Crystal structure of the integral membrane diacylglycerol kinase

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Diacylglycerol kinase catalyses the ATP-dependent phosphorylation of diacylglycerol to phosphatidic acid for use in shuttling water-soluble components to membrane-derived oligosaccharide and lipopolysaccharide in the cell envelope of Gram-negative bacteria¹. For half a century, this 121-residue kinase has served as a model for investigating membrane protein enzymology⁴–⁶, folding⁷,⁸, assembly⁹–¹² and stability¹³–¹⁴. Here we present crystal structures for three functional forms of this unique and paradigmatic kinase, one of which is wild type. These reveal a homo-trimeric enzyme with three transmembrane helices and an amino-terminal amphiphilic helix per monomer. Bound lipid substrate and docked ATP identify the putative active site that is of the composite, shared site type. The crystal structures rationalize extensive biochemical and biophysical data on the enzyme. They are, however, at variance with a published solution NMR model¹⁴ in that domain swapping, a key feature of the solution form, is not observed in the crystal structures.

Diacylglycerol kinase (DgkA) is a unique enzyme, structurally and functionally. A solution NMR model of wild-type DgkA revealed a compact, domain-swapped trimer¹⁴. This, along with extensive biochemical and biophysical data, was used to rationalize its stability, folding, active site architecture, catalytic mechanism and substrate selectivity. Because it was a backbone-only model, with motional disorder over its first 25 residues, there was a need for a high-resolution crystal structure. Traditional crystallization approaches had failed to produce quality crystals¹⁵. Given the success of the lipidic cubic phase (in meso) method with similarly sized proteins¹⁶, it was considered a worthwhile crystallization strategy.

Initial trials yielded minute crystals with monoolein (9.9 MAG) as host lipid at 20 °C. ROUNDS of optimization with shorter chain monoacylglycerols (MAGs)¹⁷,¹⁸ using rationally designed mutant forms of the kinase provided quality crystals at 4 °C. Molecular replacement with the published model¹⁴ as a template failed to provide a solution. To phase the X-ray data, seleno-methionine (Se-Met), as well as heavy-atom pre-labelling, co-crystallization and soaking, and engineered single-cysteine mutants for mercury labelling, were tested. Phases were obtained using Se-Met single-wavelength anomalous diffraction (SAD) from crystals of a stable active mutant, Δ7 (seven changes relative to wild type; Supplementary Figs 1 and 2). Structures for the wild type and a thermo-stabilized Δ4 (four changes relative to wild type)¹⁹ form of DgkA were obtained by molecular replacement using the Δ7 structure (Methods). Here, we report on structures of the wild-type, Δ4 and Δ7 constructs obtained with 7.8 MAG. The highest resolution was for Δ7 DgkA at 2.05 Å (Supplementary Table 1).

The crystal structure of all three constructs reveals a trimer with layered packing²⁰ (Fig. 1 and Supplementary Fig. 3). Across constructs, subunits and folds are alike (Supplementary Figs 4–6). This, coupled
with the fact that the three constructs are active (Supplementary Fig. 2), indicates that all three are structurally and functionally similar. Δ4 provided the most complete model and is used for the structure description that follows.

Each subunit (identified A–C) within a trimer forms a bundle of three transmembrane helices, H1–H3 (Fig. 1 and Supplementary Fig. 7). When viewed from the cytosol, the centres of mass of the three helices within a subunit roughly describe an isosceles triangle (Fig. 1c). H1 is the shortest at 19 residues. It is preceded by an N-terminal amphiphilic surface helix (Supplementary Fig. 6b), which is expected to reside on the cytosolic side of the membrane. H2 and H3 extend into the cell by about ten residues and are connected by a ~five-residue cytosolic loop, likely to be quite mobile (Supplementary Fig. 5). On the other side of the membrane, H1 and H2 are connected by a four-residue periplasmic loop.

The three subunits are arranged around an approximate three-fold symmetry axis that passes through the centre of the trimer normal to the membrane plane (Fig. 1c). The core of the trimer is made up of H2 from each subunit forming a parallel three-helix bundle. Extending away from the core, H1 and H3 from each subunit form the sides of an equilateral triangle that inscribes the trimer with H1 occupying apex positions. Viewed from the cytosol, the surface helix, which is N-terminal to H1, angles away from the trimer core contacting H3 from an adjacent subunit. Together, the surface helix N terminus of one subunit and H1–H3 of an adjacent subunit create the putative active site (Fig. 1). Thus, for example, active site AB (asAB) is formed by surface helix (SH)A, H1B, H2B and H3B, consistent with the composite, shared site model5.

Separate studies have shown that the DgkA trimer has three active sites6, with moderate heterololostry1,3 (Supplementary Discussion). The enzyme has one amphiphilic substrate, diacylglycerol (DAG), and a water-soluble substrate, Mg2+-ATP. Thus, DgkA catalyses phosphoryl transfer expected to take place at a polar/apolar interface. Given that the reaction involves direct phosphorylation1 via a pentavalent transition state, the two substrates must come into close proximity with the γ-phosphate of ATP next to the primary hydroxyl of DAG. There is only one location (per active site) on the kinase where this can happen. That is in the pocket created by the surface helix of one subunit and the polar/apolar regions of H1–H3 of an adjacent subunit (Fig. 1). This prediction matches well the active site residues mapped in previous studies1,4 (Fig. 2).

DgkA is a promiscuous enzyme. It can work with different hydroxy lipids as substrates, including the MAGs used to create the mesophase for crystallization19-21. One of the putative active sites in Δ7 includes density well described by MAG (Fig. 3 and Supplementary Fig. 8). The lipid head-group resides at the predicted membrane polar/apolar interface. Its acyl chain extends away from the interface into the hydrophobic membrane core next to H3. This tentatively identifies the lipid substrate half of a DgkA active site.

DgkA is a unique kinase with a distinctive active site. It has no recognizable nucleotide sequence or structural binding motifs22,23. With a view to identifying the ATP-binding site, crystals of DgkA were soaked with the ATP analogue, adenylyl methylidiphosphonate (AMPPCP). This caused the crystals to ‘dissolve’ (Supplementary Fig. 9). Additional soaking with ATP, ADP, AMP, ATP/S, AMPNP and dADP, but not with GTP, CTP, UTP or TTP, led to crystal dissolution (Supplementary Fig. 9). Thus, although a liganded structure did not materialize, the result is consistent with an adenine nucleotide-induced conformational and/or packing change.

![Figure 2](image2.png)

Figure 2 | Rationalizing functional biochemistry with the crystal structure of DgkA. a Residues in DgkA, in which mutations to cysteine reduce kinase activity to 7% or less that of wild-type activity4, are mapped onto the crystal structure of Δ4 DgkA. View in the membrane plane. The protein (ribbon model) is displayed with chains A, B and C coloured blue, green and orange, respectively. Residues are shown in ball and stick and colour-coded according to atom type (nitrogen, blue; oxygen, red; carbon, grey). b View from the cytosol. c An expanded view of the putative active site (asAB) of the enzyme, in which crucial kinase activity residues4,14 are clustered.

![Figure 3](image3.png)

Figure 3 | Putative active site of DgkA complete with lipid and Mg2+-ATP substrates and activating zinc. a A putative active site of Δ4 DgkA is shown superposed with 7.8 MAG lipid substrate, the zinc ion from Δ7 DgkA, and Mg2+-ATP from the docking calculation. The ATP (thick sticks, atoms coloured by type) extends ~20 Å from the cytoplasmic loop between H2 and H3 to the polar headgroup of the lipid substrate (large spheres, atoms coloured by type) at the polar/apolar interface of the membrane. Residues in the vicinity of the substrates that are known to be crucial for kinase activity4,14 are shown in ball and stick, as in Fig. 2. b Expanded view of a, with the ribbon model removed for clarity.
in the crystalline kinase. These findings support the view that the kinase adopts a functional form in the crystal.

In the absence of a nucleotide-bound structure for DgkA, ATP has been docked into the Δ7 model with the adenyly moiety interacting with the cytosolic loop (Fig. 3, Supplementary Discussion and Supplementary Fig. 10). The triphosphate extends in the direction of the lipid-binding region of the putative active site, occupied by a lipid molecule. The observation of distinct binding sites, one (modelled) for ATP and one for the lipid substrate, is consistent with the finding that the mechanism of phosphoryl transfer is of the random-equilibrium type, in which the two substrates bind independently of one another. For full activation, DgkA requires free divalent cation (magnesium, manganese, cadmium, zinc or cobalt). Here, zinc was found coordinated by the carboxyls of Glu 28 and Glu 76 (Supplementary Fig. 11), which are both vital to catalytic activity. Superposing this onto the docked ATP–AΔ7 DgkA structure positions the zinc 4.9 Å from the γ-phosphate (Fig. 3), where it may have a role in substrate placement for reaction.

The reaction product, phosphatidic acid, with its relatively bulky and charged phosphate, is expected to leave the active site for the surrounding membrane via the opening between the surface helix and H1 (Fig. 1). This may be facilitated electrostatically by Glu 69 and Glu 76 in H2 creating a push, and by Arg 9 and Lys 12 in the surface helix creating a pull on the anionic product (Supplementary Fig. 12). All four residues are important for activity.

A previous study, using subunit mixing/complementarity experiments, provided evidence for shared sites in DgkA. Ala 14 and Glu 76 were identified as active site residues contributed from one subunit, with Glu 69, Asn 72 and Lys 94 deriving from another.

The current structures show that the active sites are indeed composed of residues from two subunits consistent with the shared sites model (Fig. 1). For purposes of discussion, we focus on just one active site (asBC) (Supplementary Fig. 13). Highlighting the shared sites residues identified previously, in this active site it is clear that Glu 69 and Asn 72 on H2C and Lys 94 on H3C are on one half-site, whereas Ala 14 on SHB is on the other. Glu 76, however, is on H2. If it is to be on the same half-site as Ala 14 then it must be on H2B, which is in asAB, not asBC. The current structure is therefore consistent with Ala 14 residing on one of the shared half-sites, and Glu 69, Asn 72 and Lys 94 on the other. It is for these that maximum complementation was observed. For Glu 76, the experimental data were less convincing.

In the previous study, Asp 95 was shown to influence at least two active sites. For purposes of rationalizing this observation we focus on asBC and asAB (Supplementary Fig. 13). Asp 95 in H3C contacts SHB, which bridges two active sites. Its N terminus contributes to asBC, whereas its carboxy terminus, which extends into H1B, contributes to asAB. Thus, modifying Asp 95 is likely to affect SHB, which, in turn, will influence both asBC and asAB, consistent with the experimental observation.

The only other model for DgkA with which to compare our crystal structures was determined using solution NMR and the two are different (Fig. 4). The biggest disparity relates to domain swapping, which is present in the solution but not in the crystal structure. In the former, H3 from one subunit contacts H1 and H2 from an adjacent subunit in classic domain swapped fashion (Fig. 4a). The active site, in turn, is mapped to the space created by H1 and H3 from one subunit and H2 from another. As a result of these disparate quaternary structures, the architecture and chemistry of the active sites in the two models differ markedly (Fig. 4 and Supplementary Discussion). Notably, both are consistent with the shared sites model but for different reasons.

From the quality of the diffraction data and the corresponding refinement statistics (Supplementary Table 1) it is evident that the structures reported here represent what exists in the crystal. We must consider then if the crystal structure described is physiological, because it is possible for crystal lattice restraints to impose unusual and perhaps even unnatural conformations. However, we report here two different crystal-packing arrangements and the overall fold of the protein is the same in both. Furthermore, we have obtained a crystal structure of the enzyme with lipid-substrate-binding sites occupied, modelled ATP into the binding site, found that the enzyme is active under crystallization conditions and after crystal dissolution (Supplementary Fig. 14), and have shown that nucleotide and ATP analogue soaking cause crystals to dissolve, suggesting an adenine-specific, ligand-induced conformational change. All point to a protein with a functional form in the crystal. The solution structure, by contrast, is a low-resolution, composite model that traces only the protein backbone. It was arrived at using connectivity and distance spectroscopic and cross-linking measurements, the tolerances of which can be considerable (Supplementary Discussion, Supplementary Table 2 and Supplementary Fig. 15). Presumably, long-range distance restraints, as defined by residual dipolar couplings and paramagnetic resonance enhancements that have an inherent ambiguity, were used to assist in positioning swapped domains in the micellarized trimer. Because subunit and atom ambiguity in symmetric homo-oligomeric assemblies are complications inherent to structure determination by NMR, the possibility that the solution structure of DgkA is so affected cannot be ruled out.
DgkA is a unique kinase of which a detailed catalytic mechanism has not been established. With the crystal structures reported here, a rational testing of hypotheses relating to mechanism can now be undertaken. DgkA is probably activated in vivo under hypo-osmotic conditions. Activation contributes to enhanced production of the osmoregulatrol, membrane-derived oligosaccharide. The physico-chemical properties of membranes change in response to osmotic stress. We speculate that the surface helix connected to H1 by a short hinge (Fig. 1) acts as a sensor of osmotic stress by responding to bilayer thickness and lateral pressure. Given that the surface helix is a part of the putative active site, adjustments in its conformation and position relative to H1, and to the rest of the trimmer as the membrane responds to hypo-osmotic stress, will alter active site architecture and lead to enhanced kinase activity.

METHODS SUMMARY

Procedures for the expression, isolation, purification and functional assay of wild-type, ΔΔ and ΔΔΔ DgkA are described in the Methods. Crystals were grown in glass sandwich plates at 4 °C by the in mce method using 7.8 MAG on the as the host lipid. Crystals were harvested and snap-cooled directly in liquid nitrogen.

X-ray diffraction data were collected at beamline 23-ID-B (GM/CA-CAT) of the Advanced Photon Source, and the microfocus beamline I24 of the Diamond Light Source. Full details of diffraction data collection and processing, and structure solution and refinement are given in Supplementary Table 1 and are described in the Methods.

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**METHODS**

**Protein.** The dGkA wild-type gene and a dGkA mutant gene encoding for a thermo-stable quadruplet mutant Δ7 DgkA (Ile63Cys, Ile70Leu, Met96Leu and Val107Asp) were synthesized (Genescan) and cloned into pTRiHisB using NcoI and EcoRI restriction enzyme sites. A septuple variant, Δ7 DgkA (Ala41Cys, Cys46Ala, Ile53Val, Ile70Leu, Met96Leu, Val107Asp, Cys113Ala) was generated using PCR-based site-directed mutagenesis and verified by DNA sequencing.

DgkA native proteins were produced as described29 with an additional size-exclusion chromatography step. Se-Met labelling of Δ7 DgkA was performed following an established protocol using the methionine auxotroph B893(D3) strain in M9 minimal media supplemented with Se-Met. Selenium incorporation was subsequently covered with 800 nl precipitant solution using protein/lipid cubic mesophase onto a silanized 96-well glass crystallization plate, which was subsequently snap-cooled directly in liquid nitrogen32.

**Data collection and refinement.** In meso crystallization trials were set up by transferring 50 nl of the protein solution at 12 mg ml\(^{-1}\) into the bilayer of the lipidic cubic mesophase. This was done following a standard protocol30. The protein solution in meso was subsequently covered with 800 nl precipitant solution using protein/lipid cubic mesophase. This was done following a standard protocol30. The protein solution at 12 mg ml\(^{-1}\) was wicked away with tissue paper 32, fresh solution with or without additive was added through the window, and the well was sealed with 384-well microplates (MiTeGen) at 4–6 °C in 45–60 days for wild-type and Δ4 DgkA, respectively. For Δ7 DgkA, rectangular crystals grew to a maximum size of 10 × 50 × 150 μm\(^3\) in 45–60 days (Supplementary Fig. 9). Wells were opened with a tungsten-carbide glass cutter (Silverline) and the crystals were harvested using 30–100 μm micromounts (MiteGen) at 4–6 °C. Crystals were snap-cooled directly in liquid nitrogen32.

Crystal soaking in situ was carried out by cutting a window in the coverglass of the in meso crystallization sandwich plate and injecting into it a solution containing nucleotide, nucleotide analogue or glutaraldehyde into the precipitant solution bathing the mesophase. When a second soaking was required excess bathing solution was wicked away with tissue paper32, fresh solution with or without additive was added through the window, and the well was sealed with ClearSeal film (Hampton). Crystals were harvested as described above.

**Data collection and processing.** X-ray diffraction data were collected on the 23-ID-B beamline of the General Medicine and Cancer Institutes Collaborative Access Team (GM/CA-CAT) at the Advanced Photon Source (APS), Argonne, Illinois, and the I24 beamline at the Diamond Light Source (DLS), Didcot, Oxford. Data were collected using a 10-μm collimated microinjection at the GM/CA-CAT34, whereas a 10-μm microfocus beam was used at the DLS32. Diffraction images taken in meso were analysed automatically in Phenix.refine. Density modification and solvent flattening were recalculated on a regular basis until the maps showed minimal improvements. Se-Met phases were included throughout most of the refinement but were then excluded for the final rounds of refinement owing to marginal anisotropy between the Se-Met and native data. Non-crystallographic symmetry (NCS) restraints were not used as the polypeptide chains varied to a reasonable extent within the asymmetric unit. TLS (translation/libration/screw) groups were used for 0, 0.5, 1, 2, 3 and 10 h, 3 μl of the bathing solution was removed and transferred to 60 μl ADP assay mix (0.2 mM NADH, 1 mM phosphoenolpyruvate, 15 mM magnesium chloride, 20 μM isocitrate) each of pyruvate kinase and lactate dehydrogenase, 75 mM PIPES, pH 6.9. After incubation for 10 min at 30 °C, the drop in absorbance (A) was measured.

To assay Δ7 DgkA activity under crystallization conditions, 20 μl mesophase, formed by mixing 10 μl Δ7 DgkA solution (12 mg ml\(^{-1}\)), and 10 mg 7.8 MAG, was bathed in 1 ml crystallization precipitant (4.5% (v/v) 2-methyl-2,4-pentanediol (MPD), 0.1 M sodium chloride, 60 mM magnesium acetate, 50 mM sodium citrate, pH 5.6) supplemented with 10 mM ATP. After incubating at 4 °C for 0, 0.5, 1, 2, 3, 5 and 10 h, 3 μl of the bathing solution was removed and transferred to 60 μl ADP assay mix (0.2 mM NADH, 1 mM phosphoenolpyruvate, 15 mM magnesium chloride, 20 μM isocitrate) each of pyruvate kinase and lactate dehydrogenase, 75 mM PIPES, pH 6.9) in a 384-well plate. After incubation for 10 min at 30 °C, the drop in absorbance (A) was measured.

**Electrostatics.** The electrostatic surface potentials for DgkA were calculated using the Advanced Poisson-Boltzmann Solver (APBS) plugin, as implemented in PyMol, using default parameters49. The ion identity was confirmed by X-ray fluorescence spectroscopy of crystals and an anomalous difference map generated from X-ray diffraction data collected at the zinc edge (9.6767 keV; Supplementary Table 1 and Supplementary Fig. 11).

**Water.** In the Δ7 DgkA model, water molecules were assigned based on sigma-A-weighted F\(_{o}\) – F\(_{c}\) electron density maps contoured at 1σ using standard geometric and chemical restraints, for a total of 188 water molecules per asymmetric unit.

**Docking.** ATP is ~20 Å long, equalling the distance between the cytosolic loop and the primary hydroxy group of the putative lipid substrate in Δ7 DgkA. ATP with varying γ/β-phosphate protonation was docked into energy minimized Δ7 DgkA (Lys 12 unprotonated, Mg\(^{2+}\) coordinated to Asn 72 and Glu 76) using LigDock32. Poses with the highest LigDock scores were energy minimized to zero energy over 2,000 steps using the Smart minimizer with a Generalized Born with standard protocol30.
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