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From Kinase to Cyclase: An Unusual Example of Catalytic Promiscuity Modulated by Metal Switching

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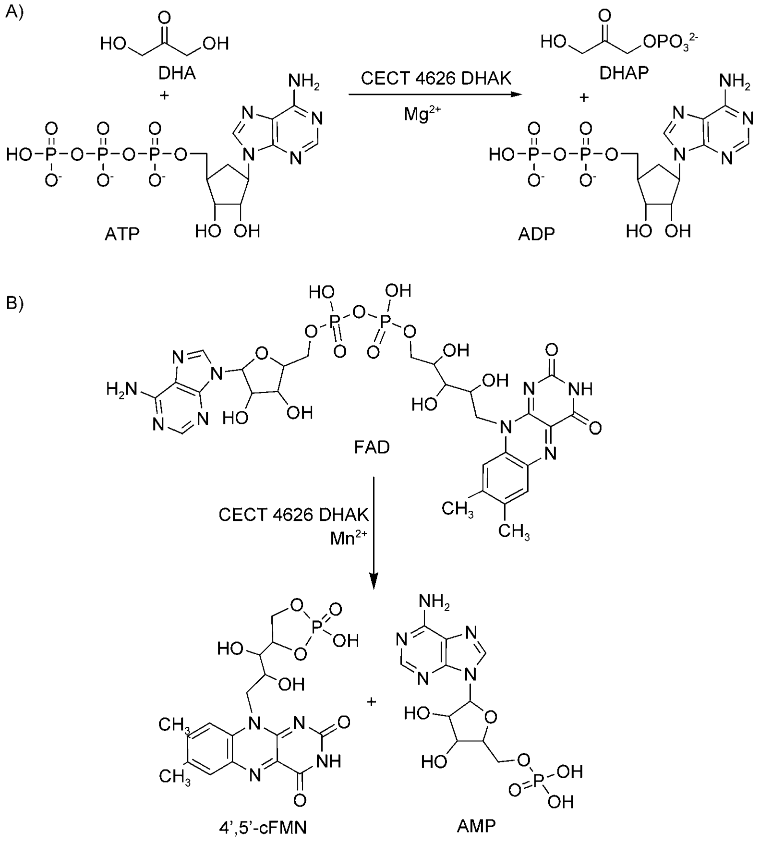
Dedicated to Professor Chi-Huey Wong on the occasion of his 60th birthday.

Enzyme promiscuity is a concept that in recent years has been gaining prominence in different fields of enzymology such as biocatalysis, enzyme engineering or enzyme evolution.[1] Catalytic promiscuity is defined as the ability of an enzyme to catalyse more than one chemical transformation.[1b,c] Naturally occurring catalytic promiscuity provides the starting point for Darwinian evolution of enzymes to perform new functions, because this process must occur gradually, with organism fitness being maintained throughout.[2] Tawfik and co-workers[3] have provided experimental evidence for the plasticity and "evolvability"[4] of promiscuous functions. These authors propose a model in which a protein acquires a new function, without losing the original one, and gene duplication may follow the emergence of a new function, rather than initiate it. Besides the intriguing implications that this theory of divergent molecular evolution has for protein evolution, its application to promiscuous enzymes allows enzymes with new catalytic activities to be designed.[5]

A special case of catalytic promiscuity is shown by metalloenzymes; the range of metallic ions that can be incorporated in the active site increases the range of chemical transformations that can be catalysed by the enzyme. Thus, there are several examples showing that protein modification, either through the covalent attachment of ligands that incorporate metal ions or through incorporation of the catalytic metal ion alone in a suitable site for coordination, is a strategy that allows enzymes with either modified or completely new catalytic activities to be obtained.[6]



Scheme 1. A) Primary reaction catalysed by DHAK from *C. freundii* CECT 4626, and B) promiscuous cyclization reaction catalysed in the presence of Mn^{2+}.

Here we describe the promiscuous behaviour of the dihydroxyacetone (DHA) kinase (DHAK) from *Citrobacter freundii* strain CECT 4626. This adenosine-5′-triphosphate (ATP)-dependent DHAK is not only able to catalyse the transfer of the γ-phosphate of ATP to DHA (Scheme 1), but also catalyses the cyclization of flavin adenine dinucleotide (FAD) (Scheme 1) to yield riboflavin 4′,5′-cyclic phosphate (4′,5′-cFMN). This catalytic promiscuity is modulated by the divalent cation that forms the complex with the phosphorylated substrate.

Although DHAKs are widely distributed in the three biological kingdoms, only their roles in the catabolism of glycerol and in methanol assimilation in microorganisms have been well characterized.[7] In the bacteria *C. freundii* strain DSM 30 040,[8] the entire *dha* regulon has been cloned and characterized at the molecular level.[9] The kinetic properties and mechanism of the corresponding DHAK have been described,[10] and the X-ray structure of the full-length DHAK in complexation with its

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substrates has been elucidated.[11] This kinase is the only one known with an all-α nucleotide-binding domain.[12]

From a biocatalytic point of view, ATP-dependent DHAKs have been given considerable attention because of their feasibility for simple and efficient production of DHAP.[13] We have reported a multi-enzyme system for one-pot C–C bond formation catalysed by DHAP-dependent aldolases, based on the use of the recombinant DHAK from *C. freundii* CECT 4626, for in situ DHAP formation.[14] This enzyme (GeneBank Accession N° DQ473522) is a new isoform that differs from the enzyme of the DSM 30040 strain by 22 amino acids (see the Supporting Information).

During the progress of our own work towards the biochemical characterization of this enzyme, Cameselle and co-workers reported the molecular identities of recombinant human and rat liver purified FAD-AMP lyases (FMN cyclases) as ATP-dependent DHAKs.[15] These authors had previously published a series of papers in which they identified and characterized this Mn^{2+}-dependent cyclase activity as the only enzymatic source of riboflavin 4′,5′-phosphate (cFMN).[16] The physiological role of this cyclase activity is unclear, because nothing is known about the biological function of cFMN apart from speculation that it may be a signalling molecule, a minor redox flavocoenzyme, or an intermediate of a degradative pathway for FAD.[15] Human FMN cyclase displays 39.4% sequence identity with the DHAK from *C. freundii* CECT 4626. We therefore decided to investigate whether the DHAK also displayed FMN cyclase activity (Scheme 1B).

The time course of the DHAK-catalysed reaction in the presence of FAD and Mn^{2+} was followed by HPLC (see the Supporting Information). Chromatograms recorded at different reaction times showed that the decrease in the FAD peak was concomitant with the emergence of a new peak, the retention time of which was coincident with that previously described for the cFMN.[16] The compound associated with this peak was purified, and its identity was unequivocally assigned by NMR and ESI-MS (see the Supporting Information). We were thus in the presence of an enzyme capable of catalysing two different reactions that were modulated by the divalent ion used as cofactor. We presume that the enzyme from the strain DSM 30040 also presents this cyclase activity because it differs only in 22 (4%) amino acids from the protein used throughout this work (see Figure S10 in the Supporting Information).

The different kinetic parameters for the two reactions and for the different substrates are summarized in Table 1. The catalytic efficiencies (k_{cat}/K_M) of DHAK for the different phosphorylated substrates were of the same order of magnitude (Table 1).

Table 1. Summary of the kinetic constants of DHAK from *C. freundii* CECT 4626 for the natural and the promiscuous activities.[a]

Activity	Substrate	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
kinase	DHA	0.0012 ± 0.0003	24.13 ± 0.92	1.98×10^7
kinase	Mg-ATP	0.35 ± 0.01	24.02 ± 0.37	6.9×10^4
kinase	Mn-ATP	0.65 ± 0.1	24.84 ± 1.1	3.8×10^4
cyclase	Mn-FAD	0.017 ± 0.001	0.47 ± 0.02	2.8×10^4

[a] Kinetic parameters calculated at 25 °C

However, the kinase- and cyclase-specific activities are drastically affected by the metal used as cofactor (Figure 1). Whereas the kinase activity was almost independent of the metal used, the cyclase activity was increased almost 50 times when the cofactor used was Mn^{2+}.

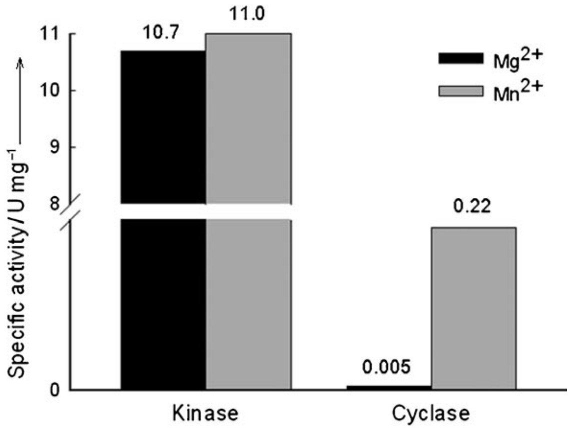

Figure 1. Specific activities (U mg^{-1}) for the natural and promiscuous reactions catalysed by DHAK in the presence of Mg^{2+} or Mn^{2+}. Activities were measured at 25 °C with metal concentrations of 12 mM. In the kinase reaction with Mn^{2+}, a concentration of 1.35 mM was used to avoid inhibition by manganese excess (see Figure 2).

A detailed analysis of the behaviour of the two activities as a function of the Mn^{2+} concentration showed that at low concentrations the DHAK is able to catalyse both the phosphorylation of DHA and the cyclization of FAD (Figure 2). Above about 1.5 mM Mn^{2+}, however, the kinase activity begins to be inhibited, while the cyclase activity reaches saturation without any detection of inhibition by an effect of Mn^{2+}. Although 1.5 mM is a concentration of manganese much higher than the intracellular concentration of 10 μM usually assumed in the litera-

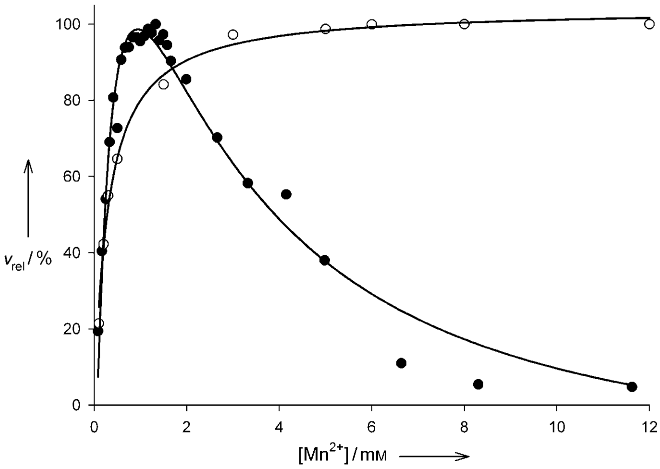

Figure 2. Natural and promiscuous activities catalysed by DHAK as function of Mn^{2+} concentration. ●) Kinase activity; ○) cyclase activity. Relative activities (v_{rel}) are represented for easier comparison.

ture,[17] the Mn^{2+} uptake systems of enterobacteria can increase cytoplasmic concentrations of Mn^{2+} over at least two orders of magnitude up to 1–3 mM on a timescale as short as a minute.[17,18] Therefore, the cytoplasmic concentration of Mn^{2+} could act as a real switch that turns the kinase activity off when the cyclase activity reaches its maximum. The observed specificity modification induced by the change from magnesium to manganese could be due to the greater flexibility of Mn^{2+}–ligand bonds—both in length and in angle—that allows the binding of FAD in the ATP active site.[17]

Because of the structural similarities between ATP and FAD, it seems to be logical to assume that the promiscuous activities may occur in the ATP binding site of DHAK. To corroborate this hypothesis, we performed a series of inhibition and docking studies (see the Supporting Information). The results, summarized in Table 2, showed that FAD and ATP were competing through the same binding site, because ATP is a strong competitive inhibitor of cyclase activity, whereas DHA is a poor uncompetitive inhibitor. Furthermore, adenosine monophosphate (AMP)—a moiety shared by both ATP and FAD—is a competitive inhibitor of both activities.

Table 2. DHAK inhibition studies.

Activity/ substrate	Type of inhibition[a] (k_i [mM]) DHA	ATP	FAD	AMP
kinase/Mg-ATP	N.I.	–	–	C.I. (10.2)
kinase/Mn-ATP	N.I.	–	M.I. (k_{ic}=0.5) (k_{iu}=3.0)	C.I. (71.8)
cyclase/Mn-FAD	U.I. (576)	C.I. (0.2×10^{-3})	–	C.I. (3.52)

[a] C.I. = competitive inhibition; U.I. = uncompetitive inhibition; M.I. = mixed inhibition; N.I. = no inhibition.

On the other hand, the behaviour of FAD as an inhibitor was more complex, and showed a mixed inhibition pattern in which the competitive component was stronger than the uncompetitive one (Figure 3).

This behaviour could be explained if FAD were bound to the active centre in two sub-sites, one of them coincident with the ATP binding site and the other probably associated with the bound isoalloxazine ring of the FAD. This interpretation was supported by docking studies. Figure 4 shows the minimized FAD bound in the ATP binding site of the DHAK from *C. freundii* strain DSM 30040 (PDB ID: 1un9).[11] The phosphate groups of FAD are positioned similarly to the ATP phosphates in the crystal structure, and are coordinated by two Mg^{2+} centres together with the γ-carboxyl groups of aspartyl residues (Asp380, Asp385 and Asp387). Asp385 is at hydrogen bond distance (3.0 Å) from the –OH(5) involved in the cyclization of FAD.[16b] Besides these residues, Val434 and Gly113, which do not participate in the binding of ATP, could be interacting with the isoalloxazine ring of the FAD.

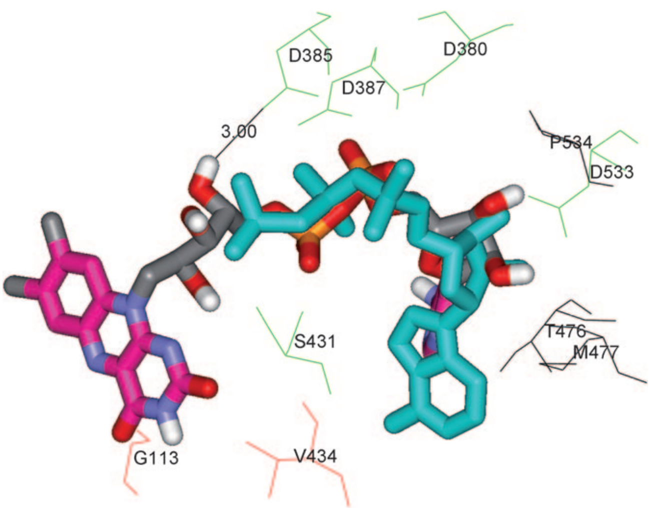

Figure 4. FAD (colour by element) and ATP (cyan) docked into the ATP binding site of DHAK enzyme (stick) from *C. freundii* strain DSM 30040 (PDB ID: 1un9).[11] Green: The amino acids that interact both with ATP and with FAD. Black: Those that only interact with ATP, and red: those residues that interact only with the FAD.

Interestingly, the kinetic parameters of the FMN cyclase activity determined for the DHAK from *C. freundii* CECT 4626 are quite different from those reported by Cameselle and coworkers for the rat liver enzyme.[16b] The results, summarized in Table 3, clearly shown that the rat liver enzyme is much more efficient as a cyclase that the bacterial enzyme. Thus, the k_{cat}/K_M value for the rat enzyme is more than one order of magnitude higher than the value for the *C. freundii* enzyme. On the

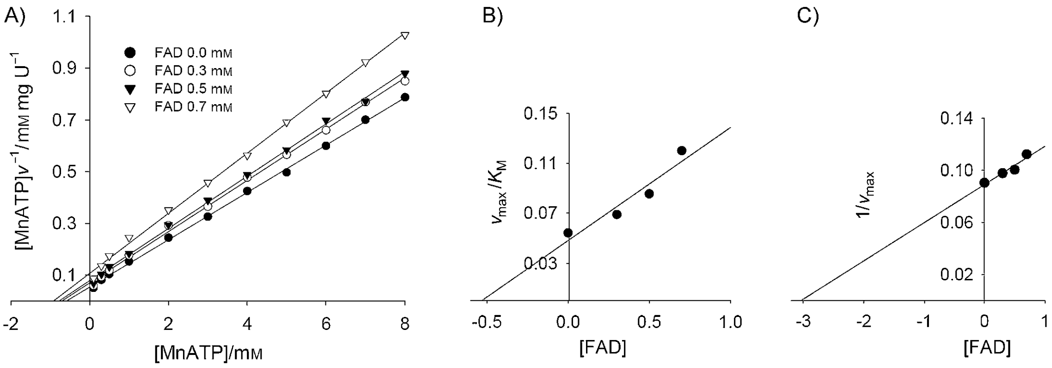

Figure 3. A) Hanes–Woolf plot from DHAK kinetics in the presence of several FAD concentrations. B) and C) are secondary plots used to calculate k_{iu} and k_{ic}.

Table 3. Kinetic parameters of 4′,5′-cFMN cyclase from *C. freundii* CECT 4626 and from rat liver.

DHAK source	Specific activity [U mg^{-1}]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1} M^{-1}]
C. freundii CECT 4626[a]	0.5	0.022 ± 0.004	0.68 ± 0.09	3.1×10^4
rat liver[b]	3.2	0.009 ± 0.001	4.82 ± 0.1	5.3×10^5

[a] Kinetic parameters calculated at 37 °C for purposes of comparison. [b] Data taken from ref. [16b].

other hand, rat liver DHAK maintains a level of kinase activity of the same order as that of the *Citrobacter* enzyme,[15] indicating that the change in the promiscuous activity does not come at the expense of the native function.[3]

From their amino acid sequences, the DHAKs from *Citrobacter*, plants and mammals are grouped into the same branch (Figure 5 and ref. [7]), although the relationships between classes are unreliable, as shown by the branching order at the centre of this unrooted tree. Our results could suggest that enzymes in this separate branch have been undergoing a process of divergent molecular evolution, along which the promiscuous function acquired higher activity and specificity. To support this hypothesis, however, it would be necessary to analyse enzymes on the other branches to see whether they exhibit cyclase activity and at what levels.

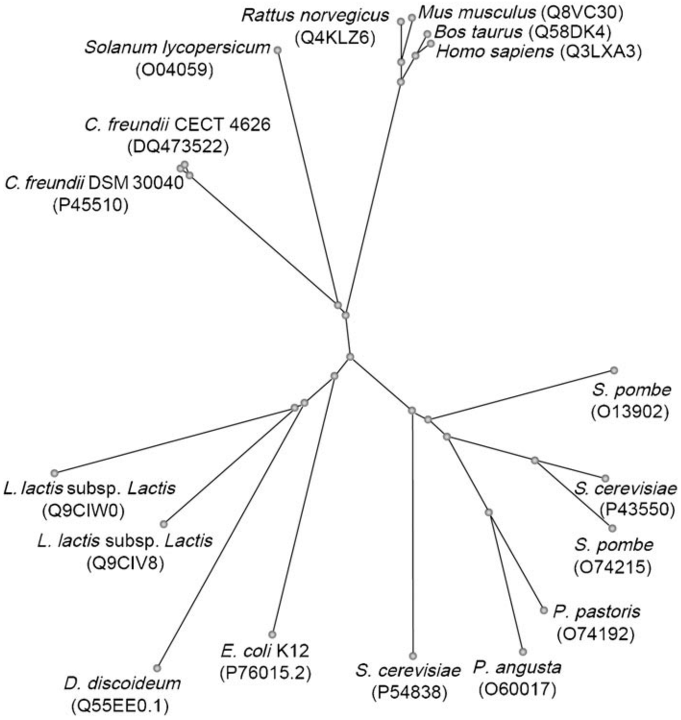

Figure 5. Unrooted tree showing the sequence relationships between DHAK from *C. freundii* CECT 4626 and DHAK-like proteins in the Swiss-Prot database (for details see the Supporting Information).

In conclusion, we have described the natural promiscuous behaviour of the DHAK from *Citrobacter freundii* CECT 4626. This catalytic promiscuity is modulated by the divalent cation that forms the complex with the phosphorylated substrate. Through kinetic and docking studies, we have demonstrated that both activities are linked to the same binding site. These results suggest that a new function has evolved in nature through recruitment of an existing promiscuous activity and its gradual improvement[2,3] and is currently maintained as a secondary function of DHAK.[19]

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Keywords: biotransformations · catalytic promiscuity · cofactors · enzyme evolution · metalloenzymes

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