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Host Lipid and Temperature as Important Screening Variables for Crystallizing Integral Membrane Proteins in Lipidic Mesophases. Trials with Diacylglycerol Kinase

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Abstract

A systematic study of the crystallization of an α -helical, integral membrane enzyme, diacylglycerol kinase, DgkA, using the lipidic cubic mesophase or *in meso* method is described. These trials have resulted in the production of blocky, rhombohedron-shaped crystals of diffraction quality currently in use for structure determination. Dramatic improvements in crystal quality were obtained when the identity of the lipid used to form the mesophase bilayer into which the protein was reconstituted as a prelude to crystallogenesis was varied. These monoacylglycerol lipids incorporated fatty acyl chains ranging from 14 to 18 carbon atoms long with *cis* olefinic bonds located toward the middle of the chain. Best crystals were obtained with a lipid that had an acyl chain 15 carbon atoms long with the double bond between carbons 7 and 8. It is speculated that the effectiveness of this lipid derives from hydrophobic mismatch between the target integral membrane protein and the bilayer of the host mesophase. Low temperature (4 °C) worked in concert with the short chain lipid to provide high quality crystals. Recommended screening strategies for crystallizing membrane proteins that include host lipid type and low temperature are made on the basis of this and related *in meso* crystallization trials.

Keywords

α -helical membrane protein structure; DAGK; DgkA; integral membrane enzyme; lipid cubic phase; screen; mesophase; sponge phase; X-ray crystallography

1. Introduction

Crystallizing membrane proteins for use in macromolecular X-ray crystallographic structure determination is a challenge. Despite the many crystallogenesis methods that are available, the overall success rate, as judged by numbers of published structures, is still quite low by comparison with water-soluble proteins. A method that is growing in popularity, in part because of its success with G protein-coupled receptors, makes use of a lipidic mesophase, the cubic phase in particular. This has variously been referred to as the lipidic cubic phase or *in meso* method. In addition to proving useful with receptors and receptor complexes the *in meso* method has been shown to be a generally effective approach having provided structures for at least seven distinct membrane protein classes.¹ These include bacterial and

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eukaryotic rhodopsins, a light harvesting complex, photosynthetic reaction centers, cytochrome oxidases, β -barrels, an exchanger, and an integral membrane peptide.¹

The cubic phase is a lyotropic liquid crystal. It consists of a highly curved lipid bilayer, the mid-plane of which is draped over a periodic minimal surface with cubic symmetry. The bilayer separates two interpenetrating but non-contacting aqueous channels. Both the aqueous and bilayer compartments are continuous in three dimensions. Accordingly, the mesophase is described as being bicontinuous (Fig. 1). A bicontinuous mesophase of this type is particularly appealing as an environment in which to carry out crystallization because it offers the prospect that the process takes place from within a lipid bilayer, akin to the native milieu encountered in a biomembrane. This is in contrast to the more traditional *in surfo* crystallogeneses methods which involve using potentially destabilizing surfactant or detergent micelles. Crystallogeneses by the *in meso* method has been proposed to involve a precipitant-induced local destabilization of the bicontinuous in favour of a lamellar phase (Fig. 1). It is into this planar, bilayered domain that the proteins partition preferentially and concentrate locally in a way that leads to nucleation and crystal growth.²

The benchmark lipid used to create the hosting bilayer and mesophase in which crystallization occurs is the monoacylglycerol (MAG), monoolein, also referred to as 9.9 MAG¹. Despite the fact that MAGs are not common membrane lipids some do form a bilayer into which the target membrane protein can be reconstituted as a prelude to *in meso* crystallization. Further, it has been noted that monoolein is unlikely to be the preferred (universal) hosting lipid for all crystallogeneses targets given that such proteins derive from native membranes with diverse biochemical and biophysical characteristics such as hydrophobic thickness, fluidity, and lipid and protein composition. For this reason, a host lipid screen was developed recently for use with integral membrane proteins of the β -barrel type.³ The screen includes MAGs that vary in fatty acyl chain length and in the position of the *cis*-olefinic bond along the chain. A requirement for the development of a successful screen was that all MAGs on the panel form the cubic phase under conditions of full hydration at 20 °C which are those that prevail for most crystallization trials.

Here, we describe an extension of the *in meso* lipid screen concept to α -helical proteins which, in nature, overwhelmingly outnumber their β -barrel counterparts. The target used in this study is diacylglycerol kinase, DgkA. It is a small, hydrophobic enzyme that resides in the plasmamembrane of *Escherichia coli* and is responsible for the Mg^{2+} -ATP-dependent synthesis of phosphatidic acid (PA) from diacylglycerol (DAG). DgkA plays a role in the production of membrane-derived oligosaccharide for the periplasm and of lipopolysaccharide for the outer membrane.⁵ The protein exists as a trimer, each subunit of which has three transmembrane helices. Two of these extend directly out of the membrane by about 10 residues. The third bends on exiting the bilayer to form a membrane-associated amphiphilic helix, up to 28 residues long, that is prone to motional disorder. This structure description is based on a recent solution NMR model.⁶ The latter is a backbone only structure and is incomplete in the sense that the first 25 residues or so are disordered, as noted. We are in pursuit of a high-resolution crystal structure for DgkA to complement and to extend the solution NMR work. Considerable progress in this regard has been made using

¹The bulk of the *in meso* crystallogeneses performed to date employs MAGs containing *cis*-monounsaturated fatty acids. A shorthand system for describing the chemical constitution of these MAGs makes use of the N.T notation introduced previously.⁴ This is based on a rather simplistic view of the MAG molecule as an object consisting of a head, a neck, and a tail with the latter two joined by a trunk. The head is the glycerol head group. It is in ester linkage to the neck corresponding to that part of the acyl chain extending from its carboxyl carbon to the first carbon of the olefin. The trunk is the *cis*-double bond. The tail extends from the second carbon of the olefin to the chain's methyl terminus. In the N.T MAG notation, N and T correspond to the number of carbon atoms in the neck and tail, respectively. The total number of carbon atoms in the chain is the sum of N and T. Thus, 11.7 MAG represents monovaccenin, a MAG with a fatty acyl chain 18 carbon atoms long where the *cis*-double bond resides between carbon atoms 11 and 12. It is an olefinic isomer of 9.9 MAG also known as monoolein.

the *in meso* method for crystallization. More traditional, surfactant-based or *in surfo* methods have been used to crystallize DgkA but structure quality crystals by this solution approach have not materialized. The objective now is to use the *in meso* method to produce a structure for a liganded DgkA which is in progress.

In this paper we describe the practical approaches and strategies used to succeed in producing diffraction quality crystals of DgkA by the *in meso* method. Briefly, this began with a broad screen based on the benchmark lipid, monoolein. Low temperature was explored as a screening tool and, for the first time, has been shown to work despite the fact that the cubic phase formed by monoolein is thermodynamically unstable below 17 °C. To this end, the intrinsic capacity of the cubic phase to undercool and the extraordinarily robust nature of the ‘metastable’ mesophase were exploited. However, significant progress toward the goal of producing large, diffraction quality crystals of the kinase was realized only upon screening systematically with host lipids. In all, seven MAGs were screened at 4 and 20 °C. The condition producing crystals that are being used for on-going structure work included 7.8 MAG as the hosting lipid with trials performed at 4 °C.

2. Material and Methods

2.1. Materials

Monoolein (9.9 MAG, lots M239-JY27-T, M239-F15-U, M239-M27-U), diolein (lot D236-MA10) and monopalmitolein (9.7 MAG, lot M219-JY22-N) were purchased from Nu-Chek Prep (Elysian, MN). 7.7 MAG (lot DY-4-65), 7.8 MAG (lots SS2-71, Tas12, Tas21, ACN15, Tas53), 8.7 MAG (lot SS3-23), 8.8 MAG (lot JMM2-61-012804), 8.9 MAG (lot JMM-1-73) and 7.9 MAG (lot JMM2-16a) were synthesized and purified in-house following established procedures.^{7,8} Empigen BB (Cat. 45165, lot 1384611 33408313), DL-dithiothreitol (DTT) (Cat. 43815, lot 1333018 63007006), magnesium acetate (Cat. M0631, lot 069K03391), tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Cat. C4706, lot 030M1523), imidazole (Cat. I0250, lot 068K5303), DNase I, (Cat. DN2, lot 056K7680), lysozyme (Cat. L6876, lot 094K1454), Tris (Cat. T8,760-2, lot 1133AH-437), lithium chloride (Cat. 62486, lot 134700814107043), lithium hydroxide (Cat. 402974, lot MKBC0399), HEPES (Cat. H3375, lot 038K5429), EDTA (Cat. E5134, lot 087K0049), EGTA (Cat. 03777, lot 0001426960), butylated hydroxytoluene (BHT) (Cat. W21,840-5-K, lot 15796PH-478), phenylmethanesulfonyl fluoride (PMSF) (Cat. 78830, lot 1419691 31509037), chloroform (Cat. 650496, lot 09690HJ), hydrochloric acid (Cat. 258148, lot. 1403487 23808281), phosphomolybdic acid (Cat. 31,927-9, lot 50281-448), MPD (Cat. 68340, lot 1261783 32007025), sodium nitrate (Cat. 71752, lot. 0001416801, KNO₃ (Cat. 204110, lot. 29298LJ), sodium citrate tribasic (Cat. S4641, lot. 047K0097), sodium chloride (for crystallization, Cat. 71376, lot. 0001429188), β , γ -methyleneadenosine 5'-triphosphate disodium salt (AMP-PCP) (Cat. M7510, lot. 068K5050) were obtained from Sigma (St. Louis, MO). SDS-PAGE (Cat. NP0302) and MES SDS running buffer (Cat. NP0002) were purchased from Invitrogen (Carlsbad, CA). Acetone (Cat. A/0606/7, lot 095939), methanol (Cat. M/4056/PB17, lot 1074898), and sodium chloride (Cat. BP358-10, lot 060875) were from Fisher Scientific (Loughborough, UK). 1,1',2,2'-tetraoleoyl-cardiolipin (Cat. 710335P, lot. 181CA-44), 1-(9,10-dibromooctadecanoyl)-*rac*-glycerol (18:0 dibromo monoglyceride, bromo-MAG, Cat. 790777, lot 180BRMG-15) were from Avanti Polar Lipids, Inc. (Alabaster, AL). n-Decyl- β -D-maltopyranoside (DM, Cat. D322LA, lot 120455) was from Affymetrix (Santa Clara, CA). Ni-NTA resin (Cat. 1018142, lot 136231239) was from Qiagen (Hilden, Germany). Isopropyl β -D-1-thiogalactopyranoside (IPTG) (Cat. MB1008, lot A21345), glycerol (Cat. G1345, lot A21344), and ampicillin (Cat. A0104, lot 20614) were purchased from Melford Labs (Ipswich, UK). 96-well plates (Cat. 265301, lot. 655700; Cat. 265302, lot. 658021) were purchased from Nunc (Roskilde, Denmark). *Nco*I (Cat. R0193) and *Eco*RI (Cat. R0101) were from New England Biolabs (Ipswich, MA).

Crystallization screens: Index HT (Cat. HR20144, lot 214407), MemFac HT (Cat. HR2-137, lot 213103-24-22), StockOption Salt (Cat. HR2-245, lot 224508), StockOption pH screen (Cat. HR2-241, lot 224111) and Additive Screen HT (Cat. HR2-138, lot 213803) were purchased from Hampton Research (Aliso Viejo, CA); JBScreen Membrane HTSS (Cat. JBS 00011630, lots 2004/01 and 2003/02) was from Jena Bioscience GmbH (Jena, Germany); MemGold (Cat. MD1-39, lot 004-1-39), HT96 PACT Premier (Cat. MD1-36, lot BN002) and MemStart & MemSys (Cat. MD1-33, lot 011121) were from Molecular Dimensions (Newmarket, Suffolk, UK); Wizard I, II and III (Cat. EBS-WIZ-1/2/3, lot EBS 0006152009299) were from Emerald Biosystems (Bainbridge Island, WA); Mbclass (Cat. 130711) was obtained from Qiagen (Hilden, Germany). Syringes (Cat. 81030) were sourced from Hamilton (Bonaduz, GR, Switzerland). Superdex 200 16/60 was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Amicon Ultracel-50 membrane concentrators (Cat. UFC905008) were from Millipore (Billerica, MA). All buffers were prepared using Milli-Q water (Cartridge Cat. PROG000S2, lot F9HN95000, Filter Cat. MPGL04001, lot F5PN18060) (Millipore, Billerica, MA).

2.2. Methods

2.2.1. Molecular Biology, Protein Production and Purification—The *dgkA* gene was synthesised (Genscript, Piscataway, NJ) based on the DNA sequence of *dgkA* in *E. coli* K12 and was cloned into pTrcHisB (Cat. V360-20, Invitrogen, Carlsbad, CA) using *NcoI* and *EcoRI*. The amino acid sequence of the expressed, wild-type (WT) protein is the same as that reported for pSD0005 by Bowie,⁹ where the N-terminal methionine is replaced by a hexa-His tag-containing decapeptide (MGHHHHHHEL) to facilitate purification. The coding sequence of a thermostable mutant variant of DgkA⁹ was also synthesised and cloned, as described above. This mutant, referred to as $\Delta 4$ DgkA, incorporated four mutations with respect to WT DgkA, as follows: I53C, I70L, M96L, and V107D.

DgkA production and purification, primarily from inclusion bodies, were carried out following published procedures with minor modifications.^{10,11} Briefly, BL21 cells carrying the pTrcHisB-DgkA plasmid and grown in Luria-Bertani broth were induced at an absorbance at 600 nm of 0.6 for 3 h at 37 °C with 1 mM IPTG. Biomass was harvested by centrifugation at 5,000 g for 15 min at 4 °C. The cell pellet from 1 L of culture was suspended in 0.1 L of Lysis Buffer (0.2 mM TCEP, 0.3 M NaCl, 0.2 mg/mL lysozyme, 50 μ g/mL DNAase, 10 μ M BHT, 1 mM PMSF (toxic), 0.2 mM EDTA, 5 mM MgCl₂, 75 mM Tris/HCl pH 7.8) and gently stirred at 25 °C for 30 min. The suspension was cooled on ice and cells were broken using a probe sonicator at 4 °C for 10 min with a 0.3 s on/0.7 s off duty cycle and a power setting of 30 % (Model HD2200, Probe KE76, Bandelin, Berlin, Germany). The zwitterionic detergent, Empigen BB, was added to the cell lysate at 3 % (w/v) to solubilize the protein from inclusion bodies and membrane fragments. All subsequent steps were carried out at 4 °C unless otherwise noted. After 30 min of mixing at 10 rpm on a Stuart SB3 rotator (Bibby Scientific Ltd., Staffordshire, UK), the solubilized protein was separated from cell debris and insoluble material by centrifuging the detergent-treated lysate at 9,000 g for 10 min. To the supernatant was added 4 mL Ni-NTA resin pre-equilibrated with 1.5 % (w/v) Empigen BB in Buffer A (0.3 M NaCl, 0.2 mM TCEP, 40 mM HEPES pH 7.5). After 30 min incubation in the SB3 rotator, the resin was placed in a gravity flow column (Cat. 732-1010, Bio-Rad, Hercules, CA). The resin was washed with 50 mL 10 μ M BHT, 3 % (w/v) Empigen BB in Buffer A, followed by a 50 mL wash with 40 mM imidazole and 1.5 % (w/v) Empigen BB in Buffer A. The Empigen BB detergent was exchanged to DM by washing the column with 60 mL of 0.25 % (w/v) DM in Buffer B (50 mM LiCl, 0.2 mM TCEP, 20 mM HEPES pH 7.5). Protein was eluted with 0.25 M imidazole/HCl pH 7.5 and 0.5 % (w/v) DM in Buffer C (1 mM TCEP, 50 mM LiCl, 10 mM HEPES pH 7.5) and concentrated to 12 mg/mL using a concentrator (Amicon Ultracel-50 membrane, Cat.

UFC905008, Millipore, Billerica, MA). The sample was then subjected to a size exclusion gel filtration on a Superdex 200 16/60 column equilibrated with Gel Filtration Buffer (0.25 % (w/v) DM, 0.1 M NaCl, 1 mM TCEP, 10 mM Tris/HCl pH 8.0) attached to an AKTA FPLC system (GE Healthcare). The protein was eluted at 76.2 mL as a symmetric peak. Peak fractions were pooled (8 mL total) and concentrated to 12–16 mg/mL using a spin concentrator, as above. The protein was aliquoted into PCR tubes at 10 μ L/tube (0.2 mL PCR tube, Cat. 72.737.002, Sarstedt, Numbrecht, Germany), flash-frozen in liquid nitrogen and stored in -80°C . Protein concentration was determined by measuring absorbance at 280 nm ($\epsilon_{1\text{ mg/ml}} = 2.1$)¹⁰ in a NanoDrop 1000 spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE). Protein purity was estimated at >95 % based on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using unheated protein. The typical yield was 15 mg of purified DgkA per liter of culture. The same procedure was used to produce pure $\Delta 4$ DgkA.

2.2.2. *In surfo* Crystallization—*In surfo* sitting drop crystallization trials were set up in MRC 2-well crystallization plates (Cat. HR3-083, Hampton Research) using a Mosquito robot (TTP LabTech Ltd. Royston, UK). 100 nL of protein at 16 mg/mL and 100 nL of precipitant solution were combined in a single drop over a reservoir containing 70 μ L precipitant solution. The plate was sealed with ClearSeal Film (Cat. HR4-521, Hampton Research) and incubated at 20°C . Precipitant kits used for screening included MemGold, MemSys & MemStart, and MembFac.

2.2.3. *In meso* Crystallization—*In meso* crystallization trials began with an initial reconstitution of the protein into the bilayer of the lipid cubic mesophase. This was done following a standard protocol.^{12,13} The stock protein solution, usually at 6–16 mg/mL, was homogenized with monoolein (9.9 MAG) in a coupled syringe device¹⁴ at room temperature (RT, $20 - 22^{\circ}\text{C}$) using two volumes of protein solution for every three volumes of lipid. For 7.7 MAG, 7.8 MAG and 8.7 MAG, which have slightly different phase behaviours compared to monoolein, the volume ratio used was one-to-one. With 7.9 MAG, 8.8 MAG, 8.9 MAG and 9.7 MAG, the corresponding cubic phases were prepared as for monoolein.

Crystallization trials were set up by transferring 50 nL of the protein/lipid cubic mesophase onto a silicized 96-well glass crystallization plate which was subsequently covered with 0.8 or 1 μ L precipitant solution using an *in meso* robot.^{15,16} Wells were sealed with a glass coverslide. The glass sandwich plates were stored in incubators/imagers (RockImager RI54 at $4 - 6^{\circ}\text{C}$, RockImager RI1500 at 20°C , Formulatrix, Inc., Waltham, MA) for crystal growth. Crystallization progress was monitored automatically in the two imagers and manually using normal and polarized light microscopy (Eclipse E 400 Pol, Nikon, Melville, NY).

Commercial crystallization screens are, by and large, designed for use with traditional *in surfo* methods such as vapour diffusion and batch. In such applications, the screens are prepared as concentrated solutions to compensate for the dilution (typically 1:3 to 3:1) that occurs when they are combined with protein solution in preparation for screening. *In meso* trials are performed under conditions where the precipitant undergoes little dilution (40:1; 800 nL precipitant solution, 20 nL protein solution). We have found that several of these commercial screens, at or close to full strength, destabilize the hosting lipidic mesophase and are best used, in initial trials at least, in a diluted form. Accordingly, in this study, PACT premier was used at 65 %, MemGold, Mbclass, MemSys & MemStart, Wizard I, II & III, MembFac and Index at 70 %, and JBScreen Membrane HTSS at 80 % of full strength. The diluent was Milli-Q water.

2.2.4. Crystal Harvesting—Harvesting was conducted as described.^{3,12,17} The crystallization condition for DgkA contains MPD which can trigger a conversion from the

cubic to the sponge phase.^{18,19} The sponge phase, in contrast to the cubic phase, does not stick to the glass plate. As a result, it, along with its cargo of crystals, can move to the side of the well as the coverslip is removed in preparation for crystal harvesting. Invariably when this happens the crystals are no longer visible and are lost. By wicking away most of the bathing precipitant solution before lifting the coverslip off the well it is possible to prevent the loss and for harvesting to proceed. This procedure is demonstrated in an open access on-line video.¹⁹ Once harvested, crystals were snap-cooled in liquid nitrogen without added cryo-protectant.¹⁹

2.2.5. Diffraction Data Collection—Diffraction data were collected on GM/CA CAT beamline 23ID-B, the Advanced Photon Source (APS), beamline I24, the Diamond Light Source (DLS), and PX II at the Swiss Light Source (SLS). At the APS, data were collected with a 1° oscillation and a 1 s exposure per image, a collimated beam size of 10 μm²⁰ and a sample-to-detector distance of 350–500 mm, with a MAR 300 CCD detector using 1.033 Å X-rays. At the DLS, data were collected with a 0.2° oscillation and a 0.2 s exposure per image, a microfocus beam size of 10 μm and a sample-to-detector distance of 500–650 mm, with a Pilatus 6M detector using 0.978 Å wavelength X-rays.²¹ At the SLS data were collected with a 0.1° oscillation and 0.1 s exposure per image, a collimated beam size of 30 × 15 or 10 × 15 μm² and a sample-to-detector distance of 450–600 mm, with a Pilatus 6M detector using 1.033 Å X-rays. Best diffraction to 3.1 Å was observed with crystals grown in the lipidic cubic phase prepared with 7.8 MAG (Table 1).

3. Results

3.1 Purified Protein

DgkA from *E. coli* exists as a trimer with a total molecular weight of 42 kDa.²² In this study, both the WT and Δ4 mutant forms of DgkA were produced as 131-residue proteins incorporating an N-terminal His-tag.^{11,22} Expressed protein was purified by sequential nickel affinity and size exclusion chromatography. The elution profile from the size exclusion column in both cases took the form of a symmetric, Gaussian-shaped peak with an elution volume corresponding to a trimer with an apparent molecular weight of 99 kDa (Fig. 2A, 2B). This is similar to the value reported previously^{22,23} which accounts for the presence of 46 ± 5 DM molecules per DgkA subunit in the protein-detergent micelle.²²

Purified WT and Δ4 DgkA ran on SDS-PAGE as monomers (14 kDa) and trimers (26 kDa), respectively (Fig. 2C, 2D), as observed previously.⁹ Both proteins were estimated to have a purity of >95% as judged by Coomassie-Blue staining in a loading series on SDS-PAGE (Fig. 2C, 2D). In a separate study, we have shown that the two DgkA constructs, purified in this way, are active in functional assays performed with the enzyme reconstituted into detergent micelles and into the lipidic cubic mesophase.¹¹ The protein could be concentrated producing an optically clear solution at 16 mg/mL. As such, it was deemed suitable for use in crystallization trials.

3.2. *In surfo* Crystallization

Bowie and colleagues reported that crystals of DgkA grown using standard *in surfo* methods were not of good enough quality to provide a structure.²⁵ Similar attempts were made to produce *in surfo* crystals as part of this study and indeed crystals of WT DgkA could be grown (Fig. 3). However, they diffracted to no better than 6 Å. Our next option was to explore the *in meso* method for crystallogenesis.

3.3 *In Meso* Crystallization

3.3.1 Initial Trials—Initial trials were conducted using both WT and $\Delta 4$ DgkA. Ten commercial screen kits (listed under Methods) were employed for trials carried out at 20 °C with monoolein as the hosting lipid. Within 16 hours of set up non-birefringent crystals less than 15 μm long were obtained with both isoforms (Fig. 4). Several alternative conditions were tested with a view to producing bigger crystals. These included doping the hosting lipid with cardiolipin (0.1, 0.2 and 0.5 mol%),²⁶ a known activating lipid, and with diolein (1 mol%),²⁷ a DgkA substrate, to no effect. The non-hydrolyzable ATP analog, AMP-PCP was also investigated by incubating the protein with ligand at 6 mM (7:1 by mol) ahead of the *in meso* reconstitution step. Again, this treatment did not produce bigger crystals.

In the course of initial trials, a MAG with two bromine atoms on the acyl chain (bromo-MAG) was tested, with and without AMP-PCP, with a view to aiding experimental phasing should lipid-protein co-crystals form. Bromo-MAG is very similar to monoolein in that it forms the cubic phase at 20 °C under conditions of full hydration. Further, it is entirely miscible with monoolein. Bromo-MAG had previously been shown to be a DgkA substrate¹¹ and to produce crystals of bacteriorhodopsin. However, when used with DgkA no crystals were obtained.

3.3.2. Optimization

3.3.2.1. Precipitant and Protein Concentration: As noted, initial trials produced small crystals. In addition, the mesophase boluses often included a diffuse brownish material likely to be either microcrystals or aggregated protein. This is not an uncommon observation with the *in meso* method and crystals can often be seen to grow at the expense of the non-crystalline protein.²⁸ With Dgk however, the ‘aggregated’ protein did not progress to crystals and the sense was that bigger and better diffracting crystals might be obtained by reducing precipitant and/or protein concentration and by further screening precipitant components.

To this end, the primary precipitant, MPD, was screened with both DgkA isoforms in the range from 5 to 12 % (v/v) in increments of 0.1 %. Best results, as evidenced by biggest crystals, were obtained with 7.2 – 8.2 % (v/v) MPD. Higher concentrations failed to produce crystals; lower concentrations gave smaller crystals. Sodium citrate buffer and sodium chloride concentrations were adjusted in the 0–200 mM range in increments of 25 mM. For both, 100 mM gave best crystals. pH was screened from 2.4 to 9.0 in units of 0.2 (HR2-241, Hampton Research, see Materials) and best crystals grew in the range pH 5.4 – 5.8 with sodium citrate as the buffer. Alternative buffers to sodium citrate were tested that included sodium acetate and MES in the range from pH 5.4–5.8 but the citrate buffer was found to be essential for crystal growth. Finally, protein concentration was investigated in the 6 to 16 mg/mL range. Crystals did not grow at 6 mg/mL and biggest crystals formed at 10 to 12 mg/mL.

Despite these extensive optimizations, crystals no more than 20 μm in maximum dimensions were produced.

3.3.2.2. Temperature: Temperature is an important variable that can be used to control crystal growth. However, with the *in meso* method based on monoolein as the hosting lipid, temperatures below 17 °C will, under equilibrium conditions, trigger a transition from the cubic to the lamellar crystalline phase, a solid, which is not compatible with crystallization. But the cubic phase is noted for its ability to undercool where it is thermodynamically unstable.^{29,30} Accordingly, crystallization trials were conducted with DgkA in the zone of mesophase metastability at 4 and 10 °C. As expected, crystal growth was slowed at both

temperatures. At 10 °C, crystal appeared within a day and reached maximum dimensions in about 10 days. At 4 °C, crystals did not appear until 3 days; they continued to grow to 70 μm over a period of one to two months (Fig. 5).

In all of these experiments, the cubic phase undercooled and remained as a viscous, optically clear and isotropic (non-birefringent) mesophase for the duration of the trials. This observation held in the presence and absence of membrane protein crystals. At these low temperatures, best crystals were obtained at 4 °C. However, the crystals, while long in one dimension, were diminutive in the other two dimensions. Further optimization was called for and a decision was made to do this at 4 °C.

3.3.2.3. Salts: Salt is an important screening variable for protein crystal growth and was investigated in this study. Accordingly, the precipitant solution that produced best crystals in the previous optimizations, containing 7.8 % (v/v) MPD, 0.1 M sodium chloride, 0.1 M sodium citrate pH 5.6, was doped with 0.1 M and 0.4 M salt from the Hampton Salt Screen and used as the precipitant for *in meso* trials. The Salt Screen has 49 salt conditions. All were used with the exception of sodium chloride which was already in the original precipitant solution.

This salt screen identified nitrate as a critical counter-anion. For both WT and $\Delta 4$ isoforms of DgkA, significant improvements in crystal size in a second and third dimension were obtained when nitrate was present (Fig. 6). Further optimizations of the precipitant components produced $30 \times 130 \mu\text{m}^2$ plate-shaped crystal of WT DgkA and $20 \times 20 \mu\text{m}^2$ bipyramid-shaped crystals for the $\Delta 4$ isoform (Fig. 7). Both WT and $\Delta 4$ DgkA crystals grown in the presence of nitrate diffracted to 3.7 Å.

3.3.2.4. Host Lipids: The *in meso* method was developed using monoolein as the hosting lipid. As noted previously, there is no good reason that it should work for all membrane protein targets that have different sizes and topologies and come from native membranes with very different physical, chemical and biochemical characteristics. This issue was examined in a systematic way with a membrane protein of the β -barrel type, OprB. In this case, monoolein was indeed not the preferred lipid. The shorter chained lipid, 7.8 MAG, produced best crystals in terms of size and diffraction quality.³ In the current study, we set out to investigate how DgkA, an α -helical membrane protein, responds to hosting lipid identity from the point of view of crystal quality. For this purpose, seven MAGs (7.7, 7.8, 7.9, 8.7, 8.8, 8.9, 9.7) that differed in terms of acyl chain length and *cis* double bond position along the chain were tested with the $\Delta 4$ isoform of DgkA at 4 and 20 °C. At 20 °C, only micro-crystals grew in 7.8, 8.9 and 9.7 MAG and usually in a background of ‘aggregated’ protein (Fig. 8A–C). However, at 4 °C, 7.8, 8.7, 8.9 and 9.7 MAG produced bipyramid crystals (Fig. 8D–G). While 8.7, 8.9 and 9.7 MAG gave the same diminutive crystals as monoolein, 7.8 MAG produced considerably larger crystals in the 50–100 μm range. Optimization of the precipitant conditions for 7.8 MAG yielded crystals that diffracted to 3.1 Å (Fig. 8G, Fig. 9). This result indicates that host lipid screening is important not only for β -barrel but also for α -helical membrane proteins.

4. Discussion

4.1. From First Hit to Diffraction Quality Crystals

The objective of this study was to produce diffraction quality crystals of DgkA for use in high resolution structure determination with a view to establishing the mechanism of action of this atypical kinase. In addition to the WT isoform of the enzyme, the thermostable mutant $\Delta 4$ was included in the investigation on the basis of the assumption that the more stable the construct the more amenable it is to crystallogenesis.^{31,32} Initial trials were

performed using the traditional *in surfo* vapour diffusion method and crystals were obtained. Unfortunately, as observed by others, these diffracted poorly and were not suitable for structure determination. DgkA is a relatively hydrophobic integral membrane protein. Given the success of the *in meso* method with such targets,^{1,17} it was decided to subject the enzyme likewise to *in meso* crystallogenesis. First trials with the benchmark hosting lipid, monoolein, at 20 °C produced crystals 15 µm in size. This initial hit formed the basis of subsequent work that eventually led to large, diffraction quality crystals (Fig. 8G).

DgkA uses DAG as a lipid substrate. At the same time it is activated by the anionic phospholipid, cardiolipin. In anticipation that either lipid might stabilize the enzyme thereby facilitating crystallogenesis they were included separately in the hosting mesophase. Neither produced a significant improvement in crystal size or visual quality. The ATP analog, AMP-PCP, was also tested as a potentially stabilizing ligand. Again, no improvement in crystal quality was observed. Bromo-MAG, a brominated variant of monoolein, was investigated as an alternative hosting lipid that, if it produced co-crystals, could possibly be used for experimental phasing. In this instance, the protein failed to produce any crystals.

Next our attention turned to the precipitant solution that provided the initial hit. MPD was its main component and a focused screen identified a particular MPD concentration range that gave best crystals. The sodium citrate and sodium chloride ingredients were similarly evaluated and optimum concentrations identified. Proton concentration was screened over seven pH units and best quality crystals were identified to form in the range pH 5.4–5.8. Initial trials were performed at a protein concentration of 16 mg/mL which, based on the appearance of ‘aggregated’ protein in the mesophase bolus, suggested that lower concentrations should be investigated. This led to the identification of 10–12 mg/mL as the optimum DgkA concentration. At this stage in the process, best crystals measured 20 µm in maximum dimension.

Our next attempt at optimization focussed on temperature. Temperatures below ambient were screened somewhat tentatively in the knowledge that the cubic mesophase, prepared with the benchmark lipid, monoolein, turns to a solid under equilibrium conditions below 17 °C. However, we were aware of the capacity of the mesophase to undercool to quite low temperatures. Accordingly, trials were set up at 4 and at 10 °C. Fortunately and as anticipated, the mesophase remained ‘metastable’ at these low temperatures and crystals of DgkA grew but at a considerably slower pace. Best crystals, as 70 µm long needles, were obtained at 4 °C.

The needles referred to, while large in one dimension, were thin. For diffraction measurements, ideally, crystals with a reasonable second and third dimension are required. This was realized on the basis of a systematic salt screen where 48 salts at two concentrations were investigated. In the process, nitrate was identified as an important anion. When included in the screen, crystals with bulk in two and three dimensions were obtained for WT and Δ4 DgkA, respectively. All subsequent optimizations included nitrate in the precipitant solution.

At this juncture, it was decided to perform a host lipid screen where the identity of the MAG used to create the mesophase in which crystallization takes place is varied. This approach proved very successful with the β-barrel membrane protein, OprB. In separate studies, shorter chain MAGs had been shown to work with integral membrane protein complexes^{28,33} and with the pentadecapeptide, gramicidin.¹⁷ Of the seven MAGs tested with Δ4 DgkA at 4 and 20 °C, 7.8 MAG at 4 °C really stood out producing crystals with thickness in all three dimensions. Upon further optimizing the components of the corresponding precipitant solution, this condition produced large, blocky crystals of Δ4

DgkA that diffracted to 3.1 Å. These are now being used for the structure determination of DgkA.

4.2. Low Temperature *In Meso* Crystallogeneses

To our knowledge, this is the first documented report of the use of low temperature to produce membrane protein crystals of diffraction quality by the *in meso* method. As noted, for crystals to grow *in meso* the metastable or undercooled state must be maintained, at least in the case of the more commonly used hosting MAGs. This propensity to undercool is a characteristic of these liquid crystalline materials and the property that was exploited here.

While metastability has an element of uncertainty associated with it, the message from the current work is clear: Temperature should be used as a screening tool for crystallization by the *in meso* method regardless of host lipid identity. But trials conducted at reduced temperatures must be done in the knowledge that the mesophase could transform to a phase that is not compatible with target protein crystallogeneses. Experience to date however suggests that such a transformation is a low probability event. If it does happen it is painfully obvious; the mesophase turns completely opaque and is birefringent.

It is important to keep in mind that the effect of lowering temperature is not just to slow motion and transport processes, such as diffusion. Temperature also influences the hydration of the mesophase which increases as temperature is reduced. Simultaneously, the bilayer thickens and the diameter of the aqueous channels in the mesophase rises. These changes in phase microstructure will, in turn, impact on crystallogeneses by altering the mobility of the protein in the plane of the membrane and the protein's disposition across the lipid bilayer.

Separately, we have used rational approaches to design a lipid, 7.9 MAG, that is thermodynamically stable in the cubic phase at low temperatures.³⁴ With 7.9 MAG therefore, metastability is not an issue and low temperature trials can be conducted confidently knowing that the cubic phase is stable under such conditions. Despite the fact that a membrane protein reconstituted into the bilayer of the cubic phase is likely to be more stable than when it is dispersed in a detergent micelle, performing *in meso* trials at low temperature is expected to further enhance the stability, and by extension, the crystallizability of the protein.

4.3. Host Lipid Screening

This study highlights the importance of performing a crystallization screen based on the identity of the lipid used to create the hosting mesophase. Such a case has already been made convincingly for a β -barrel protein where 7.8 MAG was identified as the lipid of choice.³ The principle is clearly demonstrated here as being equally applicable to an α -helical membrane protein. It so happens that with DgkA, 7.8 MAG was the preferred hosting lipid also, but in this case at 4 °C.

The different MAGs that go to make up a host lipid screen generate mesophases with distinct bilayer thicknesses, polar/apolar interfacial curvatures and aqueous channel dimensions that, as noted, are temperature dependent and tightly coupled to one another. We note that DgkA has a predicted hydrophobic thickness of 25.5 Å³⁵ based on its solution NMR structure.⁶ The bilayer thickness of the cubic phase formed by 7.7 MAG and 9.9 MAG at 20 °C is estimated to be 28.1 Å and 35 Å, respectively.³⁶ No such mensuration has been carried out for 7.8 MAG. However, we know from separate measurements that bilayer thickness increases by about 2 Å for every methylene in the acyl chain at ambient temperatures.³⁷ Thus, a reasonable estimate for the bilayer thickness of the cubic phase in 7.8 MAG at 20 °C is 30 Å. At 4 °C, the value will rise to 31 Å. The corresponding differential of 5–6 Å (= 31 Å – 25.5 Å) may just be enough of a hydrophobic mismatch with

respect to DgkA to favour partitioning of the trimer out of the mesophase and into a site of nucleation where direct protein-protein interactions facilitate progression into a macroscopic crystal.³⁸ A more complete evaluation of this proposal must await the determination of an *in meso* crystal structure for DgkA.

Recently, another short chain lipid, 7.7 MAG, has been used to produce crystals by the *in meso* method for the high resolution structure determination of two large membrane protein complexes. These include cytochrome *caa*₃ oxidase²⁸ and the β_2 -adrenoreceptor - Gs protein complex.³³ This prompts an obvious question, why did 7.7 MAG not work with DgkA? The answer may lie in hydrophobic matching. Thus, the bilayer thickness of the 7.7 MAG mesophase better matches the hydrophobic thickness of the protein than that of 7.8 MAG. Accordingly, when the correspondence is good the protein is stable and less inclined, from an energetic point of view, to partition out of the mesophase and into a crystal. In this scenario, the enzyme is content to remain dispersed in the mesophase where protein-protein contacts, when they occur, are transient and do not progress to nucleation and beyond. In the case of the large complexes just referred to, both have relatively extensive extramembrane domains. With 7.7 MAG, the polar/apolar interface of the hosting mesophase has less curvature and the aqueous channels are large, especially in the presence of spongifying agents such as PEG. This then provides the space in which to accommodate the bulky soluble domain and for the complex as a whole to move with relative ease in the plane of the mesophase membrane to sites of nucleation and crystal growth. By contrast, very little of the DgkA trimer is available to reside outside the membrane. For this reason, diffusion is not expected to be as restricted, and the elevated curvature of the 7.8 MAG mesophase should be tolerated to a higher degree by this relatively small and apolar complex.

The bulk of the MAGs used in this study were synthesized and purified in-house. The Reader may surmise therefore that they are not generally available. However, following on from the success of these alternative host lipids in recent *in meso* crystallization and structure determination of important membrane protein targets, several are commercially available. For those that are not, detailed and robust synthesis and purification protocols have been published.^{8,34}

4.4. Precipitants and the Sponge Phase

A statistical analysis that can be performed conveniently online using the Membrane Protein Data Bank (MPDB, www.mpdb.tcd.ie) shows that MPD is a very common precipitant ingredient used for membrane protein crystallization. As a small diol, it is quite water soluble. At the same time, it is part hydrocarbon. Together, these divergent properties engender MPD with mild surface active properties. In macromolecular crystallogenes, it functions as a precipitant, in part, by competing with the target molecule for water of hydration. MPD also affects bilayer interfacial curvature and can trigger a cubic to sponge phase transition. The latter evolves from the cubic mesophase by imbibing water. This gives rise to a mesophase that is still bicontinuous but that has significantly enlarged aqueous channels, lower polar/apolar interfacial curvature and less long range order. The sponge phase figures prominently as a mesophase in which crystallogenes of membrane proteins by the *in meso* method occurs.³⁹

In the current study, MPD emerged in initial screens as an important precipitant ingredient. In monoolein, optimum MPD concentrations ranged from 7 to 8 % (v/v). Under these conditions, the sponge phase had formed as judged by visual appearance of the mesophase bolus. Following lipid screening, when 7.8 MAG was identified as supporting good quality crystals, the optimum concentration of MPD had almost halved to ~4 % (v/v) for this shorter chain lipid. It so happens that this low MPD concentration, which is sufficient to induce the sponge phase in 7.8 MAG, does not spongify the cubic phase formed by monoolein. Further,

at 7–8 % (v/v) MPD where the sponge phase of monoolein forms, that of 7.8 MAG is no longer stable and does not support crystal formation. It appears therefore that what is important to crystallogenesis, at least in the case of DgkA, is a stable sponge phase. Thus, other effects such as competition for water, which are likely to be independent of the hosting lipid, are relevant but less critical to the crystallization process. This suggests that other spongifiers such as PEG, Jeffamine, PPO, butanediol, and potassium thiocyanate might be used to advantage in further optimizing DgkA crystals.

4.5. Magnesium Plays a Role

DgkA crystals grown in monoolein and in 7.8 MAG were obtained under slightly different conditions. With monoolein, magnesium had no effect on crystal morphology or indeed, diffraction quality. However, it proved to be essential for crystal growth in 7.8 MAG. Given the disparate microstructures of the hosting mesophases formed by these two lipids the conformation of the reconstituted protein and its response to magnesium may also be different. Interestingly, in addition to using magnesium as a counter ion component of its nucleotide substrate, DgkA requires free magnesium.⁴⁰ It is possible therefore that in 7.8 MAG but not in monoolein, the conformation of DgkA is such that it can associate with the free metal ion which stabilizes it facilitating crystallogenesis. Structures obtained with crystals grown in these two hosting lipids are needed to evaluate this proposal.

4.6. An *In Meso* Screening Strategy

The current project spanned a period of almost 3 years. The path that led to diffraction quality crystals of DgkA, for eventual use in structure determination, is summarized in Figure 10. As noted, two variants of DgkA were used; wild-type and a thermo-stabilized mutant. The figure shows that with progress through the project steady, incremental improvements were made in terms of crystal size and visual quality. The most dramatic of these happened as a consequence of low temperature and host lipid screening.

The order in which the various screening was performed in the current study can now be reflected on in light of the experience gained with DgkA and with other projects in the PI's lab. Clearly, having a thermo-stabilized and functional form of the target protein available was a definite advantage and is recommended whenever possible. Given the success we have had with host lipid screening in the current and related projects, we now routinely introduce this early on in the overall screening process. Thus, for example, an initial broad screen with the benchmark (and inexpensive) lipid, monoolein, as in Step A in Figure 10, should be performed by way of gaining familiarity with the protein and how it behaves under *in meso* crystallization conditions. If hits emerge at this stage, optimization around the relevant precipitant conditions should be undertaken and soon thereafter a host lipid screen should be performed. If no hits are obtained with monoolein but the protein is behaving well in that it is providing clear mesophase without signs of aggregating, then higher protein concentrations should be tested along with an exploration of different MAGs in the form of a host lipid screen. The lipid screen should include, at the very least, 7.7 MAG, 7.8 MAG, 7.9 MAG and 8.7 MAG. As time and resources permit, the screen can be extended to include 8.8 MAG, 8.9 MAG and 9.7 MAG. Hits in any of these should be followed up on with precipitant, salt/additive and protein concentration optimizations. Soon thereafter, trials should be conducted at lower temperatures, first at 4 °C and then, or in parallel, at 10 or 12 °C. As demonstrated in this study, low temperature screening can be done regardless of host MAG identity, mindful of the forgiving nature of lipidic mesophases that conveniently undercool.

In situations where the protein target is known to have a specific lipid or cofactor requirement or can be stabilized by a ligand, these naturally should be included as part of the

screening process. The ligand or lipid can be added during protein production and purification. It might also be included with the hosting lipid during mesophase preparation as is routine now with GPCRs and other targets where cholesterol is combined with the hosting MAG at ~10 mol%.¹ The extraordinary value of screening with homologs and engineered variants of the target protein must never be lost sight of.

5. Conclusions

Host lipid screen is invaluable for α -helical and β -barrel integral membrane proteins and should be a routine part of *in meso* crystallization trials. Trials might begin with the benchmark lipid monoolein to be followed up, in short order, by screening with a series of shorter chain MAGs. MAGs suitable for this purpose have been identified. They are available commercially or can be synthesised based on published procedures. Temperature is another important screening variable that should be introduced relatively early on in the screening process. Because the cubic and sponge mesophases undercool reliably, screening at low temperatures should be investigated regardless of the host MAG, mindful that the work is probably being done under metastable conditions. Rounds of optimization focussed on the precipitant components, pH, salts/additives and protein concentration should be used judiciously throughout the process. Homologs and engineered variants of the target protein should also be considered in the quest for crystals that eventually yield high resolution structures providing insights into membrane protein mode of action and interactions.

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Fig. 1.

Cartoon representation of the events proposed to take place during the crystallization of an integral membrane protein from the lipidic cubic mesophase. The process begins with the protein reconstituted into the curved bilayer of the 'bicontinuous' cubic phase (tan). Added 'precipitants' shift the equilibrium away from stability in the cubic membrane. This leads to phase separation wherein protein molecules diffuse from the bicontinuous bilayered reservoir of the cubic phase into a sheet-like or lamellar domain (A) and locally concentrate therein in a process that progresses to nucleation and crystal growth (B). Cocrystallization of the protein with native lipid (cholesterol) is shown in this illustration. As much as possible, the dimensions of the lipid (tan oval with tail), detergent (pink oval with tail), cholesterol (purple), protein (blue and green; β_2 -adrenergic receptor-T4 lysozyme fusion; PDB code 2RH1), bilayer and aqueous channels (dark blue) have been drawn to scale. The lipid bilayer is ~ 40 Å thick. From reference³ with permission. An expanded view of the various components in the system is shown in (C).

Fig. 2.

Purification of WT and $\Delta 4$ DgkA. Size exclusion chromatographic analysis of WT DgkA (A) and $\Delta 4$ DgkA (B). V_0 and V_t mark the void and total column volumes, respectively. The elution volume (V_e) for both WT and $\Delta 4$ DgkA is 76.8 mL. The near Gaussian shaped elution profiles are consistent with a monodisperse protein preparation suitable for crystallization trials. Protein purity of the WT DgkA (C) and $\Delta 4$ DgkA (D) post-size exclusion chromatography was estimated at >95 % on the basis of a loading series analysis by SDS-PAGE and Coomassie Blue staining. The high molecular weight bands observed at the 50 μ g loading in (C) are likely oligomers of DgkA. In (C), WT DgkA migrates as a monomer at the expected molecular weight of ~ 14 kDa at low loading. At higher loading, it migrates progressively slower. This may relate to the fact that the lower loading samples were prepared by diluting concentrated samples with SDS loading buffer. Thus, the SDS/DM ratio in the latter samples and thus bound to the protein is less. This reduces the associated negative charge on the protein accounting for its slower migration on the gel. Interestingly, $\Delta 4$ DgkA was prepared in the same way as WT DgkA but did not display the same behaviour. By contrast, it migrates as a trimer with anomalously lower apparent molecular weight, as reported previously.²⁴

Figure 3.

In situ-grown crystal of WT DgkA. Crystals were grown for 10 days at 20 °C by the hanging drop vapour diffusion method using 100 nL each of reservoir and protein solutions. The reservoir solution consisted of 30 % (v/v) PEG 400, 0.1 M HEPES/Na pH 7.5. The protein solution included 16 mg DgkA/mL, 4.6 % (w/v) DM, 1 mM TCEP, 150 mM NaCl, 10 mM Tris HCl pH 7.4. Images were recorded with normal light (A) and between crossed-polarizers (B).

Fig. 4.

Initial crystallization hits observed with WT and $\Delta 4$ DgkA in 9.9 MAG at 20 °C using commercial screens. A: Day 0, WT DgkA. B: Day 1, WT DgkA. C: Expanded view of B. D: Day 0, $\Delta 4$ DgkA. E: Day 1, $\Delta 4$ DgkA. F: Expanded view of E. Brownish colored 'aggregated' protein formed almost immediately after the trials were set up. Micro-crystals appeared within about 16 h. The composition of the diluted precipitant solution is 7.8 % (v/v) MPD, 0.07 M sodium chloride, 0.07 M sodium citrate/HCl pH 5.6. Images were recorded using the RI-1500 imager (Formulatrix).

Fig. 5. Effect of temperature on the crystallization of WT DgkA in monoolein. A: 20 °C, B: 10 °C. C: 4 °C. The corresponding crystal sizes are A: 5 μm. B: 15 μm. C: 70 μm. Images were recorded 2 months post-setup. The composition of the precipitant solution is 7.8 % (v/v) MPD, 0.1 M sodium chloride, 0.1 M sodium citrate pH 5.6. Images in A and B were recorded with the RI-1500 imager at 20 °C; the image in C was recorded with the RI-54 imager at 4–6 °C (Formulatrix).

Fig. 6.

Effect of nitrate salts on the *in meso* crystallization of WT and $\Delta 4$ DgkA at 4 °C in 9.9 MAG. Crystals of WT DgkA (A–D) and $\Delta 4$ DgkA (E–H) were grown in a precipitant consisting of 7.8 % MPD, 0.1 M sodium chloride, 0.1 M sodium citrate pH 5.6 supplemented with 0.1 M nitrate salts of potassium (A, E), lithium (B, F), sodium and ammonium (D, H). A small crystal in (A) resides at the tip of the solid arrow. The dotted arrow in (A) points to one of several droplets in the mesophase that should not be mistaken for crystals. Images were recorded using the RI-54 imager at 4–6 °C.

Fig. 7.

In meso grown crystals of WT DgkA (A) and $\Delta 4$ DgkA (B) obtained at 4 °C in monoolein after extensive optimization. The precipitant in (A) contains 7.9 % (v/v) MPD, 0.08 M sodium chloride, 0.12 M potassium nitrate, 0.1 M sodium citrate pH 5.6. The condition in (B) contains 8.0 % (v/v) MPD, 0.11 M sodium chloride, 0.1 M lithium nitrate, 0.1 M sodium citrate pH 5.6. Images were recorded 60 days post-setup. Images were recorded using the RI-54 imager at 4–6 °C.

Fig. 8.

Effect of hosting lipid and temperature on the *in meso* crystallization of $\Delta 4$ DgkA. Small crystals (arrows) were grown in 7.8 MAG (A), 8.9 MAG (B) and 9.7 MAG (C) at 20 °C. Relatively large, blocky crystals grew in 8.7 MAG (D), 8.9 MAG (E), 9.7 MAG (F) and 7.8 MAG (G) at 4 °C. Images were recorded in normal light (A–F) and between crossed polarizers (G). The following precipitant conditions apply for 8.9 MAG and 9.7 MAG: 6.5–7.5 % (v/v) MPD, 0.1 M sodium chloride, 0.1 M potassium nitrate, 0.1 M sodium citrate pH 5.6; for 7.8 MAG and 8.7 MAG: 4–6 % (v/v) MPD, 60 mM magnesium acetate, 0.05–0.15 M sodium chloride, 0.1 M potassium nitrate, 0.05 M sodium citrate pH 5.6. Images in (A–C) were recorded using the RI-1500 imager at 20 °C while images in (D–G) were taken using a Nikon Coolpix 4500 camera attached to an Eclipse E 400 Pol microscope at 4–6 °C.

Fig. 9. X-ray diffraction from a $\Delta 4$ DgkA crystal grown *in meso* in 7.8 MAG at 4 °C. Data were recorded on a Pilatus 6M detector with 0.978 Å wavelength X-rays, a 0.2 ° oscillation and a 0.2 s exposure per image, a micro-focus beam size of 10 μ m and a sample-to-detector distance of 645.6 mm.

Fig. 10.

Steps taken and the order in which they were done to effect the production of diffraction quality crystals of DgkA by the *in meso* method. Wild-type and thermo-stabilized mutant variants of the kinase were used in the study. Square brackets refer to concentration.

Table 1Data collection statistics for a $\Delta 4$ DgkA crystal grown in 7.8 MAG.

Beamline	I24, Diamond Light Source
Detector	Pilatus 6M
Wavelength (Å)	0.978
Crystal-to-detector distance (mm)	645.6
Rotation range per image (°)	0.2
Exposure time per image (s)	0.2
Resolution (Å)	66.33 – 3.10 (3.18 – 3.10) ^a
Space group	P3 ₁ 21
Unit cell parameters (Å, °)	$a = b = 72.74$ $c = 199.00$, $\alpha = \beta = 90$, $\gamma = 120$
Total number of observations	45,293
Number of unique reflections	11,033
Completeness (%)	94.8 (87.6)
Multiplicity	4.1 (3.5)
$I/\sigma(I)$	10.7 (2.0)
Overall B factor from Wilson plot (Å ²)	101.79
R_{merge}	0.067 (0.423)
R_{pim}	0.036 (0.262)

^aValues in parentheses are for the high resolution shell