Dihydroxyacetone phosphate (Dha-P), an intermediate for the synthesis of pyruvate, can be generated in bacteria through glycolal fermentation by the sequential actions of glycerol dehydrogenase forming dihydroxyacetone and Dha kinase generating Dha-P. Dha kinases are divided into two classes, those using ATP as the phosphoryl donor or those using the PTS-dependent DhaM protein to provide the phosphoryl group (1). The ATP-dependent Dha kinases are present in animals, plants, and some bacteria and consist of a single chain containing two domains, whereas the PTS-dependent Dha kinases exist only in bacteria and are composed of two subunits. The most studied representative of the PTS-dependent family is the \textit{Escherichia coli} Dha kinase, which is composed of DhaK and DhaL subunits. DhaM, the phosphotransferase subunit of Dha kinase, is phosphorylated by the small phospho-carrier protein HPr of the PTS (1). Phosphorylated DhaM then forms a complex with the ADP-bound DhaL subunit and transiently phosphorylates ADP to ATP (1). The ATP-loaded DhaL subsequently associates with the DhaK subunit containing the Dha substrate covalently bound to His218\textsuperscript{K} through a hemiaminal bond (2). Finally, ATP bound to DhaL, serving as a coenzyme (3), transfers phosphate to Dha yielding Dha-P.

Crystal structures for the two individual subunits of the \textit{E. coli} PTS-dependent Dha kinase—DhaK (2) and DhaL (4)—have been previously determined, as well as the structure of an ATP-dependent single chain, two-domain Dha kinase from \textit{Citrobacter freundii} (5), which has 30\% sequence identity to the \textit{E. coli} kinase. Both ATP- and PTS-dependent Dha kinases display high structural similarity of their corresponding domains/subunits and are structurally distinct from other kinases. The active site residues are highly conserved between bacteria and eukaryotes reflecting the unique phosphotransfer mechanism. The structure of the homodimeric, ATP-dependent \textit{C. freundii} Dha kinase shows swapped K- and L-domains, with bound Dha and an ATP analogue separated by approximately 14 Å (5). It is therefore unlikely that this structure represents a phosphotransfer-competent state of this enzyme, leaving the question of the mechanism unanswered. In addition to the structures of DhaK and DhaL, the structure of the \textit{Lactococcus lactis} DhaM domain has been reported as well as its complex with DhaL, providing insight into phosphotransfer from phosphohistidine to ADP (6).

We have determined the crystal structure of the \textit{E. coli} DhaK–DhaL complex bound to ATP and Dha at 2.2 Å resolution, providing a snapshot of the final step of phosphotransfer from ATP to Dha. Site-directed mutagenesis, activity measurements, and structural data indicate that His56\textsuperscript{K} is important for formation of the covalent hemiaminal bond. We show further that the covalent attachment of Dha to His218\textsuperscript{K} optimally positions the substrate for phosphotransfer but is likely dispensable for enzyme activity. Together, these data allow us to propose a general catalytic mechanism for dihydroxyacetone kinases, consistent with the available structural and biochemical data.

Results and Discussion

Structure of the DhaK–DhaL Complex Reveals Conformational Rearrangements upon Complex Formation. Each asymmetric unit contains one molecule each of DhaK and DhaL with all residues well defined in the electron density map. The biological unit of the complex is a heterotetramer containing a central DhaK dimer and two molecules of DhaL (2:2 stoichiometry). Each DhaL subunit interacts with only one DhaK subunit of the dimer, with the two DhaL subunits being approximately 37 Å apart (Fig. 14). An ADP molecule and two magnesium ions are bound to DhaL, whereas DhaK contains a Dha molecule covalently bound to His218\textsuperscript{K} (Fig. 18).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, \textit{www.pdb.org} [PDB ID codes 3PNK (DhaK), 3PNL (DhaK–DhaL complex), 3PNM (DhaK H56A), 3PNO (DhaK H56N), and 3PNQ (DhaK H56N–Dha)].

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*Superscript letter \textsuperscript{a} (or \textsuperscript{b}) denotes kinase subunit.

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Structural and mechanistic insight into covalent substrate binding by \textit{Escherichia coli} dihydroxyacetone kinase

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DhaL binds to DhaK through the narrower end of an eight-helix barrel that harbors the ADP. This is consistent with the role of DhaL-ATP as the phosphoryl donor to the DhaK-bound Dha.

The structure of DhaL in the complex is very similar to that of free DhaL (4), with an rmsd of 0.42 Å for all Ca atoms. Binding to DhaK involves residues in loops α1/α2, α3/α4, α7/α8, and portions of helices α1, α2, α4, and α5 (Fig. 1C). In contrast, DhaK undergoes significant conformational changes in several regions as compared to the free DhaK previously reported [PDB ID code 1O12, (2)] (Fig. 2A). The rmsd for the Ca atoms of free and complexed DhaK is 1.38 Å (for 336 aligned residues).

The loop pβ8/aαK undergoes a significant relocation to accommodate the narrow tip of DhaL, with the Ca atoms of Leu142K and Tyr143K moving by approximately 13 Å. Removing this loop from the comparison results in an rmsd of 0.5 Å for 328 Ca atoms. The loop pβ8/pβ9 moves by approximately 3 Å at Pro221K to accommodate Leu182L. A consequence of the adjustment of loop pβ8/pβ9 is the ordering of the neighboring loop pβ7/pβ8, which is disordered in the free DhaK (2). This ordering effect also includes the N-terminal segment of DhaK (residues 2–9), which is now sandwiched between the β7K/pβ8 loop and the β9K/aαK loop of the other DhaK subunit and forms part of the dimerization interface. DhaK residues involved in the interface are found in loops pβ4/aαK, pβ6/aαK, pβ7/pβ8, pβ8/pβ9 and in helix aα3K (for more details see SI Text).

Although the overall structure of both DhaK and DhaL subunits in the E. coli complex are similar to the corresponding domains of the ATP-dependent C. freundii Dha kinase, with rmsd values of 1.4 Å (307 Ca atoms) and 1.7 Å (162 Ca atoms), respectively, our crystal structure reveals that the binding interface between these two subunits/domains is quite different as a result of
domain swapping in _C. freundii_ (Fig. S1A). Importantly, and in contrast to the _C. freundii_ Dha kinase structure, in the _E. coli_ structure, the orientation of DhaL relative to DhaK is consistent with a direct transfer of the γ-phosphoryl moiety from ATP to the γ-OH group of Dha (Fig. 1B).

We have also crystallized _E. coli_ DhaK in a different crystal form (Table S1) than that previously reported (2). Interestingly, in our free DhaK structure, the loop β6β7/α4αK (residues 138–145) displays a conformation intermediate between that of free DhaK (PDB ID code 1012) and that of DhaL-bound DhaK (see above), providing a snapshot of the movement of this loop (Fig. S2). The flexibility of the loop β6β7/α4αK is pivotal for the ability of DhaK to bind DhaL, because conformations of this loop in both free DhaK structures are not compatible with complex formation. The difference between the two structures of free DhaK is likely caused by changes in packing environment.

The Dha Kinase Active Site Is Formed at the Interface of DhaL-DhaK Subunits. In order to bring DhaL-bound ADP in proximity to DhaK-bound Dha, the tip of the DhaL helical barrel containing loop α17β18/α28 (residues 175–185) that caps the nucleotide-binding site, dips into the cavity formed by the β6β7/α4αK and β8β9/β9αK loops, displacing both loops from their locations observed in free DhaK (Fig. 2A). The distance between the β-phosphate oxygen of ADP and the γ-OH group of Dha is 4.8 Å, sufficient to accommodate the missing ATP γ-phosphate in our DhaK–DhaL(ADP) complex as shown by our model of DhaK–DhaL(ADT) (Fig. 2B).

Both subunits contribute residues to form the active site. The key residues in the Dha binding pocket, including His218K, covalently bound to Dha, Gly53K, His56K, Lys104K, and Asp109K, are in almost identical positions as in the free DhaK structure. Dha is further anchored by Arg178K, whose guanidinium group hydrogen bonds to the γ-OH of Dha and likely stabilizes the trigonal bipyramidal transition state during phosphoryl transfer. All of these residues are highly conserved in bacterial Dha kinases. Mutations of His218K in _E. coli_ DhaK or Arg161L in _L. lactis_ DhaL (corresponding to Arg178K in _E. coli_ ) result in inactive enzymes (2, 6).

Despite the lack of conformational changes in DhaL upon binding to DhaK, the adenine ring of ADP undergoes a syn to anti conversion (Fig. S3) when compared with the free DhaL structure (4). Our molecular dynamics (MD) simulations indicate that in the syn conformation, ADP has a potential steric clash of its adenine ring with Thr107K (Fig. S4). Flipping of the adenine ring is accompanied by formation of three additional water-mediated H bonds, two between its purine N1 and the carbonyl group of Val117L and the amide group of Ala123L and the third between its purine N6 and the OH group of Thr107K. In addition, one of the two Mg2+ ions (M2+) in the ADP binding site of DhaL moves by approximately 1.0 Å compared to free DhaL and becomes a bridging element at the DhaK–DhaL interface (Fig. S3). Its coordination number increases from three to five with the addition of the carbonyl of Phe78K and a water molecule.

**His56K and Asp109K** are Important for Dha Kinase Activity. In order to evaluate the contributions of residues in the active site for activity, we performed site-directed mutagenesis and activity measurements for several mutant enzymes, focusing on the conserved residues His56K, Asp109K, His218K, and Arg178L. Gel filtration chromatography revealed that all of the DhaK mutant enzymes (His56AK, His56NK, D109AK, D109NK, and H218K) were able to form complexes with DhaL, while no complex formation between DhaK and R178EL was observed (see SI Text). Two variants of His56K, His56AK and His56NK, show a moderate decrease in _k_ cat, but at least a 40- to 300-fold increase in _K_ m (Table 1). Both D109AK and D109NK mutant enzymes showed no measurable activity (Fig. S5). Mutation of His218K to either lysine (Fig. S5) (2) or alanine (2) also abolishes activity, consistent with its role in binding and orienting the substrate. In addition to the coupled enzyme assay, similar results were obtained by NMR spectroscopy (Fig. S6).

**Table 1. Kinetic constants of wild-type DhaK and His56K mutants for Dha substrate**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>WT</th>
<th>H56AK</th>
<th>H56NK</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>k</em> cat (min⁻¹)</td>
<td>507 ± 25</td>
<td>96.1 ± 2.0</td>
<td>223 ± 4.3</td>
</tr>
<tr>
<td><em>K</em> m (mM)</td>
<td>&lt;0.008*</td>
<td>0.302 ± 0.005</td>
<td>2.20 ± 0.15</td>
</tr>
<tr>
<td><em>k</em> cat / <em>K</em> m (mM⁻¹ min⁻¹)</td>
<td>&gt;6.3 × 10⁴</td>
<td>318</td>
<td>101</td>
</tr>
</tbody>
</table>

Standard deviations were calculated from three independent experiments.

*Due to the limited sensitivity of the glycerol-3-phosphate dehydrogenase coupled assay for WT at low substrate concentrations the _K_ m could not be accurately determined.

**Structures of DhaK H56NK and H56A Reveal the Ability of Dha to Bind Noncovalently.** To gain further insight into the reduced activity exhibited by the H56NK and H56AK mutant enzymes we determined their crystal structures. The overall structure of either the H56NK or H56AK enzyme is almost identical to that of the previously reported structure of wild-type DhaK (2), although they crystallized in a different packing environment (Table S1) with four subunits in the asymmetric unit. However, in contrast to the structures of wild-type DhaK, no electron density corresponding to the Dha substrate was observed in any of the subunits in the structures of these mutant enzymes (Fig. S7). These structures were immediately suggestive of a critical role for His56K in hemiaminal bond formation with Dha. The H56NK and H56AK structures reveal very small conformational changes in the active site as compared to wild-type DhaK with covalently bound Dha. These differences include an approximately 35° rotation of the imidazole ring of His218K, possibly resulting from the shorter Asn56K and Ala56K side chains in the mutant enzyme structures. The carbohydrate group of Asp109K also rotates approximately 35°, possibly due to the loss of its hydrogen bonding interaction with the hydroxyl groups of Dha.

To better understand how the H56NK mutant enzyme could retain some activity in the absence of covalent substrate binding, we determined the crystal structure of H56NK in the presence of Dha by supplementing the crystallization buffer with 10 mM Dha. These crystals contained four molecules in the asymmetric unit (Table S1). In one subunit there is clear electron density showing the presence of Dha bound in the active site (Fig. 3A). Although this non-covalently bound Dha molecule occupies the same binding pocket as in the covalently bound structure, its C2 and O2 atoms are displaced by approximately 2 Å and approximately 3 Å, respectively, as compared with covalently bound Dha. The absence of the covalent bond results in somewhat different interactions between Dha and the surrounding residues. The Dha carbonyl group is anchored via hydrogen bonds with the main chain amide group of Gly53K, the hydroxyl group (γ-OH) buried in the binding pocket is within H-bonding distance of Lys104K (NZ) and His218K (NE2), while the other exposed hydroxyl group (γ-OH) forms a hydrogen bond to Asp109K (OD1) and Ser80O (OG) (Fig. 3A). Although this γ-OH is still orientated toward the β-phosphate group of ADP, it is shifted approximately 1.7 Å away from its position in the covalently bound substrate (Fig. 3B). We surmise that this rearrangement contributes to the observed reduced catalytic efficiency of the mutants (Table 1). Another contribution to the lower activity of the H56NK and H56AK mutants is their weaker affinity for the substrate, as reflected by a significant increase in the _K_ m (Table 1). Although our upper estimate of _K_ m for Dha in wild-type DhaK is 8 μM, the corresponding _K_ m is 302 μM and 2.2 mM for H56AK and H56NK, respectively (Table 1). Despite the different positioning of Dha in the H56NK
We propose here a plausible chemical mechanism for the overall reaction (Fig. 4). With the exception of the demonstrated roles of His218K of DhaK (2) and Arg178L of DhaL (6), the remaining residues in the active site have not been previously implicated as having a role in catalysis.

Consistent with our structural data, we propose that formation of the initial covalent complex with Dha (Fig. 4i) involves nucleophilic attack of His218K on the electrophilic carbonyl Cβ atom of Dha. For this, the NE2 atom of His218K needs to be deprotonated. The proximal protonated His56K acts subsequently as a general acid, donating its proton to form the Dha γ-OH (Fig. 4). Although in general a water molecule could also play the role of general acid, there is no suitably located water molecule in any of the available crystal structures of wild-type DhaK. Formation of the covalent enzyme–substrate complex allows for precise orientation of the substrate with respect to other active site residues, including Gly53K, His56K, Lys104K, and Asp109K.

Following formation of the covalent DhaK–Dha complex, the next step is transfer of the γ-phosphoryl group of ATP to the γ-OH of Dha (Fig. 4ii). This reaction requires activation of the γ-OH through removal of a proton, in order to generate an anion suitable for attack at the ATP γ-phosphorous atom. This activation requires a residue acting as a general base, which we identify as Asp109K, within H-bonding distance of Dha γ-OH. This role is supported by the loss of activity observed for the Asp109K mutant enzymes (Fig. S5). In the DhaK–DhaL complex, the leaving oxygen (O2B of ADP) and the entering oxygen (Oγ of Dha) are separated by 4.8 Å (Fig. 1B). Based on molecular dynamics simulations on the DhaK–DhaL–ATP model, the Py-Oγ(Dha) distance in the Michaelis complex is estimated to be 3.9 Å (Fig. 2B). The locations of negative charges developing during phosphoryl transfer may be inferred from the location of stabilizing, positively charged groups provided by the enzyme. An invariant residue, Arg178L, is located at the active site of the DhaK–DhaL complex and has been suggested to be essential.
for activity by mutagenesis (6). This residue has been postulated to stabilize the γ-phosphoryl group during phosphoryl transfer. In the crystal structure, the guanidinium group of Arg178 is within H-bonding distance of the γ-OH of Dha while at the same time does not directly interact with the β-phosphate of ADP (3.8 Å away). As previously stated, the second magnesium ion (M2) in the complex is coordinated by five oxygens, one of which is from a water molecule located between the β-phosphate of ADP and the substrate γ-OH group and likely mimics one of the oxygen atoms in the γ-phosphate group of ATP. Such an arrangement indicates that both Arg178 and M2 are important for stabilizing the negative charges accumulated on the γ-phosphoryl group (γ-phosphate) during phosphoryl transfer. In the DhaK–DhaL complex, no positively charged residue other than Arg178 could be found at the interface.

The final step in catalysis involves dissociation of Dha-P from DhaK, requiring breaking the hemiaminal linkage. Based on the structure of the active site, His56 is well positioned to act as a general base, abstracting the proton from the β-OH of Dha-P, with concomitant formation of the carbonyl group at C2 and breakage of the Dha-P-His218NE2 bond (Fig. 4iii). The only other amino acid that could potentially act as a general base, Asp109, which interacts directly with the primary hydroxyl groups of Dha via its γ-carboxyl oxygen, is located too far to interact with the β-OH of Dha. Here, the oxygen–oxygen distance is 5.4 Å as opposed to 2.6 Å between NE2 of His56 and the β-OH of Dha. As suggested previously (8), the γ-carboxyl oxygen of Asp109 loses the ability to form an H bond with the primary hydroxyl group of Dha after the phosphoryl-ester is formed. Unfavorable interactions between the phosphate group of Dha-P and the carboxyate group of Asp109 may help release the product from the enzyme. Together our data allow us to propose a chemical mechanism for DhaK supported by several structural and biochemical studies, consistent with involvement of His56 and Asp109 in addition to the previously characterized His218Nε2 (2) and Arg178 (4). These residues are highly sequence conserved in both bacterial and eukaryotic Dha kinases suggesting their common mechanism.

Our results indicating some activity for the H56A and H56N mutants require explanation. The first possibility is that covalently linked substrate is not essential for catalysis. Noncovalent binding of Dha, as reflected by our crystal structure, leads to reduced affinity as indicated by increased \( K_{\text{app}}^{\beta} \) as well as to a suboptimal orientation of γ-OH, affecting \( K_{\text{car}}^{\gamma} \). If the covalent linking is not essential for activity, why are H218K and H218AK mutants dead? We believe that because the lysine mutant introduces a more bulky side chain it does not allow for the binding of Dha. Furthermore, the small Dha molecule could not be well positioned in the absence of the histidine imidazole ring in the H218AK mutant. The second possibility is that catalysis in H56K mutants is achieved through the covalently bound Dha, assuming that His56 determines the equilibrium between the covalently and noncovalently bound states of Dha. The covalent bond might be formed without participation of His56; for example, a water molecule could replace His56 as general acid in the H56A and H56N mutants. The fact that \( K_{\text{car}}^{\gamma} \) rather than \( K_{\text{car}}^{\gamma} \) is more affected by the His56K mutation provides some support for this idea. However, we could not identify appropriate water molecule in any of the His56 mutant structures. Moreover, despite having analyzed 40 independent subunits of the crystal structures of DhaK H56A or H56N, we were not capable of capturing the covalently bound substrate even at 40 mM Dha. Therefore, it is more likely that covalent binding of Dha is not essential for activity.

**Functional Implications of Covalent vs. Noncovalent Substrate Binding by Dha Kinases.** Formation of a transient, covalent bond between substrate and enzyme usually directly contributes to catalysis (9, 10, 11); however, covalent substrate binding by dihydroxyacetone kinases is distinctly different. Previous studies have suggested that the primary role of the hemiaminal bond formed between DhaK and Dha or Dha-P is to provide the enzyme with a way of specifically selecting for short chain ketoses and aldoses vs. polyalcohols, because the enzyme displays remarkable selectivity for Dha in comparison with the closely related molecule, glycerol (8). This is in contrast to glycerol kinase, which is able to effectively utilize both Dha and glycerol as substrates for phosphorylation (12, 13). Quantum-mechanical calculations did not provide any evidence for an influence of the hemiaminal bond on activation of the Dha γ-O atom involved in nucleophilic attack of the γ-phosphate of ATP (8). In our hands, addition of glycerol (10 mM–1 M) to phosphotransferase reactions showed no decrease in kinase activity for wild-type and both H56K mutant enzymes, indicating that the specificity of DhaK for Dha is not a consequence of hemiaminal bond formation per se. The apparent inability of glycerol to compete with Dha for binding to the DhaK active site may be a consequence of subtle structural differences between the two molecules, due to the sp²-c (in Dha vs. the sp³ β-C of glycerol, resulting in steric conflicts of the latter within the quite rigid DhaK active site (based on comparison of all available crystal structures).

Our structural and biochemical results show that covalent bond formation does contribute, in part, to orientation of the substrate for optimal phosphoryl transfer. An additional potential role for hemiaminal bond formation is that in catalysis is for it to serve as an on/off switch, thereby exerting a regulatory function. A covalent bond between Dha and DhaK would support the enzyme existing in either a “Dha-loaded” or “Dha-unloaded” form, as dictated by (i) the inherent affinity of the enzyme for substrate and (ii) the effective concentration of Dha within the cell. The hemiaminal bond, once formed, commits DhaK to the “loaded” state, regardless of further changes in intracellular Dha concentration. It also provides a form of DhaK already primed for catalysis, once an ATP-loaded form of DhaL is available. In this manner, Dha binding and phosphoryl transfer are effectively uncoupled, allowing, for example, variations in the intracellular PEP pool independent of the particular state of DhaK. In a similar manner, the covalent bond would allow for an on/off switch with regards to transcriptional activation or repression that has been shown to occur via interactions with the transcription regulator DhaR (14). In either case, one would predict that a DhaK enzyme incapable of forming the hemiaminal bond in vivo would exhibit an attenuated response as opposed to an on/off behavior, given that the substrate could bind/unbind to the enzyme active site. Importantly, His56 is as strongly sequence conserved as is the catalytic Asp109 in Dha kinases across various species, including higher eukaryotes. This suggests a physiological importance for covalent substrate binding for this class of kinase in both PEP and ATP-dependent branches. The precise nature of the physiological consequences on cellular Dha metabolism resulting from loss of hemiaminal bond formation is the subject of future studies.

**Materials and Methods**

**Protein Expression, Purification, and Characterization.** Details for cloning, mutagenesis, expression, and purification are available in SI Text. Briefly, DhaK and DhaL. N-terminal His6-fusion proteins were purified by nickel affinity chromatography and the tags on DhaK removed by cleavage with tobacco etch virus (TEV) protease. Enzyme I and DhaM GST-fusion proteins were purified by glutathione affinity chromatography and cleaved from the column by TEV. HPr was purified by solubilizing inclusion bodies in urea and refolding the protein on nickel resin. For crystallization, the His6-tag was cleaved from DhaL and the DhaK–DhaL complex was further purified on a Superdex 200 size exclusion column equilibrated with 50 mM Tris pH 8, 10 mM NaCl, 1 mM magnesium acetate, 1 mM DTT. Complex formation of kinase mutants was analyzed by size exclusion chromatography in the same manner except that 1 μM ADP was substituted for AMP-PNP.
Cryotisation. Crystals of the DhaK-DhaL complex were obtained by hanging drop vapor diffusion at 19°C by equilibrating 2 μL of protein (10 mg/ml) with 1 μL reservoir solution (0.1 M HEPES pH 7.5, 3.5 M sodium formate) over 1 mL of reservoir solution. Crystals belong to space group P8_12_1 with unit cell dimensions a = b = 74.6 Å, c = 168.8 Å. Crystals of wild-type DhaK and mutant enzymes were also obtained by hanging drop vapor diffusion by equilibrating 1 μL of protein (2.5 mg/ml of DhaK and 1.7 mg/ml of DhaL-his) with 1 μL reservoir solution (0.1 M sodium citrate pH 5.6, 20% [v/v] PEG 8000) over 1 mL of reservoir solution. Crystals of the wild-type DhaK belong to space group P2_1, with a = 49.8, b = 91.5, c = 73.2 Å, β = 98.9°. Crystals of H56A and H56N belong to space group P21 with unit cell dimensions a = 59.7, b = 82.6, c = 92.8 Å, α = 77.9, β = 78.1, γ = 71.1°, whereas that of the DhaK-H56N-DhaL complex form in space group P2_1 with a = 82.2, b = 101.1, c = 99.3 Å, β = 89.9°. For data collection, crystals were transferred to reservoir solution supplemented with 12% [v/v] ethylene glycol and flash cooled in a nitrogen stream at 100 K (Oxford Cryostreams).

X-ray Data Collection, Structure, and Refinement. Diffraction data for free DhaK and the DhaK–DhaL complex were collected to 2.2 Å at the 31-ID beamline (LRL-CAT), Advanced Photon Source, Argonne National Laboratory. Data integration and scaling were performed with the program HKL2000 (15). Structure determination was performed by molecular replacement using the program Phaser (16) from the CCP4 suite with the previously reported E. coli DhaK (PDB 101Z2) (2) and DhaL (PDB ID code 2BTD) (4) structures as the search models. Refinement was carried out with the program REFMAC (17) with final R_ref = R_free of 0.172/0.210 and 0.189/0.225 for DhaK and DhaK–DhaL, respectively. Diffraction data for crystals of DhaK mutant enzymes were collected to 2.55 Å (H56A), 1.97 Å (H56N), and 2.2 Å (H56N-DhaL) at the CMCF1 beamline at the Canadian Light Source. Data processing and structure determination was done similarly as described above. All models have good geometry as analyzed with PROCHECK (18). Final data collection and refinement statistics are shown in Table S1.

Molecular Dynamics Simulations. The DhaK–DhaL–ATP, DhaK–DhaL–syn-ADP, and DhaK–DhaL–anti-ADP complexes were sampled by 5-nm MD simulations using the AMBER10 suite of programs (19) together with the AMBER ff03 force field for the proteins and a modified force field for ATP and ADP (20). Details of the calculations are available in SI Text.

Phosphotransferase Assay. Phosphorylation of Dha was measured in a coupled assay similar to that described previously (1). To remove residual endogenous Dha that could copurify with recombinant DhaL, the latter was further purified by size exclusion (Superdex 200) in buffer containing 20 mM Tris pH 8, 300 mM NaCl, 1 mM DTT, 1 mM MgCl_2, and 0.01 mM ADP. Phosphotransferase reactions for specific activity measurements contained 1 mM Enzyme 1, 1 mM HPr, 0.5 mM DhaM, 0.5 mM DhaL, 0.5 mM DhaK, 50 mM potassium phosphate pH 7.5, 16 mM Dha, 2.5 mM DTT, 2.5 mM MgCl_2, 2 units glycerol-3-phosphate dehydrogenase (Sigma Chemical Co.), and 1 mM NADH and were initiated by the addition of 2 mM PEP. The glycerol-3-phosphate dehydrogenase coupling enzyme uses NADH to convert Dha-P to glycerol-3-phosphate. The production of Dha-P in the reaction was followed by monitoring oxidation of NADH at 340 nm in a Spectramax 250 plate reader at room temperature. The effect of glycerol on phosphotransferase activity for wild-type, H56A and H56N was examined for reactions in the presence of 10 mM, 100 mM and 1 mM glycerol. k_cat was measured for wild-type enzyme from coupled phosphotransferase reactions (0–1.6 mM Dha) containing 0.5 mM Enzyme 1, 0.5 mM HPr, 0.25 mM DhaM, 0.25 mM DhaL, 0.25 mM DhaK, 50 mM potassium phosphate pH 7.5, 2.5 mM DTT, 2.5 mM MgCl_2, 2 units glycerol-3-phosphate dehydrogenase (Sigma Chemical Co.), and 1 mM NADH, initiated by addition of 2 mM PEP. These conditions are similar to those reported previously (8), and kinetic constants were derived by varying concentration of Dha only, leading to apparent values of kinetic parameters. Due to the limited sensitivity of the assay for wild-type enzyme seen at low substrate concentrations an accurate value for K_m could not be determined. For H56A and H56N, k_cat and K_m were determined from reactions containing 1 mM Enzyme 1, 1 mM HPr, 0.5 mM DhaL, 0.5 mM DhaL, and 0.5 mM DhaK at a range from 0–16 mM and 0–64 mM Dha, respectively. Values were calculated by nonlinear regression fit to a hyperbola.

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REFERENCES
Supporting Information

Shi et al. 10.1073/pnas.1012596108

Results.

DhaK–DhaL Binding Interface. Upon complex formation, the buried surface for DhaK is 1,223 Å² or 8.6% of the total solvent-accessible surface (14,150 Å²) of the DhaK subunit. The buried surface for DhaL due to complex formation is 1,346 Å², accounting for 14.9% of the total solvent-accessible surface of DhaL (9,010 Å²).

The hydroxyl group of Tyr181L forms a water-mediated H bond with the backbone carbonyl oxygen of Asn105K, whereas the Tyr181K aromatic ring stacks against the side chains of Tyr106K and Arg148K that are otherwise buried in the uncomplexed DhaK structure. The residues preceding Tyr181L also form favorable interactions with the relocated loop β6E/α4K (Fig. 1C). For example, the side chain of Arg175K packs against the aromatic ring of Tyr143K and the hydroxyl group of Ser180L forms an H bond with the amide group of Ala145K. On the C-terminal side of Tyr181L, in addition to hydrogen bonding to the carbonyl group of Gly222K, Arg185K is neutralized by Glu125K through a salt bridge. Except for the loop α7L/α8L, other regions of DhaL also contribute H bonds to stabilize the complex (Fig. 1C). The carboxylate of Asp37L is within H-bonding distance of the hydroxyl group of Thr79K. The amide and carboxyl groups of Gly78K, which are situated in the loop α3L/α4L and are in close vicinity of the ADP β-phosphate, make hydrogen bonds to the main chain carbonyl of Ser80L and the side chain of Asn112K, respectively. The guanidinium group of Arg120L, located at the end of α5L, is anchored by the hydroxyl of Thr115K.

Comparison of the E. coli DhaK–DhaL Complex, the C. freundii Dha Kinase and the L. lactis DhaK–DhaL Complex. The overall structure of both DhaK and DhaL subunits in the E. coli complex is similar to the corresponding domains of the C. freundii Dha kinase (1). However, the binding interface between these two subunits/domains is quite different in the two kinases (Fig. S1A). The assembly in C. freundii is likely driven by the hydrophobic nature of the interface and further strengthened by the long linker between the two domains. This differs from the dominant polar nature of the corresponding contact areas in the DhaK–DhaL complex in E. coli (Fig. 1C). In contrast to the rigidity shown by the loop α7L/α8L capping the ADP binding site in both free and complexed E. coli DhaL subunits, the corresponding region in the nucleotide-binding domain of C. freundii Dha kinase is disordered even in the presence of bound AMP-PNP. This is consistent with the different roles of the nucleotide in these two systems (2): ADP in the PTS-dependent kinase functions as a coenzyme and remains permanently bound, whereas ATP is a substrate and needs to be recruited in each catalytic cycle in the C. freundii kinase. Recently, the crystal structure of the DhaL–DhaM complex from L. lactis has been reported (3). The DhaM protein has the EDD fold (4), which is also the core structure of DhaK. As expected, the DhaM subunit in this complex shares high similarity with that in the E. coli DhaK–DhaL complex with an rmsd of 1.1 Å for the aligned 179 Ca atoms (Fig. S1B). Unlike the DhaK subunit in our DhaK–DhaL complex, the DhaM subunit from L. lactis does not undergo significant conformational adjustments upon complex formation. Based on similarities between DhaM and DhaK, such as the positions of several conserved polar residues, a model for the L. lactis DhaK–DhaL complex has been proposed (3). In this model, salt-bridge interactions were predicted to play important roles in DhaK–DhaL complex formation. These salt-bridge interactions, involving a few specific residues, however, are not present in our DhaK–DhaL complex structure even though most of these charged residues are conserved, including Arg120L and Lys122L as well as Glu114K and Glu118K. In both free and DhaL-bound E. coli DhaK, the carboxylic group of Glu114K is fixed by the salt-bridge provided by Arg253K, whereas that of Glu118K is anchored by Trp262K, the latter of which is part of the well-conserved Trp zipper in the β-hairpin capping the edge of the N-terminal β-sheet of DhaK (Fig. S8). This structural arrangement prevents the involvement of Glu114K and Glu118K in interacting with DhaL. It is also very likely that L. lactis DhaK–DhaL complex formation will be accompanied by similar conformational changes for L. lactis DhaK as seen in E. coli DhaK.

Materials and Methods.

Protein Cloning, Mutagenesis, Expression, and Purification. The dhaK (gi: 87081857), dhaL (gi: 1787449) and pstH (phosphocarrier protein HPr; gi: 1788755) genes from Escherichia coli K12 were cloned into a modified pET15b vector (Novagen) that resulted in an N-terminal His₆-tag fusion protein with a tobacco etch virus (TEV) protease site used to remove the tag. The dhaM (gi: 87081856) and pst (phosphotransferase system enzyme I; gi: 1788756) genes from E. coli K12 were cloned into a modified pGEX-4T1 vector (Pharmacia) to create an N-terminal GST fusion protein followed by a TEV protease site. Site-directed mutagenesis was carried out using the QuikChange mutation kit (Stratagene) according to the manufacturer’s protocol and were confirmed by DNA sequencing. Transformed E. coli BL21 (DE3) cells were grown at 37°C in Luria-Bertani (LB) broth with 100 μg/mL ampicillin and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside at 22°C for 16–20 hours.

For DhaK constructs, cells were resuspended in buffer A (50 mM Tris pH 8 and 300 mM NaCl) with 1 mM DTT and lysed by sonication. Cleared lysates were applied to nickel-nitrilotriacetic acid (Ni-NTA) resin and washed with buffer A containing 20 mM imidazole and 1 mM DTT. Proteins were eluted with 200 mM imidazole and 1 mM DTT in buffer A. DhaL constructs were purified in a similar manner, except that 1 mM magnesium acetate and 1–100 μM ADP (2) or AMP-PNP (Sigma) were added to all buffers. DhaK constructs were dialyzed against buffer B (50 mM Tris pH 8, 150 mM NaCl and 1 mM DTT) and the His₆-tags were cleaved by TEV protease. The resulting protein was applied to Ni-NTA resin to remove His-tagged TEV protease and any uncleaved protein.

To verify that the kinase mutants (H56A, H56N, D109A, D109N, H218K, R178E) retained their ability to form the DhaL–DhaK complex, samples were analyzed by gel filtration (Superdex 200) in low salt buffer (20 mM Tris pH 8, 10 mM NaCl, 1 mM DTT) with 1 mM magnesium acetate and 1 μM ADP. Prior to gel filtration, samples containing one wild-type subunit and one mutant subunit (1:1 molar ratio) were dialyzed against the low salt buffer containing 1 mM magnesium acetate and 10 μM ADP. Complex formation was observed as an increase in mass resulting from a slight shift in elution volume (14.05–14.54 mL) when compared to the elution profile of DhaK alone (14.86 mL). Peak fractions were also analyzed by SDS/PAGE to confirm the presence or absence of both subunits.

For purification of Enzyme I and DhaM, cells were resuspended in buffer C (1x PBS with 300 mM NaCl and 1 mM DTT) and lysed by sonication. Cleared lysates were applied to Glutathione-sepharose resin and washed with buffer C. The proteins were eluted by TEV cleavage where the column was washed with buffer A followed by an overnight incubation in buffer B.
acetate and 1 mM Tris pH 8, 10 mM NaCl, 1 mM DTT, and 1 mM magnesium acetate were mixed in a similar manner similar to DhaK except that 1 mM magnesium acetate was substrate. NMR spectra were acquired at 600 MHz using an coupled assay with glycerol-3-phosphate dehydrogenase. Directly detect formation of Dha-P, alleviating the necessity of requiring AMP-PNP were added to buffer B. DhaK and DhaL were mixed in a 1:1 molar ratio and dialyzed into buffer containing 50 mM Tris pH 8, 10 mM NaCl, 1 mM DTT, and 1 mM magnesium acetate and 1 mM AMP-PNP. Protein samples were further purified on a Superdex 200 column equilibrated in the same buffer. Fractions containing the DhaK–DhaL complex were concentrated to 10 mg/mL by ultrafiltration and 0.2 mM PMSF was added. No density corresponding to AMP-PNP was observed in the complex.

NMR-based Phosphotransferase Assays. 1H NMR was used to directly detect formation of Dha-P, alleviating the necessity of using a coupled assay with glycerol-3-phosphate dehydrogenase. Reaction conditions were very similar to those for the spectrophotometric assay, including enzyme concentrations, buffers and temperature except that 3 mM PEP and 1.6 or 16 mM Dha was used as substrate. NMR spectra were acquired at 600 MHz using an AVANCE III spectrophotometer (Bruker) equipped with a cryorobe. Chemical shifts for Dha and PEP were established using pure compounds. Representative spectra are shown in Fig. S6.

Molecular Dynamics Simulations. The DhaK–DhaL–(ATP), DhaK–DhaL–(syn-ADP), and DhaK–DhaL–(anti-ADP) complexes were sampled by 5-ns MD simulations using the AMBER10 suite of programs (6) together with the AMBER ff03 force field for proteins and a modified force field for ATP and ADP (7). Starting from the crystal structure of the DhaK–DhaL–(ADP) complex, the γ-phosphate group was attached to ADP in the crystal structure to form the ATP substrate using the Leap program in AMBER10 utilizing the ATP library file of Meagher et al. (7). The syn-ADP starting structure was adapted from the ADP-bound DhaL crystal structure (2BTD) (8) and superposed onto the crystal structure of the DhaK–DhaL–(ADP) complex (PDB ID code 3PNL). RESP (Restrained ElectroStatic Potential) partial charges for Dha covalently bound to His218 were calculated. Each complex was solvated in a rectangular parallelepiped TIP3P water box (9) and the electro-neutrality of the system achieved by the addition of Na+ counter ions. For the ATP-bound complex, the nucleophilic hydroxyl Oγ atom of Dha was subjected to two harmonic distance restraints of 30 kcal mol−1 Å−2, one with respect to the reactive γ-phosphate P atom of ATP within the 3.4–3.9 Å range and the other one to the OD2 atom of Asp109 of DhaK within the 2.7–3.2 Å range. For the ADP-bound complex, only the second restraint was used. The two Mg2+ ions in the crystal structure were subjected to harmonic distance restraints with respect to their coordinating atoms from Asp305, Asp353, Asp371, Phe78β, and the α- and β-phosphate groups of ATP or ADP. For each complex, we performed a 5 ns NPT production run with snapshots collected every 1 ps, using a 2 fs time-step. The final ATP or ADP-bound complex structure was obtained by coordinate averaging over the last 2 ns of the MD trajectory, followed by 1,000 steps of energy minimization without any restraints.

**Fig. S1.** Superposition of the *E. coli* DhaK-DhaL complex with (A) Dha kinase from *C. freundii* and (B) the DhaL-DhaM complex from *L. lactis*. Secondary structures are shown in ribbon representation. The AMP-PNP, ADP, and Dha molecules are shown in stick mode and the metal ions as spheres. The *E. coli* DhaK and DhaL subunits are shown in yellow and cyan, respectively. The K- and L-domains from *C. freundii* Dha kinase in (A) are shown in light blue and deep salmon, respectively. In B, DhaL from *L. lactis* is colored in light blue and DhaM subunits are colored in green and deep salmon.

**Fig. S2.** Conformational flexibility of loop β6-α4 of DhaK as indicated by three alternate conformations from three crystal structures [DhaK in free form previously determined in space group P2₁2₁2₁, PDB ID code 1OI2 (10), shown in pale green; DhaK in free form determined in space group P2₁ from the current study (PDB ID code 3PNK) is shown in red; DhaK as bound to DhaL (PDB ID code 3PNL) shown in yellow]. Residue Y143 is shown in stick mode in all three forms. The DhaL from the DhaK–DhaL complex is shown in cyan.
Fig. S3. Conformational change of the ADP molecule (from syn to anti) and relocation of the second magnesium ion (M2' to M2) in DhaL upon complex formation. The (Fo – Fc) omit map for the ADP molecule is contoured at 4σ.

Fig. S4. MD calculation of ADP in both syn- (magenta) and anti- (cyan) conformations. In all of the resulting models with ADP in the syn- conformation, the adenosine moiety of ADP is pushed away due to a potential steric clash with Thr107ɛ.
Fig. S5. Specific activity for wild-type Dha kinase and mutants measured at 16 mM Dha. Specific activities are given as \( \mu \text{mol Dha-P detected per minute per mg of } \text{DhaL-DhaK protein.} \) The standard deviation was calculated from three independent experiments.

Fig. S6. Representative \(^1\)H NMR spectra for Dha-P production by wild-type and mutants of Dha kinase. All spectra were acquired at 23°C using 2 or 15 min time intervals. Data shown represent 1 hour after reaction was initiated by addition of 3 mM PEP.
Fig. S7. Active site of the DhaK H56N\^5 mutant. The Dha molecule is absent due to the H56N\^5 mutation. All residues are shown in stick mode and covered by the 2Fo – Fc electron density map contoured at 1.3\(\sigma\). The residues G53\^K, N56\^K, K104\^K, D109\^K, and H218\^K are labeled. Water molecules in close vicinity are shown as red spheres.

Fig. S8. Anchoring of acidic residues (Glu114\^K and Glu118\^K) by residues in the \(\beta\)-hairpin of DhaK, explaining why these residues could not participate in interactions with the DhaL subunit. DhaK is shown in yellow, DhaL in cyan.
Table S1. X-ray data collection and refinement statistics

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<th>DhaK(H56N)</th>
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*Data for the highest resolution shell are given in parentheses.
\[ R_{sym} = (\sum |I_{obs} - I_{avg}|) / |I_{avg}| \]
\[ R_{work} = (\sum |F_{obs} - F_{calc}|) / |F_{obs}| \]