Studies on thiamine diphosphate-dependent enzymes

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Abstract

The 3-deaza analogue of TPP (thiamine diphosphate), a close mimic of the ylid intermediate, has been synthesized and is an extremely potent inhibitor of a variety of TPP-dependent enzymes, binding much more tightly than TPP itself. Results using deazaTPP complexed with the E1 subunit of PDH (pyruvate dehydrogenase) have led to a novel proposal about the mechanism of this enzyme. The 2-substituted forms of deazaTPP, which mimic other intermediates in the catalytic mechanism, can also be synthesized and 2-(1-hydroxyethyl)deazaTPP is also an extremely potent inhibitor of PDC (pyruvate decarboxylase). Attachment of such 2-substituents is expected to be a way to introduce selectivity in the inhibition of various TPP-dependent enzymes.

Introduction

Thiamine (vitamin B_1) is required in the diet of humans and other mammals for the production of the coenzyme TPP (thiamine diphosphate) **1**. TPP is required by a number of enzymes that have in common the fact that they all catalyse the cleavage and formation of bonds to the carbon atom of a carbonyl group (for a recent review on TPP-dependent enzymes see [1]). The simplest mechanism is that of PDC (pyruvate decarboxylase) that decarboxylates pyruvate to give acetaldehyde, the penultimate step in the formation of ethanol in fermentation by yeast and certain bacteria. The mechanism, shown in Scheme 1, involves deprotonation of C-2 of the thiazolium ring of TPP to give the ylid **2**, followed by attack of **2** on the carbonyl group of the substrate to give 2-lactylTPP **3**. The positively charged thiazolium ring then facilitates decarboxylation to give the enamine intermediate **4** that, in the case of PDC, protonates to give 2-(1-hydroxyethyl)TPP **5**. Finally, release of acetaldehyde regenerates ylid **2**, completing the catalytic cycle.

All TPP-dependent enzyme mechanisms proceed through an enamine intermediate similar to **4**, which is produced by a decarboxylation step if the substrate is an α-ketoacid, as for PDC, but can also be formed by deprotonation (if the substrate is an aldehyde) or some other fragmentation of the initial substrate-TPP covalent adduct. The enamine intermediates then either react with a variety of different electrophiles, such as a proton (as for PDC), an aldehyde, an α-keto-acid, a lipoyl group or some other oxidizing species, or they cause expulsion of an adjacent leaving group in the substrate.

Crystal structures of a number of TPP-dependent enzymes have been determined with TPP bound in the active site. They are invariably dimers (or dimers of dimers) with the active

sites at the interface between the different subunits. TPP is bound in a conformation, called the V-conformation, which is not its lowest energy conformation in solution. This conformation is characterized by the close proximity of the 4′-amino group on the pyrimidine ring to the hydrogen atom on C-2 of the thiazolium ring that is removed to form ylid **2**. It is now thought that this amino group is in fact the base that effects the deprotonation. However, in the normal amino-form this nitrogen atom is not a good base as its lone pair of electrons is conjugated with the π-system of the pyrimidine ring. Therefore it is suggested that tautomerization to the less-stable imino form **1a** occurs at some stage and this is then the base. The best evidence for this is that oxythiamine diphosphate **6** is totally inactive when bound in place of TPP and, furthermore, whereas the exchange of the hydrogen on C-2 of TPP **1** is more than 10^5-fold faster when bound to PDC than when free in solution, the enzyme actually retarded exchange of the hydrogen at C-2 of **6** [2].

Despite the existence of several crystal structures, many of the other details of the mechanisms of TPP-dependent enzymes (e.g. how the substrate binds, which are the acid/base groups on the enzyme) remain obscure. One reason for this is that the substrate, if bound in the active site, would react and the intermediates in the reaction, e.g. **2–5** for PDC, are not stable either. An exception to this occurs with transketolase, for which the enamine intermediate (equivalent to **4**) is apparently stable in the absence of an 'acceptor' aldehyde [3] and also a crystal structure of an acceptor aldehyde bound to the TPP-form of the enzyme has been obtained [4]. In addition, many of the crystal structures have mobile loops, invisible in the X-ray structure, which are thought to close over the active site when the substrate binds and which contain some of the essential catalytic groups.

One solution to the difficulty in obtaining structures with the natural substrate bound is to use an analogue of the substrate that cannot react or cannot undergo the full catalytic cycle. Another solution is to use a mutant enzyme that cannot

Scheme 1 | Structures of TPP (1) and inactive analogues oxythiamine diphosphate (6) and deazaTPP (9) and mechanism of the PDC reaction
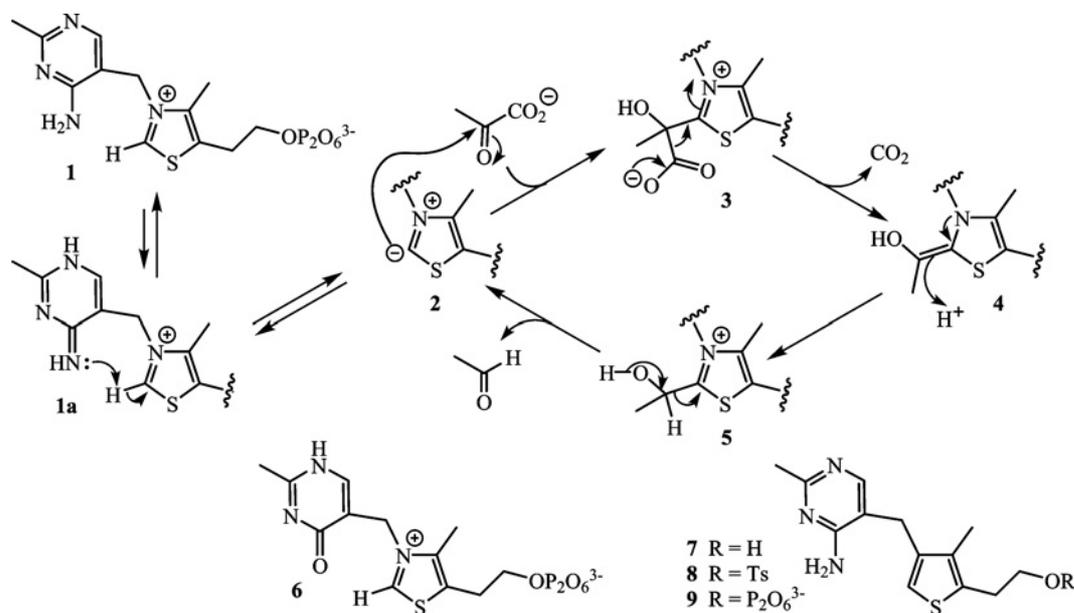
catalyse some or all of the steps in the mechanism. However, both of these are somewhat hit-or-miss approaches and even if successful, would probably only allow the structure of one state of the enzyme to be solved. Our approach has been to synthesize and use an analogue of the coenzyme that is inactive. In choosing an inactive analogue of TPP we wanted a molecule that was as close as possible to TPP and, in addition, was capable of bearing substituents at C-2 to match reaction intermediates such as **2–5**. We therefore chose deazaTPP **9**, in which the nitrogen atom of the thiazolium ring is replaced by a carbon. In fact, deazaTPP **9** is isoelectronic with TPP, and so expected to be essentially identical in shape and size, but the lack of positive charge means that formation of the anion at C-2 (as in ylid **2**) would not be possible.

## Synthesis of deazaTPP and inhibition of PDC

Our synthesis of deazathiamine **7** involved ten steps, the first six of which could be performed without chromatography, and could be performed on a multigram scale [5]. Conversion of **7** into deazaTPP **9** by heating in concentrated phosphoric acid, a published method for thiamine and several of its analogues, was unsuccessful for **7**, probably because the thiophene ring of **7** is more acid-sensitive than the thiazolium ring of thiamine. Fortunately, however, we found an alternative procedure involving formation of the tosylate **8** and nucleophilic displacement of the tosylate group by the pyrophosphate trianion as its tetrabutylammonium salt [6]. After optimization, this procedure yielded a useful 72% of the desired deazaTPP **9**, which was conveniently purified by anion-exchange chromatography.

With deazaTPP in hand, the first step was to find out how well it binds to TPP-dependent enzymes. The enzyme

that we have studied in greatest detail is PDC from the bacterium *Zymomonas mobilis*. When the holo-enzyme (with TPP bound) was incubated with deazaTPP, time-dependent inhibition was observed. However, the inhibition was very slow, with approx. 70% inhibition, at the most, being observed after 4.5 h. The reason for the inhibition being so slow is that the inhibitor, deazaTPP, cannot bind to the enzyme until TPP has unbound, which is known to be a slow process. Our inhibition data fitted very well to that expected of inhibition that is competitive with TPP, resulting in a pseudo-first-order rate constant for inhibition that followed Michaelis–Menten-like saturation kinetics with the maximum rate of inhibition equal to the rate of unbinding of the pre-bound TPP. The rate of unbinding of TPP was determined as 5.1×10^{-3} min^{-1} (similar to the rate determined by other methods [7]) and the rate that deazaTPP binds was found to be almost 7-fold faster than the rate of TPP binding.

Because of the very slow unbinding of TPP, it was necessary to study binding of deazaTPP to the apoenzyme, which can be prepared by removal of TPP (by dialysis, gel filtration or ultrafiltration) at elevated pH [8]. The extent of binding was determined by adding a large excess of TPP after a given time and measuring the residual enzymic activity. As expected, inhibition was much faster now and showed pseudo-first-order rate constants that were proportional to the deazaTPP concentration over the full range of concentrations with no sign of saturation. The derived second-order rate constant was 0.16 μM$^{-1} \cdot$ min^{-1}. A similar value (0.24 μM$^{-1} \cdot$ min^{-1}) was obtained by following the decrease in fluorescence of the protein that occurs when deazaTPP binds.

In the above assays, the enzyme activity decreased exponentially to zero and, after sufficient incubation with deazaTPP (~10 min with 5 μM deazaTPP), the enzyme showed no

Scheme 2 | Mechanism of the PDH reaction
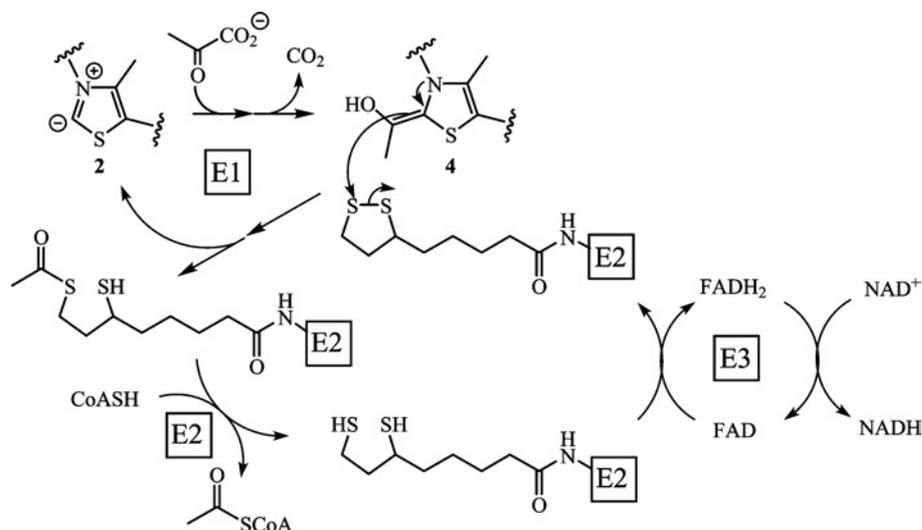
measurable activity. This shows that over the time-course of the assay the excess TPP added does not displace deazaTPP. In an attempt to measure the dissociation rate for deazaTPP, the enzyme was freed of excess deazaTPP by ultrafiltration and then incubated with a large excess (100 μM) of TPP for 24 h. After this there was still no measurable activity, indicating a dissociation rate constant of $<2.8 \times 10^{-6}$ min^{-1}. Combining this with the measured rate of association ($\sim 0.2\,\mu$M$^{-1} \cdot$ min^{-1}), the inhibition constant K_i must be <14 pM.

Preliminary studies with acetohydroxyacid synthase (the first enzyme in the biosynthesis of the branched amino acids) (assays kindly performed by Syngenta plc) and transketolase (S. Mann and F.J. Leeper, unpublished work) indicate that deazaTPP is also an extremely potent but slow acting inhibitor of these enzymes.

Studies with 2-oxoacid dehydrogenases

The other TPP-dependent enzymes that we have studied are the E1 subunits of PDH (pyruvate dehydrogenase) and α-KGDH (α-ketoglutarate dehydrogenase). These belong to a small group of multicomponent 2-oxoacid dehydrogenase complexes, which oxidatively decarboxylate 2-oxoacids and transfer the resulting acyl group to CoA.

The reaction scheme for PDH is shown in Scheme 2. The E1 subunit is the TPP-dependent decarboxylase, which transfers the acetyl group to a lipoyl group attached to the E2 subunit. At an active site that is also part of the E2 subunit the acetyl group is further transferred to CoA, giving acetyl CoA and a dihydrolipoyl group. Oxidation of the dihydrolipoyl group occurs on the E3 subunit and involves FAD and the ultimate hydride acceptor, NAD$^+$. This enzyme complex is remarkable for the fact that it uses five different coenzymes but it is even more extraordinary in the organization of the complex. The E2 subunits form a spherical core containing,

in many organisms, 60 individual subunits arranged as an eicosahedron of trimers. Attached by a flexible segment of peptide are first a binding domain that binds both E1 and E3 subunits and then one or more lipoyl domains that have the lipoyl group attached by an amide linkage to a lysine residue. The E1 and E3 subunits bind to the binding domain and form a shell around the core, leaving a gap of approx. 60 Å (1 Å $= 10^{-10}$ m) in which the lipoyl domains move between each type of active site. The whole complex has a molecular mass of approx. 10 MDa.

Our investigation of the binding of deazaTPP **9** used the isolated E1 subunit of α-KGDH (kindly supplied by R. Frank, Department of Biochemistry, Cambridge University, Cambridge, U.K.). Without the E2 and E3 subunits the complete reaction is not possible but decarboxylation of α-ketoglutarate catalysed by the E1 subunit alone does occur using the oxidizing agent DCPIP (dichlorophenol-indophenol) [6]. This oxidizes the enamine intermediate (equivalent to **4**) to 2-succinylTPP, which hydrolyses to succinate plus TPP. The colour change when DCPIP is reduced provides a convenient assay.

Inhibition studies incubating deazaTPP with the holoenzyme in the presence of 25 μM TPP showed time-dependent inhibition similar to that observed with PDC but considerably faster due to the more rapid dissociation of TPP ($k_{off} \sim 0.07$ min^{-1}). In this case deazaTPP binds approx. 75 times faster than TPP. Slow partial recovery of activity was observed on incubation of inhibited enzyme with excess TPP, consistent with a K_i value of approx. 5 nM (500-fold tighter binding than TPP itself).

The much stronger binding of deazaTPP than TPP to all the TPP-dependent enzymes tested might seem surprising until one considers that the charge state of deazaTPP more closely mimics the ylid **2**, which is overall neutral in the five-membered ring, than TPP **1** which is positively charged.

Scheme 3 | Synthesis of 2-substituted derivatives of deazaTPP
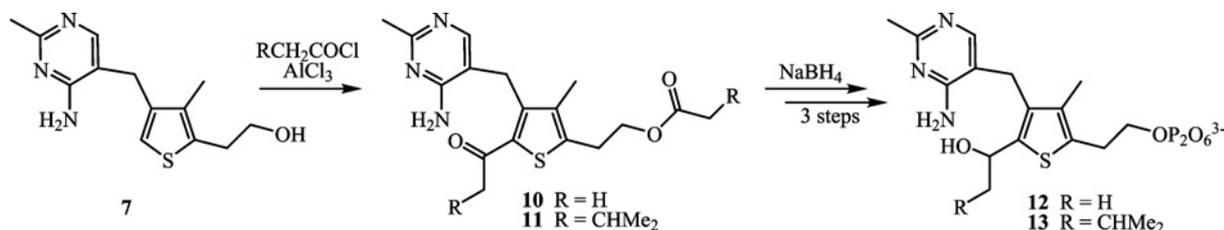
Clearly, one of the effects of binding TPP in the active site of these enzymes is to make formation of the ylid easier and the strength of binding of deazaTPP is a result of this increased stabilization of the neutral form.

In the Department of Biochemistry in Cambridge, R. Frank and B. Luisi have been using deazaTPP in structural studies of PDH E1 subunit and have obtained an X-ray crystal structure of the enzyme–deazaTPP complex (personal communication). The deazaTPP is seen in the active site, in an identical position to that normally occupied by TPP. Proteolysis studies have also provided interesting results [9]. With the E1–TPP complex, chymotrypsin causes cleavage of the α-chain (the E1 subunit is an $\alpha_2\beta_2$ tetramer) but only approximately half the α-chains are cleaved, suggesting that there is some interaction between the two active sites. The cleavage sites have been determined to be in a loop that closes over the active site. In the crystal structure, this loop is seen in the closed form in one of the two active sites but disordered in the other. With the E1–deazaTPP complex, however, both α-chains of the tetramer are protected from proteolysis. The similarity of deazaTPP **9** to the ylid **2** suggests that the loop closes when the ylid forms and, therefore, that in the E1–TPP complex one of the two TPP molecules is present as the ylid **2** while the other is in the protonated form **1**.

Frank et al. [9] have presented evidence that the interaction between the two active sites is mediated by a water-filled channel lined by acidic residues. They suggest that this channel allows a proton to be shuttled between the two active sites and have presented a novel proposal for how the reactions at the two active sites could be co-ordinated (and driven) by proton transfer between one site and the other at appropriate points in the catalytic cycle.

Progress towards deaza-analogues of intermediates in the catalytic mechanism

We have found that addition of a substituent at C-2 of deazathiamine **7** is possible by Friedel–Crafts acylation reactions (Scheme 3). Thus acetylation of **7** gives the 2-acetyl derivative **10**, from which 2-(1-hydroxyethyl)TPP **12** has been made.

Studies with PDC have shown that **12**, the deaza-analogue of intermediate **5**, is a potent inhibitor, binding with a similar rate and affinity as deazaTPP itself (S. Mann and F.J. Leeper, unpublished work).

In addition to obtaining crystal structures of the enzymes with these deaza-analogues of reaction intermediates, we hope that attachment of suitable groups at C-2 of deazaTPP will provide selectivity in binding to the various TPP-dependent enzymes. To test this hypothesis, the 2-isovaleryl derivative **11** has been made by a similar Friedel–Crafts reaction and converted into 2-(1-hydroxy-3-methylbutyl)TPP **13** (M. Wood and F.J. Leeper, unpublished work). Studies to be performed in the near future will show whether the two deaza-analogues **12** and **13** show the expected selectivity for inhibition of the E1 subunits of PDH and the branched-chain 2-oxoacid dehydrogenase respectively.

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