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Review

Small substrate, big surprise: fold, function and phylogeny of dihydroxyacetone kinases

B. Erni*, C. Siebold⁺, S. Christen, A. Srinivas, A. Oberholzer⁺⁺ and U. Baumann

Department of Chemistry and Biochemistry, University of Bern, Freiestr. 3, 3012 Bern (Switzerland),
e-mail: bernhard.erni@ibc.unibe.ch

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Abstract. Dihydroxyacetone (Dha) kinases are a family of sequence-conserved enzymes which utilize either ATP (in animals, plants and eubacteria) or phosphoenolpyruvate (PEP, in eubacteria) as their source of high-energy phosphate. The kinases consist of two domains/subunits: DhaK, which binds Dha covalently in hemiaminal linkage to the N ϵ 2 of a histidine, and DhaL, an eight-helix barrel that contains the nucleotide-binding site. The PEP-dependent kinases comprise a third subunit, DhaM, which rephosphorylates *in situ* the firmly bound ADP cofactor. DhaM serves as the shuttle for the transfer of phosphate from the bacterial PEP: carbohydrate phosphotransferase system (PTS) to the Dha kinase. The DhaL

and DhaK subunits of the PEP-dependent *Escherichia coli* kinase act as coactivator and corepressor of DhaR, a transcription factor from the AAA⁺ family of enhancer-binding proteins. In Gram-positive bacteria genes for homologs of DhaK and DhaL occur in operons for putative transcription factors of the TetR and DeoR families. Proteins with the Dha kinase fold can be classified into three families according to phylogeny and function: Dha kinases, DhaK and DhaL homologs (paralogs) associated with putative transcription regulators of the TetR and DeoR families, and proteins with a circularly permuted domain order that belong to the DegV family.

Keywords. AAA⁺-ATPase, ADP, coenzyme, glycerone, hemiaminal, phosphoenolpyruvate, phosphotransferase.

Dihydroxyacetone: the substrate

Dihydroxyacetone (Dha) is one of the simplest carbohydrates. It consists of only three carbon atoms, is non-chiral and in principle can be formed under prebiotic conditions by spontaneous condensation of three molecules of formaldehyde. Glyceraldehyde can be formed by tautomerization (hydrogen shift) of Dha, and the two trioses then can combine by aldol condensation to form sorbose

and fructose [1, 2]. The two hexoses (or hexose phosphates under biotic conditions) can serve as precursors for nearly every sugar that exists in nature (for the structural formulas of Dha and its reaction partners see Fig. 1). Solid dihydroxyacetone (C₃H₆O₃, glycerone) exists as a dimeric hemiketal that in aqueous solution dissociates into a 4:1 mixture of monomeric ketone and ketone hydrate (*gem*-diol) [3]. Dha is chemically reactive like other ketose sugars but because of its small size cannot react intramolecularly to form cyclic hemiketals. Instead, it reacts intermolecularly with proteins and nucleic acids by Maillard-type cross-linking reactions [4, 5]. Owing to its chemical reactivity Dha is an important precursor of building blocks for use in organic synthesis [2] and has many technical applications. Dha is the active ingredient of sunless tanning (self-tanning) sprays and lotions [6, 7], and 6-acetyl-

* Corresponding author.

⁺ Present address: Division of Structural Biology, The Henry Wellcome Building for Genomic Medicine, The University of Oxford, Roosevelt Drive, Oxford OX3 7BN (United Kingdom).

⁺⁺ Present address: Section of Structural Biology, The Institute of Cancer Research 237 Fulham Road, London SW3 6JB (United Kingdom).

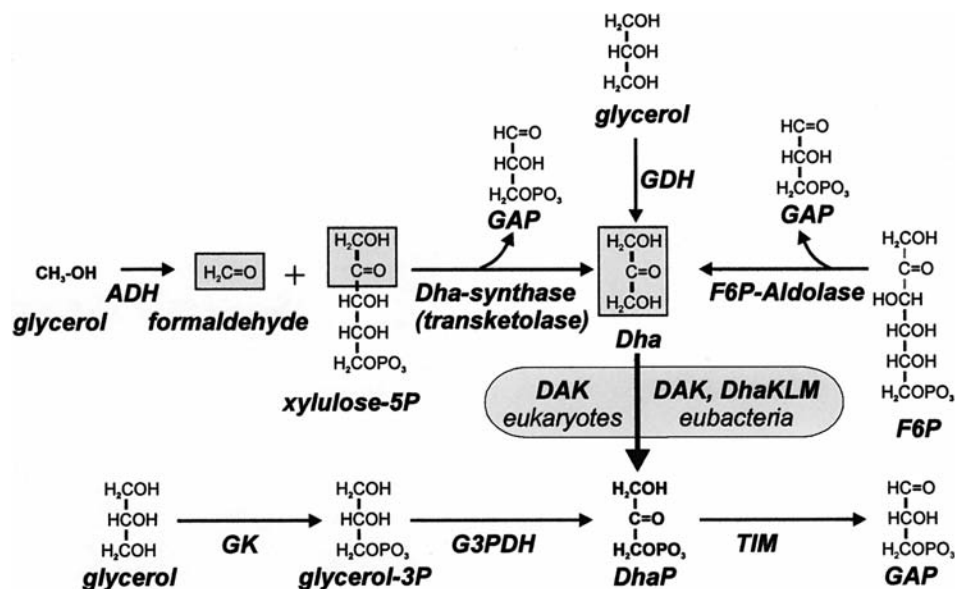


Figure 1. Metabolic origins of Dha. Alcohol dehydrogenase (ADH) and Dha-synthase are used by methylotrophic yeast for the assimilation of methanol. Membrane-bound and soluble glycerol dehydrogenases (GDH) occur in bacteria. Aldolases are ubiquitous. The Dha kinases (DAK and DhaKLM) also phosphorylate D-glyceraldehyde and possibly other short-chain ketoses and aldoses (not shown). Glycerol kinase (GK) and glycerol-3-phosphate dehydrogenase (G3PDH) are an alternative for the conversion of glycerol to dihydroxyacetone phosphate (DhaP). Triosephosphate isomerase (TIM) interconverts DhaP and glyceraldehyde-3-phosphate (GAP). The rectangular boxes highlight the carbon bond-forming reactions of Dha.

1,2,3,4-tetrahydropyridine, the product of the ‘Hunter reaction’ between Dha and L-proline, contributes to the roasty odour of crackers, popcorn and bread crust [8, 9].

Dha is utilized as a carbon source by bacteria and yeasts, and there are several pathways by which free Dha is formed in living cells (Fig. 1). (i) The yeast *Schizosaccharomyces pombe* and the bacteria *Klebsiella pneumoniae* and *Citrobacter freundii* oxidize glycerol with a soluble NAD^+ -dependent glycerol dehydrogenase under anaerobic conditions, and Dha is then phosphorylated by a Dha kinase [10–13]. (ii) *Gluconobacter oxydans* and related bacteria oxidize glycerol with a membrane-bound, pyrroloquinoline quinone (PQQ)-dependent glycerol dehydrogenase that is connected *via* ubiquinone to a respiratory chain. The oxidation reaction takes place on the outer face of the membrane, and Dha is released into the medium [14]. Industrial *Gluconobacter* strains yield up to 220 kg/m^3 of Dha because the dehydrogenase retains activity even after irreversible growth inhibition of cells by Dha [15]. (iii) Methylotrophic yeasts produce Dha during methanol assimilation. In this pathway, methanol is first oxidized to formaldehyde. To this one-carbon compound a two-carbon hydroxyethyl group is then transferred from xylulose-5-phosphate by Dha-synthase, a thiamine pyrophosphate-dependent transketolase [16–21]. Bacteria, for comparison, utilize other routes to assimilate formaldehyde and its oxidation product CO_2 , namely the ribulose monophosphate (RuMP) cycle, the serine pathway and the reductive pentose phosphate cycle [21, 22]. None of them produces Dha. Only recently have

the xylulose monophosphate cycle enzymes also been discovered in a methanol-utilizing carboxydobacterium [23, 24].

Little is known about the metabolic origin and the fate of free Dha in higher eukaryotes and plants. Dha and glyceraldehyde may be formed by slow aldol cleavage of ketopentoses and ketohexoses or ketose monophosphates [25, 26] and by the oxidation of glycerol by NAD^+ -dependent alcohol dehydrogenases and NADPH -dependent aldehyde reductases [27]. Dha stimulates the secretion of insulin [28] and is utilized as gluconeogenic precursor by mammalian tissues [28–32]. Dha and pyruvate have been claimed to increase muscle mass and aerobic endurance capacity [33, 34], and Dha thus made its way also into over-the-counter weight-loss products.

Occurrence and biochemistry of Dha kinases

Dha kinases occur in eubacteria, plants and animals, including humans. There exist two different enzymes with Dha kinase activity: (i) In the narrow sense, Dha kinases that phosphorylate Dha and D-glyceraldehyde (EC 2.7.1.28 and EC 2.7.1.29). Such kinases consist of a Dha binding α/β domain and a nucleotide-binding all α -helical domain. They are the topic of this review. (ii) Glycerol kinases (EC 2.7.1.30) that consist of two structurally homologous α/β domains. They have the binding sites for glycerol and ATP in the cleft between the two domain [35, 36]. Glycerol kinases are less specific, and in addition to

glycerol also phosphorylate Dha and glyceraldehyde [37–40]. The Dha-specific kinases have been characterized in bacteria [10, 41] and in methylotrophic yeast (for a review see [42–44]). They have been purified from yeasts [16, 17, 42, 43, 45–49], algae [50], tomato [51], rat brain [31], porcine kidney [52] and from bacteria, viz. *C. freundii* [10, 41], *K. pneumoniae* [13] and *E. coli* [53].

Dha kinases can be grouped into two structurally related families (Fig. 2) according to their source of high-energy phosphate, namely ATP and phosphoenolpyruvate (PEP). The ATP-dependent kinases occur in yeasts, animals, plants and eubacteria, the PEP-dependent only in eubacteria. The existence of a PEP-dependent kinase was first noticed by Jin and Lin [54] in an *E. coli* double mutant lacking the ATP-dependent glycerol kinase and the NAD⁺-dependent glycerol-phosphate dehydrogenase. While looking for an ATP-dependent Dha kinase, they

observed that an *E. coli ptsI* mutant, which lacked EI of the phosphoenolpyruvate:sugar phosphotransferase system (PTS; Fig. 2) and therefore did not grow on PTS sugars also no longer grew on Dha (for reviews on the PTS see [55–57]). They subsequently isolated a transposon mutant unable to grow on Dha but that grew normally on PTS sugars. The transposon-tagged gene was mapped to 26 min in the *E. coli* chromosome, termed *ptsD* and proposed to encode a Dha-specific enzyme II of the PTS [58]. However, this putatively membrane-bound PtsD activity has never been confirmed biochemically. Instead, the operon at 26 min on the *E. coli* chromosome was found to encode two proteins (DhaK and DhaL) that are homologous to ATP-dependent Dha kinases, and one multidomain protein (DhaM) that is homologous to enzyme I and HPr, two phosphotransfer proteins of the PEP:carbohydrate (PTS) [59]. The DhaK and DhaL subunits were also found as upregulated protein spots in the proteome of an *E. coli ptsI* (EI) mutant [60]. Both findings confirmed that Jin and Lin indeed discovered a Dha kinase and that this kinase was functionally and genetically associated with the PTS.

ATP-dependent Dha kinases are single polypeptide two-domain proteins, while the PEP-dependent forms consist of three subunits, DhaK, DhaL and DhaM (Fig. 2). DhaK and DhaL are homologous to the amino terminal K- and the carboxy terminal L-domain of the ATP-dependent kinases. DhaK contains the binding site for Dha, and DhaL contains the nucleotide-binding site. DhaM is a phosphohistidine protein that transfers phosphoryl groups from a phosphoryl carrier protein of the PTS (HPr or EI) to the DhaL-ADP complex [53, 61, 62].

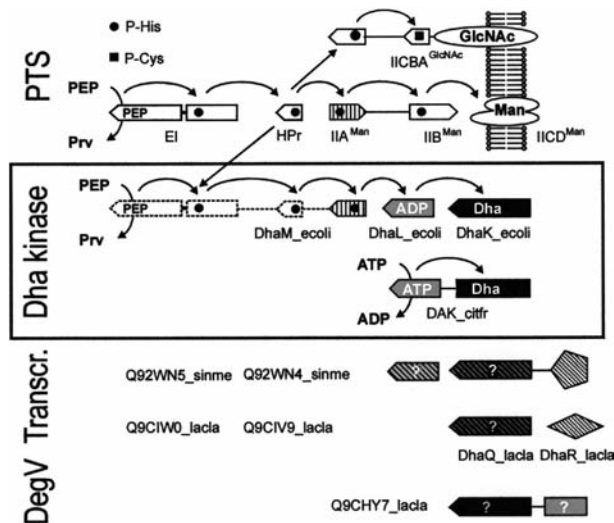


Figure 2. Dha kinases and the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS). Center: a PEP-dependent heterotrimeric Dha kinase (DhaK, DhaL, DhaM), and an ATP-dependent two-domain Dha kinase. Top: Domain structure of the PTS. Shown are the core components of the *E. coli* PTS (EI and HPr) with two transporters for mannose (IIA^{Man}, IIC^{Man}, IID^{Man}) and GlcNAc (IICBA^{GlcNAc}). Bottom: Transcription regulators from *S. meliloti* consisting of a SorC-DhaK fusion protein and a DhaL-like subunit. Transcription regulators from *L. lactis*: DhaQ is a DhaK paralog; DhaR belongs to the TetR-family of transcription factors. Domain structure of the DegV protein YfhG (Q9CHY7) of *L. lactis*. DNA-binding domains/subunits are shown diagonally striped. Domains homologous to EI and HPr (white), IIA (vertically striped), DhaL (grey) and DhaK (black) are vertically aligned. DhaM subunits always have the IIA^{Man}-like domain (vertically striped) and, depending on the strain, between one and three HPr and EI-like phosphotransferase domains (white). DhaM of *E. coli* has three domains (IIA^{Man}, EI, and HPr-like). Active site His and Cys are indicated as circles and squares. Arrows indicate the phosphoryl flow from the core PTS proteins (EI and HPr), to the PEP-dependent carbohydrate transporters and the PEP-dependent Dha kinases. The '?' indicates that the ligands are not known. Proteins of unknown function are identified by their Swiss-Prot accession numbers.

Structure and molecular function of Dha kinases

The structures of the ATP-dependent *C. freundii* Dha kinase (DAK) and of the homologous DhaK subunit of the *E. coli* kinase have been solved by X-ray crystallography [62, 63]. The two kinases assume the same fold but differ in details (Fig. 3). The structure of the *C. freundii* DAK will serve as a frame of reference for the following discussion. This kinase is a homodimer of two intertwined subunits. Each subunit consists of two domains (K and L), which are connected by a 20-residue-long, extended linker. The domains are swapped such that the amino terminal K-domain of one subunit is complexed with the carboxy terminal L-domain of the other (Fig. 3a).

The amino terminal K-domain consists of two six-stranded α/β folds that together form a three-layered mixed α/β sandwich (Figs. 3a, c). The core of the first fold consists of a four-stranded open pleated sheet with the same topology as the IIA subunit of the mannose family of the PTS transporters [64, 65]. The core of the second domain has a topology similar to the carboxy terminal domain of the

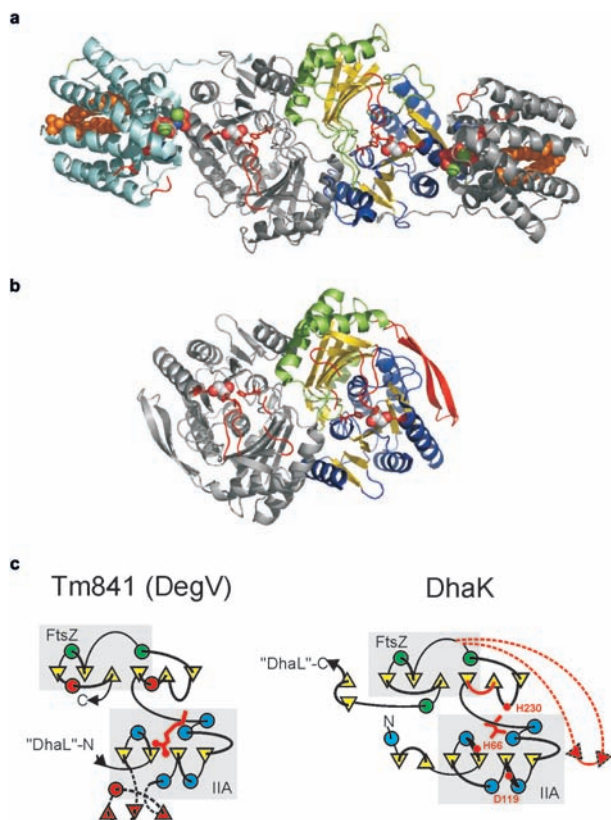


Figure 3. Structure of the Dha kinase from *C. freundii* (a) and *E. coli* (b). Cartoon representation of the Dha kinase dimers. The subunits of the dimers are coloured in blue/green and grey and the DhaL domains in cyan. The α -helices of the first and second DhaK subdomain are coloured in blue and green, respectively, β -sheets in yellow. The active site His (red sticks) of DhaK with Dha (spheres) are shown. The nucleotide (AMPPNP, grey), the two Mg^{2+} ions (green) bound to the DhaL-domain, and the phosphatidic acid molecule which occupies the interior of helix-barrel (orange) are shown as spheres. The β -hairpin of *E. coli* DhaK is shown in red. A loop which is ordered in the *C. freundii* DhaK domain and disordered in the *E. coli* DhaK subunit is shown in red. The IIA and FtsZ core folds are boxed. PDB accession codes: *C. freundii* 1UN9; *E. coli*, 1O12; TM841, 1MGP.

cell division protein FtsZ. The K-domain provides the dimerization interface consisting of two pairs of helices. The substrate, Dha, is bound covalently in hemiaminal linkage to the K-domain (Fig. 4a). The C-N bond is formed between the carbonyl carbon of Dha and the imidazole ring (N ϵ 2) of His-220, and the hemiaminal is stabilized by a hydrogen bond between the *geminal* hydroxyl group and N ϵ 2 of His-61. Dha is oriented by additional hydrogen bonds extending from the C1- and C3-hydroxyl groups of Dha to the γ -carboxyl group of Asp-114 and to the main chain amide of Gly-59. There is no evidence for a role of the hemiaminal in covalent catalysis [66]. Rather,

hemiaminal formation is a ‘clever strategy’ to discriminate between a short-chain carbonyl compound (Dha or glyceraldehyde) that can form a hemiaminal and the isomeric alcohol (glycerol) that cannot form such a covalent bond. The *E. coli* Dha kinase, in fact, is not inhibited even by as high as 2 M glycerol [66].

The carboxy terminal L-domain of the *C. freundii* DAK (Fig. 3a) consists of an eight-stranded α -helical barrel of regular up-down topology [62] with the nucleotide-binding site in a shallow depression at the narrow end of the barrel. This fold is novel and unlike any other kinase with known structure (for reviews see [67, 68]). The hydrophobic interior of the barrel is filled with a phospholipid molecule which was retained during all purification steps. The lipid headgroup protrudes from the wider end of the barrel, where it is accessible to phospholipase D, and the lipid can be removed with a mild detergent. Detergent-washed Dha kinase remains active, suggesting that the lipid plays no role in catalysis but may stabilize the structure of the barrel. The four helix-connecting loops at the narrow end of the barrel form the side wall of the ATP-binding site. The first and second loops embrace the phosphate groups of ATP, the third contacts the adenine base. The fourth loop, which is disordered in the *C. freundii* structure, most likely forms a flexible cap over the binding site. The three phosphate groups of ATP are coordinated by two Mg^{2+} ions, which in turn are complexed by three γ -carboxylates from aspartates in the first loop (Fig. 4b). This novel ATP-binding site differs in two aspects from those of other nucleotide-binding proteins

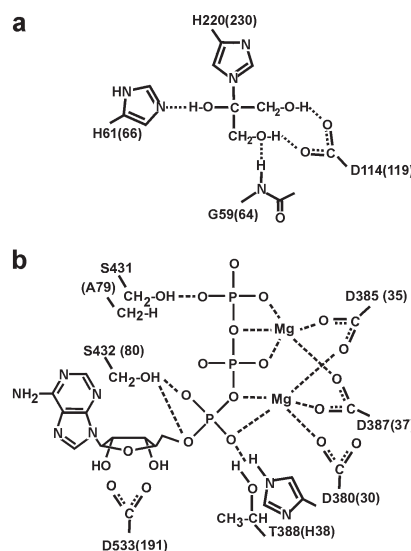


Figure 4. Ligand-protein interactions. (a) Structure of the hemiaminal between Dha and N ϵ 2 of histidine, and hydrogen bonds to invariant His and Asp. (b) Hydrogen- and metal-binding interactions between ATP and conserved residues of the nucleotide binding site [62]. Numbers refer to residues of the *C. freundii* kinase, numbers in parenthesis to the *E. coli* kinase. H38 is invariant in the DhaL subunits of PEP-dependent kinases, T388 is conserved in most ATP-dependent kinases.

and protein kinases [69, 70]. First, the four loops are centred between α -helices and not in β -hairpins or β - α loops. And second, the loops do not contain aromatic residue or carboxy terminal lysines, which in the other ATP-binding proteins are in contact with the nucleotide.

The binding sites for Dha and ATP are 14 Å apart in the crystal structure (Fig. 3a) and it is not clear how they reorient for in-line phosphoryltransfer. One way of bringing ATP and Dha together is that the two domains are freely mobile in solution [62]. Another possibility is tilting of the nucleotide-binding site by a shear deformation of the helix bundle along the barrel axis combined with the elliptical compression of the barrel in the direction of the K-domain.

The DhaK subunit of the PEP-dependent *E. coli* Dha kinase assumes the same fold as the *C. freundii* counterpart [63]. The only obvious difference is a β -ribbon of 25 residues inserted in the carboxy terminal subdomain of *E. coli* DhaK. The β -ribbon folds back to cap the edge of the β -sheet in the amino terminal α/β fold (Fig. 3b). It has been proposed that β -ribbons prevent the association between hydrophobic edge strands and concomitant protein aggregation [71, 72]. The β -ribbon is present only in a small subgroup of PEP-dependent Dha kinases, which utilize large multidomain DhaM subunits as phosphoryl-group donors (see below).

The *E. coli* DhaL subunit is expected to assume the same fold as the L-domain of DAK (Fig. 3a). DhaL also binds a nucleotide, which, however, is retained during all purification steps. The exchange half-life of ADP in complex with DhaL is 100 min, five times the generation-time of a fast growing *E. coli*! And the removal of the nucleotide by sequestration of Mg^{2+} with EDTA reduces the thermal unfolding temperature of DhaL from 65 to 40 °C [61]. ADP is not exchanged for ATP in *E. coli* DhaL – unlike in any other kinase with known function – but is rephosphorylated *in situ* by the DhaM subunit. The nucleotide no longer is a substrate of DhaL but a coenzyme with the function of a phosphoryl group binding amino acid. This conversion of a substrate-binding site into a cofactor-binding site is a remarkable example of functional evolution [61].

DhaM is the third subunit of PEP-dependent Dha kinases [53]. In most species DhaM consists of a single domain that is homologous to the amino terminal domain of the IIAB^{Man} subunit of the *E. coli* mannose transporter [64, 73, 74]. Occasionally and as shown below, DhaM contains additional domains similar to the phosphotransferase subunits HPr and enzyme I of the PTS [64, 73, 74].

Control of *dha* operon transcription by Dha kinases

DhaK and DhaL homologs also occur in operons for putative transcription factors. These operons are adjacent

and often divergently oriented to *bona fide dha* operons suggesting that they control Dha kinase expression. The molecular mechanisms by which Dha kinase subunits function as coactivators of transcription has been elucidated in *E. coli* (Fig. 5). Genetic and biochemical studies showed that the *dha* operon is controlled by DhaR and the two kinase subunits DhaK and DhaL [60, 75]. DhaR belongs to the family of enhancer-binding proteins (EBPs) that activate the sigma RNA polymerase complex by direct contact and by causing DNA looping [76–78]. EBPs usually consist of three domains, a DNA-binding, a AAA⁺ ATPase and a receiver domain for low-molecular and protein ligands. *E. coli* DhaR stimulates the transcription of the *dha* operon from a σ 70 promoter and autorepresses transcription of the *dhaR* gene. DhaK and DhaL antagonistically act as corepressor and coactivator of DhaR (Fig. 5). In the presence of Dha, when the phosphoryl group is transferred from DhaL::ATP to Dha, the now dephosphorylated DhaL::ADP binds to the DhaR receiver domain and activates the expression of the *dha* operon. In the absence of Dha, DhaL::ADP is rephosphorylated by DhaM to DhaL::ATP, which does not bind to DhaR. If DhaM or EI are inactivated by a mutation DhaL::ADP remains in the dephosphorylated state and the *dha* operon is expressed constitutively [60, 75]. DhaK competes with DhaL for binding to DhaR and thus tunes down the effect of DhaL::ADP. Therefore transcription of the *dha* operon overshoots when DhaK and DhaM or DhaK and EI are inactivated together [60, 75]. The novelty of this mechanism is (i) the formation of a complex between a transcription factor and two enzyme subunits and (ii) the coupling of transcription activation with turnover and not only binding of the substrate. The dou-

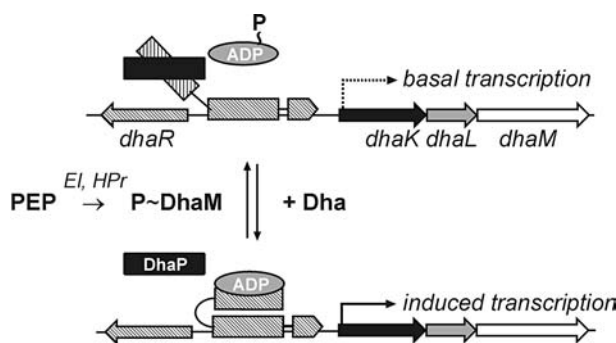


Figure 5. Model of Dha kinase mediated transcription control. *E. coli* DhaR (diagonally striped), the activator of the *dhaKLM* operon, consists of a receiver domain, an AAA⁺-ATPase domain and a DNA-binding helix-turn-helix domain. DhaL::ADP (grey) is the coactivator of DhaR, DhaK (black) an antagonist of DhaL::ADP. By binding to DhaK, the inducer Dha reduces the affinity of DhaK for DhaR. By dephosphorylation of DhaL::ATP, Dha increases the affinity of DhaL::ADP for DhaR. The σ 70 initiation factor that directs the RNA polymerase to the promoter site and the RNA polymerase are not shown. The *dhaR* gene is divergently transcribed from a constitutively active promoter which in *E. coli* is repressed by DhaR [75].

ble-check for binding and turnover may increase the selectivity and the sensitivity of control [75]. It is not known whether the DhaL-DhaR complex acts directly by allosteric activation of the σ initiation factor and the RNA polymerase or indirectly by DNA bending.

In *Lactococcus lactis* an operon for a DhaK homolog (Swiss-Prot entry Q9CIW0), and a protein (Q9CIV9) with the predicted fold of a tetracyclin repressor [79] is found next to the *bona fide dha* operon. In *Sinorhizobium meliloti* an operon for a homolog of DhaL (Q92WN5) and a two-domain protein, Q92WN4, follow the genes for *bona fide* DhaK (Q92WN2) and DhaL (Q92WN3) subunits. The amino terminal domain of Q92WN4 has 27% sequence identity with SorC (P37078), the regulator of the L-sorbose operon of *K. pneumoniae*, and with DeoR (P39140), the regulator of the deoxyribonucleoside and deoxyribose utilization operon *dra-nupC-pdp* of *Bacillus subtilis*. The carboxy terminal DhaK-like domain of Q92WN4 has 41% sequence identity with the *S. meliloti* DhaK (Q92WN2) subunit, and the DhaL paralogs (Q92WN5 and Q92WN3) share 33% sequence identity. It is not known how these transcription regulators function or what the role of the associated kinase subunits may be.

Distribution and phylogeny of Dha kinases

A protein-protein BLAST (blastp at NCBI) with DhaK (DhaL) of *E. coli* as the input generated 169 (161) hits from 115 (117) bacterial species and 70 (56) hits from 39 (34) eukaryotes. With the single-domain DhaM (Q9CIV6) of *L. lactis* as input, the output contained 87 hits from 78 bacterial species and none from eukaryotes. 60 hits from bacterial and eukaryotic species were selected for closer inspection and classified into three families (1–3) according to the organization of the operon, the subunit composition of the gene product (Fig. 6), the phylogenetic relationship (Fig. 7) and the predicted function: (i) Dha kinases, (ii) DhaK and DhaL homologs (paralogs) associated with putative transcription regulators and (iii) proteins of unknown function with circularly permuted domain order.

The kinase family (i) can be further divided into four groups (a–d, Figs. 6 and 7): (a) Single-subunit ATP-dependent Dha kinases like the *C. freundii* kinase described above. This group also includes all Dha kinases of yeasts, plants and animals (EC 2.7.1.28) and the triokinases (EC 2.7.1.29), which were defined based on their specificity for glyceraldehyde but probably are not an enzyme class of their own. The *C. freundii* kinase displays more sequence identity (40%) with human than with the yeast (*Pichia pastoris*) kinases (30%). Altogether the yeast kinases form a large, separate branch within group A, which for lack of space has been omitted from Figure 7. (b) PEP-dependent Dha kinases that consist of DhaK,

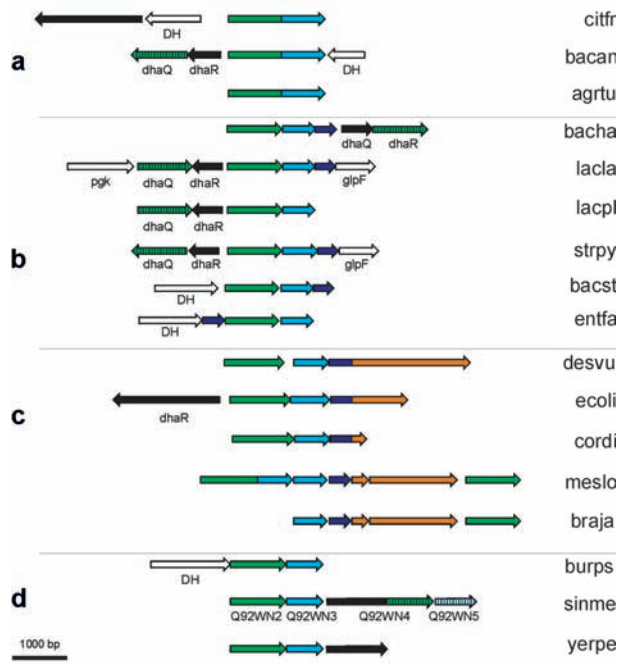


Figure 6. Structures of *dha* operons. (a) Genes for ATP-dependent Dha kinases with and without divergently transcribed genes for DhaR. (b) Genes for PEP-dependent kinases with and without genes for Tet-R-like (*dhaR*) and DhaK-like (*dhaQ*) transcription regulators. (c) PEP-dependent Dha kinases with large multidomain DhaM subunits and genes for EI and HPr of the PTS. The *M. luti* locus contains genes for PTS- and ATP-dependent kinases. (d) Operons for Dha kinases without a DhaM subunit and associated transcription regulators of the SorC/DeoR-type. The domains/subunits and their colours are: DhaK (green), DhaL (cyan), DhaM IIA-domain (blue), PTS domains (orange), DNA-binding proteins (black), DhaK-like (*dhaQ*; green striped). DH refers to dehydrogenase, *glpF* to glycerol facilitator. The labels a–d refer to the clusters in the comparative tree (Fig. 7). For abbreviations, see Fig. 7.

DhaL and a ‘small’ single-domain DhaM subunits. They form the largest group of bacterial Dha kinases. (c) PEP-dependent Dha kinases that contain ‘large’ multidomain DhaMs. Their DhaMs contain in addition to the obligatory IIA^{Man}-like domain additional domains similar to HPr and enzyme I (EI) of the PTS (Fig. 6). Two-domain (IIA-HPr) DhaMs occur in some Actinobacteria [*Corynebacterium diphtheriae* (Q6NED9)], three-domain (IIA-HPr-EI(N) DhaMs in *E. coli*, and four-domain [IIA-HPr-EI(N)-EI(C)] DhaMs in *Desulfovibrio vulgaris* (Q72DE8) and some other strains of the δ and γ subdivision of proteobacteria. It is noteworthy that all the β -hairpin-containing DhaKs (Fig. 3b) are associated with such three- and four-domain DhaMs. (d) The fourth group contains two types of Dha kinases: (i) Two-subunit kinases (DhaK and DhaL) encoded by discistronic operons without a gene for a DhaM subunit. Their DhaLs have an invariant histidine in loop 1 of the nucleotide-binding site (LGDGDHG, Fig. 4b) like the PEP-dependent kinases of groups B and C. (ii) Kinases that by their domain architecture resemble ATP-dependen-

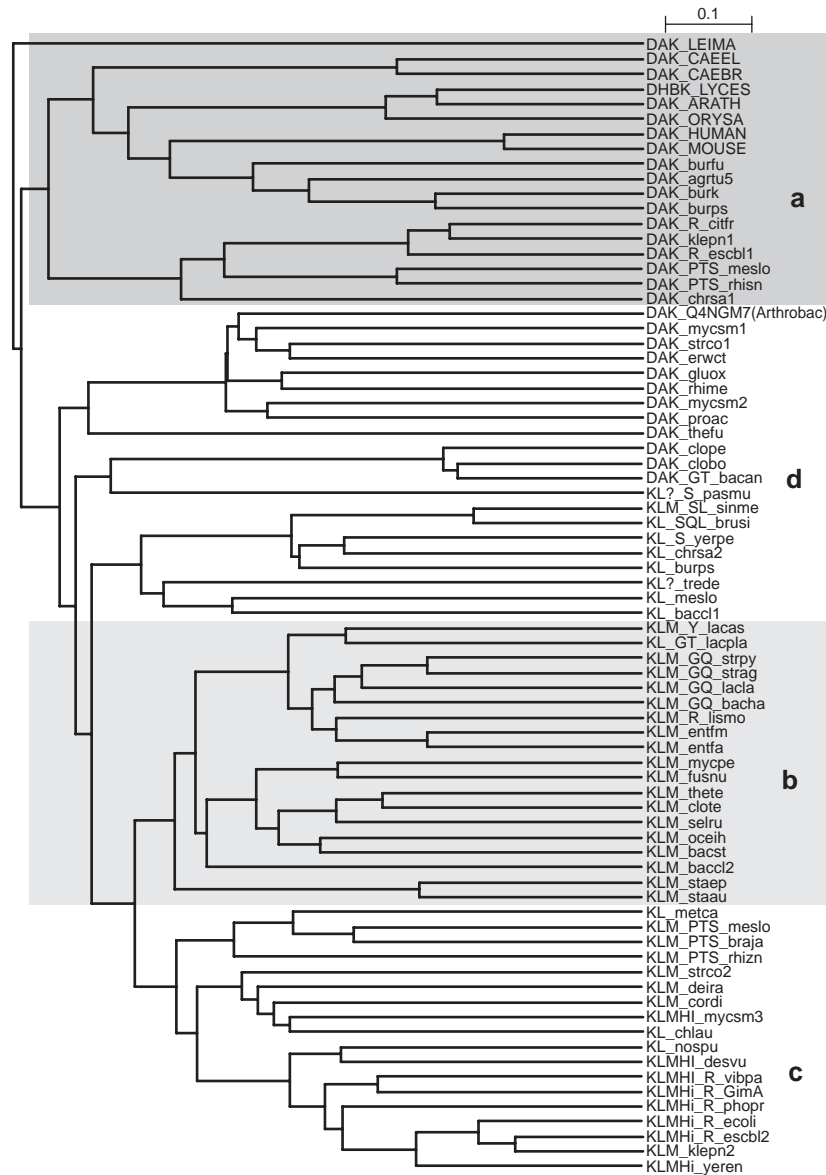


Figure 7. Phylogenetic tree of Dha kinases. DhaK and DhaL subunits encoded in an operon were treated as a unit. DAK refers to two-domain Dha kinases, DHBK to 3,4-dihydroxy-2-butanone kinase [51], KL to two-subunit Dha kinases without a DhaM subunit and KLM to three subunit kinases. M indicates single-domain (IIA) DhaM subunits, MH two-domain (IIA-HP), MHi three-domain [IIA-HP-EI(N)] and MHi four-domain [IIA-HP-EI(N)-EI(C)] DhaM subunits. PTS indicates that genes for PTS proteins (EI and HPr) are associated with the *dha* operon as shown in Figure 6. G (DhaR, TetR-like), Q (DhaK-like), R (AAA+), S (SorC-like) and L (DhaL-like) indicate that transcription factors are associated with the *dha* operon as shown in Figure 6. X, Y and ? refer to genes of unknown function. Numbers after the species abbreviation indicate that their genomes encode more than one Dha kinase. Bar, 0.1 replacement per position. The sequences were aligned with the program MULTALIGN (www.toulouse.inra.fr/multalin.html) [93] and clustered with TREETOP (www.genebee.msu.su/services/phtree_full.html) [94] with alignment parameters set to default values. The species abbreviations are: *Leishmania* (LEIMA); *Caenorhabditis* (CAE); *Arabidopsis* (ARATH); tomato (LYCES); rice (ORYSA); *Agrobacterium tumefaciens* (agrtu); *Bacillus anthracis* (bacan); *Bacillus halodurans* (bacha); *Bacillus clausii* (baccl); *Bacillus stearothermophilus* (bacst); *Bradyrhizobium japonicum* (braja); *Brucella suis* (brusi); *Burkholderia pseudomallei* (burps); *Chloroflexus aurantiacus* (chlau); *Chromohalobacter salexigens* (chrsa); *Citrobacter freundii* (citfr); *Clostridium perfringens* (clope); *Clostridium botulinum* (clobo); *Clostridium tetani* (clote); *Corynebacterium diphtheriae* (cordi); *Deinococcus radiodurans* (deira); *Desulfovibrio vulgaris* (desvu); *Erwinia carotovora* (erwct); *Escherichia coli* K1 genetic island (GimA); *Escherichia coli* K12 (ecoli); *Escherichia blattae* (escbl); *Enterococcus faecalis* (entfa); *Enterococcus faecium* (entfm); *Fusobacterium nucleatum* (fusnu); *Gluconobacter oxidans* (gluox); *Klebsiella pneumoniae* (klepn); *Lactococcus lactis* (lacla); *Lactococcus plantarum* (lacpl); *Listeria monocytogenes* (lismo); *Pasteurella multocida* (pasmu); *Photobacterium profundum* (phopr); *Mesorhizobium loti* (meslo); *Methylococcus capsulatus* (metca); *Mycoplasma penetrans* (mycpe); *Mycobacterium smegmatis* (mycsm); *Nostoc punctiforme* (nospu); *Oceanobacillus iheyensis* (oceih); *Rhizobium meliloti* (rhime); *Rhizobium* sp. (rhizn); *Selonomonas ruminantium* (selru); *Sinorhizobium meliloti* (sinme); *Staphylococcus aureus* (staa); *Staphylococcus epidermidis* (staep); *Streptomyces coelicolor* (strco); *Streptococcus agalactiae* (strag); *Streptococcus pyogenes* (strpy); *Thermoanaerobacter tengcongensis* (thete); *Treponema denticola* (trede); *Vibrio parahaemolyticus* (vibpa); *Yersinia enterocolitica* (yeren); *Yersinia pestis* (yerpe).

dent kinases, but which by their sequence are more similar to the PEP-dependent forms. The bacterial strains encoding such 'incomplete' Dha kinases often also contain only a reduced PTS with genes for homologs of EI, HPr and IIA proteins [80] but not for carbohydrate transporters (enzymes II). The function of these incomplete kinases and whether other IIA-like proteins can complement the missing DhaMs is not known.

The second family of DhaK and DhaL paralogs associated with transcription regulators (DhaRs) of the TetR and DeoR families has been described above. They form a small branch of their own within the D group (not shown in Figure 7). The 'regulatory' orthologs (termed DhaQ) from different strains are more closely related to each other (50–65% sequence identity) than to the catalytic DhaK paralogs (identity 40–45%). It thus appears that the duplication and the functional diversification of the ancestral *dhaK* gene preceded the evolutionary separation of the modern carriers of these paralogous genes.

The third family comprises the DegV-like proteins (Pfam accession number 02645), whose function is not known. They have a circularly permuted domain order. The fold of the amino terminal domain is similar to DhaL. The fold of the carboxy terminal domain is similar to the fatty acid binding protein TM841 of *Thermotoga maritima* (PDB code 1MGP [81]), and TM841 in turn shares the core fold with DhaK [62, 63], as shown in the topological diagrams of Figure 3c. The difference between DhaK and TM841 lies in two extra secondary structure elements and in the chemistry of their ligand binding sites. TM841 and – by prediction – the carboxy terminal domains of DegV proteins have an extra α/β fold inserted between β_2 and α_2 of the core, while DhaK has two extra β -strands and one α -helix (a dimerization helix) appended to the amino terminus. The ligands, Dha in DhaK and a fatty acid in TM841, occupy the same binding pocket and are both hydrogen bonded to the first topological switch point of the amino terminal α/β subdomain. The residues, involved in ligand binding are, however, different. It is not known whether the DhaL-like domain of DegV proteins has kinase activity or what the substrate and molecular function of the DhaK-like domain might be.

Biological function of Dha kinases and open questions

While the structure and molecular function of the PEP-dependent *E. coli* Dha kinase and of the ATP-dependent *C. freundii* kinase are reasonably well understood, much less is known about the physiological role of Dha kinases in living cells. Two well-characterized functions, namely (i) catabolism of glycerol and (ii) methanol assimilation (methylotrophy), have been described above (Fig. 1). Two additional functions (iii) detoxification (chemical stress

response) and (iv) control of gene expression will be discussed in this section. Glyceraldehyde and Dha can cross-link proteins and by autooxidation produce superoxide radicals [82, 83], and Dha has been shown to enhance the mutation rate in SOD-deficient *E. coli* [84–86]. Molin et al. [87] demonstrated that Dha is toxic to *Saccharomyces cerevisiae* and that detoxification is dependent on a functional Dha kinase. Deletion of the two genes for Dak1p and Dak2p made cells hypersensitive, while overexpression made them more tolerant to Dha. Dha toxicity was dependent on cosubstrates and salt conditions, which led to the proposition that detoxification of Dha might be a part of the physiological response to Dha-dependent stress conditions. It is therefore reasonable to assume that Dha kinases clean up the short-chain carbonyl compounds that may be formed as side products of the carbohydrate metabolism (aldol cleavage of ketohexoses and ketopentoses or oxidation of glycerol). The rate of Dha formation by non-specific oxidation may be increased in organisms that under osmotic stress accumulate glycerol in high concentrations [88, 89]. Owing to hemiaminal formation, Dha kinases can remove traces of Dha even in the presence of an excess of glycerol [66], which glycerol kinases, for comparison, cannot.

The substrate spectrum of some 'Dha kinases' may be broader than suggested by most of the published reports. A recent and unexpected finding is that FMN-cyclase, an enzyme that catalyses the formation of cyclic FMN (riboflavin 4',5'-cyclic phosphate) from FAD, is identical with the human Dha kinase (SWISS-Prot Q3LXA3, Q9BVA7) [90]. Cyclase and kinase activity were copurified from rat liver extracts as well as with the human recombinant protein. Kinase activity was six times stronger than cyclase activity, but FAD, ATP and Dha were mutually competitive inhibitors [90]. While the ADP moiety of FAD in principle can be accommodated in the nucleotide binding site, it is not clear at all how the riboflavin moiety can bind to the L- or K-domain.

The physiological role of Dha kinases in transcription control is still far from understood. One answer may be found in bacteria that contain both a PEP-dependent and an ATP-dependent kinase, for instance *Escherichia blattae* [91], *K. pneumoniae*, *Streptomyces coelicolor* and probably also *C. freundii*. DhaR of *E. coli* controls not only the expression of the *E. coli* but also of the *C. freundii* kinase [75], suggesting that the PEP-dependent form functions as 'sensor kinase' for the control of the ATP-dependent 'metabolic kinase'. A further hint that this sensor kinase may also control other biological processes is provided by the 20-kb genetic island of meningitic *E. coli* K1 (GimA) [92]. This island contains among others the *ibeA* virulence gene (invasin) that contributes to the pathogenesis of *E. coli* meningitis, three genes for a PEP-dependent Dha kinase (*ptnC*, *ptnK*, *ptnP*) and the gene *ibeR* for a homolog of the *E. coli* DhaR. Of all the potential promoter/

operator sequences on GimA, the putative promoters of *ibeA* (Q9FCY5) and *ptnC* (Q8VP37) display the strongest similarity to the promoter/operator region of the *E. coli dha* operon [75]. It is therefore possible that IbeR controls the *ibeA* virulence gene and that the PEP-dependent Dha kinase serves as the sensor for an environmental stimulus at the blood-brain barrier. However, this hypothesis has not yet been tested.

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