From ATP as Substrate to ADP as Coenzyme

FUNCTIONAL EVOLUTION OF THE NUCLEOTIDE BINDING SUBUNIT OF DIHYDROXYACETONE KINASES*

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Dihydroxyacetone kinases are a family of sequence-related enzymes that utilize either ATP or a protein of the phosphoenolpyruvate-sugar phosphotransferase system (PTS) as a source of high energy phosphate. The PTS is a multicomponent system involved in carbohydrate uptake and control of carbon metabolism in bacteria. Phylogenetic analysis suggests that the PTS-dependent dihydroxyacetone kinases evolved from an ATP-dependent ancestor. Their nucleotide binding subunit, an eight-helix barrel of regular up-down topology, retains ADP as phosphorylation site for the double displacement of phosphate from a phospho-histidine of the PTS protein to dihydroxyacetone. ADP is bound essentially irreversibly with a $t_1/2$ of 100 min. Complexation with ADP increases the thermal unfolding temperature of dihydroxyacetone L from 40 (apo-form) to 65 °C (holoenzyme). ADP assumes the same role as histidines, cysteines, and aspartic acids in histidine kinases and PTS proteins. This conversion of a substrate binding site into a cofactor binding site reflects an instance of parsimonious evolution.

Few compounds are as ubiquitous and highly connected as adenine nucleotides (1). ATP functions as a carrier of chemical energy, ADP, AMP, ADP-ribosyl, adenylyl moieties as enzyme regulators, and cAMP as a second messenger. The nucleotide coenzymes NADH, FAD, and coenzyme A contain an adenosyl group that without direct participation in catalysis assists in binding to the apoenzyme. Here we report on ADP acting as phospho-histidine in the double displacement phosphoryl transfer reaction catalyzed by the *Escherichia coli* dihydroxyacetone (Dha)$^1$ kinase. Dha kinases are a family of sequence-related enzymes that can be divided into two groups according to their phosphate donor, namely ATP or a phosphoprotein of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) (2) (Fig. 1A). ATP-dependent kinases occur in bacteria, yeast, animals, and plants; Dha kinases dependent on the PTS, a dedicated energy transducing system involved in carbohydrate uptake and control of carbon metabolism (3, 4), occur only in bacteria. In methylo trophic yeast, free Dha is the product of a transketolase reaction between ribulose-5-phosphate and formaldehyde derived from methanol. In bacteria, Dha is formed by oxidation of glycerol (5–7). For yeast it has been shown that Dha kinases fulfill a “housecleaning” function by removing chemically reactive (8) and potentially hazardous short chain carbohydrates (9).

The Dha kinases of *C. freundii* (DAK) and of *E. coli* (DhaK, DhaL) are prototypes of ATP- and PTS-dependent kinase, respectively (2, 10, 11). The former consists of two domains that are connected by a long flexible linker, the latter of two subunits (DhaK and DhaL) that show homology with DAK throughout their combined lengths. DhaK contains the Dha binding site, DhaL the nucleotide binding site (12, 13). The DhaL fold, an eight-helix barrel of regular up-down topology, constitutes a new scaffold of nucleotide-binding proteins (Fig. 1, B and C). The PTS-dependent kinases utilize an additional subunit (DhaM) that serves as a phosphate shuttle between the general phosphoryl carrier protein HPr of the PTS and the kinase (Fig. 1A). DhaM subunits are made up of one domain that delivers the phosphate to the kinase and (species-dependent) one or two additional domains that form a phospho-histidine relay to the PTS. All three domains are homologous to known proteins of the PTS (2).

The sequence and functional similarity between PTS- and ATP-dependent kinases points to their common origin. A phylogenetic tree generated from 41 DhaL subunits and domains shows three distinct protein clusters (Fig. 1D). The first contains the DhaL-like domains from ATP-dependent kinases of animals, plants, yeast, and bacteria. The second contains three types of DhaL subunits: (i) DhaL encoded by operons containing genes for DhaK subunits and multidomain (M) or single-domain (m) DhaM, (ii) DhaL encoded in dhaKL operons without a *dhaM* gene (x), and (iii) DhaL paralogs encoded in an operon together with a protein that itself consists of a SorC-type transcription factor and a DhaK-like domain (12). The two subunits are presumed to control Dha kinase expression (14). The third cluster contains domains from proteins of unknown function that are related to DhaL only by similarity of fold (12). The evolutionary relationship between ATP- and PTS-dependent kinases is elucidated by the observation of an isotope exchange between Dha and Dha phosphate (DhaP). It pointed to a double displacement mechanism, and together with our previous failure to detect a covalent phospho-histidine or phospho-aspartate intermediate (2) it eventually led to the identification of a tightly bound ADP acting as phosphate acceptor in the kinetically active intermediate.

MATERIALS AND METHODS

*Protein Purification and Activity Assays—* Proteins were purified as previously described (2, 15). All activity assays and analytical gel filtrations of DhaL were done in salt buffer (1 mM MgCl$_2$, 50 mM NaCl, 10...
mm HEPES, pH 7.5, and 10 μM ADP unless indicated otherwise. Equilibrium phosphate exchange assays contained per 0.1 ml: 1 μM DhaK, 0.1 mM [14C]Dha, 0.8 mM DhaP, and DhaL or DhaM as indicated. After 30 min of incubation at 37 °C, the reaction was stopped by dilution with 2 ml of ice-cold water, and [14C]DhaP was separated by ion exchange chromatography and determined by liquid scintillation counting as described (16). Dha kinase assays contained per 0.1 ml: 0.1 mM [14C]Dha (1000 cpm/nmol) as substrate, 0.5 mM P-enolpyruvate, 0.08 mM EI, 3.5 mM HPr, 1.5 mM DhaM, 1 mM DhaK, and 0.03 mM DhaL (or as indicated). After 30 min of incubation at 37 °C the reaction was stopped and analyzed as indicated above. DhaLADP (10 μM in 0.2 ml) was phosphorylated with 60 μM [γ-32P]ATP, then radiolabeled proteins were separated by 10% SDS-PAGE and quantified with Fuji FLA-3000 phosphorimaging. After 10 min of incubation at 30 °C, DhaL-[γ-32P]ATP and apo-DhaL were separated from [32P]P-enolpyruvate and free [γ-32P]ATP on a Hi Trap desalting column at 20 °C in the absence and presence of 10 mM EDTA, respectively. ATP and DhaP were separated by thin layer chromatography (polyethyleneimine cellulose, eluent 1:1 (v/v) mixture of 0.84 M KH2PO4, pH 3.4, and 0.25 M LiCl, 1 mM formic acid). Initial burst assays for (i) ATPase activity of DhaL and (ii) the formation of DhaL-ATP from DhaLADP were done as follows. (i) 2 μM apo-DhaL (wild-type or mutants) were incubated with 10 μM [α-32P]ATP in 20 μl of standard buffer at 30 °C for the indicated time, and the reaction was stopped by the addition of 80 μl of 1 M formic acid. [α-32P]P and [α-32P]ATP produced were separated by thin layer chromatography (polyethyleneimine cellulose, 0.4 M K2HPO4, 0.7 M BOH, 1 M formic acid). After incubation at 37 °C for the time indicated, the DhaL-[α-32P]P complex was then separated from free [α-32P]P by gel filtration on a Superdex-75 column in buffer A at room temperature. Protein unfolding—Temperature-induced unfolding was monitored by circular dichroism spectroscopy at 222 nm in a Jasco spectropolarimeter (J715-A) using a 0.5-mm light-path cell. 20 μM DhaL and 100 μM indicated nucleotide were heated at a rate of 60 °C h⁻¹ in buffer A (5 mM MgCl₂, 150 mM NaCl, 10 mM Tris-HCl, pH 8.5, 1 mM dithiothreitol, 10% glycerol). Data were normalized to the difference of the linear changes above and below the transition region.

Isotope Exchange Experiments—The DhaL-[α-32P]P complex was prepared by incubation of 0.67 μl of 80 μM DhaL-ADP in buffer A containing 100 μM free ADP and 2 μCi of [α-32P]ATP overnight at 20 °C. During this time [α-32P]ADP was formed by the intrinsic ATPase activity of DhaL, and isotopic equilibrium with ADP was established. The DhaL-[α-32P]P complex was then separated from free [α-32P]P by gel filtration on a Superdex-75 column in buffer A at room temperature. Peak fractions containing DhaL-[α-32P]P were pooled. The ADP/DhaL molar ratio in the peak fractions was between 0.9 and 0.95. ADP exchange was initiated by the addition of a 166-fold molar excess of cold ADP (10 μM of 20 mM ADP) to 190 μl of 6 μM DhaL-[α-32P]P. After incubation at 37 °C for the time indicated, the DhaL-[α-32P]P complex was separated from free [α-32P]P by gel filtration on a Hi Trap desalting column (Amersham Biosciences) in buffer A. The koff rate was calculated by non-linear least square fitting of the radioactivity counts in the two fractions to the equation cpm(t) = cpm₀ exp(−kₐoff t) and cpm(0) = cpm₀ (1 − exp(−kₐoff t)), respectively.

Sequence Analysis—Amino acid sequences were aligned with CLUSTALW. Analyses were performed with 41 of 61 representative sequences from bacteria and eukaryotes. Sequences of paralogs were included; orthologs of closely related organisms were omitted. The phylogenetic tree was constructed with Gene-Bee (13) using the default parameters. Swiss-Prot primary accession numbers are given. The sequences of Klebsiella pneumoniae and Mycobacterium smegmatis were obtained at genome.wustl.edu/projects/bacterial/kpneumoniae/ and tigrblast.tigr.org/umfg/index.cgi?data base = m_smeqsmegatis sec.

RESULTS AND DISCUSSION

The DhaL Subunit of the PTS-dependent Dha Kinase Is Transiently Phosphorylated—The Dha binding subunit DhaK,
[14C]-labeled Dha, and unlabeled DhaP were incubated without and with DhaL. [14C]DhaP was formed only in the presence of DhaL. Addition of the phosphotransferase subunit DhaM did not support phosphate transfer or stimulate the DhaL-dependent reaction (Fig. 2), indicating that DhaL was necessary and sufficient as the phosphate transferring subunit. His-38 of DhaL appeared as a prime candidate for a protein phosphorylation site (12) because (i) it is invariant in all PTS-dependent kinases, (ii) the H38T and the H38A mutants were inactive, (iii) His-38 corresponds to the nucleotide binding Thr-388 of the Citrobacter freundii kinase (Fig. 1, B and C), and (iv) PTS proteins generally are phosphorylated at histidines (3, 4).

After incubation of DhaL with [32P]P-enolpyruvate (phosphoenolpyruvate) and catalytic amounts of EI, HPr, and DhaM, the 32P label comigrated with DhaL on gel filtration in EDTA-free buffer while it was released in the presence of EDTA (Fig. 3, A and B). 32P-labeled, histidine-tagged DhaL could be affinity captured with Ni2+, nitritrolactic acid beads but could not be detected in non-denaturing polyacrylamide gels (not shown).

The EDTA sensitivity, and the fact that the nucleotide is coordinated by two well ordered magnesium ions in the x-ray structure of the C. freundii kinase (Fig. 1C) (12), suggested that a nucleotide may also play a role in the PTS-dependent Dha kinases. To demonstrate that the [32P]phosphate comigrating with DhaL is the γ-phosphate of ATP and that this γ-phosphate is actually transferred to Dha, the peak fraction from the gel filtration (Fig. 3A) was split in two. DhaK and Dha were added to one half and EDTA (5 mM) to the other. Phosphate was transferred quantitatively to Dha in the presence of DhaK while γ-32PATP was released in the presence of EDTA (Fig. 3C, lanes a and b). This demonstrates that DhaL-ATP is the catalytically competent intermediate and that hydrolysis of ATP to ADP does not represent a control mechanism, for instance to turn off the enzyme.

DhaL Contains ADP as a Coenzyme—To further support this proposition, histidine-tagged DhaL was purified by metal chelate affinity chromatography followed by gel filtration in the presence of EDTA. DhaL thus purified is inactive, severely unstable, and prone to precipitate at room temperature. Addition of ADP or ATP restored activity, whereas the non-hydrolyzable analogue AMPPNP, the structural analogue ADPβS, AMP-β-mannate, and AMP did not (Fig. 4A). The ADP concentration necessary for half maximal activity (AC50) is 18 mM. The H38T mutant also could be activated by ADP, but it had a 1000-fold higher AC50 of 38 mM (Fig. 4B), indicating that His-38 is critical for strong binding of the ADP cofactor and not, as presumed, as a phosphorylation site. The dissociation rate of the DhaL-ADP complex (koff rate) was measured by isotope exchange between the purified DhaL[α-32P]ADP complex and a 166-fold molar excess of unlabeled ADP (Fig. 5A). The koff rate calculated from the decay curve is 11.2 ± 1.3·10−5 s−1. This corresponds to a half-life (t1/2) of 100 min for the Dha-ADP complex, which is three times the generation time of a rapidly dividing E. coli cell. Taking the AC50 value of 18 mM (Fig. 4B) as an estimate for Kd, the calculated koff rate for the DhaL ADP association thus is 6.2·10−9 M−1 s−1. This value is three orders of magnitude slower than the average association rate constants of enzymes that bind nucleotides as substrates and after each turnover release them as products (19). The slow association rate may be due to desolvation requirement (19) or, more likely, to a major conformational adjustment required for the opening of the ADP binding site.

Complexation with ADP increases the thermal unfolding temperature of the DhaL apoenzyme from 41.5 to 65.7 °C (Fig. 5B), providing additional evidence for an essentially irreversible apoenzyme-coenzyme complex and the important role of ADP in stabilizing the protein. AMPPNP and ATPβS increase the unfolding temperature of DhaL by 9 and 12 °C, respectively, relative to the nucleotide-free form. The H38T and H38A mutants, which have a 1000-fold lower affinity for ADP, are moderately stabilized by the nucleotide (Fig. 5C).

The fact that ADP and ATP have the same cofactor activity whereas AMPPNP has none (Fig. 4A) supports the proposition that ADP is the active moiety. DhaL has a weak ATPase activity that produces a burst of one [α-32P]ADP/DhaL upon incubation of apo-DhaL with [α-32P]ATP (Fig. 6A). Conversely, a stoichiometric amount of [γ-32P]ATP is rapidly formed but not released when the DhaL:ADP complex is incubated with [32P]P-enolpyruvate in the presence of DhaM and the general phosphoryl carrier proteins of the PTS (Fig. 6B). The two burst...
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FIG. 5. ADP binding to DhaL is essentially irreversible. A, isotope exchange reaction between DhaL-[\(\alpha\text{-}^{32}\text{P}\)]ADP and a 166-fold molar excess of free ADP. DhaL-[\(\alpha\text{-}^{32}\text{P}\)]ADP and released [\(\alpha\text{-}^{32}\text{P}\)]ADP were separated by gel filtration. Shown are the fraction of DhaL-[\(\alpha\text{-}^{32}\text{P}\)]ADP remaining (circles) and the fraction of [\(\alpha\text{-}^{32}\text{P}\)]ADP released (squares) at time \(t\) (for details see “Materials and Methods”). B and C, temperature-induced unfolding of DhaL holo- and apoenzyme. The DhaL-ADP holoenzyme is stable; the apoenzymes and complexes with other nucleoside phosphates are destabilized. The DhaL H38T (dashed line) and DhaL H38A (dotted line) mutants are only marginally stabilized by ADP. For details see “Materials and Methods.”

FIG. 6. Initial burst of DhaL-ATP hydrolysis and slow nucleotide exchange in the His-38 mutants. A, incubation of wild-type DhaL with ATP results in the rapid formation of one ADP per DhaL. In the presence of the H38A and H38T mutants ADP is formed continuously, pointing to nucleotide exchange. B, PTS-mediated phosphorylation of DhaL-ADP. One ATP is formed per DhaL. The mutants H38T and H38A after an initial burst lose ATP continuously. Conditions were: 2 \(\mu\text{M}\) DhaL, 10 \(\mu\text{M}\) [\(\alpha\text{-}^{32}\text{P}\)]ATP (A) and 2 \(\mu\text{M}\) DhaL, 10 \(\mu\text{M}\) [\(\alpha\text{-}^{32}\text{P}\)]ADP (B) in standard assay buffer. C and D, comparison of the PTS- and ATP-dependent kinase activities of wild-type and H38T mutant. C, PTS-dependent activities are identical. D, the ATP-dependent activity of H38T is 2-fold increased relative to wild-type. The \(k_{\text{cat}}\) values are calculated from the curves and given in inserts. Conditions were 0.2 \(\mu\text{M}\) ADP in assay buffer (C) and 1 \(\mu\text{M}\) ATP, [\(^{14}\text{C}\)]Dha, and DhaK in salt buffer (D). For details see “Materials and Methods.”

ATP- and ADP-dependent DhaL Have a Common Origin—

The tightly bound ADP participates in a double displacement phosphoryl transfer reaction and thus plays the same role as histidines, cysteines, and aspartic acids in other phosphoprotein intermediates. Such a cofactor function of ADP has to the best of our knowledge not been described before. Retaining ADP as cofactor instead of evolving an amino acid such as a histidine into a phosphorylation site is an unprecedented example for parsimony in functional evolution during the switch of a kinase from ATP to the PTS as source of high energy phosphate. Which modifications of the nucleotide binding site accompanied this switch? In loop 1 (GDXXGDGD/T/H/GXNM) of the helix barrel, the threonine that is in hydrogen bonding distance to the \(\alpha\) phosphate of ATP (12) in the ATP-dependent kinase of \(C.\) \textit{freundii} is replaced by a histidine that is invariant in the PTS-dependent forms. Although interactions of nucleotide oxygens with aspartate, serine, threonine, lysine, and arginine are frequent, interactions with histidine were not detected in over 3000 contacts analyzed (1), adding evidence to our finding (Fig. 4B) that this histidine may be important for tight binding of ADP. In loop 3 (GC/S/A/GXLYG) a serine/threonine that coordinates with the \(\gamma\) phosphate in the ATP-dependent kinase (12) is replaced by an alanine in most PTS-dependent kinases. In the conserved sequence of loop 5 (GXXAXXGDRTKM) no conspicuous differences between the two forms can be detected. The most remarkable of the four loops surrounding the nucleotide binding site is loop 7 (GRASYL/DPGA). The middle section of this long loop is highly variable in the ATP-dependent kinases, and the loop is disordered in the x-ray structure of the \(C.\) \textit{freundii} kinase in complex with AMPPNP (12). In the PTS-dependent DhaL, however, not only the flanking region (GRASYL and DPGA) but also the central portion (GERSLG) is almost invariant. Loop 7 is long enough to latch over the nucleotide binding pocket, and the rigidity of this loop could determine the rate of nucleotide exchange. Instead of being exchanged after each round of phosphate transfer, ATP is regenerated from ADP in \textit{situ} by DhaM, a dedicated phosphotransferase of the PTS. Evolution of DhaM must have occurred by the more “conventional” path involving gene duplication, fusion, and specialization of protein modules of the PTS that already have the intrinsic property of multiply interacting with proteins as diverse as carbohydrate transporters, enzymes, and transcriptional regulators. It is noteworthy that some bacteria (\textit{Sinorhizobium}, \textit{Mycobacterium}, \textit{Listeria}, and \textit{Klebsiella}) have genes for more than one DhaL-like subunit or domain that according to the phylogenetic tree (Fig. 1D) arose by gene duplication from a common ancestor. The association of these paralogs with transcription regulators indicates that they act as coactivators or corepressors of transcription. It thus appears that the use of ADP as cofactor has been invented only once in
evolution and is now being used for both catalysis and signaling. A double function as enzyme subunit and autoactivator of transcription has been described for DhaL of *E. coli* (20).

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