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Review

Structure, mechanism and catalytic duality of thiamine-dependent enzymes

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Abstract. Thiamine is an essential cofactor that is required for processes of general metabolism amongst all organisms, and it is likely to have played a role in the earliest stages of the evolution of life. Here, we review from a structural perspective the enzymatic mechanisms that involve this cofactor. We explore asymmetry within homodimeric thiamine diphosphate (ThDP)-dependent enzyme structures and discuss how this may be correlated with the kinetic properties of half-of-the-sites reactivity, and negative cooperativity. It is likely these structural and kinetic hallmarks may arise through reciprocal coupling of active sites. This mode of communication between distant active sites is not unique to ThDP-dependent enzymes, but is widespread in other classes of oligomeric enzyme. Thus, it appears likely to be a general phenomenon reflecting a powerful mechanism of accelerating the rate of a chemical pathway. Finally, we speculate on the early evolutionary history of the cofactor and its ancient association with protein and RNA.

Keywords. Enzyme, thiamine diphosphate, molecular evolution, allostery, structure and function, catalysis, cooperativity.

Introduction

If chemistry is the interplay between the states of matter and their rates of change, then biology is a finely tuned orchestration of such chemical transformations, in which timing is the essence. The kinetic conductors of this biochemical change are enzymes and ribozymes, made of a limited repertoire of amino acids or nucleotides that have acquired accumulatively the capacity to catalyze at least 6800 different reactions [1]. Nonetheless, the range and catalytic power of biological enzymes are increased greatly by the assistance of organic and metallic cofactors, recruited repeatedly since the earliest stages of enzyme evolution.

One key organic cofactor of all extant life is thiamine diphosphate (ThDP), which is the active form of vitamin B_1. The early evolutionary emergence of this molecule is suggested by its essential role in most, if not all, organisms and its requirement at several central points of anabolic and catabolic intermediary metabolism, such as the pentose-phosphate pathway and the Krebs cycle, also known as the tricarboxylic acid (TCA) cycle. In its varied metabolic roles, ThDP assists in making and breaking bonds between carbon and sulfur, oxygen, hydrogen, and nitrogen; and most

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remarkably, the breaking and making of carbon-carbon bonds.

The chemical structure of ThDP, shown in Figure 1a, comprises three groups: a diphosphate-terminated side-chain, a five-membered thiazolium ring, and a six-membered aminopyrimidine ring. In bacteria, thiamine is generated from amino acids, 1-deoxyxylulose 5-phosphate, and aminoimidazole ribotide building blocks that are used to form thiazole and aminopyrimidine precursors, the adduct of which is then phosphorylated before it is recruited within enzymes for catalysis. Variations of this pathway are found in eukaryotic organisms [2–4]. The biosynthesis of ThDP is highly regulated at different levels, in part through a "riboswitch" (Fig. 2a, b), *i.e.*, a conformational transition in the RNA transcripts encoding the synthetic enzymes that is induced by the binding of the ThDP itself [5]. This mode of regulation is highly conserved, which again implicates an early evolutionary origin [6]. One of the mechanisms of action of riboswitches is described in the caption for Figure 2.

Returning to the catalytic properties of the ThDP, it was shown nearly 50 years ago that the activity of ThDP in thiamine-dependent enzymes resides in the C-2 carbon, which must be activated by deprotonation to form a potently nucleophilic 'ylide' (Fig. 1c) [7]. It might at first seem surprising that ThDP by itself is a very poor catalyst under physiological conditions, but the limited activity could be expected because the hydrogen atom on C-2 has a relatively high p*K*a of 17–19 [8]. However, when the cofactor is accommodated into the tailored pocket of its globular protein partner, the effects of conformation and electrostatics conspire to allow the C-2 proton to be extracted. There is evidence that ThDP first tautomerizes into the unusual imino-form (Fig. 1b) and the nitrogen atom of the imine is then responsible for abstracting the C-2 proton [9–11]. Another major factor that is thought to favor formation of the ylide, and subsequently the decarboxylation step, is the non-polar nature of the thiazolium environment (estimated dielectric constant of 13), which helps to stabilize species that are neutral relative to the positively charged thiazolium ring [12, 13]. The globular environment of the protein provides not only an activating environment, but also a nurturing one that defines substrate specificity, guides the formation of defined products and protects this highly reactive nucleophile from inappropriate quenching or from engaging in potential mis-reactions.

Although ThDP-dependent enzymes catalyze diverse reactions, all share certain mechanistic similarities (Fig. 3). These reactions may be categorized broadly into those that are decarboxylating and those that are non-decarboxylating, the latter of which are 'transferase'-type reactions. The first half-reactions of both the 'decarboxylase' and 'transferase'-type thiamine enzymes involve the chemically challenging breaking of a C-C or C-H bond adjacent to a carbonyl group in the substrate; this forms a covalently bound enamine intermediate (shown in Fig. 3). Once the metastable enamine intermediate is formed, a specific second substrate (which may in the simplest cases be nothing more than a proton) binds and reacts to form one of the many diverse products (summarized in Table 1). As an example, the decarboxylation of pyruvate, a major high-energy starting material used for the synthesis of many biomolecules, requires ThDP enzymes. The decarboxylations of other 2-oxo acids are also catalyzed by ThDP-dependent enzymes, each defined by precise substrate specificity.

The ThDP-dependent 'transferase' enzyme transketolase (TK) transfers a ketol group from a ketose to an aldose sugar proceeding through a non-oxidative mechanism. More exotically, ThDP is utilized as a cofactor in the first step of the clavulanate pathway for synthesizing β-lactam antibiotics [14] (CEA synthetase, Table 1).

A vast canon of research has illuminated the structure,

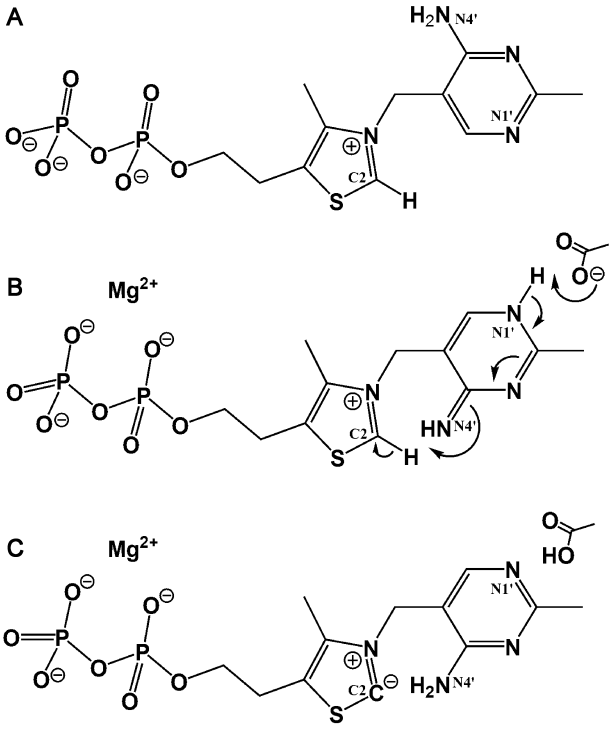

Figure 1. The chemical structures of thiamine diphosphate (ThDP) and its activated form. (A) ThDP in solution. (B) ThDP bound to enzyme, coordinated by Mg^{2+}. The cofactor takes a V-conformation placing the N-4′ near to the C-2. The aminopyrimidine ring is in the rare imino form. Upon activation the C-2 proton is abstracted via N-4′ of the aminopyrimidine ring and relayed to an invariant glutamate within the enzyme. (C) The activated ylide form of ThDP.

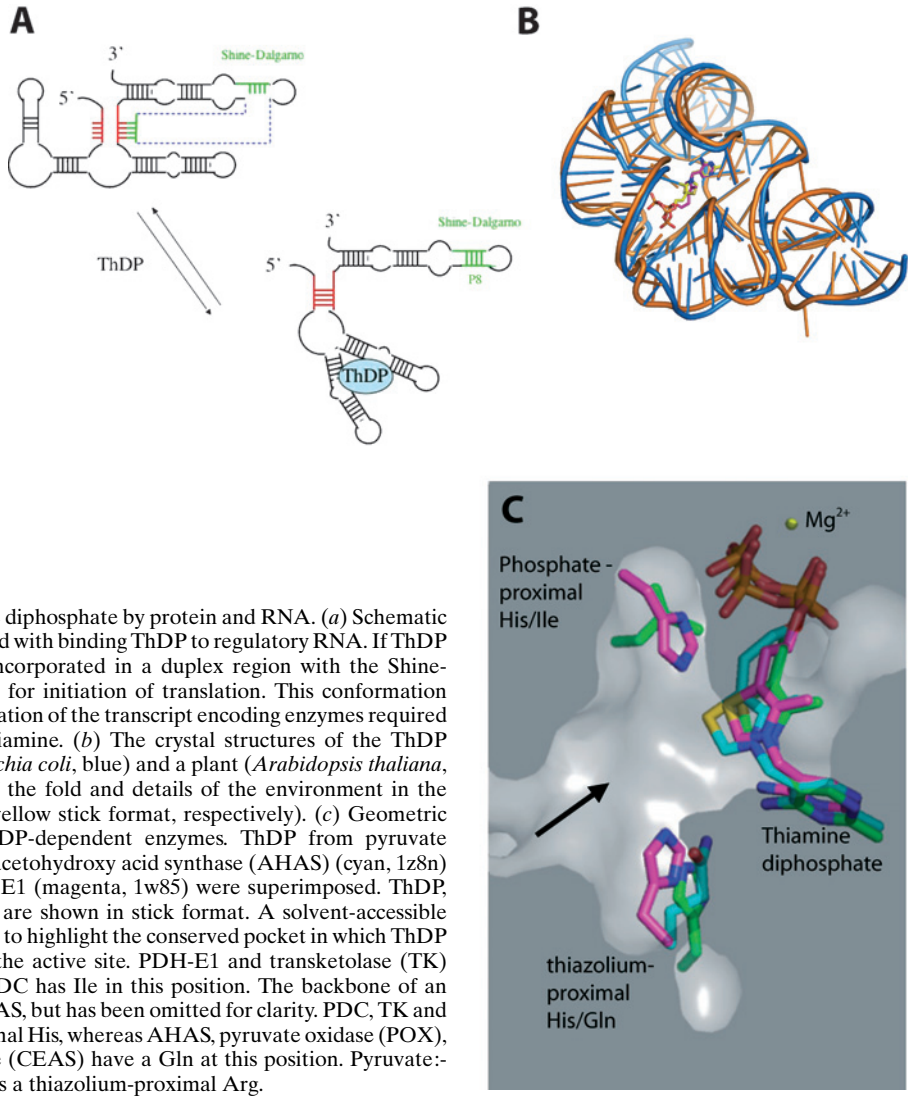

Figure 2. The recognition of thiamine diphosphate by protein and RNA. (*a*) Schematic of the conformational switch associated with binding ThDP to regulatory RNA. If ThDP is present, the region P8 becomes incorporated in a duplex region with the Shine-Dalgarno element, which is required for initiation of translation. This conformation masks the element and impedes translation of the transcript encoding enzymes required for the biosynthesis or import of thiamine. (*b*) The crystal structures of the ThDP riboswitch from a bacterium (*Escherichia coli*, blue) and a plant (*Arabidopsis thaliana*, orange) showing the conservation of the fold and details of the environment in the vicinity of the ThDP (magenta and yellow stick format, respectively). (*c*) Geometric conservation of residues within ThDP-dependent enzymes. ThDP from pyruvate decarboxylase (PDC) (green, 1pyd), acetohydroxy acid synthase (AHAS) (cyan, 1z8n) and pyruvate dehydrogenase (PDH)-E1 (magenta, 1w85) were superimposed. ThDP, phosphate and C-2 proximal residue are shown in stick format. A solvent-accessible surface (gray) is shown using PDH-E1 to highlight the conserved pocket in which ThDP binds. Arrow indicates entrances to the active site. PDH-E1 and transketolase (TK) possess a phosphate proximal His. PDC has Ile in this position. The backbone of an active site loop takes this place in AHAS, but has been omitted for clarity. PDC, TK and PDH-E1 all possess thiazolium-proximal His, whereas AHAS, pyruvate oxidase (POX), N2-(2-carboxyethyl)arginine synthase (CEAS) have a Gln at this position. Pyruvate:-ferredoxin oxidoreducase (PFOR) has a thiazolium-proximal Arg.

function and mechanism of ThDP-dependent enzymes in the last 50 years. Excellent historical reviews of thiamine and a detailed examination of other current interests surrounding ThDP may be found elsewhere [8, 15]. In this report, we turn our attention to the large number of ThDP enzymes structures that have several unexplained properties, which are now beginning to be resolved both by kinetic and X-ray crystallographic approaches. We focus on the molecular mechanisms of ThDP-dependent enzymes from a structural standpoint, and explain how thiamine may be used in communication between active sites in oligomeric ThDP-dependent enzymes. Finally, we discuss the wider implications for communication of distant active sites in other classes of enzyme.

An ancient and conserved structure

If the ThDP-dependent enzymes are grouped according to reaction catalyzed, and the sequences compared within the respective groups, it is found that these proteins generally have limited amino acid sequence similarity (usually less than 20% identity). Notwithstanding the sparse conservation of the sequence, many of the tertiary folds of these enzymes are remarkably similar, as seen in more than 20 structures of thiamine-dependent enzymes, from eukaryotic, eubacterial and archeal sources, and encompassing many of the known catalytic processes performed by this cofactor. The greater conservation of folds than of sequences is not unusual or unexpected and may be regarded as a general property of proteins, particularly those that arose early in evolution. Thus, the structural homology of ThDP-dependent enzymes indicates that the ThDP-binding fold emerged, most likely, by divergent evolution from a common ances-

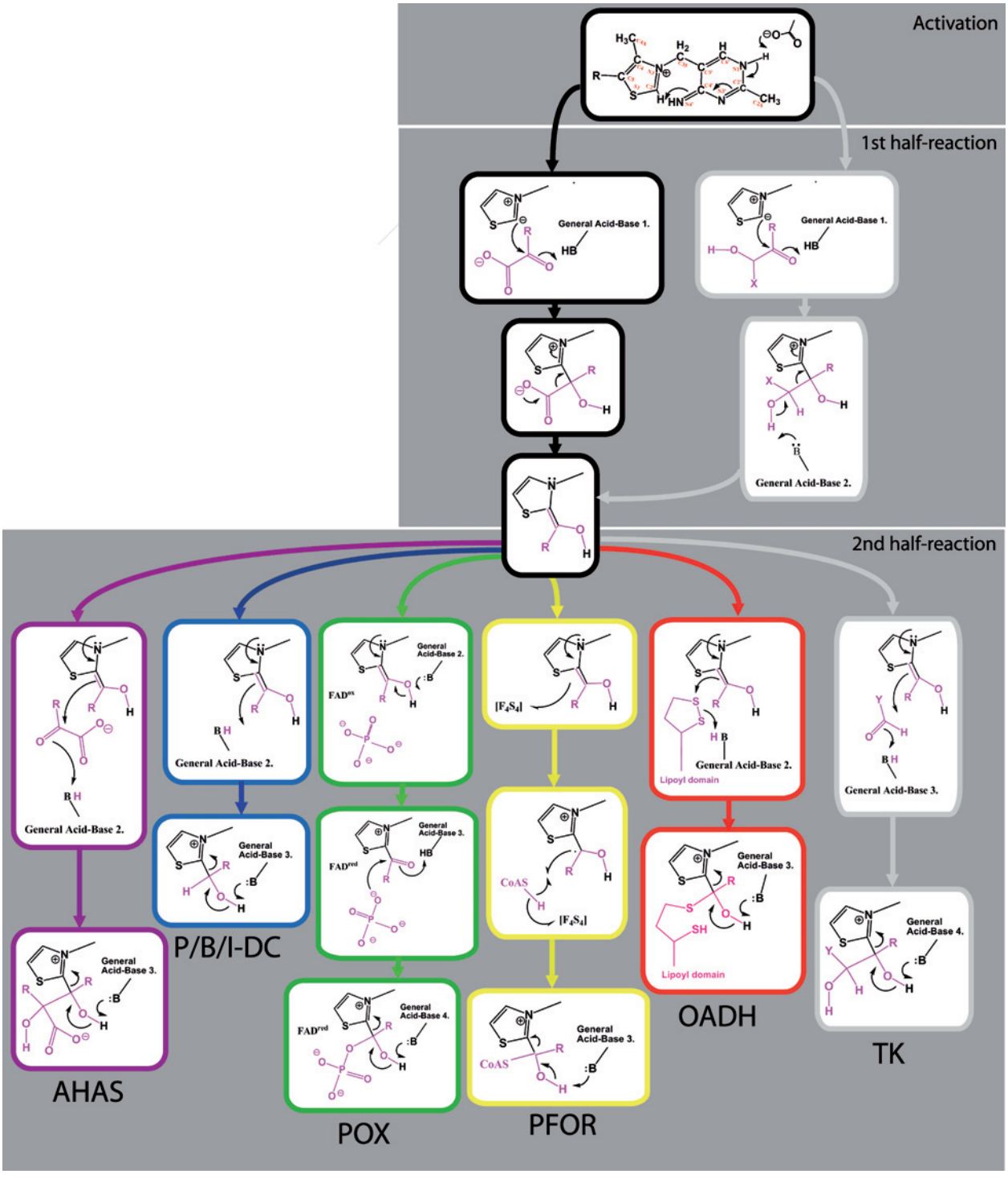

Figure 3. The catalytic mechanisms of ThDP-dependent decarboxylase and transferase enzymes. ThDP is activated by deprotonation forming an ylide on the ThDP C-2 atom. Substrate atoms are shown in magenta. The first half-reaction involves nucleophilic attack on a substrate carbonyl and general base catalysis. The second half-reaction is specialized for each category of ThDP-dependent enzyme: AHAS (EC 2.2.1.6), pyruvate/benzoylformate/indolepyruvate decarboxylase (P/B/I-DC; EC 4.1.1.1, EC 4.1.1.7, EC 4.1.1.74, respectively), POX (EC 1.2.3.3), PFOR (EC 1.2.7.1), 2-oxo acid dehydrogenase (OADH: EC 1.2.4.1, EC 1.2.4.2, or EC 1.2.4.4) and TK (EC 2.2.1.3). The catalytic mechanisms of a few ThDP-dependent enzymes (*e.g.*, carboxyethyl arginine synthase) have been omitted.

Table 1. Properties of thiamine diphosphate (ThDP)-dependent enzymes. Ionizable residues lining the acidic tunnel are listed for structures of each type of ThDP-dependent enzyme.

Enzyme	1st and 2nd substrate	1st and 2nd product	Acidic tunnel	Structural asymmetry	Comment
Pyruvate decarboxylase (PDC and other paralogous decarboxylases)	2-oxo acid and H^+	CO_2 and aldehyde	**1pyd, 1bfd, 1ovm**: 2 Glu; **1zbd**: 2 Glu, 2 Asp, 2 His	Substrate analogue (pyruvamide) bound in half of the active sites (1qpb) [36]	Penultimate step of anaerobic fermentation in the production of ethanol.
Acetohydroxyacid and acetolactate synthase (AHAS)	Pyruvate and 2-oxo acid	CO_2 and acetolactate or 2-aceto-2-hydroxy-butyrate	**1jsc, 1ozf, and 1n0h**: 2 Glu, 2 His	Herbicide bound in half of the active sites and order-disorder asymmetry of active site loops* (1noh).	First step of Leu, Ile and Val biosynthesis in plants and microorganisms.
Deoxyxylulose 5-phosphate synthase (DXPS)	Pyruvate and glyceraldehydes 3-phosphate	CO_2 and deoxyxylulose 5-phosphate	No crystal structure available	N/A	First step in non-mevalonate pathway to isoprenoids in bacteria and chloroplasts.
Pyruvate oxidase (POX)	Pyruvate and phosphate	CO_2 and acetyl phosphate	**1pox**: 4 Glu, 2 His and Na^+	No structural asymmetry found so far.	FAD is an electron acceptor and re-oxidized by O_2.
Pyruvate ferredoxin-oxidoreductase (PFOR and other oxidoreductases)	2-oxo acid and coenzyme-A	CO_2 acyl-CoA	**1bop**: 6 Glu and 2 Asp	Radical intermediate only present in half the active sites (1kek) [38]	Mechanism involves a radical intermediate that has been observed in a crystal structure and by EPR.
E1 subunit of pyruvate dehydrogenase (PDH-E1 and other paralogous enzymes, e.g. branched-chain 2-oxo acid and 2-oxoglutarate dehydrogenases)	2-oxo acid and oxidized lipoyl group attached to E2 subunit	CO_2 and reductively acylated lipoyl domain	**1w85**: 6 Glu, 2 Asp and Mg^{2+}; **1ni4 and 1um9**: 6 Glu, 2 Asp; **1la8**: 6 Glu, 2 Asp and 2 Arg; **1qs0 and 1dtw**: 4 Glu, 4 Asp	Order-disorder asymmetry* (1ni4 and 1w85) and substrate analogue binding (1umc) [28]	Overall, the PDH converts pyruvate to acetyl-CoA and reduces NAD.
Transketolase	Ketose and aldose	Aldose and ketose	**1trk and 1itz**: 6 Glu, 2 His; **1qgd**: 6 Glu	Order-disorder asymmetry of active site loops* (1trk) [17, 35]	Used in the pentose phosphate pathway and the Calvin cycle to interconvert sugars of different sizes.
N^2-(2-Carboxyethyl) arginine synthase (CEAS)	Glyceraldehyde 3-phosphate and arginine	H^+ and N^2-(2-carboxyethyl) arginine	**1upa**: 2 Glu, 2 Asp and 2 His	No structural asymmetry found so far.	In the biosynthesis of the β-lactam antibiotic clavulanic acid. Elimination of water and then phosphate from the enamine intermediate occurs before attack of arginine.

* Order-disorder asymmetry is implicated by temperature factors.

tor. In this context, the selective pressures on the evolution of ThDP-binding folds appears to have maintained the geometric position of the small number of residues that bind the ThDP, while tolerating many other substitutions elsewhere. Since several of the interactions between the ThDP diphosphate and the enzyme are mediated by the peptide backbone and not by side chains, it is not surprising that the tertiary structure of all ThDP-binding domains is conserved.

Well before the abundant structural data became available, the simple motif G-D-G-(X)-N was identified and successfully predicted to demark the ThDP binding site [16]. The cofactor is bound primarily through its diphosphate group, which coordinates a divalent cation (usually Mg^{2+}) and is situated at the switch-point of an α/β-type domain in the protein. The aminopyrimidine ring is held in a pocket lined with hydrophobic residues and in proximity to an invariant glutamate [17]. Although the thiazolium group is not pinioned by many direct contacts with the protein, it is positioned at the center of the active site through restraints placed on the adjoining diphosphate and aminopyrimidine ring.

Conserved aspects of the catalytic mechanism

The structural data explain how the protein environment of the enzyme activates the ThDP. One salient observation is that the overall conformation of ThDP is maintained so as to favor activation by positioning N-4′ of the aminopyrimidine group in close proximity to the C-2 of the thiazolium ring (Figs 1b and 2c) [17]. The proton from the C-2 is relayed via the positioned aminopyrimidine in its imino form (Fig. 1b) to the invariant glutamate, thus generating the reactive ylide (Fig. 1c). The dynamics of this process have been probed by NMR, which elegantly showed how activation manifests itself as an increase in the rate of proton exchange at C-2. Thus, the enzyme is shown to exert 'kinetic control' of ThDP activation [18].

Thiamine activation is not the only mechanistic property shared in this family of proteins. All ThDP enzymes catalyze two successive half-reactions, with the consequence that the reactions have two distinct kinetic processes. Such two-step enzyme mechanisms are referred to as "ping-pong". In the first reaction the substrate is cleaved, releasing the first product and leaving behind an enzyme-intermediate complex. This metastable intermediate is 'trapped' in the enzyme perhaps as the resonance state between the enamine and carbanion configurations [19]. The second half-reaction involves the nucleophilic attack of the enamine intermediate onto the incoming second substrate, resulting in ligation to generate the final product. Unless the ThDP enzyme also houses an Fe-S or flavin adenine dinucleotide (FAD) redox center (to which we will return in the subsection on radicals), the nucleophilic power of the enamine intermediate is directed solely at the second substrate. In some cases the second substrate is merely a proton, whereas in other cases it is a carbonyl-containing compound and a proton donor is required to neutralize the resultant negative charge on the new intermediate. Therefore, an ionizable residue is expected to be present in a position in which it may protonate either the enamine intermediate or the incoming second substrate during ligation in the second half-reaction.

Common to all ThDP-dependent enzymes is the need for a proton donor to initiate the first half-reaction and a proton acceptor to complete the second half-reaction (Fig. 3). This proton donor-acceptor is required in order that the reaction may pass via the metastable enamine intermediate. The wealth of crystal structures of thiamine-dependent enzymes suggests that the proton donor-acceptor for the first step is the aminopyrimidine N-4′ in concert with an invariant Glu (Figure 1) [17, 20, 21]. However, there are no residues that are universally conserved among the thiamine-dependent enzymes that could have the same role for the second half-reaction; such residues may differ according to enzyme class. Other active site residues are expected to play additional roles in catalysis, including helping to stabilize the enamine intermediate and the transition states along the reaction coordinate [22].

One of the most studied enzymes in this context is the pyruvate dehydrogenase E1 component (PDH-E1, EC 1.2.4.1), which initially decarboxylates pyruvate and then reductively transfers the enamine group to a lipoyl group, forming a product with a thioester bond. PDH-E1 is a paradigm of the reductive decarboxylase-type of ThDP enzyme and has been investigated by a combination of mutagenesis, kinetics and structure determination, revealing several residues that are essential for its catalytic function [23–25]. The active site of all PDH-E1 contains a thiamine flanked by two His residues (Fig. 2c) [26]. One histidine is hydrogen bonded to the diphosphate and is also within hydrogen-bonding distance of the S atom of the ThDP, whereas the second is partially buried in a hydrophobic pocket, near the C-2 carbon, and the 4′-amino group of the aminopyrimidine ring. We refer to these histidines as phosphate-proximal and thiazolium-proximal, respectively. Mutation of the phosphate-proximal His still allows decarboxylation in the first half-reaction, but disrupts the reductive acetylation in the second half-reaction. The selective disruption of the second half-reaction when this residue is mutated supports the view that the phosphate-proximal His is the proton donor for the second substrate, the lipoyl group [23]. A phosphate-proximal His is also found in the closely related paralogue TK, which is consistent with the requirement of a proton donor for both the TK aldehyde substrate and the PDH-E1 lipoyl substrate. Mutation of the thiazolium-proximal His significantly inhibits both decarboxylation and reductive acylation by PDH-E1 [23, 24]. Thus, the role of the thiazolium-proximal His, though clearly important, is harder to dissect since its removal causes 'blanket' inactivation. We have attempted to deduce a function for this residue by comparison with other ThDP enzymes.

When thiamine enzyme structures are superimposed using the cofactor as a reference point, the geometric conservation of residues within the active sites can be analyzed. One of the first features noticed in this overlay is that the active sites of different thiamine enzymes show a high degree of variation in shape and composition. This is not so surprising given the significant differences in substrate specificity, particularly in the second half-reaction, where the substrate may be as small as a proton, as in the case of pyruvate decarboxylase (PDC, EC 4.1.1.1), or as large as an entire protein domain, as seen in pyruvate dehydrogenase [27]. Interestingly, the site occupied by the

phosphate-proximal His is a potential proton donor in PDH-E1 and TK exclusively. Other 'decarboxylase'-type enzymes, such as PDC (Table 1) contain instead a non-polar residue at this position (Fig. 2c), and this likely is a reflection of where the second substrate, a proton, is required to complete the ligation.

The thiazolium-proximal histidine is conserved only in PDH-E1, TK and PDC. However, all other thiamine enzymes appear to contain a polar residue (either Arg or Gln) at this position (Fig. 2c). Moreover, several crystal structures are available for the complex between enzyme and the enamine intermediate, which suggests that the polar residue at the site corresponding to the thiazolium-proximal His may hydrogen bond to either the enamine intermediate or the transition state(s) of the reaction(s) [28]. It is likely therefore that the residue at this position is not a proton donor, but instead serves as an electrostatic catalyst in all ThDP enzymes. Corroborating this hypothesis, mutagenesis of the thiazolium-proximal His to Gln in *B. stearothermophilus* PDH-E1 had no effect on the decarboxylase activity of the enzyme, (unpublished work within our laboratory, Chris Titman, Xue Yuan Pei, René Frank and Ben Luisi).

We argue that ThDP-catalyzed reactions require the assistance of at least one ionizable group to function as a proton donor at the start of the reaction, and a proton acceptor at the end of the cycle. The question remains, what is the identity of the functional group that acts as the universal proton donor in the first half-reaction and proton acceptor in the second half-reaction? Given that all ThDP-dependent enzymes use the same partial reaction to arrive at an enamine intermediate, it may perhaps come as a surprise that no single residue is universally common to ThDP-dependent enzymes. It has been suggested that the ThDP itself provides the universal proton donor-acceptor [15]. This possibility appears even more likely since the discovery of ThDP enzymes that have no residues that are expected to be capable of donating or accepting a proton in the active site, leaving only the N4′ of the aminopyrimidine group as the sole possible source of a general acid-base [14].

Access to the active site

Some of the functions of thiamine enzymes require more than the proximity of specific residues in the active site, which is reflected in other notable structural differences between thiamine enzymes. Some ThDP-dependent enzymes [PDH-E1, pyruvate:ferredoxin oxidoreductase (PFOR, EC 1.2.7.1) and N2-(2-carboxyethyl)arginine synthase (CEAS, EC 2.5.1.66)] house the cofactor at the bottom of a long, narrow funnel-shaped cleft [14, 29]. The opening to the active site in these enzymes is therefore highly restricted. In contrast, the active site of other ThDP enzymes is relatively exposed to the bulk solvent [*e.g.*, TK, PDC, acetohydroxy acid synthase (AHAS), pyruvate oxidase (POX) EC 1.2.3.3] [30–33]. The difference in accessibility to the active site is determined by the size and conformation of several active site loops. For example, TK and PDH-E1 both share a very similar fold, yet in TK the active site loops are shorter, resulting in greater solvent exposure of the thiamine. It appears that size and conformation of the loops forming a lip around the entrance to the active site is correlated with the size of the second substrate, which in the case of PFOR, PDH-E1 and CEAS contain long aliphatic groups well suited to binding in a long narrow cleft (coenzyme A, lipoyl group, and arginine, respectively).

Many ThDP enzymes have acquired ancillary functions in the course of evolution. For example, PDH-E1 contains a recognition site that guides its assembly into a multienzyme complex. Although PFOR and TK are not known to assemble as part of multienzyme complexes, they too contain a similar domain for which a function has not yet been identified. Interestingly, the *Azotobacter vinelandii* PDH-E1 may be able to make specific interactions with a DNA promoter element, which has opened a fascinating potential avenue for the coordinated regulation of gene expression [34]. However, a domain or surface for this putative interaction has not been shown for this or any other PDH-E1.

Coupled active sites

Although ThDP-dependent enzymes are either tetramers or dimers, they all function as a 'dimer of active sites'. This is in keeping with our observation that most if not all types of enzymes that function by a ping-pong catalytic mechanism are composed of a dimer of active sites. However, over the last 30 years evidence has accumulated suggesting that the two active sites of each pair are neither equivalent nor independent. This property appears to be common among enzymes that operate by a ping-pong catalytic mechanism and is manifested as asymmetry in crystal structures and by distinctive kinetic properties (see below). The earliest structural evidence of non-equivalence was noted in yeast TK, where the active sites differ in relative disorder (implicated by temperature factors) [17, 35]. Asymmetry was also noted in the structure of yeast PDC bound with a substrate analogue, pyruvamide, which only binds in one of the two active sites from each 'catalytic dimer' [36, 37]. This is evidence of 'half-of-the-sites activity', in which only one active site at a time may engage the substrate. Non-equivalence was also described in the report of the PFOR structure in complex with a radical intermediate, which is only

present in one of the two active sites of the homodimer [38].

The *Thermus thermophilus* branched-chain 2-oxo acid dehydrogenase (E1) crystallized alone and in complex with substrate analogues show a similar pattern of asymmetry [28]. In each structure the homodimer is asymmetric with respect to the conformation of polypeptide loops within the active site and for the binding of substrate analogue. Our crystal structure of PDH-E1 from another thermophilic species, *Bacillus stearothermophilus* reveals a similar structural asymmetry of active site loops [26, 39]. This asymmetry may account for earlier observations from solution studies, which show that one set of loops is preferentially susceptible to protease [40].

The structures of several other members of the ThDP enzyme family, available in the protein databank, also reveal conformational non-equivalences, hitherto unreported (summarized in Table 1). Thus, structural asymmetry appears to be a widespread, though an unexplained, feature of many ThDP-dependent enzymes. These structural examples of non-equivalence can be correlated with kinetic data that implicates a communication between each pair of active sites. Early studies showed that thiamine enzymes possess unusual hysteretic kinetics of cofactor binding. The first ThDP binds rapidly, while the second active site takes up the cofactor orders of magnitude more slowly than the first [41]. More recently, Jordan and coworkers [42] made incisive discoveries showing that the extent of interactive site communication is not just an incidental part of cofactor binding but is fundamental to the overall mechanism of catalysis. Their kinetic and spectroscopic evidence suggests that the dimer of active sites in ThDP-dependent enzymes is out of phase with one another and contain intermediates at different stages of the catalytic cycle. This and other kinetic information suggests that these enzymes have an alternating sites mechanism [43].

Despite its widespread occurrence, the cause, mechanism, and most importantly, function of this asymmetry have not been understood until recently. How is it that the asymmetry is communicated from one protomer to the other? The order-disorder asymmetry within these structures cannot be easily attributed to allosteric conformational changes.

Recently, we turned our attention to the *B. stearothermophilus* PDH-E1 in our attempts to explain the structural non-equivalence found in ThDP-dependent enzymes. Although this PDH-E1 contains two active sites, one is ordered while the other is not both in the crystal structure [26] and in solution [40]. Our attention was drawn to the middle of the protein, which contains a long cavity filled with at least fourteen hydrogen-bonded water molecules (Fig. 4a). At each end of the cavity are the inward facing aminopyrimidine rings of both ThDP cofactors. The cavity spans ~20 Å from the closest atoms of each ThDP. Of particular note is the apparent enrichment of acidic residues lining this channel and hydrogen bonding to the water molecules. Four aspartates and six glutamates line the cavity, and only one Mg^{2+} ion is present to provide a positive charge to neutralize this concentration of acidic residues. Thus, the water molecules and acidic residues lining the cavity provide a network of hydrogen bonds that bridge the cofactors at each end of the tunnel.

Moreover, a similar water-filled cavity is found linking the cofactors in all the available structures of ThDP-dependent enzymes and thus appears to be an evolutionarily conserved feature of all ThDP-dependent enzymes. However, the acidic character of the channel found in E1 is not conserved and in some cases His residues are also prevalent (Table 1).

The function of this channel was probed by mutagenesis of several of the acidic residues lining the cavity in *B. stearothermophilus* PDH-E1. The catalytic activity of the enzyme was severely perturbed by these changes and together with other biochemical evidence a function was attributed to the channel as a 'proton wire' [26]. It is likely that the network of water molecules connecting the ThDP at each end is an integral part of the wire. A proton may thus be transduced by a Grotthus mechanism from one water molecule to the next [44, 45]. Unlike other proton translocators, such as the proton pump in cytochrome c oxidase, only one proton is transduced back and forth in the proton wire of ThDP enzymes, like the motion of a Newton's cradle. This mechanism is likely to be highly efficient since transfer of just a single proton does not require significant conformational rearrangement of molecules in the tunnel during each proton transfer event, although it remains to be investigated whether or not this is indeed kinetically barrierless. The preponderance of acidic residues plays an important part for the activity of the proton translocation in PDH-E1; however, the use of a proton wire has yet to be tested in other ThDP enzymes, particularly those with fewer acidic residues.

We were able to connect the conformational asymmetry of PDH-E1 with the operation of the proton wire. This was demonstrated by testing the conformation of the active site loops of the acidic channel mutant in solution, which showed that the conformation of the loops is determined by information transfer via the proton wire and the changing charge state of the ThDP cofactor [26]. The evidence for a molecular switch involving the activation of ThDP and a proton wire connecting the active sites can be used to propose a new mechanism for ThDP-dependent enzyme

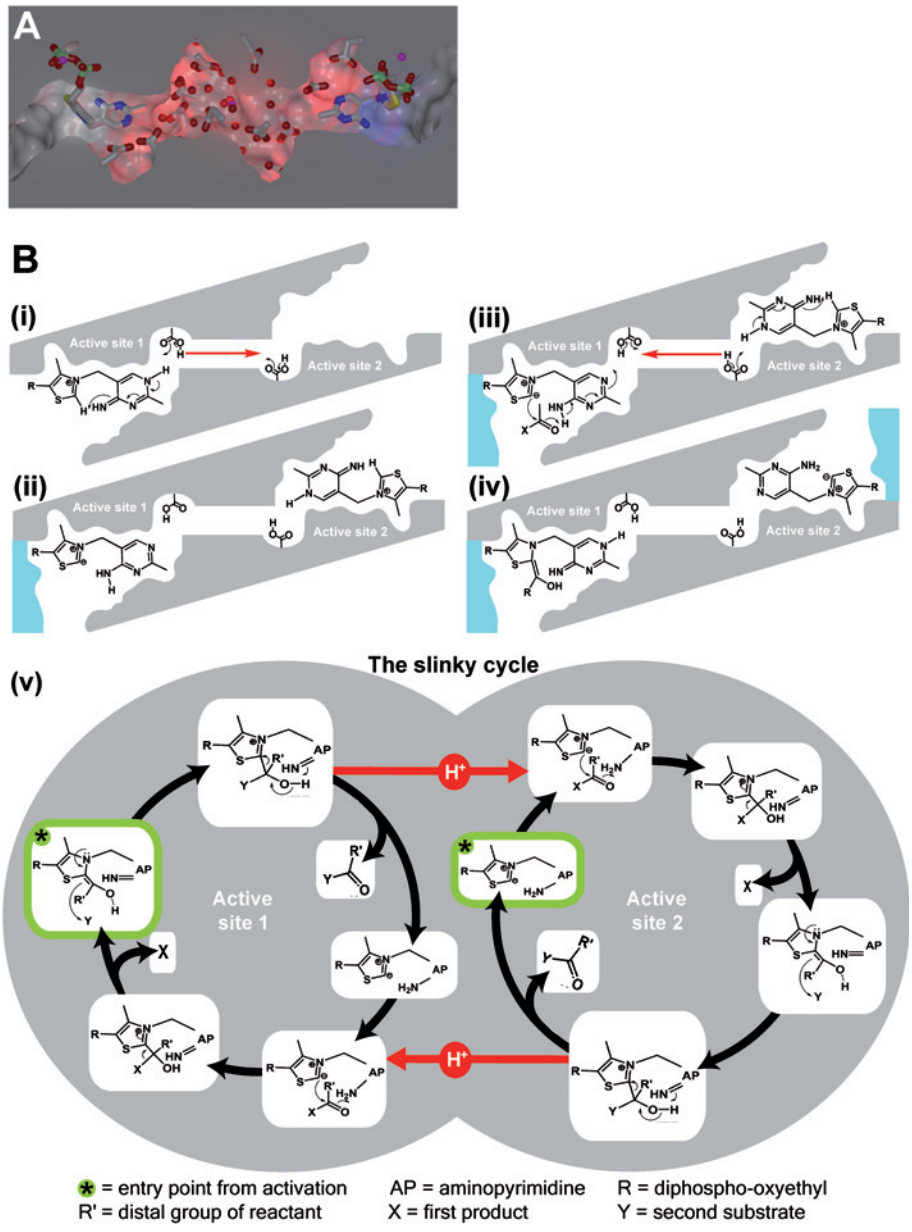

Figure 4. (*a*) The solvated, acidic tunnel from *Bacillus stearothermophilus* PDH-E1. Two ThDP cofactors, one in each active site, and the acidic residue (Asp or Glu) side-chains are shown in stick format. Entrained solvent molecules and a single Mg^{2+} ion are shown as red and magenta spheres, respectively. The protein is rendered with a solvent accessible surface and cut with a bounding plane to expose the interior cavity linking each active site. (*b*) A postulated universal mechanism for ThDP-dependent enzymes. (*i-iv*) ThDP binding and activation. (*v*) The slinky cycle. The final step in each cycle is not applicable to several ThDP-dependent enzymes such as PFOR, which employs a radical mechanism. However, the chemical necessity for a general acid at the last step of catalysis is common to all ThDP-dependent enzymes and so we speculate that PFOR also functions by a slinky catalytic mechanism.

activation. In this proposal the first ThDP binds to the active site and is immediately activated with the concomitant folding of loops around the cofactor, and the proton released in forming the carbanion is relayed via the aminopyrimidine ring, the invariant Glu and along the proton wire to the second apo-active site. Accordingly, when the second ThDP is bound, it remains in a dormant, protonated state because the first activated ThDP is already bound (Fig. 4b, i and ii). Activation of the second ThDP will drive a proton back along the proton wire, which causes the first site to become re-protonated and so the active site asymmetry is maintained. It is a formal possibility that in solution the active sites are in dynamic equilibrium, each exchanging between the activated and dormant states. A dynamic equilibrium could also be consistent with the kinetics of ThDP activation shown by NMR [18]. Once the holoenzyme has been formed, with both ThDPs bound in place, this will be the state of the enzyme *in vivo* before the catalytic cycle starts, as it is in the PDH-E1 crystal structure.

The model of active site communication through a 'molecular switch and proton wire' explains not only the basis of thiamine activation but also suggests a molecular means of generating an alternating, coupled action in the two catalytic sites of this ThDP-dependent enzyme (Fig. 4b). This molecular coupling of the active sites model is consistent with the kinetic model suggested by Jordan and co-workers [42] to

account for active site communication. After substrate-dependent activation of the cofactor (Fig. 4b, i–iv), the first site enters the catalytic cycle as the enamine intermediate (green box on left of Fig. 4b), whereas at the second site the ThDP is poised to start the reaction as an activated carbanion (highlighted in green on the right of Fig. 4b). On one side, the incoming second substrate reacts with the enamine intermediate, which requires a general acid. Concomitantly, the second site starts the catalytic cycle with the participation of a general base. Through the proton wire, both sites reciprocate their catalytic needs by long-range general acid-base catalysis and thus become synchronized in a mutually obligated cycle. This back and forth communication is metaphorically similar to the movement of a wave in the 'slinky' toy composed of a helical spring; for this reason we refer to it as the 'slinky cycle'.

Although the slinky cycle catalytic mechanism has yet to be shown to apply for all ThDP enzymes, it accounts for several previously perplexing properties found in many of them, such the hysteresis of cofactor binding, active site non-equivalence, the conserved solvated cavity linking the active sites, and the kinetic data suggesting an alternating sites mechanism. Important questions, however, remain regarding the limitations of such a mechanism. Can one active site function in the absence of substrate, while the other active site engages the substrate?

This question has been indirectly addressed by kinetic studies by the group of Jordan [46] using yeast PDC, a close homologue of the PDH-E1. During the PDC reaction cycle, the enamine intermediate is protonated to form the hydroxyethyl-ThDP intermediate, which is released to form acetaldehyde (Fig. 5). However, if an apo-PDC is soaked with hydroxybenzyl-ThDP (an analogue of the post-enamine intermediate) the enzyme partitions, with some molecules releasing the benzaldehyde product and some molecules reverting to the enamine intermediate [46]. The activity of the apo-PDC towards pyruvate is regained at the same rate as the formation of an enamine in the active site of PDC. This partitioning to form the enamine intermediate in addition to product is surprising since formation of the "enamine should be by all rules uphill!" [8]. Partitioning of the hydroxybenzyl-ThDP may be explained by the involvement of a molecular switch and proton wire connecting the active sites (Fig. 5). We thus propose that the apo-enzyme binds the first hydroxybenzyl-ThDP in an analogous fashion to ThDP activation; the enzyme catalyses general base catalysis forming the benzaldehyde product. The enzyme favors the completion of the reaction only in the first site because the coenzyme returns to a net neutral state, which allows the active site loops to stabilize in an ordered conformation. Subsequently, hydroxybenzyl-ThDP binds in the second active site, in which completion of the reaction is unfavorable because it would require reprotonation of ThDP in the first site and concomitant unfavorable unfolding of the active site loops. Instead, the usual reaction of PDC is reversed in this active site, expelling the second substrate, a proton, and forming the enamine intermediate. This may be favored by the enzyme because it neutralizes the ThDP thiazolium group, allowing the loops of the second site to fold also.

The reversion to the enamine intermediate illustrates two important limitations of the slinky cycle. First, it is not likely to be favorable to form product in the second site if it results in reprotonation of the distant ThDP. Consequentially, neither ThDP is able to function as long-range general acid-base in the absence of substrate. Furthermore, the inability of the hydroxybenzyl-ThDP to form product spontaneously in both the PDC active sites suggests that the enamine is more stable than the product, at least in the context of the active site. Thus, we speculate that, in the normal catalytic cycle, the formation of product from the enamine intermediate may be directly coupled to and driven by decarboxylation in the adjacent active site.

Coupling in other ping-pong enzymes

Many other ping-pong enzymes are also multimeric with at least two active sites and share the kinetic properties found in ThDP-dependent enzymes, including negative cooperativity of substrate binding, half-of-the-sites activity or hysteretic coenzyme activation. Mechanisms have been proposed for these processes that are conceptually similar to the proton-wire model proposed for ThDP-dependent enzymes. Although structural asymmetry has been discovered in some of these other ping-pong enzymes, communication is likely to take some other form than that found in PDH-E1 because the hydrated acidic cavity, a hallmark of ThDP-dependent enzymes, is not found in the structures of other ping-pong enzymes. Instead conformational changes between a pair of active sites may couple their catalytic activity.

For example, all pyridoxal-5-phosphate (PLP)-dependent enzymes are composed of one or more 'catalytic dimers' and use an invariant Lys residue to anchor the PLP coenzyme ([47] and references therein). Two members of this family of PLP-dependent enzymes, aspartate aminotransferase (A-AT, EC 2.6.1.1) and glutamate-1-semialdehyde aminomutase (GSA-AT, EC 5.4.3.8) [48] have been crystallized with non-equivalent active sites within each catalytic dimer. The GSA-AT asymmetry has been corrobo-

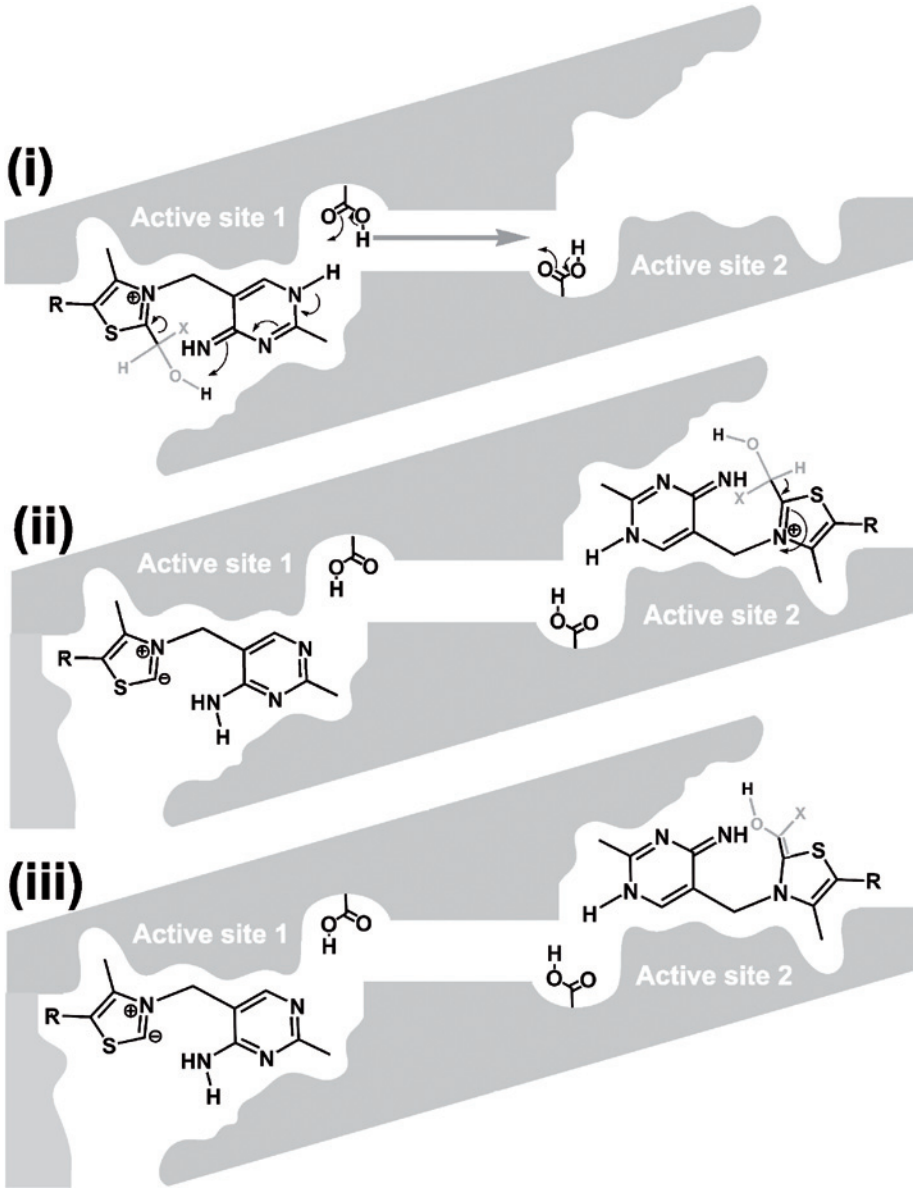

Figure 5. How the proton wire might help stabilize the enamine intermediate of thiamine-dependent enzymes in PDC treated with hydroxybenzyl-ThDP (X = phenyl, R = diphospho-oxyethyl). (*i*) Hydroxybenzyl-ThDP binds the first apo-active site. The reaction goes to completion in an analogous fashion to ThDP activation; benzaldehyde is released and the active site loops close around the activated ThDP. (*ii*) The second hydroxybenzyl-ThDP binds and is stabilized by reverting to the enamine intermediate; the PDC second substrate, a proton, is removed. (*iii*) The enamine intermediate is more stable because the ThDP thiazolium is neutral, which causes the second active site loops to fold. In the presence of substrate, both active sites can now re-enter the slinky cycle.

rated by solution studies. Analogous to PDH-E1, GSA-AT is asymmetric with respect to the conformation of an active site loop, and this is thought to help select substrate at the correct phase of ping-pong catalysis. Since the catalytic mechanism of the two half-reactions from most PLP-dependent enzymes are related by inversion, one Lys could be prepared to act as a general acid if the other active site Lys is acting as a general base. The free energy of the first stage of catalysis, which is favorable [49], can be transduced to raise the pK_a of the second active site and drive the second 'uphill' stage of catalysis. Active site lysines reciprocally obligated via conformational changes may thus accelerate the rate of PLP-dependent reactions.

Similarly, the enzymes that catalyze the ATP-dependent selective attachment of amino acids to their cognate tRNAs, acyl-tRNA synthetases, are also half-of-the-sites-activity, ping-pong enzymes whose catalytic centers are coupled ([50] and references therein). In a manner reminiscent of the asymmetry in PDH-E1, several acyl-tRNA synthetase crystal structures possess asymmetrically arranged active site loops in which one is folded while the other is disordered. Consequently, several authors have proposed an alternating sites mechanism, in which the active sites are coupled [51–53]

An elegant demonstration of the dependence of active site coupling was shown for the homodimeric enzyme thymidylate synthase (TS, EC 2.1.1.45). When an inactive TS mutant is expressed, a "thymineless" death ensues for the bacterial host, because the

mutant TS forms an inactive heterodimer with the cell's endogenous wild-type TS [54]. This dominant negative phenotype of the mutant implies an obligatory coupling of the active sites in the dimeric TS enzyme.

Active site coupling can in principle also occur in certain homo-oligomeric enzymes catalyzing chemical substitution by a 'concerted' mechanism that is not of the ping-pong type. For example, the negative cooperativity associated with the malic enzyme has been interpreted as 'catalysis-driven product release', whereby completion of the reaction is rapid but expulsion of the product in one active site is slow and dependent on conformational changes that occur as a result of catalysis in the second active site [55]. More recently, kinetic isotope effects on the malic enzyme have been interpreted as evidence for proton tunneling or active site coupling [56].

Thiamine, radicals and an ancient anoxic world

It is becoming increasingly appreciated that ThDP not only facilitates reactions through its induced polarity, but may also serve as a catalyst of radical-mediated reactions. In these cases, the ThDP enzymes also house additional redox cofactors required for the activity of the enzyme. An example is provided by PFOR, which proceeds through single electron transfer to the four-iron-four-sulfur coordination cluster(s) situated near the thiamine. Another example is POX, which contains the cofactor FAD in the vicinity of the ThDP. In both cases these cofactors provide redox centers to receive two electrons from the reductive decarboxylation catalyzed by ThDP. In both, an intermediate ThDP radical was either detected [57] or inferred [58] after decarboxylation of the substrate. It remains to be seen whether a radical mechanism exists for other members of the ThDP-dependent enzyme family, although such a mechanism is consistent with the apparent ancient origin of this cofactor. Thus, POX and PFOR are usually found in anaerobic micro-organisms, and it can be envisaged how the type of radical-mediated redox chemistry they use could have been more widespread in the early life forms before the appearance of an oxygen-rich biosphere, which might otherwise interfere with a radical intermediate [59].

We mentioned in the introduction that organisms from all three domains of life (archae, eubacteria and eukaryotes) use ThDP-sensing riboswitches to regulate genes related to thiamine synthesis or uptake. The crystal structure of a plant ThDP riboswitch shows how the ligand is sequestered in a defined pocket and is specifically recognized [5]. Virtually the same interaction is seen in the bacterial ThDP riboswitch (Fig. 2b) [60]. The recognition raises the possibility that the cofactor might have been incorporated into an RNA enzyme structure in the ancient past. Given our current understanding of the role of the protein in the activation of the cofactor, it is intriguing to ask whether the ThDP could be activated when bound to RNA and whether the riboswitches may be derived from such a primordial ribozyme. This, however, seems unlikely for the two available structures, since the C-2 has limited accessibility and the cofactor does not adopt the canonical V-conformation required for activation. However, it is not difficult to envisage an RNA fold that might provide an interior pocket, nonetheless accessible for potential substrates, in which substituents of the nucleic acid serve as general acid and general bases, or as metal chelating centers for radical chemistry.

Conclusions

Although the chemistry and catalytic mechanisms of ThDP-dependent and many other ping-pong, homodimeric enzymes may be disparate, they appear to be related by specific enzymatic properties both in solution and crystal structures, which includes half-of-the-site reactivity, negative cooperativity or conformational non-equivalence, especially of active site loops. This behavior is consistent with the notion of reciprocally coupled active sites, as exemplified in the 'slinky cycle' of ThDP-dependent enzymes, and may reflect a more general mechanism of traversing challenging chemical pathways and accelerating the rate of chemical transformations.

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Note added in proof: While this review was in proof a paper appeared which provides strong direct evidence of half-of-sites activity in PDH-E1 [Seifert, F., Golbik, R., Brauer, J., Lilie, H., Schröder-Tittmann, K., Hinze, E., Korotchkina, L. G., Patel, M. S. and Tittmann, K. (2006) Direct Kinetic Evidence for Half-Of-The-Sites Reactivity in the E1 Component of the Human Pyruvate Dehydrogenase Multienzyme Complex through Alternating Sites Cofactor Activation. Biochemistry, 45, 12775 – 12785].