20/C-Q-0.1) were obtained from Starna. TLC silica gel 60 F₂₅₄ plates (Cat. 1055540001, lot HX068423) were from Merck. Gas-tight syringes (100 μ L 1710RN, Cat. 81030/00, lot 1451119) were purchased from Hamilton. RNase inhibitor (Cat. AM2694, lot 1104020) was obtained from Ambion. 24-well tissue culture plates (Cat. 662160, lot E10070AA) were purchased from Greiner Bio-One. Cell-free reaction containers (85 μ L and 3 mL) were home-built. Glass baseplates (127.8 \times 85.5 \times 1 mm, Cat. 1527127092) and coverslips (77 \times 112 \times 0.1 mm, Cat. 01029990911) for *in meso* crystallisation were from Marienfeld. Tungsten glass cutter (Cat. 633657) was obtained from Silverline Tools Ltd. 7.8 MAG (lot TasA53) was synthesised and purified in-house [9, 10]. Micromounts (Cat. M2-L18SP-30/50) for crystal harvesting were purchased from MiTeGen. *E. coli* A19 cells [*rna19 gdh A2 his95 relA1 spoT1 metB1*] were obtained from the *E. coli* Genetic Stock Center (CGSC No. 5997, New Haven, CT, USA).

Methods

S30 extract

The S30 extract was prepared from a 10-L culture of *E. coli* A19 cells as described [1]. A 350-mL overnight culture of *E. coli* A19 cells, grown at 37 °C and 180 rpm in an Innova 42 incubator shaker (New Brunswick Scientific, Enfield, CT, USA), was used to inoculate 10 L of sterile doubly concentrated YPTG broth (16 g/L bacto tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, 100 mM glucose, 22 mM potassium phosphate monobasic and 40 mM potassium phosphate dibasic) in a 15-L B. Braun Biostat fermenter. The culture was grown at 37 °C, 500 rpm and an air aeration rate of three volumes of air per volume of liquid per minute to an optical density at 600 nm (OD₆₀₀) of 4–6. At that point, the culture was cooled within 30 min to 18 °C using a circulating water bath set to 10 °C. All subsequent

steps, unless otherwise noted, were carried out on ice or at 4 °C. Cells were harvested at 5,000×g for 10 min. The wet cell pellet was weighed (~80 g) and resuspended in 88 mL of 14 mM magnesium acetate, 0.6 mM potassium chloride, 1 mM DTT, 0.1 mM PMSF and 10 mM Tris–acetate pH 8.2. The cells were lysed with three passes through an EmulsiFlex-C5 High Pressure Homogeniser at 15,000 psi. The lysed cell suspension was centrifuged at 30,000×g for 30 min. The upper, non-turbid two-thirds (~120 mL) of the supernatant containing lysed cell extract was transferred to 40-mL centrifuge tubes and centrifuged at 30,000×g for 30 min. The upper, non-turbid two-thirds of the supernatant (~90 mL) was transferred to a 250-mL bottle. A solution of 5 M sodium chloride was added to the supernatant to a final concentration of 0.4 M and incubated in a water bath for 45 min at 42 °C to dissociate endogenous tRNA and ribosomes. The cloudy solution (~100 mL) was dialysed (14 kDa MWCO) against 5 L of S30C Buffer (14 mM magnesium acetate, 60 mM potassium acetate, 0.5 mM DTT and 10 mM Tris–acetate pH 8.2) to reduce the sodium chloride concentration which is inhibitory to the cell-free reaction. After 2 h, the dialysis buffer was replaced with fresh buffer and the dialysis was continued overnight (16 h). The next day, the cloudy dialysate was centrifuged at 30,000×g for 30 min. The upper, non-turbid two-thirds of the supernatant (~90 mL) containing the extract to be used in cell-free expression (the S30 extract) was removed, snap-frozen in liquid nitrogen in aliquots of 0.3 or 1.2 mL in 1.5-mL Eppendorf tubes and stored at –80 °C for up to 12 months. The preparation, as described, takes 3 days.

The activity of the each new S30 extract was determined by quantifying the expression of green fluorescent protein (GFP) in the pIVEX vector under standard conditions [1]. This involved expressing GFP over a range of magnesium concentrations from 5 to 26 mM in 50 µL reaction volumes. The fluorescence of the GFP produced in the reaction reflects the amount of GFP expressed, and was used to judge the optimal magnesium concentration for each new batch and to assess batch-to-batch variability. Because the final magnesium concentration has a significant impact on protein expression [1], optimisation must be carried out on each batch of S30 extract and on new membrane protein targets.

T7 RNA polymerase purification

The T7 RNA polymerase was purified as described [1]. An overnight culture of BL21 (DE3) Star cells containing the pAR1219 expression plasmid for the T7 RNA polymerase was used to inoculate 2 L of Luria Broth (10 g/L bacto tryptone, 5 g/L yeast extract, 10 g/L sodium chloride). The cultures were grown at 37 °C and 180 rpm in an Infors HT Multitron shaker (Infors HT, Basel, Switzerland) to

an OD₆₀₀ of 0.6. The expression of the T7 RNA polymerase was induced with 0.8 mM IPTG for 5 h at 37 °C. Subsequent steps were performed on ice or at 4 °C. The cells were harvested by centrifugation at 5,000×g for 15 min and resuspended in 60 mL of Buffer A (50 mM sodium chloride, 10 mM EDTA, 10 mM β-mercaptoethanol, 5 % (v/v) glycerol, 1 protease inhibitor tablet and 30 mM Tris–HCl pH 8.0). The cells were lysed by passage 4 times through an EmulsiFlex-C5 High Pressure Homogeniser at 15,000 psi, followed by centrifugation at 20,000×g for 30 min to remove cell debris. The DNA in the supernatant was removed by precipitation using streptomycin sulphate at a final concentration of 2 % (w/v) followed by centrifugation at 20,000×g for 30 min. The supernatant was loaded onto a 40-mL Q-Sepharose column pre-equilibrated with two column volumes (CV) of Buffer A at 1 mL/min. The column was washed with Buffer A at 4 mL/min until the A₂₈₀ dropped below 0.1 (typically 6 CV). The T7 RNA polymerase was eluted using a gradient from 50 to 500 mM sodium chloride in Buffer A over 9 column volumes (CV) at 3 mL/min. Fractions containing T7 RNA polymerase, identified as a 99-kDa band on SDS-PAGE, were pooled (~140 mL) and dialysed (cellulose membrane, 14 kDa MWCO) against 5 L of 10 mM sodium chloride, 0.5 mM EDTA, 0.5 mM DTT and 10 mM potassium phosphate buffer pH 8.0 overnight (16 h). After dialysis, glycerol was added to a final concentration of 10 % (v/v). The protein was concentrated using an Amicon 30-kDa concentrator to 20 mL. Glycerol was added to a final concentration of 50 % (v/v). The concentrated T7 RNA polymerase at 3 mg/mL was flash frozen in liquid nitrogen in aliquots of 1 mL in 1.5-mL Eppendorf tubes and stored at –80 °C for up to 12 months.

Cell-free protein production and purification

The Δ7 *dgkA* gene for use in cell-free expression was amplified using pTrcHisB-Δ7 *dgkA* [8] as the template and the following primer pairs 5'-GGAATTCCATATGGGT CACCACCACCAC-3' and 5'-CCGCTCGAGTTATC CAAAATGCGACCATAAC-3'. The PCR product was purified using a PCR Purification Kit and cloned into an *NcoI/XhoI*-digested pIVEX vector using the In-Fusion HD Cloning Kit. The correct sequence was verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany). The plasmid, carrying the Δ7 *dgkA* gene encoding the same sequence as reported [8], was purified from 100 mL of recombinant DH5α cells using the Qiagen Plasmid Midiprep Kit. This generally yields 200 µL of plasmid DNA with a concentration between 0.5 and 0.9 mg/mL based on measurement of absorbance at 260 nm (A₂₆₀) with a Nanodrop 1000 (Wilmington, DE, USA).

Optimisation of magnesium concentration for DgkA expression was carried out in an *E. coli*-based continuous

Table 1 Composition of the feeding and reaction mixes used for continuous exchange cell-free expression

Components	Stock solution	Final solution
Feeding mix		
RCWMDE mix ^a	16.67 mM	1 mM
Amino acids, all 20	4 mM	0.55 mM
Acetyl phosphate lithium	1 M	20 mM
Phosphoenolpyruvic acid	1 M	20 mM
NTP mix ^b	75×	1×
DL-dithiothreitol	0.5 M	2 mM
Folinic acid	10 mg/mL	0.1 mg/mL
Complete protease inhibitor	50× (1 tablet/mL)	1×
Buffer (0.5 M EDTA, 2.5 M HEPES pH 8.0)	24×	1×
Magnesium acetate	1 M	5–30 mM
Potassium acetate	4 M	0.21–0.33 M
PEG 8,000	40 % (w/v)	2 % (w/v)
Sodium azide	10 % (w/v)	0.05 % (w/v)
Reaction mix, composed of feeding mix and additional components below		
S30 extract	1×	0.35×
Plasmid	0.5 mg/mL	20 µg/mL
RNAse inhibitor	20 U/µL	0.3 U/µL
T7-RNA polymerase	1.4 mg/mL	50 µg/mL
<i>E. coli</i> MRE600 tRNA	40 mg/mL	0.5 mg/mL
Pyruvate kinase (200 U/mg)	10 mg/mL	40 µg/mL

^a Arginine, cysteine, tryptophan, methionine, aspartic acid and glutamic acid each at 16.67 mM in Milli-Q water

^b CTP, UTP and GTP, each at 240 mM, and ATP at 360 mM in 160 mM sodium hydroxide

exchange, precipitate-generating cell-free (P-CF) expression system on a 50-µL scale, as described [1]. Briefly, expression trials that included 50 µL reaction mix and 825 µL feeding mix (Table 1) were carried out at magnesium acetate concentrations ranging from 5 to 26 mM for 16 h at 30 °C. Post-expression the reaction mix was transferred to a 1.5-mL Eppendorf tube and centrifuged at 12,000×*g* for 10 min at 4 °C. The pellet, composed of precipitated protein, was washed twice with 0.2 mL S30C Buffer. The final pellet was resuspended in 50 µL S30C buffer. 3 µL of the suspension was loaded onto a 12 % (w/v) SDS-PAGE to visualize and to quantify DgkA expression.

The optimal magnesium concentration, established based on small-scale expression as above, usually between 14 and 20 mM depending on S30 extract batches, was used subsequently for preparative-scale (3 mL reaction mix, 42 mL feeding mix) cell-free expression [1]. Expression, carried out over a 16-h period with continuous shaking (150 rpm, Model 311DS, Labnet, Edison, NJ, USA) at 30 °C, produced a cloudy white precipitate that included, among other things, Δ7 DgkA. The reaction mix containing

the precipitated protein was transferred into 1.5-mL Eppendorf tubes and centrifuged for 10 min at 20,000×*g* at 4 °C. The pellet was washed twice with 1 mL of S30C Buffer and stored at −20 °C or used directly for protein purification.

The Δ7 DgkA purification protocol implemented in this study was adapted from a procedure developed for use with *in vivo*-expressed DgkA [11, 12]. All steps were performed on ice or at 4 °C. The combined protein precipitate from 8 preparative-scale cell-free expression trials (24 mL total) was solubilised in 50 mL of Solubilisation Buffer (3 % (w/v) Empigen BB (detergent), 1 mM TCEP, 300 mM sodium chloride and 20 mM HEPES pH 7.5) for 30 min by rotation at 10 rpm on a wheel mixer (Model SB3, Stuart, Stone, UK). The resulting solution was centrifuged at 20,000×*g* for 10 min to remove unsolubilised material. To the clear supernatant containing detergent-solubilised material was added 5 mL of Ni-NTA resin pre-equilibrated with Equilibration Buffer (0.5 % (w/v) Empigen BB, 1 mM TCEP, 300 mM sodium chloride and 20 mM HEPES pH 7.5) to selectively bind the His-tagged Δ7 DgkA. Binding was facilitated by mixing on a wheel mixer, as above, for 30 min, after which the resin was packed into a gravity column (1 × 15 cm) and washed with 10 CV of Solubilisation Buffer followed by 15 CV of Solubilisation Buffer supplemented with 40 mM imidazole to wash out non-specifically bound protein. The Empigen BB was exchanged to DM *in situ* by washing the column with 12 CV of Detergent Exchange Buffer (0.25 % (w/v) DM, 1 mM TCEP, 150 mM sodium chloride and 20 mM HEPES pH 7.5). The protein was eluted with Elution Buffer (250 mM imidazole, 0.25 % (w/v) DM, 1 mM TCEP, 150 mM sodium chloride and 10 mM HEPES pH 7.5). This procedure typically yields 10 mL of protein solution at 2.4 mg protein/mL, as judged by A₂₈₀ ($\epsilon_{1 \text{ mg/ml}} = 2.1$ [6, 11]). The protein was concentrated to 12 mg/mL using an Amicon 50-kDa concentrator. The concentrated protein solution was loaded onto a Superdex S200 16/60 gel-filtration column pre-equilibrated with Gel-Filtration Buffer (0.25 % (w/v) DM, 1 mM TCEP, 100 mM sodium chloride and 10 mM Tris/HCl pH 7.4). Peak fractions (4.5 mL at 2.6 mg/mL) were combined, concentrated to 12 mg/mL, as above, and flash frozen in liquid nitrogen in 15-µL aliquots in 0.2-mL PCR tubes and stored at −80 °C for up to 6 months. A typical crystallisation trial uses 1–2 aliquots (180–360 µg protein) so the protein solution is not subjected to repetitive freeze-thaw cycles.

The reducing agent, TCEP, used in the above procedure contains phosphorus. To facilitate phosphorous-based phospholipid analysis on expressed protein, TCEP was replaced with the phosphorous-free reducing agent, DTT. To this end, Δ7 DgkA at 12 mg/mL, purified as above, was diluted fivefold with 0.25 % (w/v) DM, 1 mM DTT, 100 mM sodium chloride and 10 mM HEPES pH 7.5. The

diluted $\Delta 7$ DgkA was concentrated to 12 mg/mL using an Amicon 50-kDa concentrator. This dilution/concentration process, repeated five times, should reduce the TCEP concentration in the protein sample from 1 mM to 0.3 μ M.

In vivo-expressed protein

The production and purification of *in vivo*-expressed WT and $\Delta 7$ DgkA were carried out as described [11]. To facilitate quantitation of residual phospholipid in the purified protein, the phosphorous-containing TCEP used in the Detergent Exchange Buffer, the Elution Buffer and the Gel Filtration Buffer was replaced with 0.2 mM DTT.

SDS-PAGE analysis

Protein solutions were mixed with two volumes of SDS-PAGE loading buffer (0.35 M Tris-HCl pH 6.8, 30 % (w/v) glycerol, 10 % (w/v) SDS, 0.6 M β -mercaptoethanol, 0.01 % (w/v) bromophenol blue) at 20 °C and 15 μ L of the mix was loaded onto 12 % (w/v) acrylamide SDS-PAGE without prior heating. The gels were run at 170 V for 50 min at room temperature (RT, 20–22 °C). Gels were stained with GelCode Blue Safe Stain and visualised using the auto exposure setting on the FlourChem SP Imager with AphaView (version 1.3.0.7) imaging software (Alpha Innotech, Santa Clara, CA, USA).

Identification of cell-free-expressed $\Delta 7$ DgkA

The cell-free expression method used here produces membrane proteins that appear in the reaction mix as a precipitate. The precipitate can also include contaminant proteins from the expression system (Fig. 1a). A precipitate alone therefore is not proof that expression has worked. As confirmation, the SuperSignal West HisProbe Kit which employs a horseradish peroxidase (HRP)-tridentate chelator- Ni^{2+} conjugate to detect expressed His-tagged protein was used. Accordingly, the proteins were separated on SDS-PAGE and transferred onto a nitrocellulose membrane using the iBlot instrument (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. All subsequent manipulations were performed at RT. The nitrocellulose membrane was blocked with 10 mL BSA/TBST (Bovine serum albumin/Tris-Buffered Saline and Tween 20) for 1 h with shaking at 60 rpm. The blocked membrane was washed twice with 15 mL TBST for 15 min with shaking at 60 rpm. The HRP-tridentate- Ni^{2+} conjugate was allowed to bind to the His-tag of $\Delta 7$ DgkA for 1 h with shaking at 60 rpm. To remove non-specifically bound protein, the membrane was washed 4 times with 15 mL TBST for 15 min. The membrane, with the conjugate bound to His-tagged $\Delta 7$ DgkA, was incubated with an enhanced chemiluminescent

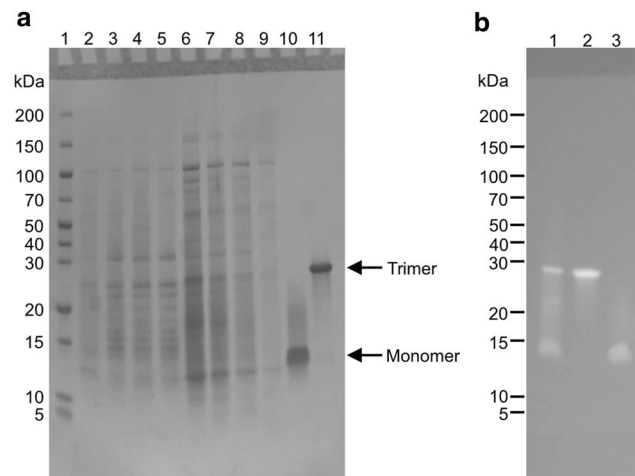


Fig. 1 Cell-free expression and expression optimization of $\Delta 7$ DgkA. **a** SDS-PAGE analysis of $\Delta 7$ DgkA expression at different concentrations of magnesium acetate (5, 8, 11, 14, 17, 20, 23, 26 mM, lanes 2–9). *In vivo*-expressed wild type (WT) and $\Delta 7$ DgkA are included in lanes 10 and 11, for reference. WT and $\Delta 7$ DgkA run predominantly as monomers (apparent MW 13 kDa) and trimers (apparent MW 28 kDa), respectively [27]. Molecular weight markers are in lane 1. The gel was stained with Coomassie Blue. **b** Identification of His-tagged DgkA using the SuperSignal West HisProbe kit [48]. Cell-free expressed $\Delta 7$ DgkA (lane 1), and *in vivo*-expressed $\Delta 7$ (lane 2) and WT DgkA (lane 3) were blotted and detected as described under "Methods"

detection substrate for HRP (provided with the SuperSignal West HisProbe Kit) for 5 min. The bands were visualised directly using a FlourChem SP Imager with the auto-exposure setting (Alpha Innotech, Santa Clara, CA, USA).

In meso kinase assay

The kinase activity of $\Delta 7$ DgkA was monitored using a coupled assay with monoolein as the lipid substrate. Thus, the consumption of ATP in the phosphorylation of monoolein was coupled through the sequential action of pyruvate kinase and lactate dehydrogenase to the oxidation of NADH monitored by a change in A_{340} , as described [11, 13]. Briefly, the $\Delta 7$ DgkA (66 μ g protein/mL in 0.25 % (w/v) DM, 1 mM TCEP, 150 mM sodium chloride and 10 mM HEPES pH 7.5) was homogenised with monoolein (2:3 (v/v) ratio, protein solution to lipid) at 20 °C using a coupled syringe mixing device [14]. This spontaneously forms the lipid mesophase containing reconstituted protein. For assay, 5 μ L of protein-laden mesophase was placed on the wall of a well in a 96-well plate and covered with 200 μ L of assay mix (20 mM ATP, 0.1 mM EDTA, 0.1 mM EGTA, 55 mM magnesium acetate, 1 mM PEP, 0.2 mM DTT, 50 mM lithium chloride, 0.4 mM NADH, 20 U/mL of pyruvate kinase and lactate dehydrogenase and 75 mM PIPES pH 6.9). Immediately, the plate was placed in a

micro-plate reader (Molecular Devices SpectraMax M2^e, Sunnyvale, CA, USA) at 30 °C and shaken (autosshaker function setting on) for 5 s. A₃₄₀ readings were recorded every 15 s typically for 30 min with shaking for 2 s between each read. Protein-free controls were run where buffer-alone was used in place of the protein solution.

Phospholipid assay

A published wet ashing protocol was used to assay for the phospholipid content of protein samples generated in the course of this study [15]. Briefly, potassium dihydrogen phosphate standards (0–1 µg phosphorus), 5 µg of dioleoyl phosphatidylethanolamine and 100 µg test protein samples were transferred to 6-mL glass tubes pre-washed with 1 % (v/v) nitric acid. Where appropriate, organic solvent was removed by flushing with a stream of nitrogen. Wet ashing was carried out by adding 130 µL 70 % (v/v) perchloric acid followed by incubation in a heat block at 180 °C for 50 min. After cooling the samples to RT, sequentially 0.66 mL milliQ-water, 100 µL 2.5 % (w/v) ammonium molybdate and 100 µL 10 % (w/v) L-Ascorbic acid were added with mixing. Colour was developed by incubating the samples in a water bath at 97 °C for 5 min. Upon cooling to RT, 0.35-mL aliquots were placed in 96-well plates and A₈₁₀ was recorded in a plate reader (Molecular Devices SpectraMax M2^e, Sunnyvale, CA, USA). All samples were assayed in triplicate and controls that lacked added phosphate were included with each set of analysis. Assays were performed in the range of 0–30 nmol phosphorous.

Thin-layer chromatography (TLC)

TLC was used to verify that lyso-phosphatidic acid (LPA) was the phosphorylated product of cell-free-expressed $\Delta 7$ DgkA activity when the protein was reconstituted into the lipid cubic phase prepared with monoolein in the presence of ATP [11].

TLC analysis was also used to detect and identify lipids in *E. coli* membranes, S30 extract, and purified in vivo-expressed and cell-free $\Delta 7$ DgkA. To prepare *E. coli* membranes for TLC analysis, cell pellet (~1 g) from 1 L of culture grown to an OD₆₀₀ of 1.0 was resuspended in 20 mL of 10 mM HEPES pH 7.5 and lysed by passing 3 times through the Emulsiflex C5 cell disruptor at 4 °C. The lysed cell suspension was centrifuged for 15 min at 8,500×g at 4 °C to remove cell debris. The supernatant was centrifuged at 150,000×g for 45 min at 4 °C (Beckman Optima L-100 XP). The membranes in the pellet were resuspended in 1 mL of 10 mM HEPES pH 7.5 and were used directly for lipid extraction.

Phospholipid was extracted following an established procedure [16] that involved solubilizing the lipid in a mixture

of chloroform and methanol (2:1 by vol.). With *E. coli* membranes (1 mL) and S30 extract (0.3 mL), each as a suspension or solution was extracted with organic solvent. In the case of pure protein, the 80 µL of sample was extracted with 0.5 mL solvent. Extraction was carried out by vigorous vortexing for 30 s followed by incubation on ice for 10 min. The aqueous and organic solvent layers were separated by centrifugation at 3,000×g at RT for 15 min. The lower, chloroform-rich layer was carefully removed, evaporated to dryness with a stream of nitrogen and the extracted lipid dissolved in a defined volume of chloroform as follows: 20 µL, 40 µL and 10 mL for S30 extract, purified protein and *E. coli* membranes, respectively. TLC analysis was performed by spotting on the plate fixed volumes (2–5 µL) of the chloroform solutions containing extracted lipid. Plates were pre-run in chloroform and run in a solvent system containing chloroform:methanol:acetic acid (65:10:15 by vol). Plates were dried on a hot plate at 150 °C, immersed in phosphomolybdate (20 % (w/v) in ethanol) staining solution and organic matter was visualized by charring on a hot plate at 150 °C. Phospholipid and detergent standards, run in parallel, included phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and DM.

Absorption and fluorescence spectroscopy

All spectrophotometric measurements were carried out at 20–22 °C using $\Delta 7$ DgkA protein samples solubilised in Gel-Filtration Buffer. Spectra were baseline corrected using protein-free buffers. The average of three consecutive spectra recorded using aliquots from the same solution is reported.

UV–visible spectroscopic analysis was performed at 0.5 mg protein/mL in a 1-cm pathlength quartz cuvette (Sigma Aldrich, St. Louis, MO, USA) with a UVIKON_{XL} spectrophotometer (Northstar Scientific, Leeds, UK). Spectra were recorded from 400 to 250 nm at a scanning speed of 200 nm/min.

Fluorescence measurements were carried out at 0.1 mg protein/mL in a 3-mm pathlength quartz cuvette (Hellma, Jena, Germany) with a FluoroMax-3 spectrofluorometer (Horiba, Kyoto, Japan). Emission spectra were recorded from 470 to 300 nm at 10 nm/s with an excitation wavelength of 280 nm and slit widths corresponding to a spectral width of 2 nm.

Circular dichroism (CD) spectroscopy

CD analysis was carried out at 0.57 mg protein/mL in a 0.1-mm pathlength quartz cuvette (Starna, Hainault, UK) with a Jasco J-815 spectrometer (Jasco, Easton, MD, USA) at 20 °C. Spectra from 250 to 190 nm were recorded at 20 nm/min in 1-nm steps with a bandwidth setting of 1 nm.

Spectra were smoothed using the binomial function included in the Jasco spectra analysis software package (version 1.54.03). The K2D3 web server was used to calculate secondary structure content (Louis-Jeune et al. [17]).

Crystallisation

In meso crystallisation trials began with the reconstitution of the protein into the bilayer of the lipid cubic mesophase. This was done following a standard protocol [18]. The protein solution at 12 mg/mL was homogenised with 7.8 MAG [9, 10] in equal parts by volume using a coupled syringe mixing device at RT. Crystallisation trials were set up by transferring 50 nL of the protein/lipid cubic mesophase onto a silicised 96-well glass crystallisation plate which was subsequently covered with 800 nL precipitant solution using an *in meso* robot [19]. Wells were sealed with a glass cover slide and the plates were stored in a walk-in refrigerator at 4–6 °C for crystal growth. Crystallisation progress was monitored using normal and cross-polarised light microscopy (Model Eclipse E 400 Pol, Nikon, Tokyo, Japan) at 4–6 °C. Precipitant solutions consisted of 3–6 % (v/v) MPD, 100 mM sodium chloride, 60 mM magnesium acetate and 50 mM sodium citrate pH 5.6. Rectangular-shaped crystals grew to a maximum size of $20 \times 60 \times 180 \mu\text{m}^3$ in 6–7 days. Wells were opened with a glass cutter and the crystals were harvested with 30–50- μm MiTeGen micromounts at 4–6 °C. Crystals were snap-cooled directly in liquid nitrogen without added cryo-protectant [20].

Diffraction data collection and processing

X-ray diffraction measurements were made on beamline 23ID-B of the Advanced Photon Source (APS), USA, beamline I24 of the Diamond Light Source (DLS), UK, and beamline PX-II of the Swiss Light Source (SLS), Paul Scherrer Institute, Switzerland. Diffraction images, taken with a tenfold attenuated beam, were used to locate crystals in the mesophase and to centre on highly ordered regions of the crystal. A complete data set was obtained by merging data recorded at APS from two crystals at a total of seven different locations. Data were reduced with xia2 [21] using XDS [22], XSCALE and SCALA [23].

Structure solution and refinement

Initial phases were obtained by molecular replacement using Phaser [24] with the protein component of a published structure (PDB 3ZE3) [8] as the searching model. In subsequent cycles of iterative model building and refinement, the program Coot [25] was used for model building while the program Phenix [26] was used for refinement.

Results

Cell-free expression and protein purification

The cell-free expression system chosen for use in this study employed direct synthesis of $\Delta 7$ DgkA in the absence of a receiving membrane mimetic in the reaction mix. Consequently, the protein ended up as a precipitate which, under ideal circumstances, can be solubilised and refolded. Further, as the protein is synthesised *in vitro* it aggregates with other proteins in the system. These show up when the harvested protein is analysed by SDS-PAGE with a non-specific stain such as Coomassie Blue (Fig. 1a). To establish that $\Delta 7$ DgkA was definitively among the precipitated proteins, the gel was subjected to further analysis using a HRP-tridendate- Ni^{2+} conjugate to detect His-tagged proteins (Fig. 1b). The data showed that $\Delta 7$ DgkA was, indeed, among the precipitated proteins and that it was expressed in relatively large amounts. It migrated, under conditions of SDS-PAGE analysis, as a monomer and a trimer, as has been observed previously with *in vivo*-expressed protein [27].

Magnesium plays a key role in a host of nucleotide-based biochemical reactions and is a critical component of the translation process in any cell-free expression system [1]. Accordingly, it is an important variable that needs optimising, a process that can be performed efficiently on an analytical scale requiring miniscule amounts (50 μL) of reaction mix. The data in Fig. 1a show that the production of cell-free expressed $\Delta 7$ DgkA peaked at 17 mM magnesium acetate. In addition to $\Delta 7$ DgkA, the latter yield included contaminant proteins that were pulled down from the reaction mix with the aggregated kinase. At a best estimate, the enzyme was 50 % pure at this stage.

Since milligram quantities of pure protein are usually needed for crystallisation trials, it was necessary to scale up the level of cell-free expression. This was done using chambers that hold 3 mL reaction mix (Fig. 2a), and sufficient protein was obtained by running eight reactions in parallel. The total yield of protein under these preparative-scale conditions was 24 mg corresponding to an output of 1 mg/mL reaction mix (Fig. 2b).

The harvested and washed precipitated protein from preparative-scale P-CF expression was successfully solubilised in the detergent, Empigen BB, using a protocol developed for *in vivo*-expressed DgkA [8]. With the protein immobilised on a Ni-NTA column the Empigen was exchanged for decylmaltoside (DM) in preparation for crystallisation, and the protein was eluted in a single step with concentrated imidazole. The $\Delta 7$ DgkA-enriched fractions from the Ni-NTA column were pooled and concentrated to 12 mg/mL. To further purify the protein, the sample was subjected to size-exclusion chromatography (SEC). The protein eluted

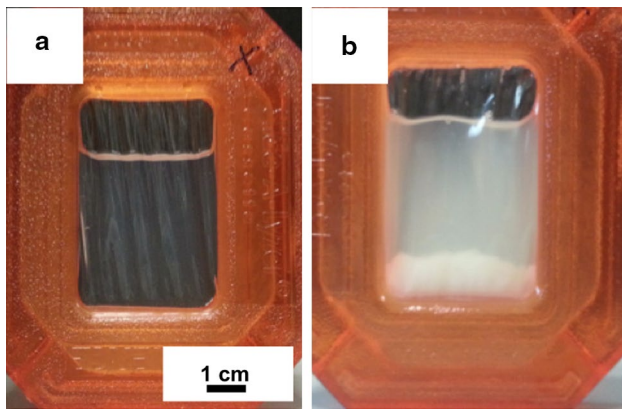


Fig. 2 Preparative-scale expression of $\Delta 7$ DgkA. **a** Cassette in which the 3-mL reaction mix is contained photographed before expression begins. The reaction mix solution is housed between two dialysis membranes, held approximately 3 mm apart, and is seen as a clear solution. **b** Photograph of the same cassette as in **a** after 16 h of expression at 30 °C. The reaction mix solution is cloudy with precipitated protein. Bulk precipitated protein can be seen settled at the bottom of the cassette. The yield of pure $\Delta 7$ DgkA from such an expression trial was typically 3 mg

as a sharp, Gaussian-shaped peak (Fig. 3a), indicative of a monodisperse sample, as observed previously with in vivo-expressed DgkA [8]. Fractions collected from the SEC peak were concentrated to 12 mg protein/mL and shown by SDS-PAGE in a loading series to be ~95 % pure (Fig. 3b). This was comparable to the in vivo-expressed $\Delta 7$ DgkA (Fig. 3c) and therefore suitable for further characterisation and crystallisation.

Absorption and fluorescence spectroscopy and circular dichroism

Three different types of spectroscopic analysis were performed on cell-free and in vivo-expressed $\Delta 7$ DgkA with a view to identifying differences, if any, in protein conformation and local environment. All spectroscopic work was done using protein dispersed in a solution of detergent (DM) micelles. The UV-visible absorption spectra of the two protein types were identical (Fig. 4a). They show peaks in the vicinity of 280 nm dominated by tryptophan absorption reflecting the aromatic amino acid composition of $\Delta 7$ DgkA (5 tryptophans, 2 tyrosines and 3 phenylalanines).

The electronic excitation spectra of the two $\Delta 7$ DgkA types were identical in shape and superimposable when normalised (Fig. 4b). With an excitation wavelength of 280 nm, the emission profile contains contributions from both tyrosine and tryptophan fluorescence and showed a maximum at 337 nm. A difference in fluorescence intensity between the two protein types was observed. Specifically, the fluorescence yield of the cell-free protein was 81 % that of its cell-based counterpart. This could be attributed to a

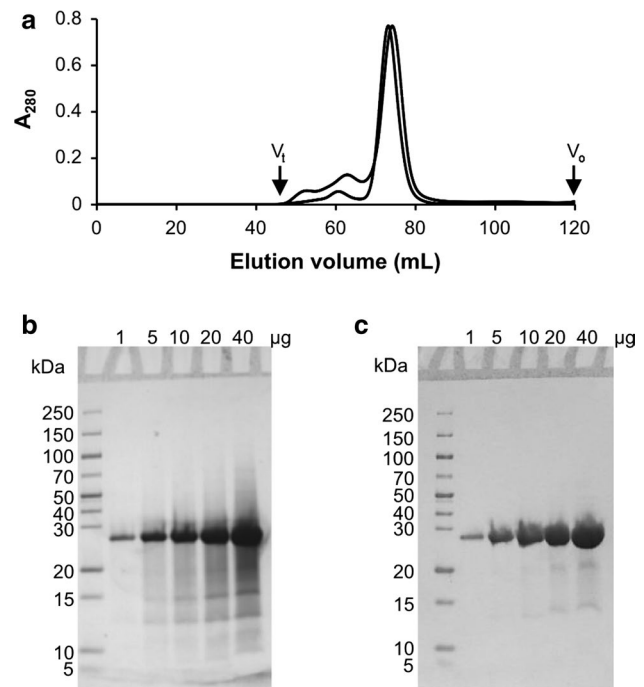


Fig. 3 Purification and characterization of $\Delta 7$ DgkA. **a** Size exclusion chromatography analysis of in vivo- (solid line) and cell-free-expressed $\Delta 7$ DgkA (dashed line). The elution volume (V_e) for the in vivo and cell-free $\Delta 7$ DgkA were 73.1 and 74.2 mL, corresponding to apparent molecular weights of 97.7 and 92.5 kDa, respectively. The main peak has a near Gaussian shape indicating that both samples are monodisperse. The void and total volume are indicated as V_0 and V_t , respectively. To assess purity, the indicated amounts of cell-free (**b**) and in vivo-expressed (**c**) $\Delta 7$ DgkA as a loading series were subjected to SDS-PAGE analysis. Both forms run predominantly as a trimer with an apparent molecular weight of 28 kDa. Less than 1 % of the protein exists as a monomer (apparent MW, 13 kDa). The band at 15 kDa in (**b**) is likely a contaminant. The two, low molecular weight bands at 9 and 11 kDa in (**b**) are either contaminants or degraded DgkA. The high molecular weight bands in (**c**) are likely oligomers. Gels were stained with Coomassie blue

more polar environment for the tryptophan and tyrosine chromophores in the cell-free sample.

Circular dichroic behaviour of the two protein types was virtually identical with strong negative peaks at 209 nm for both in vivo and cell-free samples (Fig. 4c). This type of spectrum is characteristic of an α -helical protein. The spectra can best be fit with the following secondary structure elements: 91.8 % α -helix, 0.2 % β -strand and 8 % random coil.

Lipid analysis

As the names imply, cell-free and in vivo-expressed $\Delta 7$ DgkA are made in entirely different environments. We expected therefore that the two samples, whilst containing the same $\Delta 7$ DgkA protein, might differ especially in residual lipid content and that this, in turn, might alter the

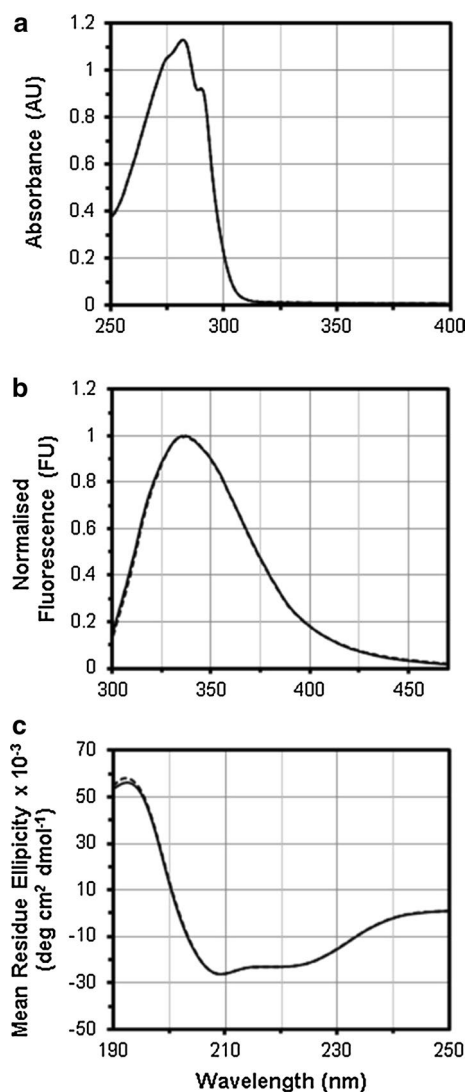


Fig. 4 Spectrophotometric analysis of $\Delta 7$ DgkA. **a** UV-visible, **b** fluorescence and **c** circular dichroism spectra of in vivo- (solid line) and cell-free-expressed $\Delta 7$ DgkA (dashed line) in a detergent micellar solution. Spectra were recorded in Gel-Filtration Buffer. The fluorescence yield of the cell-free-expressed protein was 81 % that of its in vivo-expressed counterpart. To better compare spectral profiles, the two spectra in (b) have been normalised to the same value at the emission maximum, 337 nm

properties of the corresponding protein. For purposes of lipid analysis, two methods were used. Firstly, as a measure of bound phospholipid, inorganic phosphate content was assayed after wet ashing the purified protein. The result indicates that no phospholipid could be detected in the protein sample despite the fact that 120 μg (8.4 nmol) of $\Delta 7$ DgkA was subjected to analysis.

The second lipid analysis method employed was thin-layer chromatography (TLC). For this purpose, pure protein samples were extracted with a mixture of chloroform and methanol [16]. The organic phase containing

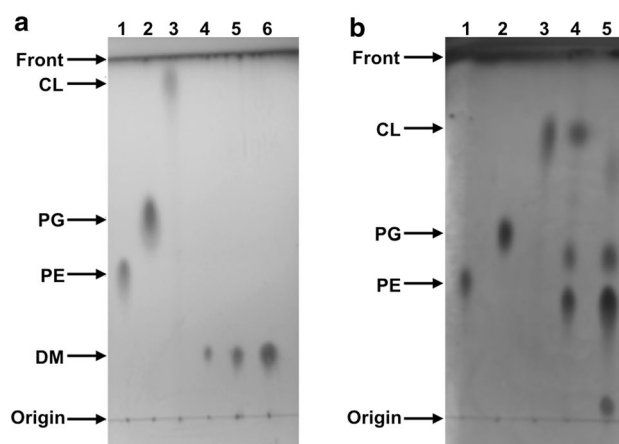


Fig. 5 Phospholipid content of cell-free and in vivo-expressed $\Delta 7$ DgkA and of *E. coli* membranes and S30 extract as determined by thin-layer chromatography. **a** Analysis of lipid extracted from purified cell-free (lane 5) and in vivo-expressed $\Delta 7$ DgkA (lane 6). Reference lipid and detergent standards include phosphatidylethanolamine (lane 1 1 μg), phosphatidylglycerol (lane 2 1 μg), cardiolipin (lane 3 1 μg), and DM (lane 4 1 μg). **b** Analysis of lipid extracted from *E. coli* S30 extract (lane 4) and isolated outer and inner membranes (lane 5). Lipid standards are as described in **a**. The faint spot close to the origin in lane 5 was not identified but is likely to be quite polar

the extracted lipid was separated, dried and used for TLC analysis. The results (Fig. 5a) show that no lipid could be detected despite the fact that the lipid extract, representing a relatively large amount of protein (120 μg , 8.4 nmol), was spotted on the TLC plate.

The detection limits of the phosphate content assay and staining/visualisation method used with TLC each correspond to ~ 1 μg (1.3 nmol) lipid under conditions of measurement. Assuming an average phospholipid molecular weight of 750 g/mol these data indicate that the lipid content of either sample was less than 0.16 mol lipid per mole $\Delta 7$ DgkA or less than one lipid molecule per six molecules of protein.¹

For reference, TLC analysis was also performed on isolated *E. coli* membranes and S30 extract (Fig. 5b). The analysis showed that both contained PE, PG and CL which is to be expected [1, 28, 29]. The data suggest that the S30 extract had a lipid content of ~ 100 $\mu\text{g}/\text{mL}$,² as reported pre-

¹ The detection limit for lipid using TLC is ~ 1 μg (1 μg lipid = 1×10^{-6} g/750 g mol⁻¹ = 1.3×10^{-9} mol = 1.3 nmol lipid) assuming an average lipid molecular weight of 750 g mol⁻¹. The amount of $\Delta 7$ DgkA sample used in the analysis was 120 μg (120 μg = 120×10^{-6} g/14,318 g mol⁻¹ = 8.4×10^{-9} mol = 8.4 nmol DgkA). The lipid content of the protein is therefore less than (1.3/8.4) 0.16 mol lipid per mol $\Delta 7$ DgkA.

² By comparing the staining intensity in the S30 sample lane (Lane 4) with the lipid standards (Lanes 1–3) in Fig. 5, it is possible to estimate that the lipid in the S30 extract in Lane 4 corresponds to ~ 3 μg total lipid. 2 μL of extracted S30 lipid dissolved in chloroform was spotted in Lane 4. The total volume of the S30 lipid extract was 20 μL representing 30 μg total lipid derived from 0.3 mL S30 solution. This corresponds to 100 μg lipid/mL S30 extract.

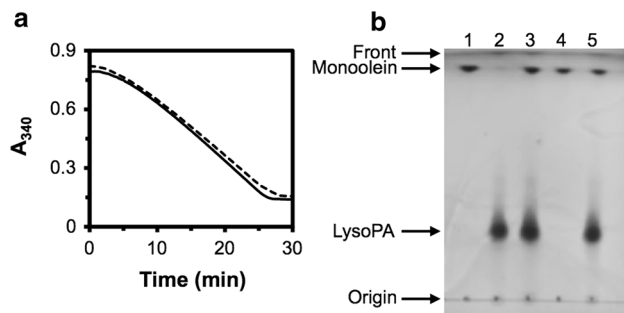


Fig. 6 Kinase activity of $\Delta 7$ DgkA reconstituted in the lipid cubic mesophase at 30 °C. **a** Coupled spectrophotometric assay of kinase activity recorded for in vivo-expressed (solid line) and cell-free DgkA (dashed line). The average of three replicates is shown. **b** Thin-layer chromatographic evidence that cell-free expressed DgkA is active and can convert the hosting mesophase lipid, monoolein, to lyso-phosphatidic acid in the presence of ATP. Lanes 4 and 5, respectively, show lipid extracted from assays performed with and without cell-free-expressed $\Delta 7$ DgkA reconstituted into the lipid mesophase. Incubations lasted for 30 min. Lanes 1–3 show standards that include monoolein and lyso-phosphatidic acid

viously [1]. Interestingly, Berrier et al. [29] reported the presence of membrane vesicles in S30 but not in S100 extracts. *E. coli* membrane lipids amount to 10 % of cellular dry weight.³

Cell-free $\Delta 7$ DgkA is enzymatically active

The data obtained to this point indicated that $\Delta 7$ DgkA can be produced in large quantities by the cell-free method, and that the as-synthesised P-CF protein can be solubilised and purified by affinity and size exclusion chromatography. Further, the P-CF protein is biophysically/structurally analogous to its cell-based counterpart. However, before entering crystallisation trials it was important to demonstrate that the protein in hand was functionally active.

Activity was measured with the enzyme reconstituted into the lipid membrane of the cubic mesophase of the type used for crystallisation. DgkA is a promiscuous enzyme; it works not only with diacylglycerols but also with monoacylglycerols (MAGs) as lipid substrate [11]. Monoolein is the MAG used to create the mesophase for reconstitution. In the presence of Mg^{2+} -ATP, the reconstituted enzyme was shown to be catalytically active (Fig. 6a). The specific activities of the cell-free and cell-based proteins were similar at 12.9 and 12.3 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively.

³ Following the procedure outlined in Footnote 2, the membrane lipid content in dry *E. coli* cells can be estimated. Lane 5 in Fig. 6 corresponds to $\sim 6 \mu\text{g}$ total lipid from 2 μL lipid extract. The total volume of the lipid extract was 10 mL which corresponds to 30 mg membrane lipid derived from 1 g wet cells (0.31 g dry cell mass [30]). Thus, the membrane lipid content of dry *E. coli* cells is estimated at 10 %. A value of 9.1 % lipid has been reported [31].

That the mesophase reconstituted enzyme was catalytically active was further demonstrated by monitoring directly, using TLC, the kinase lipid product, lyso-phosphatidic acid (Fig. 6b).

Crystallisation and structure determination of cell-free $\Delta 7$ DgkA

A structure for the in vivo-produced $\Delta 7$ DgkA at 2.05-Å resolution was obtained recently using crystals grown by the lipid mesophase method [8]. With a view to crystallising the cell-free enzyme, the same crystallisation protocols and conditions known to work with the in vivo-expressed protein were implemented. These included using i) 7.8 MAG as the hosting mesophase lipid, ii) precipitants containing sodium citrate pH 5.6, sodium chloride, magnesium acetate and methyl-2,4-pentanediol (MPD), and iii) a growth temperature of 4 °C [8]. Within 7 days of set-up, crystals with a maximum dimension of 180 μm were obtained (Fig. 7). Due to the relatively high concentration of MPD in the precipitant, the cubic mesophase, as expected, converted to the more fluid sponge phase from which harvesting crystals can be a challenge. However, using specialized methods developed for this purpose [20], crystals were successfully recovered, snap-cooled in liquid nitrogen and used for X-ray diffraction data collection with a synchrotron micro-beam (Table 2). Phasing was done by molecular replacement with the model of the corresponding cell-based $\Delta 7$ DgkA construct. The structure of cell-free produced DgkA to a resolution of 2.28 Å is shown in Fig. 8.

Discussion

This paper reports the X-ray structure of an integral membrane enzyme, a kinase, solved using cell-free produced protein and crystals grown by the lipid mesophase method. Prior to entering crystallisation trials the cell-free enzyme was shown to be biochemically and biophysically similar to its in vivo counterpart. The fact that it crystallised under conditions analogous to those used successfully with the in vivo protein supports this view. Indeed, the crystal structures of the two proteins are virtually identical with an RMSD of 0.313 Å over 570 residues. The protein exists as a trimer with three active sites of the shared sites type. Each straddles at least two subunits with one half in the membrane and the other cytoplasmic. The amphiphilic nature of the active site reflects the contrasting amphiphilicities of the substrates (diacylglycerol, ATP) and products (phosphatidic acid, ADP) of this diminutive kinase.

The $\Delta 7$ DgkA construct used in the current study incorporated design features for stability and heavy atom

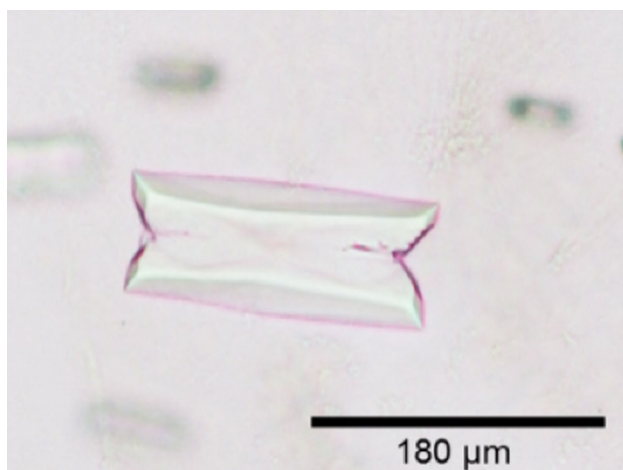


Fig. 7 Crystal of cell-free-expressed $\Delta 7$ DgkA growing at 4 °C in the lipid mesophase formed by 7.8 MAG viewed with normal light. Trials were conducted as described under “Methods” using a protein solution with a concentration of 12 mg DgkA/mL. The precipitant included 3–6 % (v/v) 2-methyl-2,4-pentenediol, 0.1 M sodium chloride, 60 mM magnesium acetate and 50 mM sodium citrate pH 5.6

labelling [8]. Accordingly, it has seven residues that differ from the wild-type enzyme (A41C, C46A, I53 V, I70L, M96L, V107D, C113A). Despite these primary structure differences the two have been shown to be functionally and structurally alike [8]. However, the *in vivo*-expressed protein gave rise to crystals that diffracted optimally to 2.05 Å. By contrast, the cell-free enzyme provided a structure with a resolution of 2.28 Å. The disparity in resolution is worthy of comment. To begin with, we acknowledge that while some optimisation was carried out with the cell-free target, optimization was nowhere near as extensive as that performed with the cell-based enzyme [33]. Thus, further optimisation may well have delivered a comparable or perhaps even higher resolution structure. We are also mindful that the proteins entering crystallisation trials, whilst structurally and functionally alike, could have slight compositional differences that gave rise to crystals differing in diffraction quality. These include differences in protein heterogeneity and lipid profile. Heterogeneity could come about as a result of proteolysis, and since the proteases active during *in vitro* and *in vivo* expression and purification are likely to be different (although protease inhibitors were used in both), the disparity may well show up in the heterogeneity of the corresponding proteins impacting, in turn, on crystal quality. Lipid could not be detected in purified protein produced by the cell-free and *in vivo* methods; both had less than one molecule of phospholipid for every six molecules of DgkA. Since both derive primarily from aggregated protein, precipitate and inclusion bodies in the case of cell-free and *in vivo*-expressed protein, respectively, such a finding is perhaps not surprising. Regardless, at this level of

sensitivity, lipid profile cannot be identified as a reason for the difference in resolution of the solved structures.

Attractive features of cell-free expression

The *E. coli*-based cell-free system implemented in this study is particularly easy and inexpensive to set up and simple to use. The S30 extract, which contains the ribosomes and other components required for protein synthesis, can be prepared conveniently with, for the most part, standard laboratory equipment and supplies. Biomass for cell lysate production is ideally done in a fermenter with a working volume of at least 10 L. This will provide enough S30 extract for 200 mL of reaction mix corresponding to 300 mg expressed and purified $\Delta 7$ DgkA protein. The cell lysate takes, in total, 3 days to produce and can be stored in aliquots at -80 °C where it is stable for at least 12 months. It is estimated that each 1 mL of cell-free reaction costs about €82.⁴ Equivalent material sourced commercially can cost 20 times this figure.

Cell-free expression is extremely efficient in that milligrams of protein can be produced overnight in 1 mL of reaction mix. Further, because it is an open system the protein can be harvested directly without the need for all that goes with culturing live cells and isolating membranes or inclusion bodies from harvested biomass. The cell-free synthesised protein is relatively pure to begin with requiring, in the case of DgkA and EmrE [32], little more than sequential nickel affinity and size-exclusion chromatographic steps to generate protein of a quality suitable for crystal structure work. Indeed, with a different DgkA construct, $\Delta 4$ DgkA [33], diffraction quality crystals were obtained using protein that had only been purified by nickel affinity chromatography (unpublished observations). It is, of course, necessary in the cell-free system of the precipitate type to solubilise the precipitated protein and to refold it as a prelude to purification and crystallisation. The option is always there, however, to perform expression in the presence of detergent micelles [34], liposomes [35] or nanodiscs [36], which act as membrane mimetic receptacles for the nascent protein, with a view to diverting it, partly at least, away from aggregation. These alternative approaches, however, usually require additional steps where the ‘reconstituted’ protein is isolated from aggregated material and the protein is removed or exchanged from its membrane

⁴ The approximate cost for a 1-mL cell free reaction is as follows: S30 extract: €0.43, plasmid: €2.14, T7 polymerase: €0.34, amino acids: €0.35, folic acid: €0.07, RNA inhibitor: €12.43, tRNA: €6.46, pyruvate kinase: €0.28, AcP: €18.33, PEP: €18.40, NTP mix: €8.81, protease inhibitor: €3.33, HEPES: €0.22, PEG 8,000: €0.02, DTT: € 0.02, magnesium acetate, potassium acetate and sodium azide: € 0.04, dialysis cassette: €9.87. Total of €81.54.

Table 2 Data collection and refinement statistics

Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	75.29, 91.82, 143.92
α , β , γ (°)	90, 90, 90
Wavelength (Å)	0.97933
Beamline	23-ID-B, APS
Number of crystals	2
Resolution (Å)	75.29–2.28 (2.34–2.28) ^a
<i>R</i> _{merge}	0.094 (0.814) ^a
<i>R</i> _{pim}	0.042 (0.479) ^a
<i>I</i> / σ <i>I</i>	15.9 (1.9) ^a
Completeness (%)	98.4 (96.9) ^a
Redundancy	9.6 (5.4) ^a
Wilson B-factor (Å ²)	39.04
Refinement	
Resolution (Å)	52.02–2.28
Number of reflections	45,339
<i>R</i> _{work} / <i>R</i> _{free}	0.1886/0.2298
Number of atoms	5,344
Protein	4,688
Ligand/ion	656
Number of chains	6
Number of residues	597
Chains (residues present)	A (6–121), B (23–120), C (29–121), D (14–121), E (19–26, 40–121), F (29–120)
B-factors (Å ²)	
Protein	58.46
Ligand	76.40
R.m.s deviations	
Bond lengths (Å)	0.013
Bond angles (°)	0.969
Ramachandran	
Favoured region (%)	99.67
Allowed region (%)	0.33
Outlier region (%)	0.0
MolProbity clash score	3.07

^a Highest resolution shell is shown in parenthesis

mimetic and reformulated in preparation for crystallisation screening.

The P-CF expression system used here, when optimised with regard to magnesium ion alone, yielded sufficient $\Delta 7$ DgkA in 24 mL of reaction mix to conduct functional assays and productive crystallisation trials. More protein, if required, could be produced simply by scaling up the expression. A more complete expression optimisation can always be implemented with a view to increasing protein yield. This typically involves adjusting potassium

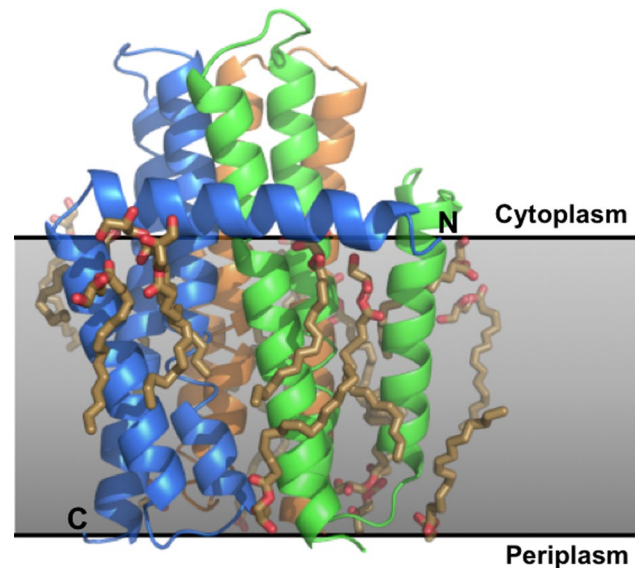


Fig. 8 The structure of cell-free-expressed $\Delta 7$ DgkA is shown as a ribbon model viewed from the membrane plane. Individual subunits are coloured *blue*, *green* and *orange*. Eighteen lipid molecules are shown as stick models with carbons and oxygens colored *yellow* and *red*, respectively. The N- and C-termini of one subunit are indicated. Membrane boundaries are based on hydrophobic thickness calculations from the PPM server [49]

ion concentration, plasmid concentration, incubation time, feeder mix-to-reaction mix volume ratio and temperature.

Producing foreign proteins *in vivo* can run into difficulties when the expressed protein, for a myriad of possible reasons, turns out to be toxic to the host cell. This is particularly true with integral membrane proteins where yield can be severely compromised. In such circumstances, cell-free expression should certainly be considered.

Uniform labelling with SeMet is commonly used for experimental phasing [37]. However, depending on the identity of the host organism and the expressed protein, including SeMet in the growth medium can impact negatively on biomass and yield of expressed protein [38]. To our knowledge, this is not usually a problem with cell-free expression [7]. The same approach can be extended to seleno-cysteine. It can also be used with non-standard/natural amino acids [39] [photo probes (photo-leucine, photo-methionine, cross-linkers, etc.)] provided the corresponding aminoacylated tRNA can be made and is acceptable to the translational machinery *in vitro*. The use of suppressor tRNA approaches enables the site-specific incorporation of non-native residues [40]. When the latter technology is used in combination with aminoacylating ribozymes, also known as flexizymes [41], the limits to residue identity dictated by the genetic code no longer exist enabling wide-ranging, systematic studies relating to residue chemistry to be performed.

Cell-free expression is useful when isotopically labelled protein is called for. These can include radionuclides for tracer applications or stable isotopes as used in NMR analysis. The advantages of doing this *in vitro* are many but particularly important is the avoidance of isotope dilution and scrambling, processes that plague cell-based labelling [42].

Where cell-free expression is carried out in the presence of a membrane mimetic, be that a micelle, a liposome or a nanodisc, the addition of a stabilising ligand may prove advantageous. Thus, as the protein folds and incorporates into the membrane mimetic it has something with which to associate, thereby stabilising it in a functional form. Given that cell-free expression takes place in an open system, ligand addition is simple. Interestingly, in the current study, DgkA was expressed in the presence of a co-substrate, ATP, there to fuel the translation process. So despite it ending up as an aggregate, the enzyme may well have used ATP as a ligand about which to fold as it emerged from the ribosome and to assume some semblance of a native state. This, in turn, may have facilitated the generation of material that was more tractable with regard to subsequent solubilisation and refolding.

Cell-free expression for structure work

To our knowledge, there are only two other integral membrane proteins expressed by the cell-free method that have been successfully crystallised yielding structures. Both are α -helical proteins. They include the multidrug transporter in *E. coli*, EmrE [2], and rhodopsin II (ARII), a light-driven proton pump from the unicellular green alga, *Acetabularia acetabulum* [3]. The EmrE structure reveals a dimer with antiparallel orientation consistent with the view that the protein functions with dual topology. Structure determination was done using both *in vivo* and cell-free N-terminally hexahistidine-tagged protein. Cell-free expression was similar to that implemented in the current study and was based on an *E. coli* lysate without a membrane mimetic. Performed on a preparative scale with 20–100 mL reaction mix, the product was SeMet-labelled EmrE which yielded structures to 3.8- and 4.5-Å resolution based on anomalous SeMet data. The authors reported that the generation of SeMet-labelled protein *in vivo* was problematic and that the cell-free alternative was important to the project [32]. ARII is a eukaryotic-derived microbial rhodopsin and its structure was obtained to a resolution of 3.2 Å using cell-free produced protein. Phasing of the ARII structure was done by molecular replacement with a model for bacteriorhodopsin from *Halobacterium salinarum*. The cell-free system used in this case was again *E. coli*-based and was optimised for bacteriorhodopsin expression. However, the protein was translated in the presence of the steroid

detergent, digitonin, and phosphatidylcholine, likely to create a hosting membrane mimetic for the direct production of functional protein.

The human voltage-dependent anion channel 1, hVDAC1, is a 19-stranded β -barrel protein that shuttles ions and small molecules across the outer mitochondrial membrane. Solution and crystal structures for this protein are available [43, 44]. hVDAC1 has also been expressed in an *E. coli*-based cell-free system supplemented with detergents (C_8E_4 , $C_{12}E_8$, β -DDM, α -DDM, LDAO, fos choline-12, cymal-5) for the generation of functional protein. The cell-free produced protein was successfully crystallised by vapour diffusion in hanging-drop mode. Unfortunately, the crystals diffracted to no better than 6 Å in α -DDM [45].

Conclusions

Cell-free expression as a means for producing functional integral membrane proteins is highly efficient, versatile and cost effective. Milligrams of protein can be produced overnight using millilitre volumes of reaction mix at low cost. Because the system is open, harvesting protein is straightforward and the newly synthesised protein is already in a relatively pure condition. Functional protein can be obtained directly when expression is done in the presence of a membrane mimetic. Alternatively, it can be obtained when the protein, expressed as an aggregate, is subsequently solubilised and folded, as in the current study. To date, four membrane proteins expressed by the cell-free method have progressed to successful crystallisation and three of these have yielded X-ray structures. These include a transporter to 3.8 Å, a light-driven pump to 3.2 Å and, as reported here, a lipid metabolising kinase 2.3 Å. The disparate nature of these assorted proteins attests to the potential and generality of the method that should be considered for adoption in membrane structural and functional biology laboratories and centers.

In this study, we chose to carry out expression in the absence of a membrane mimetic, in part, because the aggregated protein so formed resembled the inclusion bodies that the protein, overexpressed *in vivo*, forms naturally and that has been used successfully for crystallography. However, it is possible to do the expression in the presence of a membrane mimetic for the direct production of functional protein. To date, detergent micelles [45], liposomes [35], nanodiscs [46] and bicelles [47] have been used for this purpose, and each has its own pros and cons. As a logical extension to the current work, we are proposing to use the bicontinuous lipid mesophase as an alternative receiving membrane mimetic, for several reasons. The cubic phase comprises an essentially limitless reservoir for the expressed membrane

protein throughout which it can diffuse. The mesophase includes a familiar bilayered membrane in which the newly synthesised protein is likely to feel at home and to retain a native, functional fold. The bicontinuous nature of the mesophase means that both sides of the membrane embedded protein are accessible which is important for functional characterisation and assay development. Should certain proteins prove refractory to unaided insertion into the mesophase, translocon proteins can be added to facilitate the process. Because of its sticky and viscous nature, the mesophase is readily harvested for subsequent use as a system with which to perform biochemical, pharmacological and biophysical characterisation. Another consequence of the mesophase's unique rheological properties is that it lends itself to miniaturisation and to microarray-type applications for high-throughput screening. One of our immediate objectives is to use the protein-laden mesophase for direct *in meso* crystallisation. Thus, by performing cell-free expression and *in meso* crystallisation in tandem, the need to separately isolate and purify the protein is avoided rendering the process from gene to crystal highly efficient in terms of time and cost whilst eliminating the potential damaging effects of solubilising detergents. The work described in the current study is a step toward this long-term objective.

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