Thiamine models and perspectives on the mechanism of action of thiamine-dependent enzymes

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Thiamine dependent enzymes catalyze ligase and lyase reactions near a carbonyl moiety. Chemical models for these reactions serve as useful tools to substantiate a detailed mechanism of action. This *tutorial review* covers all such studies performed thus far, emphasizing the role of each part around the active site and the conformation of the cofactor during catalysis.

1 Introduction

1.1 General

Thiamine pyrophosphate (TPP) serves as a cofactor in a number of enzymic processes found in almost all major metabolic pathways. In living organisms, thiamine dependent enzymes are mainly involved in the decarboxylation of α-ketoacids, by both non-oxidative and oxidative mechanisms. The study of the non-enzymic decarboxylation of α-ketoacids by either thiamine or by simple thiazolium derivatives has provided detailed information on thiamine chemistry. Based on these data Breslow[1] proposed a mechanism for the catalytic action of thiamine that remains as the general accepted model today. Decarboxylation is initiated by formation of a C(2) carbanion of TPP, that interacts with the C(2) of the substrate α-ketoacids to form a nucleophilic adduct, followed by CO_2 release and formation of the C(2α)-carbanion/enamine (Fig. 1). Subsequent protonation leads to the 'active aldehyde' intermediate whose metabolism depends on the specific enzyme involved. Finally, decarboxylation with subsequent release of an aldehyde molecule is the main reaction of all α-ketoacid decarboxylases, which form part of many different metabolic pathways (Fig. 1).[2] However, in the presence of a further aldehyde or 2-ketoacid molecule acting as an acceptor, the C(2α)-carbanion/enamine yields 2-hydroxyketones or hydroxyketoacids (Fig. 1).[2]

All thiamine-dependent enzymes require Mg^{2+} or Ca^{2+} for coenzyme binding. While other bivalent metal ions can replace them *in vitro*, the native metals remain the most active.[3] We

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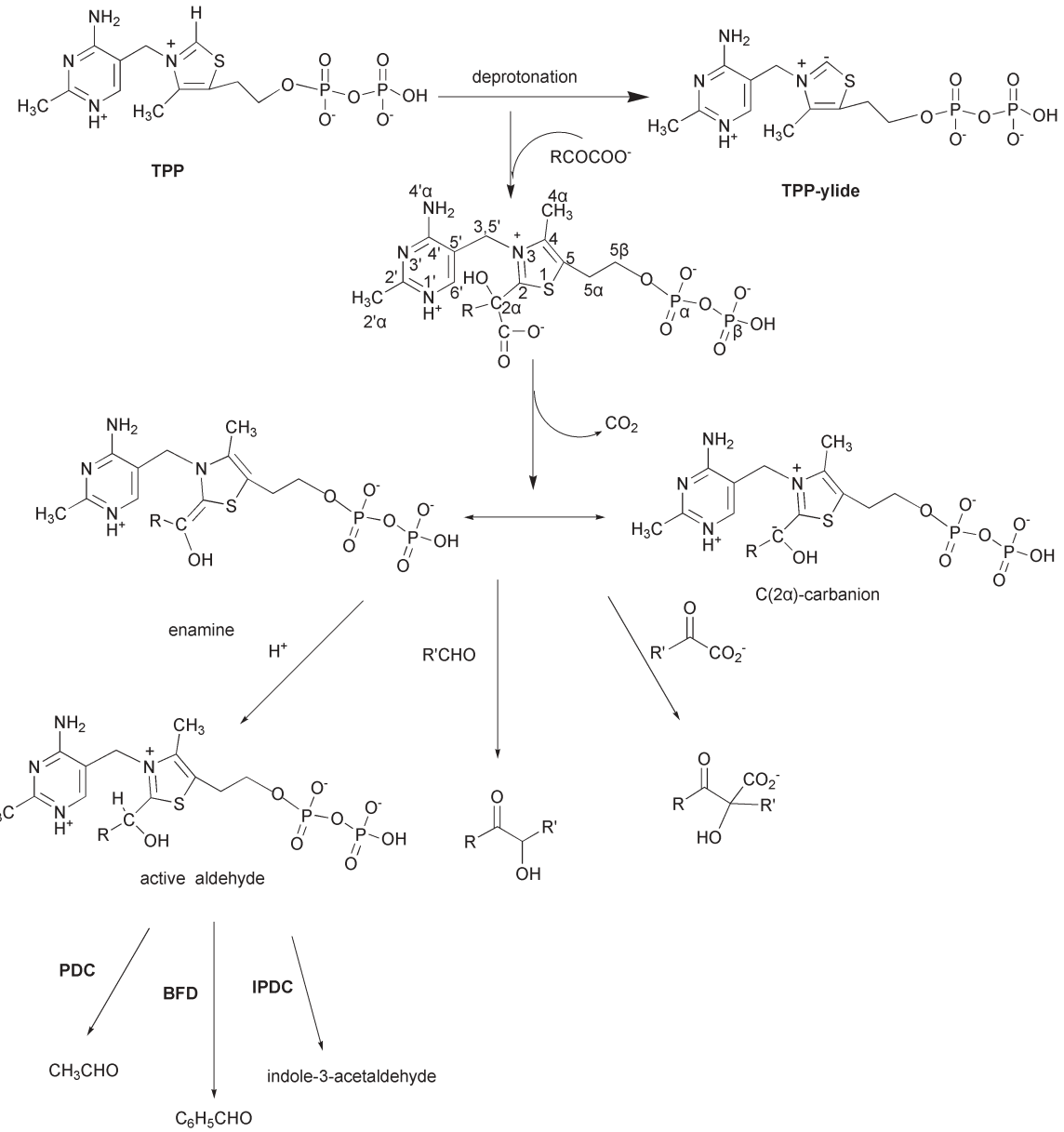


Fig. 1 Reactions involving TPP.

intend to review and clarify the features of the interactions of metal ions with simple thiamine models, emphasizing the mechanism of thiamine-dependent α-ketoacid decarboxylases. Investigation of the detailed catalytic mechanism of action of thiamine enzymes and more particularly of α-keto decarboxylases is a very important issue and involves the elucidation of the possible role of all conserved residues located near the active site during the catalytic cycle.

1.2 α-Keto decarboxylases

The three most important non oxidative thiamine-dependent enzymes are pyruvate decarboxylase (PDC), benzoylformate decarboxylase (BFD) and indolepyruvate decarboxylase (IPDC).[4] PDC is present in fungi (*S. cerevisiae* (yPDC), *S. uvarum*) and rarely in prokaryotic cells (*Z. Mobilis* (ZmPDC) and *C. botulinum*).[5] BFD is a component of the mandelate pathway and it is present in only a few organisms (*Pseudomonas*)[6] while IPDC is present in plants.[7] They catalyze the decomposition of α-keto derivatives (pyruvic acid, benzoylformate, indolepyruvate) to the respective aldehydes and CO_2.[4] It must be noted that α-keto decarboxylases are also powerful biocatalysts able to catalyze carboligase reactions, utilizing aldehydes as donor substrates and producing α-hydroxy ketones. The stereocontrol of this reaction is only strict when aromatic or heterocyclic aldehydes are used as acceptors.[8]

In aqueous solutions near physiological pH (6.0–6.5) the enzymes are present as tetramers with an average molecular weight of 240–250 KDa. The tetramer consists of four identical subunits divided in three domains named α, β and γ. Each subunit contains one TPP (thiamine pyrophosphate) molecule and Mg(II) which tightly interact to form dimers. The active tetramer is formed by two dimers through the β-domains *via*

relatively weak interactions in yPDC and stronger interactions in ZmPDC, BFD and IPDC.

In yPDC, the optimum pH value for catalytic activity is 6.0, while at pH ~6–8.5 a dimer–tetramer equilibrium is established. At pH > 8.5, dissociation of the cofactors is observed with loss of catalytic activity.[6,7,9,10] This is the case also for IPDC in which the tetramer is stable at pH from 5.6 to 7.5, while at lower or higher pH values an equilibrium between dimers–tetramers or monomers–dimers is established respectively.[11] On the other hand, there is no equivalent equilibrium between oligomeric forms of the enzyme ZmPDC as the tetramer is stable in a wide pH range (from 5 to 9).[12]

The TPP and Mg(II) cofactors are located in the interface between two identical subunits. Each tetramer contains four Mg(II) ions and four TPP molecules.[7,9,10] In the case of BFD two additional metal ions are present, a Mg(II) which is located in the interface between two subunits and a Ca(II) ion at a crystal contact.[6] The cofactors are buried deep in the enzyme, but in ZmPDC, IPDC there are active site cavities connecting the thiazolium ring of TPP with the surface of the enzymes.[7,9,10] The binding properties of the cofactors and the possible catalytic role of the residues located in the active center of the enzymes, are presented in Table 1.

The metal ion Mg(II) is octahedrally coordinated with the carboxyl/carbonyl oxygen atoms of the conserved residues Asp, Asn, Gly, Gln and the pyrophosphate group of TPP. Other conserved residues (Table 1) interact through hydrogen bonds with N(1′) and N(4′α)H_2 of the pyrimidine ring and the N(3) of thiazole.[4,6,7,9–11,13–15] Although Mg(II) ions are essential in thiamine catalysis *in vivo*, other bivalent metals (Zn(II), Co(II), Ni(II)) can also act similarly *in vitro*.[3]

2 Model studies

2.1 Coordination sites

Over the years, there has been a particular interest in thiamine metal complexes to identify the role of metal ions in thiamine enzymes.[16] Early studies on the chemical reactions of thiamine with metal ions had suggested that the N(1′) site and the pyrophosphate group are the potential binding sites for TPP.[16] However, despite these original indications, the first isolated thiamine–metal complexes were found to be ionic compounds without direct metal–thiamine bonds.[16] This was due to a) the positive charge on N(3) of thiazole, b) the easy protonation of pyrimidine N(1′) with a pK_a value about 5 and c) the fact that the cofactor is unstable at pH > 7.[16]

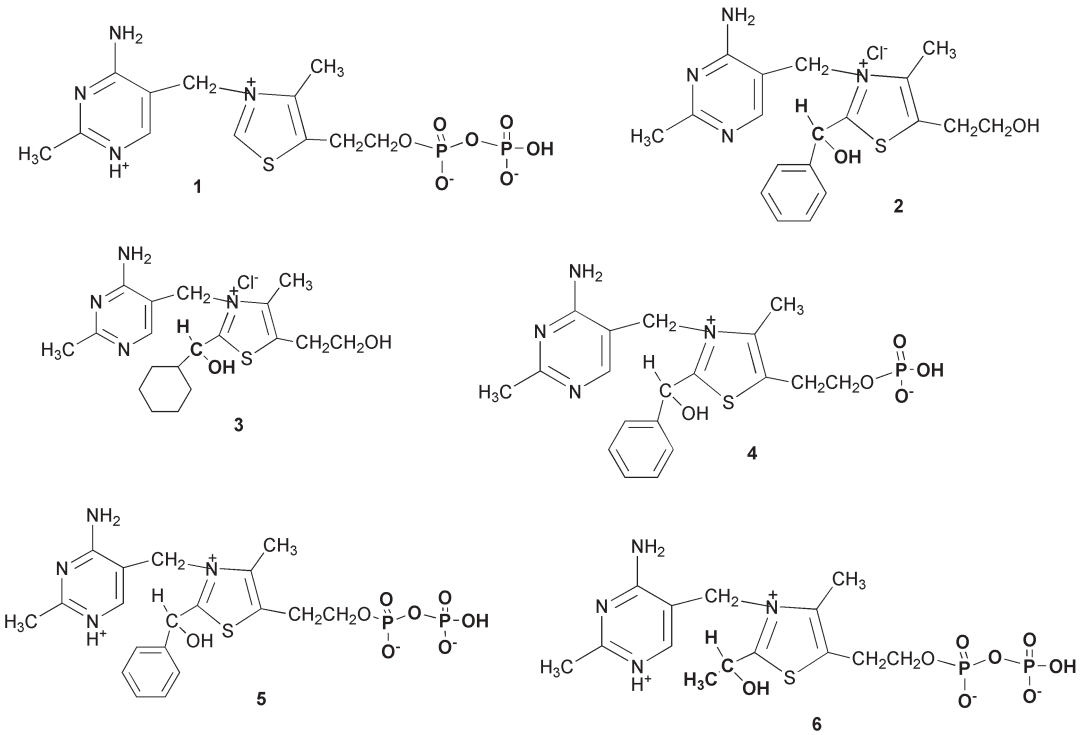
Early in our research, no examples of a direct metal–thiamine bond existed in the literature. Interactions of Pt(II) and Pd(II) with thiamine and derivatives resulted in the first examples of such complexes with direct M–ligand bonding, namely Pt–N and Pd–N. The results were interpreted spectroscopically and explained by the high Pt–N and Pd–N bond strengths.[17] The proposed zwitterionic structure with MCl_3^- moieties at N(1′) neutralized by the net positive charge on thiazolium, was later proven by an X-ray crystal structure determination.[16] Subsequent crystal structure determinations of thiamine–metal complexes (for example: Zn(II), Cd(II)) confirmed the N(1′) tendency to react with bivalent metals,[16] although pyrophosphate binding was also found.[18] It thus became clear that the reason for the difficulties in isolating metal–thiamine complexes with bivalent metal ions presenting direct metal–ligand bonds was most likely the net positive charge on the thiazolium ring and only heavy metals like Pt(II) and Pd(II) with high bond strengths could offset this.[16] This difficulty might be overcome if an internal neutralization of this charge was attained and this was indeed taking place in the 'active aldehyde' derivatives of thiamine.[19] Hogg had already proposed a low pK_a = 12 for the HO-C(2α) hydroxylic proton, affecting the final step of the catalytic cycle of the α-ketoacid decarboxylases.[20]

The strong electrostatic interaction observed between the S atom and the oxygen on the C(2α), manifested from the relatively short S…O-C(2α) distance, was impressive and was helpful in achieving this goal.[19] The success of this hypothesis was proven subsequently by the isolation of many such complexes of metals with 'active aldehyde' derivatives of thiamine resulting in direct metal–ligand bonds.[16,19]

In an effort to address the importance of $S^{(+)}…O^{(-)}$ interaction, we have studied the reactivity of various 'active aldehyde' thiamine derivatives towards metal ions. Our approach involved thiamine pyrophosphate (TPP) (**1**) itself, 2-(α-hydroxybenzyl)-thiamine chloride (HBT) (**2**), 2-(α-hydroxy-α-cyclohexylmethyl)-thiamine chloride (HCMT) (**3**), 2-(α-hydroxybenzyl)-thiamine monophosphate (HBTMP) (**4**) , 2-(α-hydroxybenzyl)-thiamine pyrophosphate (HBTPP) (**5**) and finally 2-(α-hydroxyethyl)-thiamine pyrophosphate (HETPP) (**6**), the latter two being the 'active aldehyde' intermediates of BFD and PDC respectively (Scheme 1).

Table 1 Cofactor properties and temporary groups of residues involved in catalysis of the enzymes yPDC, BFD and IPDC as revealed by crystal structure analysis and site directed mutagenesis studies[3,6,7,9–11,13–15]

	yPDC	BFD	IPDC
Cofactors	TPP, Mg(II)	TPP, Mg(II), Ca(II)	TPP, Mg(II)
Metal binding residues	D(444), N(471), G(473), H_2O, pyrophosphate	D(428), Q(455), T(457), H_2O, pyrophosphate	D(435), N(462), G(464), H_2O, pyrophosphate
Cofactor's aminopyrimidine and thiazole moiety binding residues	G(413), E(51)	G(401), L(403), N(23), E(47), Y(433), P(24)	E(52)
Imino tautomeric form stabilization and ylide formation	E(51), D(28), H(114), H(115), G(413)	E(47), G(401)	E(52), D(29)
Carboxyalkyl-TPP formation, decarboxylation	D(28), E(477), Y(290), I(415)	S(26), H(281), H(70)	D(29), E(468)
Product release	D(28), E(471), H(114), H(115)	H(70)	D(29), E(468), H(115), H(116)
V-conformation stabilization	I(415)	L(403)	—

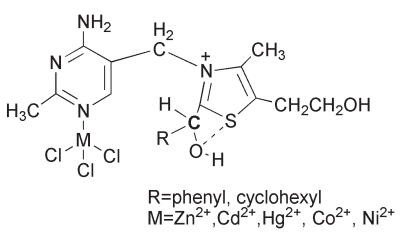


Scheme 1 Molecular structures of TPP (**1**) and various 'active aldehyde' derivatives of thiamine (**2–6**).

The reactions of **2** and **3**, where N(1′) is deprotonated, with metal chlorides such as $HgCl_2$, $CdCl_2$, $ZnCl_2$, $CoCl_2$ and $NiCl_2$ always lead to the easy formation of metal thiamine complexes with direct metal–N(1′) bonds.[19,21] Furthermore, in all cases the N(1′) was found to be the only reactive site. In all cases pseudotetrahedral structures with metal ions coordinated through the N(1′) atom and three chloride anions were found. A common structural feature was also the close $S^{(+)}\ldots O^{(-)}$ proximity (Scheme 2).[19,21]

Interestingly, reaction of **2** with $CuCl_2$ produced Cu(I) and thiochrome as a result of 2-(α-hydroxybenzyl)-thiamine oxidation to thiochrome. From this reaction mixture, crystals of Cu^{II}(thiochrome)Cl_2 were isolated. Their crystal structure comprised an infinite array of Cu^{II}(thiochrome)Cl_2 neutral units linked together through the N(4′α) and N(1′) atoms of thiochrome coordinated to Cu(II) (Scheme 3).[22]

In the case of compound **4**, we have demonstrated the ability of the monophosphate side chain to act as a potential coordination site, in addition to the N(1′) of the pyrimidine. In **4**, at pH 6, both sites, *i.e.*, N(1′) and the phosphate group, were coordinated to the metals, with the sole exception of Hg(II), reacting only through N(1′).[23]



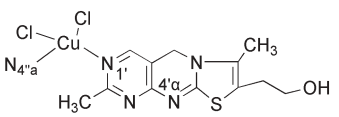
Scheme 2 Molecular structure of $MLCl_3$ complexes (L = HBT or HCMT).

As a part of our modeling work, we have synthesized various metal coordinated compounds with **5** and **6**, representing the 'active aldehyde' intermediates of BFD and PDC respectively.[24,25] It was found that **5** and **6** may exist in as many as three different protonation states, depending on pH (Scheme 4).

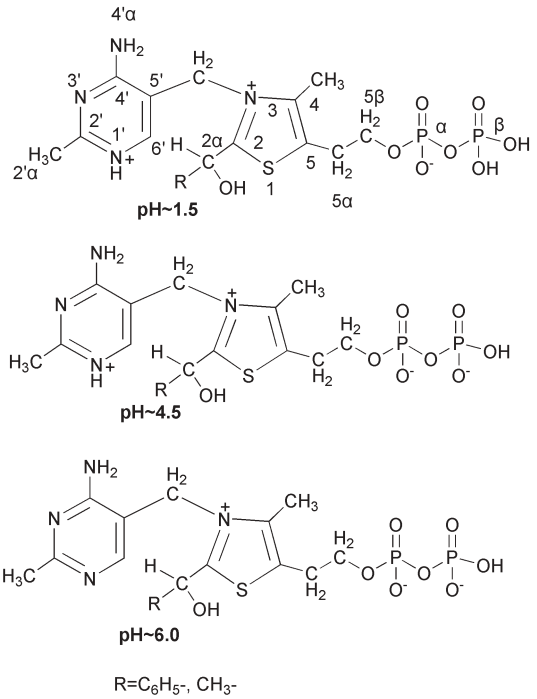
At pH values around 6, Zn(II) and Cd(II) formed complexes with **5**, with both metals being coordinated through both the N(1′) and the pyrophosphate group. The same structure was found for the zinc and cadmium complexes with **6**. Once again, Hg(II) was coordinated only through N(1′).[24,25] ^{31}P NMR data indicated an unequal α,β bidentate coordination of the diphosphate group to the metal ions, with the P_β–O^- apparently bound more strongly than the P_α–O^- site. Thus, at near physiological pH, both **5** and **6** ligands form 2 : 2 adducts with Zn(II) and Cd(II) ions consistent with N(1′) and phosphate coordination.[17,18] The formation of such dimeric species was detected by ESI-MS and supported by potentiometric studies (Scheme 5).[25]

At lower pH values, the N(1′) atom was protonated and the reaction of metal ions with the 'active aldehyde' derivatives **5** and **6** resulted in the formation of 1 : 2 (M : ligand) complexes, corresponding to metal coordination only with the pyrophosphate groups.[24,25]

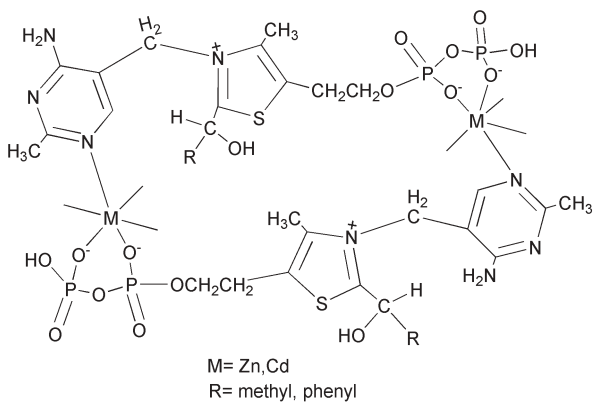
When thiamine pyrophosphate (**1**) itself was used as a ligand, a similar coordination with metal ions (Zn(II),Cd(II))



Scheme 3 Molecular structure of Cu^{II}(thiochrome)Cl_2.



Scheme 4 The 'active aldehyde' intermediates of BFD and PDC at different pH values.

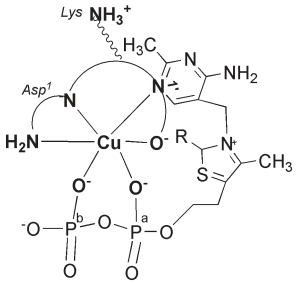


Scheme 5 Proposed structure of $M_2L_2H_2$ complexes (M = Cd, Zn, L = TPP, HBTPP, HETPP).

was observed.[26] That is, at physiological pH, coordination with the metal ions took place *via* both the N(1′) atom and the pyrophosphate group. Stability constant measurements showed the formation of dimeric complex species, with **6** forming more stable metal complexes than **1**.[26] $\beta_{Zn(II)–HETPP}/\beta_{Zn(II)–TPP} = 7.94$, $\beta_{Cd(II)–HETPP}/\beta_{Cd(II)–TPP} = 3.72$.

This finding was consistent with the observation of the easy formation of metal complexes with 'active aldehyde' derivatives.[26]

In the crystal structures of thiamine enzymes, the diphosphate moiety is embedded in an elaborate network of salt bridges and hydrogen bonds, formed between protein residues, water molecules and the metal ion.[6,27–30] Despite the numerous crystallographic data,[6,27–30] direct structural information on the active site of thiamine-dependent enzymes *in solution* is scarce. For this reason, we have attempted to construct and



Scheme 6 Proposed structure of the tertiary metal–'active acetaldehyde'–peptide complex, at physiological pH.

characterize a pertinent model system in solution. In order for such a model to be credible, it has to incorporate the structural key components *i.e.* the protein, the cofactors (the thiamine and the metal ion) and the substrate. In this context we have synthesized: a) the pentapeptide Asp-Asp-Asn-Lys-Ile which mimics the metal binding site Asp185-Asp186-Asn187-Lys188-Ile189 of transketolase and surrounds the pyrophosphate moiety[29,30] and b) HETPP **6**, involving TPP and substrate as a covalent adduct.[27,28] In this manner we have studied the tertiary system [Cu(II)]–[pentapeptide]–[HETPP] by pulsed-EPR spectroscopy in aqueous solutions, at physiological pH.[31]

Our data showed the formation of a tertiary Cu^{2+}–pentapeptide–HETPP complex and moreover, the stability constants of this tertiary system were found to be higher than that of the simple Cu(II)–HETPP and Cu(II)–peptide systems.[31,32] The peptide backbone offers three coordination sites to the metal ion. The coordination sphere is completed by two additional phosphate oxygens of the coenzyme as in the crystal structures of the enzymes[6,27–30] and instead of a water molecule, the N(1′) atom of the pyrimidine ring is also bound to the metal (Scheme 6).[31,32] A similar structural model for tertiary [Zn(II)]–[pentapeptide]–[HETPP] and [Cd(II)]–[pentapeptide]–[HETPP] systems was also proposed involving zinc or cadmium coordination to phosphate oxygens, N(1′) of pyrimidine and the functional groups of the Asp^1 residue.[33]

2.2 Conformation

TPP can adopt three different conformations (F, S and V,†[34]) in terms of the relative orientation of the thiazolium and pyrimidine rings. In all crystal structures of TPP-dependent enzymes, TPP adopts the V conformation which brings 4′α-NH_2 close to C(2).[6,27–30] It is believed that at this position the 4′α-NH_2 group is able to act as an efficient proton acceptor for the C(2) proton, initializing the catalytic cycle[4,6,27–30] and creating the ''ylide''. On the other hand, it should be noted that without exception all the C(2)-substituted thiamine intermediates which have been either isolated from enzymic systems or synthesized *in vitro* adopt the S conformation.[26] In this

† The relative orientations of the pyrimidine and thiazole rings in thiamine are determined by the torsional angles Φ_p = N(3)–C(3,5′)–C(5′),C(4′) and Φ_T = C(5′)–C(3,5′)–N(3)–C(2). These angles have positive values in the clockwise direction and according to their values there are three main conformations of thiamine and its derivatives: the F-conformation when $\Phi_T = 0°$ and $\Phi_p = \pm 90°$; the S-conformation when $\Phi_T = \pm 100°$ and $\Phi_p = \pm 150°$; the V-conformation when $\Phi_T = \pm 90°$ and $\Phi_p = \pm 90°$.

context, over the past fifteen years, more than twenty different metal complexes of 'active aldehyde' derivatives of thiamine have been studied by our group in the solid state and in solution.[19,21–26,31–33] In all cases, even in the case of the ternary [M^{2+}]–[Asp-Asp-Asn-Lys-Ile]–[HETPP] systems (M = Cd, Zn, Cu) the 'active aldehyde' intermediates adopt the S conformation.[19,21–26,31–33]

2.3 Perspectives

In summary, we have highlighted the following points: (i) in either the solid phase or in solution, the metal binding sites of 'active aldehyde' derivatives of thiamine are both the pyrophosphate group and the N(1′) atom of the pyrimidine moiety and (ii) the free ligands as well as the metal-coordinated ligands ('active aldehyde' derivatives of TPP) always adopt the S conformation.

These observations concern the model compounds. However, the validity of these conclusions for *in vivo* systems requires further investigation. In this context the key questions to be addressed are: (a) During the catalytic cycle *in vivo*, would the protein environment allow the coenzyme–substrate adduct to obtain the stable S conformation? (b) Is the enhanced reactivity of the N(1′) position towards the metals used in nature?

To address these questions, we have evaluated the coenzyme activity of TPP–metal complexes and HETPP–metal complexes by performing enzymic studies in the presence of the pyruvate decarboxylase (PDC) apoenzyme.[26] These studies showed that TPP–metal complexes with direct N(1′)–metal bonds did *not* present cocatalytic activity. Within this context methylation on the N(1′) atom results in the same behavior, *i.e.* no activity.[35] In contrast the model HETPP–metal complexes with direct metal–N(1′) bonds (in the S conformation) exhibit coenzyme activity.[26]

Based on these data we suggested that the N(1′)–metal bond in the TPP–metal complexes acts as an inhibitor preventing the H-bond formation between the N(1′) atom and the side chain of Glu418, one of the few conserved interactions of the cofactor with the protein environment in all thiamine enzymes. This finally affects the 4′α-NH_2 group which cannot act as a proton acceptor for the C(2) proton, initializing in this way the catalytic cycle.[26]

With the model HETPP–metal-complex, it appears that, in this catalytic step, neither the N(1′)–metal bond nor the S conformation inhibits the enzymic procedure. This suggests that (i) the 4′α-NH_2 group is not involved in the last steps of catalysis and (ii) the coenzyme–substrate adduct formed during the intermediate catalytic steps may adopt the S conformation, as *in vitro*, without inhibiting the enzymic procedure.

It has been suggested that the tendency of N(1′) to react with metal ions, as observed in the model compounds, may not be used in thiamine enzymes.[6,27–30] This was based on the observation that the only role of the metal ion is to bridge the pyrophosphate group of the cofactor to a conserved group of side chains of the pyrophosphate binding area of the different apoenzymes.[6,27–30] However, if by any chance the N(1′) atom is used as a metal coordination site *during* the catalytic cycle, the coordination to N(1′) should take place after the formation of the 'active aldehyde' intermediates.[19]

3 Catalysis and active sites

A unique feature of the TPP cofactor is its relative importance in catalysis, given that TPP alone can perform the reaction, although over a million times less efficiently than the holoenzyme. The X-ray structures of several α-ketoacid decarboxylases[6,7,28,36] showed that despite the similar mode of cofactor binding, the only conserved residues in the active site are those directly bound to the cofactor or metal ion. This supports the suggestion that it is the cofactor, its conformation and its environment that determine the catalytic efficiency.[15]

A possible mechanism for TPP-dependent catalysis by α-ketoacid decarboxylases,[37] and related enzymes, that takes into account the results of both biochemical[2–15,27–30,36–44] and model studies[16,17,19,21–26,31–33] is presented in Fig. 2 and includes several steps in which proton transfers are needed. In the initial step, deprotonation of the C(2) atom of TPP is required, resulting in the formation of an ylide. Crystallographic[6,7,28,29,36] and NMR[39] data suggest that the 4′α-amino group of the cofactor itself participates in the abstraction of the C(2)-H proton involving an imino tautomer, as has been previously postulated.[40] Two conserved interactions between the enzymes and the cofactor presumably operate as follows:[6,28] (a) a glutamic acid close to N(1′) (Glu51 in yPDC, Glu50 in ZmPDC, Glu47 in BFD and Glu52 in IPDC) maintains this nitrogen atom in a protonated state; the induced positive charge serves to drive the 4′α amino group, by lowering its pK_a, to an imino tautomer, and (b) the H-bond between the resulting imino group and a glycine carbonyl (Gly413 in yPDC, Gly413 in ZmPDC, Gly401 in BFD and Gly463 in IPDC) likely helps to localize the lone pair of electrons on the imino nitrogen for proton abstraction. These aspects are further substantiated by our studies showing that a TPP–metal complex with a direct N(1′)–metal bond does not have any catalytic activity. This catalytic deficiency was attributed to the N(1′)–metal bond, which acts as an inhibitor preventing the hydrogen bond formation between the N(1′) atom and the side chain of glutamic acid.[26]

For this intramolecular catalysis, the cofactor is forced into an energetically unfavorable V conformation by the enzyme, forcing N(4′α) and C(2) into close proximity and assuring that the imino nitrogen is poised to serve as a base to deprotonate the C(2) position. In the active site of PDC and BFD, the distance between N(4′α) and C(2) is not long enough to accommodate two protons.[6,36] That is, if C(2) is protonated, N(4′) must exist in the singly protonated form. It was suggested that in the resting state of the enzyme, the aminopyrimidine is likely to exist in the imino tautomeric form, poised to accept the C(2) proton.[6,36]

The C(2) carbanion of the cofactor, once formed, is able to attack the carbonyl carbon of the substrate (Fig. 2), in the second step of catalysis. For covalent bonding between these two atoms to occur, stabilization of the negative charge developed at the carbonyl oxygen is required in order to form the first tetrahedral intermediate (**1**, Fig. 2). Based on the crystal structures of α-ketoacid decarboxylases, it was suggested that one histidine and one glutamic acid should play a functional role in the active site (His115, Glu477 in yPDC,

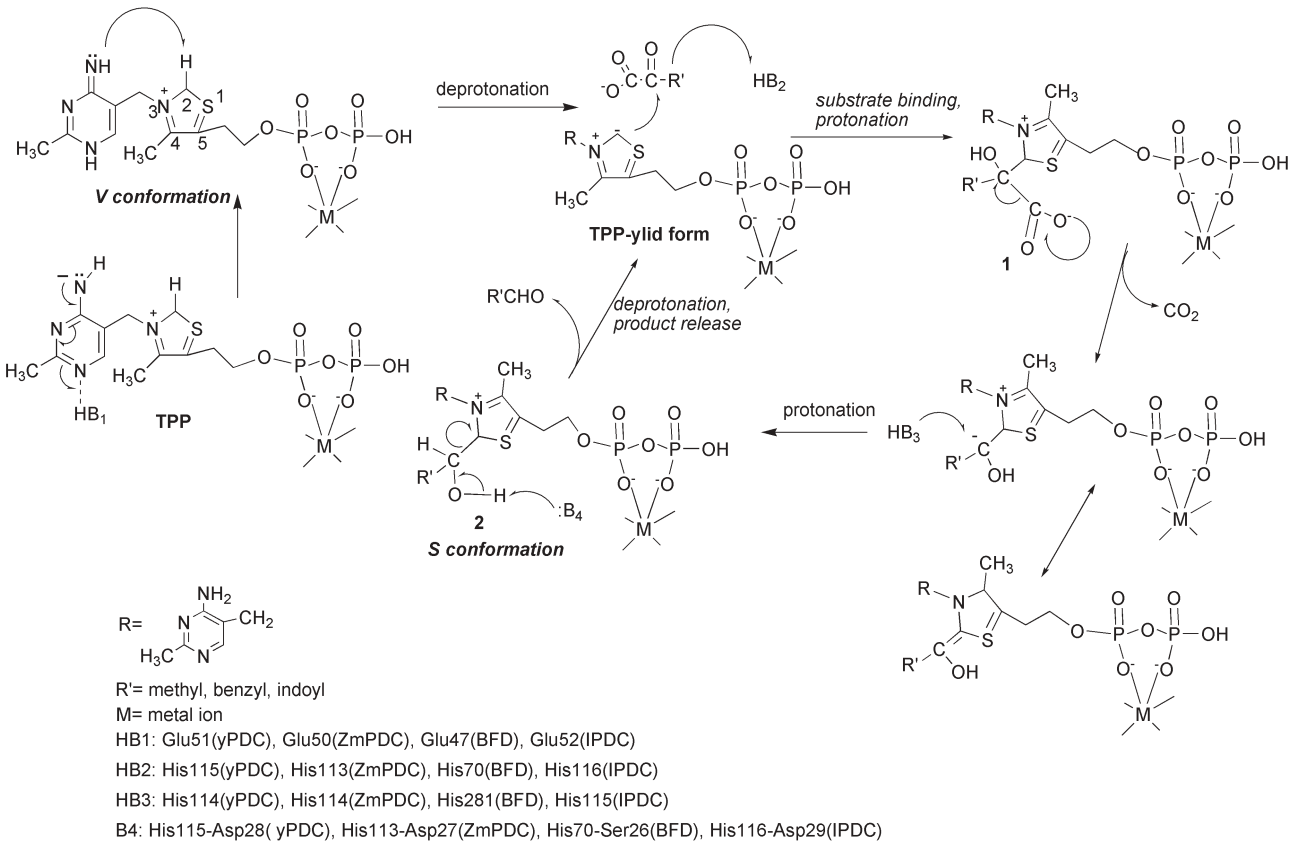


Fig. 2 A possible detailed mechanism of action of α-ketoacid decarboxylases.

His113, Glu473 in ZmPDC, His70, Glu28 in BFD and His116, Glu468 in IPDC). Structural and kinetic data combined with site-directed mutagenesis studies ensure the contribution of this histidine to the protonation of the carbonyl oxygen of the substrate. Since His must be protonated prior to substrate binding, the ionization state of His is controlled by glutamic acid. That is, this pair of residues apparently forms part of a proton relay.[6,15,28]

Subsequent decarboxylation of **1** results in a C(2α)-carbanion/enamine intermediate.[41,42] When catalysis is allowed to proceed in D_2O, the decarboxylation step is virtually unaffected, whereas the substrate binding and product release, both steps requiring proton transfer, show primary isotope effects.[43]

Protonation of the C(2α)-carbanion/enamine provides a second tetrahedral intermediate (**2**, Fig. 2). The proton transfer rate of the steps leading to and from the C(2α)-carbanion/enamine was measured in H_2O and the pK_a for this dissociation was found to be ~15.4. At pH 6.0, formation of the enamine on the enzyme indicates a lowering of the pK_a by greater than nine units by the enzyme environment. The stabilization of this zwitterionic enamine intermediate at the active center was sufficient to account for a 10^9-fold rate acceleration by the enzymes.[42] Based on the X-ray structures of several α-ketoacid decarboxylases, a second histidine residue is also well positioned to act as catalytic acid/base. It has been proposed that this conserved His (His114 in yPDC, His114 in ZmPDC, His281 in BFD and His115 in IPDC) is involved in the protonation of the C(2α)-carbanion/enamine.[15]

Finally, aldehydes are eliminated with the assistance of an enzymic base (Fig. 2). Based on steady-state kinetic data, a complete deficiency in catalyzing acetaldehyde elimination from HETPP (**2**, Fig. 2) is observed in both His113Lys and Asp27Glu variants of ZmPDC. Since His113 is within hydrogen bonding distance of Asp27, it was suggested that this pair of residues constitutes a functional dyad to assist product release.[43] In BFD, His70 likely removes the proton from HBTPP (**2**, Fig. 2) to allow the release of benzaldehyde in conjunction with Ser26.[15] His70 of BFD has a counterpart of His115 in yPDC,[15] while Asp28 is also important in the deprotonation of HETPP and concominant product release.[44] The corresponding functional amino acid-dyad in IPDC is His116-Asp29.[7]

Of note, HETPP–metal complexes with a direct N(1′)–metal bond and the cofactor in the S conformation, do not inhibit the enzymic procedure, since they are able to release acetaldehyde in the presence of pyruvate decarboxylase (PDC) apoenzyme.[26] These findings showed that the product release is assisted by an enzymic base, instead of a putative intramolecular catalysis *via* the imino N(4′α) atom.

Moreover, in the final step, the S conformation of the cofactor obviously does not obstruct the catalytic cycle. This is why, while the V conformation is active in the initial step of catalysis, the S conformation should also be favorable during

the catalytic cycle. This may be adopted after the formation of the 'active aldehyde' intermediates (Fig. 2).[16,19]

4 Immobilization of TPP on a silica surface

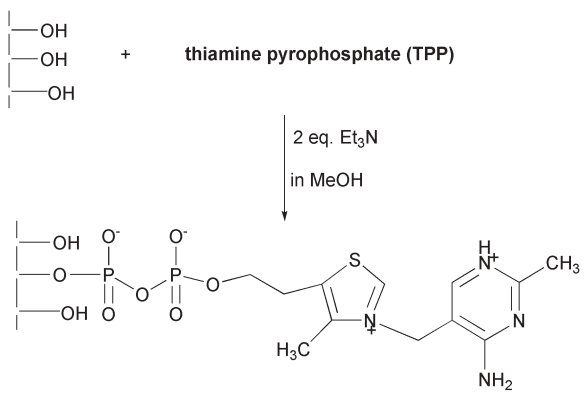
Integration of chemo- and bio-catalysis together with materials science provides the opportunity to design and develop new materials for innovative applications. Hybrid organic–inorganic composite materials are among the most attractive targets achievable by this co-operative process. We have developed recently a convenient, one-step synthesis for the tethering of vitamin B_1 on a silica surface *via* the phosphate group (Scheme 7).[45]

Thiamine enzymes and thiamine itself in protein-free model systems, catalyze pyruvate decarboxylation. Evaluation of the catalytic properties of the novel biomimetic material showed that the catalytic activity of the immobilized TPP remained intact after its mild anchoring procedure and, moreover, it is a very active biocatalyst, even more efficient than the homogeneous one.[45]

Conclusions

Model studies of thiamine–bivalent metal complexes in combination with biochemical ones have provided a good insight into the elucidation of the mechanism of action of thiamine dependent enzymes. Summarizing the key points of the catalytic mechanism: TPP binds to the apoenzyme through the pyrophosphate group and bivalent metal ions, and is forced to adopt the V conformation within the enzymes, bringing the 4′α-NH_2 group (in the imino tautomeric form) near C(2) of thiazole, attracting a proton, creating the ''ylide'' and initiating the catalytic cycle. This is followed by addition of the substrate, decarboxylation of the formed adduct and formation of the 'active aldehyde' intermediate which most probably adopts the S conformation. This conformation favors an important $S^+ \ldots O(2\alpha)^-$ interaction facilitating the release of the main aldehyde product and finally regenerating the TPP-''ylide'' form.

With regard to the immobilization of TPP on a silica surface, further studies are required to evaluate non enzymic thiamine catalysis in homogeneous and heterogeneous systems and are currently in progress in our group.[46]



Scheme 7 Reaction of TPP immobilization on a silica surface.

At this point, as regards future model studies, it is essential to recognize that too few model studies have been carried out to properly reflect the conformation of TPP *during* the catalysis. Based on this, one can conclude that modeling the active site of TPP-dependent enzymes still remains an active research field.

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