Crystal Structure of the *Citrobacter freundii* Dihydroxyacetone Kinase Reveals an Eight-stranded α-Helical Barrel ATP-binding Domain*

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Dihydroxyacetone kinases are a sequence-conserved family of enzymes, which utilize two different phosphoryldonors, ATP in animals, plants and some bacteria, and a multiphosphoprotein of the phosphoenolpyruvate carbohydrate-phosphotransferase system in bacteria. Here we report the 2.5-Å crystal structure of the homodimeric *Citrobacter freundii* dihydroxyacetone kinase complex with an ATP analogue and dihydroxyacetone. The N-terminal domain consists of two αβ-folds with a molecule of dihydroxyacetone covalently bound in hemimianial linkage to the Ne2 of His-220. The C-terminal domain consists of a regular eight-helix α-barrel. The eight helices form a deep pocket, which includes a tightly bound phospholipid. Only the lipid headgroup protrudes from the surface. The nucleotide is bound on the top of the barrel across from the entrance to the lipid pocket. The phosphate groups are coordinated by two Mg2⁺ ions to γ-carboxyl groups of aspartyl residues. The ATP binding site does not contain positively charged or aromatic groups. Paralogs of dihydroxyacetone kinase also occur in association with transcription regulators and proteins of unknown function pointing to biological roles beyond triose metabolism.

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The atomic coordinates and structure factors (code 1UN8 and 1UN9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡‡ The abbreviations used are: Dha, dihydroxyacetone; PTS, phosphoenolpyruvate:sugar phosphotransferase system; ANP, adenyllyl-imidodiphosphonate; DhaK, Dha-binding subunit of the *E. coli* Dha kinase; DhaL, small subunit of the *E. coli* Dha kinase; MAD, multi-wavelength anomalous dispersion; H1, helix 1.

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Dha kinase (C.f.)

\[
\text{ATP} + \text{Dha} \rightarrow \text{DhaP} + \text{ADP}
\]  

(Eq. 1)

Enzl,HPr (E.c.)

\[
\text{PEP} + \text{DhaM(His)} \rightarrow \text{DhaM(His−P)} + \text{Prv}
\]  

(Eq. 2)

DhaK::DhaL (E.c.)

\[
\text{DhaM(His−P)} + \text{Dha} \rightarrow \text{DhaP} + \text{DhaM(His)}
\]  

(Eq. 3)

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The K- and L-domains display 36 and 28% amino acid sequence identity with the DhaK and DhaL subunits, respectively. DhaM is a multiphosphoryl protein of the PTS with sequence similarity to the IIA<sub>man</sub> domain of the mannos transporter (Protein Data Bank code entry 1PDO) (30), the phospho-ryl carrier protein HP<sub>r</sub> (Protein Data Bank code entry 1PTF) (31), and the N-terminal domain of enzyme I (Protein Data Bank code entry 1ZYM) (32).

The x-ray structure of the DhaK subunit of <i>E. coli</i> in complex with its substrate has recently been solved at 1.75 Å (33). Dha is bound in hemiaminal linkage to the imidazole nitrogen of an invariant histidine. It has been proposed that Dha kinases exploit the chemical reactivity of the carbonyl group to discriminate between the potentially toxic Dha and the structurally similar compatible solute glycerol (33). In contrast, glycerol kinases cannot discriminate between the two substrates (10). Here we present the x-ray structure of the full-length Dha kinase of <i>C. freundii</i> in complex with Dha, an ATP analogue and a phospholipid.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Site-directed Mutagenesis**—The dhaK gene was PCR-amplified with <i>C. freundii</i> genomic DNA as template and primers encoding NsiI and BglII restriction sites. The PCR fragments were digested with NsiI and BglII and ligated with a plasmid vector obtained by digestion of pTSGH11 (35) with the same enzymes. The recombinant dhaK gene encodes a Dha kinase with a C-terminal His-tag. The point mutations D380A, D385A, D387A, and T388H in DhaK were obtained by digestion of pTSGH11 (35) with the same enzymes. The PCR fragments encoding NsiI and BglII restriction sites. The PCR fragments were used for plasmid purification, restriction analysis, ligation, successful mutagenesis was verified by DNA sequencing. Standard procedures were used for plasmid purification, restriction analysis, ligation, and transformation.

**Protein Purification and Activity assay**—The Dha kinase of <i>C. freundii</i> was overexpressed in <i>E. coli</i> K12 WA2157A HIC (ΔmanXYZ ΔptoH)(36). Cells (3 liters) were grown at 37 °C and induced with 200 µM isopropyl-1-thio-β-D-galactopyranoside at A<sub>600</sub> = 0.8, and incubation continued for 5 h. Cells were collected by centrifugation, and the sediment was resuspended in buffer A (25 ml, 50 mM NaP, pH 8.0, 10 mM β-mercaptoethanol, 500 mM NaCl), broken by two passages in a French pressure cell, and fractionated by differential centrifugation into cell debris (10 min, 3000 × g), membrane fraction (90 min, 150,000 × g), and cytoplasmic fraction (supernatant), which was mixed with 10 ml of N<sup>2</sup>-nitrilotriacetic acid resin (Qiagen) and allowed to adsorb for 30 min. The resin was transferred to a column washed with 100 ml buffer A containing 0 and 50 mM imidazole. The His-tagged protein was eluted with 200 mM imidazole in the same buffer, concentrated, and purified by gel filtration over Superdex 200 (Amersham Biosciences) with a buffer containing 10 mM HEPES, pH 7.5, 400 mM NaCl, and 2 mM dithiothreitol. Seleno-Met-Dha kinase was prepared using the methionine pathway inhibition method (37) and purified like the native protein. The presence of seleno-Met was confirmed by electrospray ionization mass spectrometry (results not shown). Dha kinase was concentrated to 50–500 mg ml<sup>−1</sup> in 5 mM HEPES, pH 7.5, 2 mM dithiothreitol. Kinase activity was measured in a coupled assay by reduction of dihydroxyacetone phosphate with glycero-l-3-phosphate dehydrogenase as described previously (1) with ATP instead of phosphoenolpyruvate plus PTS proteins as phosphoryl donor. The disappearance of NADH was monitored continuously in a SpectraMax-250 plate reader at 30 °C.

**Crystallization and Data Collection**—(Seleno-Met)-Dha kinase was crystallized in buffer B (100 mM HEPES, pH 7.3, 50 mM NaCl, 9% (v/v) polyethylene glycol 3000, and 25% (v/v) glycerol) using sitting drop vapor diffusion. Crystals belong to the space group C<sub>222</sub>, with cell dimensions of a = 100.4 Å, b = 124.6 Å, and c = 236.4 Å and contain one dimer per asymmetric unit. For ANP<sup>−</sup> and Dha complexed Dha kinase, native protein crystals were soaked with 5 mM ANP, 10 mM MgCl<sub>2</sub>, and 2 mM Dha for 3 h at 20 °C in buffer B. The best crystals of the native protein diffraction to 2.5 Å after flash-freezing at 105 K in buffer B. Native diffraction data were collected at the Swiss Norwegian Beamline at European Synchrotron Radiation Facility (Grenoble, France) employing a MAR345 image plate detector (Xrayresearch, Hamburg, Germany). The seleno-Met x-ray data sets were measured at beamline BW17A at the EMBL Hamburg Outstation at the DESY using a MAR CCD-165 detector. Because of the smaller area of the detector, which caused reflection overlap, and also because of the inferior quality of the selenomethionine crystals, data were only collected to a resolution of 3.5 Å. Three data sets were collected at peak (0.9743 Å), remote (0.9252 Å), and inflection point (0.9750 Å). The ANP/Dha-associated Dha kinase crystals were measured at the SLS beamline X06SA at the PSI Villigen using again a MAR CCD-165 detector. All of the datasets were collected employing a MAR CCD-165 detector (Xrayresearch, Hamburg, Germany) and were processed using the HKL program package (38).

**Structure Solution and Refinement**—The Dha kinase structure was determined by MAD methods. Of the 28 methionines expected in the asymmetric unit, the positions of 20 were readily determined using Shake-and-Bake (39). Phases were computed using SOLVE (40) and density modification, and phase extension to 2.5-Å resolution using the native data set was effected by the program RESOLVE (40). The resulting map was of high quality and allowed the tracing of the whole polypeptide chain. An initial protein model was built into the electron density using the program O (42). Non-crystallographic symmetry restraints were used to refine the model to a 2.5-Å resolution using the programs CNS (43) and REFMAC (44). All of the data within the resolution range were included during refinement. Data collection and resolution range were included during refinement. Data collection and structure refinement are summarized in Table 1.
refinement statistics are given in Table I. A Ramachandran plot as defined by Kleywegt and Jones (45) showed no residues in disallowed regions but showed nine outliers in generously allowed regions (1.0%). The last four C-terminal residues and the additional 8-residue C-terminal histidine tag are not visible in the structure, and the protein model ends with the glutamate 550.

**Lipid Analysis**—Phospholipids were separated by thin layer chromatography on silicic acid. 10 μl of concentrated DhaK (15 mg/ml) were applied, the chromatogram was developed with CHCl₃:MeOH:H₂O 76:21:3, and lipids stained with 40% sulfuric acid, ninhydrin, or phosphate stain (46).

**Sequence Analyses**—To retrieve DhaL sequences from the non-redundant protein data base at NCBI (www.ncbi.nlm.nih.gov/entrez/), the combined DhaK and DhaL sequences of *E. coli* and *C. freundii* were used as queries (Swiss Protein Data Base accession numbers P45510, P76015, and P76014). The alignments were performed with MultiAlin (47) using the default parameters. Phylogenetic trees were constructed with Gene-Bee (48) using the default parameters.

**Coordinates**—Crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (accession codes 1UN8 (apo form); 1UN9 (Dha/APN form)).

**RESULTS**

**Structure Determination and Overall Architecture**—Dha kinase was purified by nickel-nitrilotriacetic acid affinity and size-exclusion chromatography from where it eluted as a symmetrical peak with the retention time expected of a 2,60-kDa homodimer. It was crystallized with polyethylene glycol 3000 as precipitant, and the structures of the apoprotein and the complex with Dha and ANP were solved. Phases were determined using selenomethionine-substituted protein and multiwavelength anomalous dispersion at 3.5-Å resolution. Refinement of the native protein structure against the 2.5-Å data resulted in an R-factor of 19.3% and an R_free of 24.5% with reasonable stereochemistry (Table I). The asymmetric unit contains one dimer. The Dha kinase monomer is well ordered (residues 1–550) with the exception of the loop 518–528.

The K-domain (residues 1–330) and the L-domain (residues 350–550) are separated by an extended linker (residues 331-335).
and swapped such that the L-domain of one kinase subunit is bound to the K-domain of the other (Fig. 1). The linker is well ordered, and it contains a short β-strand, which is hydrogen-bonded to the edge strand of the N-terminal β-sheet of the K-domain.

The K-domain of *C. freundii* has the same structure as the DhaK subunit of *E. coli* kinase (33). It consists of two α/β-folds, each containing a six-stranded mixed β-sheet surrounded by a two-stranded antiparallel α-helix barrel enclosing a deep pocket. The helices are between 3.5 and 6 Å turns long with their axes tilted at an angle of −15° with respect to the barrel axis. The first and longest helix (amino acids 355–386) is kinked at an angle of 45° between Leu-372 and Glu-373. The helices are amphipathic with the hydrophobic side chains directed into the pocket of the barrel and with the polar residues surface exposed. All of the loops are well defined with the exception of the longest one (amino acids 513–533) between helix 7 and 8 where residues 518–527 are not visible in the electron density map. The four loops at the top of the barrel contain highly conserved residues (Fig. 2), whereas the loop sequences on the bottom side (defined by the N and C termini of the chain) are not conserved.

**Lipid-binding Pocket**—Strong electron density remained in the native protein model that could not be explained by protein atoms and became the predominant feature in *Fo* − *Fc* maps after sequence fitting and initial model refinement (Fig. 3A). This density has the shape of a 2-myristoyl(C14)-3-palmitoyl(C16)-phospholipid. The two acyl chains extend ∼15 Å into the pocket where they are surrounded by apolar side chains. Residues in a distance of <4 Å are depicted by open circles and footnotes (results not shown). The lipids can be removed by incubation with the detergent phospholipase D. Both spots were stained phosphate-positive, and the phospholipase-sensitive spot also was ninhydrin-positive, indicating that the lipid is not essential for kinase activity (results not shown). Given the hydrophobic lining of the pocket, the phospholipid is probably replaced by one or two detergent molecules.

**The ATP- and Dha-binding Sites**—Crystals were soaked in a solution containing ANP, MgCl₂, and dihydroxyacetone, and a complete data set was collected to a 3.1-Å resolution from a single crystal (Table I). A difference Fourier map calculated with phases derived from the native model revealed strong extra positive electron density into which one molecule of ANP...
and one molecule of Dha per monomer could be fitted unambiguously. After rigid body and positional refinement using the model of the apo-enzyme, two magnesium ions complexed to the ANP molecule were also identified (Fig. 3C, peaks at 8σ and 6σ in the Fo - Fc map). To further confirm their position, the crystals were soaked with ANP, Dha, and Mn2+ instead of Mg2+, and a dataset of a single crystal was collected to a 4.0-Å resolution (Table 1). Isomorphous and anomalous difference Fourier maps showed clear peaks at the putative Mg2+ positions, confirming the original interpretation (Fig. 3B). The two magnesium atoms have a 3.99-Å center to center distance. ANP binds to the L-domain at the top of the α-barrel (Fig. 3, C and D). The bottom of the nucleotide-binding site is formed by Met-428, Met-436, Met-477, and Phe-392, which septum-like separate the ANP-binding pocket from the lipid-binding pocket. The side walls are formed by the helix termini and the connecting loops. The adenine base is packed between the connecting loops. The adenine base is packed between the helix termini and the connecting loops. The adenine base is packed between the helix termini and the connecting loops. The adenine base is packed between the helix termini and the connecting loops.

The Asp-380, Asp-385, and Asp-387 mutants of the ATP-dependent C. freundii kinase are completely inactive (Fig. 4B), whereas the corresponding mutants of the PTS-dependent E. coli kinase have reduced activity (Fig. 4C). The reduced activity may be caused by protein instability rather than by catalytic inefficiency because the E. coli mutants were unstable and expressed only in minute amounts compared with the wild-type protein. The conserved Thr-388 is of particular interest. Mostly two-domain Dha kinases (presumably ATP-dependent) have a Thr in this position, whereas the two-subunit kinases (presumably PTS-dependent) always have a His (Fig. 2). Whereas Thr-388 is not essential for activity of the C. freundii enzyme, His-38 is essential for the E. coli enzyme (Fig. 4, B and C). This invariant histidine in the first loop of a DhaL subunit might thus be diagnostic for the PTS dependence of a Dha kinase.

Dha is bound to the K-domain at the same site as reported for the 1.75-Å resolution structure of the homologous E. coli DhaK subunit (33). The details of bonding are not revealed at the lower resolution of this structure, but given the similarity of sequence (Fig. 2) and structure, an identical binding mechanism can be inferred (Fig. 1C). The binding sites for Dha and ATP are 14 Å apart in the crystal structure presented here (Fig. 1), indicating that the contacts between the K- and L-domains are induced by the crystal lattice. The solvent-accessible surface buried between H3 of the K-domain and H4 and H5 of the L-domain is 1340 Å², which is less than the strongest crystal...
contact of 2300 Å² that stretches across the K-L interface. Moreover, the contact area does not contain conserved residues as would be expected of a functional interface (helices 4 and 5 in Fig. 2). We envisage the K- and L-domains as mobile in solution and connected only by a long flexible linker as it was shown for the dimeric IIABMan subunit of the *E. coli* mannose transporter (51). Attempts to induce alternative conformations by co-crystallization instead of soaking with Dha/ANP and with the heterobifunctional substrate ATP-dihydroxyacetone² were unsuccessful.

**Amino Acid Sequence and Structure Comparison of the *C. freundii* and *E. coli* Dha Kinase Domains** — Overlay of the *C. freundii* K-domain with the *E. coli* DhaK subunit using the DALI server (52) yields a root mean square distance of 1.32 Å for 287 C/H9251 atoms (Fig. 1D, red and blue backbone traces). The only prominent difference is the extended β-ribbon that caps one edge of the N-terminal β-sheet of the *E. coli* DhaK. This ribbon occurs only in the DhaK subunits of Gram-negative bacteria, which contain large multidomain DhaM subunits and where it might prevent edge-to-edge aggregation between apolar β-sheets (53, 54). Amino acid sequence comparison reveals that DhaK subunits without this β-ribbon always contain a charged residue (Arg, Lys, or Glu) inserted at an invariant position of the edge strand, which is a dominant strategy for blocking edge strand aggregation (53).

The L-domain, an eight-stranded α-helical barrel, represents

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² L. F. Garcia-Alles, unpublished data.

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**Fig. 4. Phospholipid analysis and Dha kinase activity.** A, sulfuric acid stained thin layer chromatogram of phosphatidylethanolamine (PE) and phosphatidic acid (PA) bound to the L-domain before and after treatment with phospholipase D (PLD). No lipid can be detected after treatment of DhaK with β-octylglucoside (OG). B and C, titration of wild-type and mutant forms of *C. freundii* (DhaK (C.f.)) (B) and *E. coli* DhaL subunit (C). Wild-type (○), D380A and D30A (●), D385A and D35A (▲), D387A and D37A (▼), and T388H and H38T (●) are shown. H38C and H38A are also inactive (data not shown). Means ± S.D. of seven (wild type) and three (mutants) independent experiments. In B, the concentrations of ATP and Dha were 1.1 and 1.0 mM, respectively. In C, the concentrations were as follows: 1 mM phosphoenolpyruvate, 1 mM Dha, 0.15 μM EI, 2.0 μM HPr, 0.33 μM DhaM, and 0.33 μM DhaK.

**Fig. 5. Phylogram of representative DhaL homologues.** The operon organization with coding regions is indicated for each group as follows: DhaL, black; DhaK, gray; DhaM and IIA domains, vertically hatched; HPr and enzyme I domains, cross-hatched; SorC-like (transcription) domain, cross-hatched; and domain structurally related to TM841 of *T. maritima*, diagonally hatched. Swiss Protein accession numbers are given where available. The proteins mentioned in the text are boldface underlined.
a novel fold without a match in the protein structure data bank. A BLASTP search (56) revealed genes for L-domain-like protein subunits and domains in over 80 Gram-positive and Gram-negative bacteria as well as in many eukaryotes. A phylogram of representative L-domain homologues and paralogues is shown in Fig. 5. The prokaryotic L-domains and DhaL subunits cluster in six different groups, which reflect operon structure and possibly different protein function. These groups consist of the following subunits and domains. (a) This group contains L-domains of ATP-dependent Dha kinases biochemically proven for C. freundii (25), predicted for Mesorhizobium loti and Bacillus anthracis. This group also includes the L-domains of all of the eukaryotic Dha kinases (data not shown but see Fig. 2 for alignment). (b) This group contains DhaL subunits of PTS-dependent Dha kinases from Gram-positive bacteria, which contain single domain DhaM (IIA Man-fold only) (1). This group contains DhaL subunits of Gram-negative bacteria, which contain a multidomain DhaM (1) and a β-ribbon in DhaK (33). (d) This group contains two-subunit putative Dha kinases without an associated DhaM subunit. Neither the dhaKL operon itself nor the adjacent operons contain genes for a DhaM-like subunit. For lack of experimental evidence, it is not clear which phosphoryl donor these proteins utilize. (e) This group contains DhaL-like subunits encoded in an operon together with a putative transcription factor. The latter consists of two domains, a (Dha)K-like domain and a domain belonging to the SorC family of transcription regulators (57). (f) This group contains two-domain proteins with a L-like domain at the amino terminus rather than the C terminus. These proteins of unknown function often are encoded in operons with RecG or alkaline shock proteins.

DISCUSSION

The x-ray structure of the C. freundii Dha kinase revealed several properties that are unusual and new for a soluble protein: (i) an eight-stranded α helix barrel (L-domain); (ii) a phospholipid molecule included in the barrel; (iii) an ATP-binding site located at the top of the barrel; and (iv) covalent binding of the substrate by a hemiaminal bond, which does not participate in the enzyme-catalyzed reaction (33). The lipid-protein complex is reminiscent of lipid transfer proteins of plants (55) and a recently described pollen allergen (41). In lipid transfer proteins, the lipid is enclosed by four amphipathic helices in the pollen allergen by two interlocked EF-hand folds. However, whereas in these binding proteins the lipids are completely shielded from the medium, the polar headgroup remains solvent-exposed in the L-domain (Fig. 3B).

To our knowledge, lipids bound in such a way have so far been observed in membrane proteins only where they are supposed to determine the packing of transmembrane helices (34). The cellular function of the lipid in the L-domain of the Dha kinase is not known, nor how it gets incorporated during protein folding. We favor the hypothesis that the lipid stabilizes the helix barrel and, in comparison with the non-annular membrane lipids, may play a purely structural role.

The amino acid sequence comparison indicates that the ATP-binding site of the L-domain is well conserved in all of the Dha kinases (Fig. 2), regardless of whether they utilize ATP or a phosphoprotein of the PTS as phosphoryl donor. Mutations of the conserved asparyl residues not only completely abolish ATP-dependent activity of the C. freundii enzyme but also compromise the activity and stability of the PTS-dependent E. coli kinase. Although the function of these invariant asparyl residues appears obvious from the x-ray structure, namely to provide binding sites for the ATP-magnesium complex, their molecular function in the PTS-dependent kinases is unknown. Here they may participate in the binding of the phosphoryldonor subunit DhaM. In this case, DhaM, the component of the PTS, would have evolved toward binding to the already pre-
formed binding site of the DhaL ancestor rather than vice versa. Indeed, the DhaL domains display a higher degree of amino acid sequence similarity than the IIA domains. For instance, the DhaL subunit of E. coli has 26 and 30% sequence identity with the C. freundii and Homo sapiens DhaL domain, respectively, whereas the IIA domains of DhaM and the mannos transporter of E. coli only have 15% sequence identity. Thr-388 is another residue of interest in the nucleotide-binding site. The equivalent position in the PTS-dependent kinases contains an H9251 but differs in how these cores are modified (Fig. 6). (i) The K-domains of Dha kinases contain an IIAMax and the FtsZ corefolds but differ in how these cores are modified (Fig. 6). (ii) TM841 has an additional group of Dha bound to the K-domain are both oriented and transiently phosphorylated at this histidine (His-38 of the E. coli subunit). This intermediate is unusually liable for a PTS protein and therefore escapes detection by autoradiography on polyacrylamide gels (1). Taken together, this finding suggests that the nucleotide-binding site is important not only for phosphoryl transfer from ATP but also for the transfer of the phosphoproteins of the PTS. A phylogenetic analysis of prokaryotic and eukaryotic L-domains indicates that the ATP-dependent Dha kinases are deeper rooted than the PTS-dependent ones. Consequently, it is tempting to speculate that the PTS-dependent forms evolved from an ATP-dependent ancestor to tap the PTS as source of high energy phosphate.

The α-helix barrel fold appears not only as C-terminal domain in Dha kinases but also as N-terminal domain in a family of two-domain proteins with unknown function (Fig. 5). One representative example is YhG of Lactococcus lactis (Swiss Protein Data Bank code Q9CHY7). Whereas the amino acid sequence similarity of its N-terminal domain with the L-domain of Dha kinases is obvious (Fig. 2), the sequence of its C-terminal domain bears no similarity to the K-domain. How-
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