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Mini review

X-ray crystallographic snapshots of reaction intermediates in pyruvate oxidase and transketolase illustrate common themes in thiamin catalysis

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ABSTRACT

Thiamin diphosphate (ThDP)-dependent enzymes play pivotal roles in intermediary metabolism of virtually all organisms. Although extensive mechanistic work on cofactor models and various enzymes has served as a guide to understand general principles of catalysis, high-resolution structural information of reaction intermediates along the catalytic pathway was scarcely available until recently. Here, we review cryocrystallographic studies on the prototypical ThDP enzymes pyruvate oxidase and transketolase, which provided exciting insights into the chemical nature and structural features of several key intermediates and into the stereochemical course of substrate processing. The structures revealed a conserved (*S*)-configuration at the C2alpha stereocenter of the initially formed tetrahedral intermediate in the different enzymes with the scissile C2alpha–C2beta bond being directed perpendicular to the aromatic ring plane of the thiazolium portion of ThDP confirming the proposed maximum overlap mechanism. Elimination of the respective leaving groups (carbon dioxide, sugar phosphates) appears to be driven – amongst other factors such as stereoelectronic control – by strain relief as the C2–C2alpha bond, which connects C2 of ThDP with the carbonyl of the substrate, substantially deviates from planarity and relaxes to an in-plane conformation only after bond fission to give an enamine-type intermediate with considerable delocalization of the free electron pair onto the thiazolium ring. Except for the apparent flexibility of the cofactor itself, no major structural rearrangements are detectable indicating that the enzyme active centers are poised for catalysis. The structures also provide the basis for understanding the origins of substrate and reaction specificity.

Even though it was discovered as early as 1937 that "cocarboxylase" [1], the essential cofactor of yeast pyruvate decarboxylase, is identical with ThDP as the biologically active derivative of vitamin B1, the first three-dimensional structure of a ThDP enzyme, transketolase (TK), became available only in the early 1990s by the pioneering studies of Lindqvist et al. at Karolinska [2]. This early achievement was subsequently followed by structural studies on pyruvate oxidase (POX) and pyruvate decarboxylase [3,4]. To date, 30-odd structures of different ThDP enzymes in the resting state could be solved, mostly by X-ray crystallography, and in a few instances as in case of 2-keto acid multienzyme complexes by electron microscopy at non-atomic resolution. Despite these enormous advances in elucidating the molecular architecture of the different enzymes and their active sites, which harbor the cognate ThDP cofactor in the canonical *V* conformation, there had remained a paucity of high-resolution structural data on reaction intermediates formed at the active centers in the course of catalysis. Initially, this lack of data was partly due to the fact that the catalytic sequences of ThDP enzymes mostly involve a multitude of related inherently unstable intermediates [5], so that co-crystallization of a ThDP enzyme with a chemically synthesized native intermediate would be an unsuccessful prospect. And although substrate/product soaking in combination with freeze-trapping of intermediates has become a routine tool in modern structure-based enzymology more recently, the structural analysis of a defined single on-pathway intermediate at high occupancy in ThDP enzymes is intricate due to the complexity of the reactions, the plurality of intermediates present at steady state and the marginal accumulation of some short-lived key intermediates [6,7].

In 1997, the first structure of a ThDP enzyme in a non-covalent complex with a substrate was reported [8]. After co-crystallization of TK with its donor substrate D-fructose 6-phosphate (F6P), aimed to visualize the tetrahedral F6P–ThDP or post-elimination adduct 2-(1,2-dihydroxyethyl)-ThDP (DHEThDP) carbanion/enamine, unambiguous electron density for the product D-erythrose 4-phosphate could be observed at the active center of

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TK as a result of enzyme-catalyzed decomposition of F6P. These studies foreshadowed that even those ThDP intermediates suspected to be thermodynamically stabilized at the active centers may undergo futile side reactions preventing a discrete covalent intermediate to be accumulated. A few years later Fontecilla-Camps and associates reported the X-ray structure of a radical ThDP intermediate trapped at the active center of pyruvate:ferredoxin oxidoreductase (PFOR) after soaking protein crystals with the substrate pyruvate and flash-cooling the crystal [9]. Noteworthy, PFOR converts pyruvate and CoA in an oxidative manner to acetyl-CoA and CO_2, and omitting CoA from the reaction or soaking mixture yields a stable AcThDP-type radical that accumulates to high occupancy both in solution and in the crystalline state thus constituting an adequate (yet technically demanding) system to structurally characterize a discrete intermediate [10].

Over the last 5 years, a variety of different approaches and techniques have been successfully developed to enable structural characterization of many in part unstable reaction intermediates trapped at the active centers of several ThDP enzymes. It is not the scope of this mini review to retrace the whole trail of research of that field but rather to focus on structural studies on TK and an acetyl phosphate-producing pyruvate oxidase as prototypical members of the ThDP enzyme family. For each enzyme conditions could be established, which resulted in the accumulation of key intermediates at high occupancy in the crystalline state.

1. Reaction pattern and intermediates of transketolase and pyruvate oxidase

Although transketolase and pyruvate oxidase act on different substrates and catalyze different transformations, the intermediate pattern and the underlying chemistry of some elementary reactions are similar [5,11,12] (Fig. 1).

Transketolase utilizes ThDP and Ca^{2+} as cofactors and catalyzes the reversible transfer of a 2-carbon dihydroxyethyl fragment from a donor ketose (D-xylulose 5-phosphate, X5P; D-fructose 6-phosphate, F6P; D-sedoheptulose 7-phosphate, S7P) to the C1 position of an acceptor aldose (D-glyceraldehyde 3-phosphate, G3P; D-erythrose 4-phosphate, E4P; D-ribose 5-phosphate, R5P) [13]:

xylulose 5-phosphate + ribose 5-phosphate

⇄ sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate

xylulose 5-phosphate + erythrose 4-phosphate

⇄ fructose 6-phosphate + glyceraldehydes 3-phosphate

Transketolase acts in tandem with the Schiff-base forming transaldolase as key enzymes in the pentose phosphate pathway of cellular carbohydrate metabolism.

Pyruvate oxidase from *Lactobacillus plantarum* converts pyruvate, inorganic phosphate and oxygen to the high-energy metabolite acetyl phosphate, carbon dioxide and hydrogen peroxide [14]. As opposed to the equilibrium reaction catalyzed by TK, the oxidative decarboxylation of pyruvate by POX is essentially irreversible [15]:

pyruvate + phosphate + oxygen

→ acetyl phosphate + carbon dioxide + hydrogen peroxide

Besides ThDP and Mg^{2+}, POX contains FAD as an additional cofactor, whose mechanistic role is to oxidize ThDP-bound pyruvate after decarboxylation permitting a chemical coupling of oxidation–reduction and acyl transfer to phosphate thereby generating the energy-rich acetyl phosphate acid anhydride linkage.

The oxidative decarboxylation of 2-keto acids such as pyruvate by ThDP enzymes is a central reaction in intermediary metabolism with the 2-keto acid dehydrogenase multienzyme complexes [16] and PFOR [10] as the physiologically most important members of this enzyme family.

The reaction sequences of TK and POX (Fig. 1) commence with the carbonyl addition of the 2-keto substrate (TK: donor ketose; POX: pyruvate) to the C2 carbanion of ThDP yielding a covalent substrate–ThDP adduct I_1 (TK: e.g., X5P–ThDP; POX: 2-lactyl-ThDP) followed by elimination of either an aldose (TK) or CO_2 (POX) to give the central carbanion/enamine intermediate I_2 (TK: DHEThDP; POX: 2-(1-hydroxyethyl)-ThDP, HEThDP). All catalytic steps encompassing substrate addition and subsequent product elimination are analogous reactions in TK and POX. Reaction sequences diverge at the carbanion/enamine intermediate stage. In TK, the carbanion/enamine adds to the C1 aldo function of an acceptor aldose to give a tetrahedral adduct, whereas it formally undergoes two-electron oxidation by FAD in POX yielding 2-acetyl-ThDP (AcThDP). Please note that TK-catalyzed elimination from the initial donor–ThDP adduct is a reversible reaction, whereas decarboxylation of 2-lactyl-ThDP (LThDP) proceeds quasi-irreversibly.

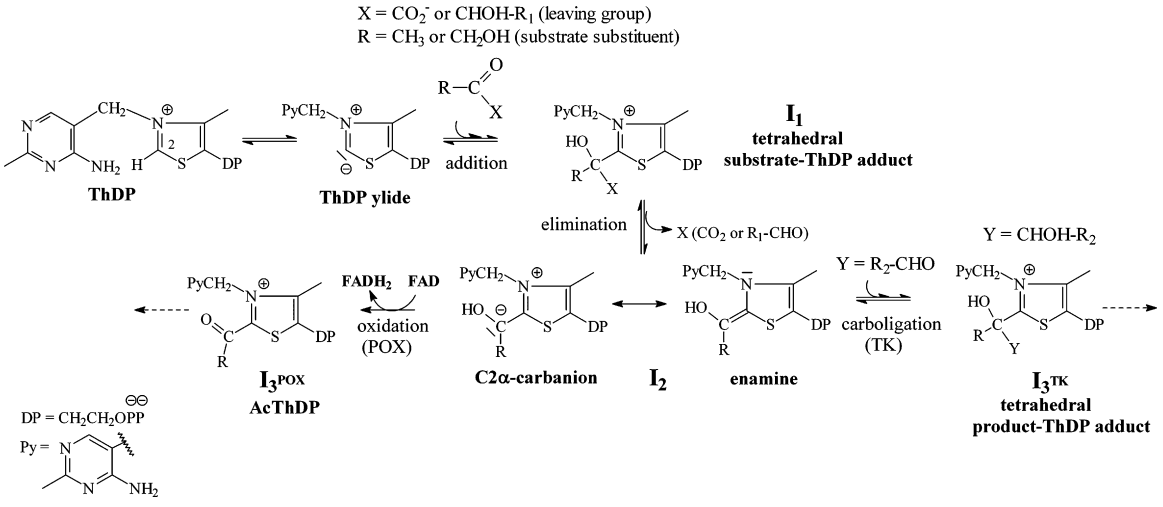

Fig. 1. Reaction sequences and involved intermediates of POX and TK.

2. General strategies for the observation of reaction intermediates by crystallography

There are different approaches to visualize a single intermediate species using protein crystallography [17]. On the one hand, the reaction can be synchronized throughout the entire crystal. On the other hand, reaction conditions can be adjusted such that one discrete intermediate accumulates to high occupancy. While the former approach works only for a few systems due to eventual loss of an initially established synchronization, the latter technique has enabled the structural characterization of many intermediates in enzymatic transformations. The most commonly used technique is 'freeze-trapping' that is to flash-cool a protein crystal after soaking with substrate or product at temperatures, at which discrete intermediates are readily formed. Data collection may then occur at temperatures as low as 100 K, where collective protein motions are frozen out and no further processing of intermediates will take place in the crystal. This technique is often combined with the utilization of enzyme variants with substitutions of functional groups required for discrete catalytic steps and thus leading to the accumulation of an intermediate, the further processing of which would require catalysis by the replaced functional group. Also, substrate analogs, which may shift the equilibrium position of a reaction or undergo processing until a stable intermediate or intermediate analog is being formed, have found widespread application.

When enzymes act on multiple substrates in a ping-pong-type mechanism such as TK, omission of one substrate from the reaction mixture will stop turnover halfway through the catalytic cycle and the enzyme will settle to equilibrium. No such straightforward method is available for enzymatic systems involving unimolecular reactions. Yet, in a large number of ThDP-dependent enzymes, elimination reactions of covalent substrate–ThDP adducts are unimolecular, and in the common case where this reaction is a decarboxylation, this step is also virtually irreversible. Hence, the structural visualization of these intermediates is not as easily achieved as of those, which are thermodynamically stabilized on the enzyme under equilibrium conditions.

3. Conditions for the accumulation of covalent intermediates in TK

3.1. The tetrahedral sugar–ThDP intermediates X5P–ThDP and F6P–ThDP

NMR-based intermediate studies on *Escherichia coli* transketolase (*Ec*TK) in solution revealed that enzymatic processing of the donor substrate in the absence of an acceptor aldose resulted in the discrete accumulation of the initial X5P–ThDP adduct (I_1 in Fig. 1) under equilibrium conditions with negligible amounts of the carbanion/enamine intermediate (I_2) [18]. Substrate soaking of *Ec*TK crystals with X5P at ambient temperatures, which allowed the equilibrium of the donor half-reaction to be established and subsequent flash-cooling of the crystal also resulted in the accumulation of the X5P–ThDP adduct to high occupancy in the crystalline state [18]. The same observation holds true for freeze-trapping the F6P–ThDP adduct (I_3 in Fig. 1) after soaking TK crystals with the ketose substrate F6P, the enzymatic reaction product of X5P and E4P conversion [18].

3.2. The DHEThDP carbanion/enamine intermediate

As discussed in the previous section, the equilibrium position of the donor half-reaction of TK disfavors an accumulation of the DHEThDP carbanion/enamine intermediate to any significant extent when using native substrates such as X5P or F6P [6,18]. As opposed to this strong stabilization of donor–ThDP adducts on the enzyme, the donor substrate analog β-hydroxypyruvate ($CH_2OH–CO–CO_2^-$) is quasi-irreversibly converted to CO_2 and enzyme-bound DHEThDP carbanion/enamine intermediate at high occupancy with a life-time sufficient for freeze-trapping and structural characterization using cryocrystallography [19].

4. Conditions for the accumulation of covalent intermediates in POX

4.1. The pre-decarboxylation intermediate 2-lactyl-ThDP

The initially formed LThDP adduct (I_1 in Fig. 1) is a key intermediate in all ThDP enzymes that act on pyruvate and serves as a prototype for thiamin-based pre-decarboxylation intermediates [5]. Quantitative analysis of the intermediate distribution of POX and of many other pyruvate-processing ThDP enzymes by NMR spectroscopy revealed LThDP to be extremely short-lived and marginally accumulated at steady state rendering a structural characterization of LThDP under steady-state turnover or single turnover conditions almost impossible [6,15,20,21]. However, through a rigorous kinetic and spectroscopic examination of POX mutant enzymes, active site variant Phe479Trp was serendipitously found in which decarboxylation of LThDP is rate limiting for the overall reaction as evidenced by the almost exclusive accumulation of LThDP in the active sites (occupancy >90%) under steady-state conditions. On account of this accumulation under turnover conditions, LThDP could be trapped in the POX variant at steady state by flash-cooling the crystal after a short soak with high pyruvate concentrations [22].

4.2. The HEThDP carbanion/enamine intermediate

The catalytic cycle of POX can be specifically blocked at the HEThDP carbanion/enamine stage when POX with a fully reduced flavin is reacted with pyruvate under anaerobic conditions [6]. Under these conditions, the redox reaction between the carbanion/enamine and the already two-electron reduced flavin is impeded, and catalysis at the thiamin site stops before electron transfer that is at the carbanion/enamine intermediate stage (I_2 in Fig. 1). Practically, POX crystals can be soaked with pyruvate in mother liquor, until the dissolved dioxygen is quantitatively depleted due to enzymatic turnover. When quasi-anaerobic conditions are established, the flavin will become fully reduced (evident by bleaching of the bright yellow crystal) as it cannot be reoxidized by O_2. The ThDP cofactor works independently of the FAD redox state and is still capable to bind and decarboxylate pyruvate, hence HEThDP will be readily formed [22].

5. The structures of the tetrahedral X5P–ThDP and LThDP intermediates reveal a conserved stereochemical course and strain in the adducts

The high-resolution X-ray crystallographic snapshots of X5P–ThDP bound to *Ec*TK and of LThDP trapped at the active center of *Lp*POX variant Phe479Trp (shown in Fig. 2) suggest a common substrate binding mode in ThDP enzymes. Both covalent substrate–ThDP conjugates adopt a similar conformation at the active centers and exhibit similar structural features [18,22].

The absolute configuration and spatial orientation of the three substituents at the C2alpha stereocenter of enzyme-bound X5P–ThDP and LThDP are identical as both adducts are being formed as the (*S*)-stereoisomer. This implies that the C2 carbanion, when considered as a point nucleophile, attacks the substrate carbonyl from the *re* side in the preceding carbonyl addition.

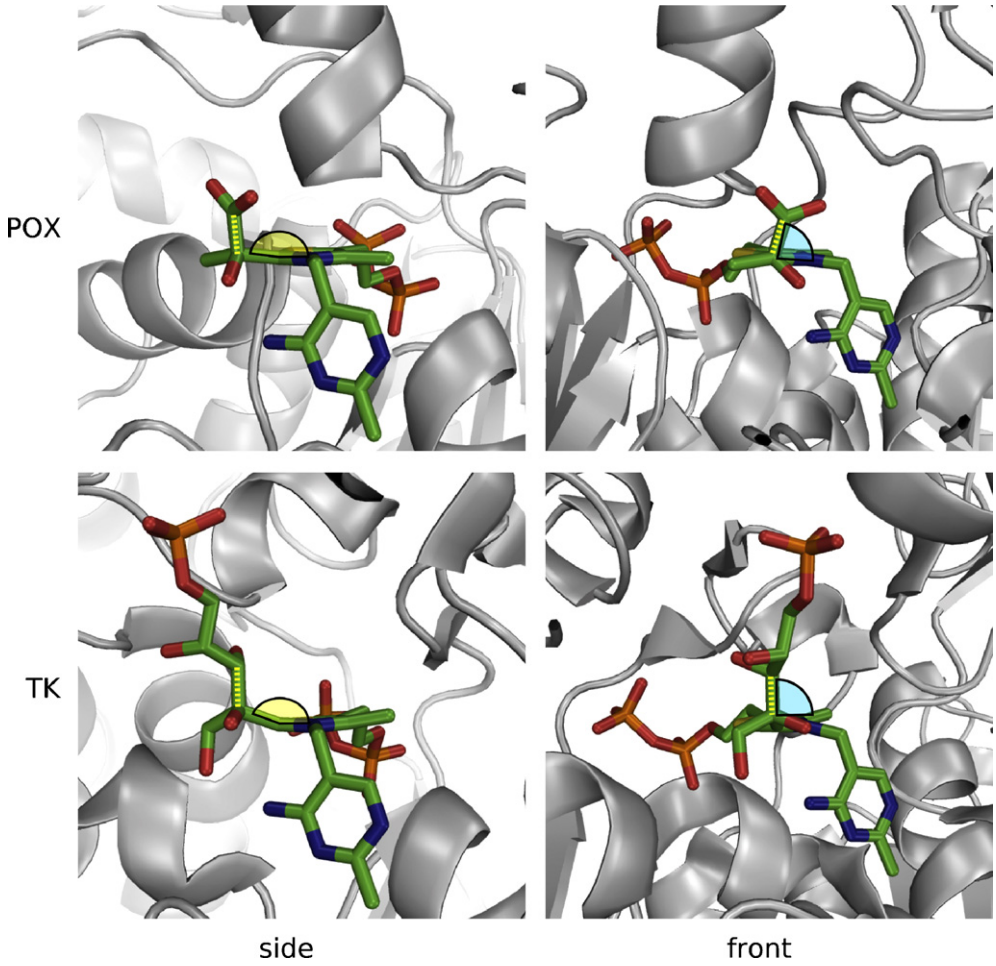

Fig. 2. The X-ray structures of the initial tetrahedral reaction intermediates LThDP in the active site of POX from *Lactobacillus plantarum* (top row) and X5P–ThDP bound to TK from *Escherichia coli* (bottom row). The cofactor adducts are shown as sticks and the proteins in cartoon representation. The out-of-plane distortion of the C2–C2alpha bond is indicated by the yellow angles in the side view. The scissile C2alpha–C2beta bond is marked with yellow dots, and the almost perpendicular angle that this bond forms with the thiazolium ring plane is highlighted by the light blue angle in the front view.

In both enzymes, the respective leaving groups (sugar phosphate in X5P–ThDP, carboxylate in LThDP) are positioned above and at the same side of the thiazolium ring with the scissile C2alpha–C2beta bond being directed perpendicular to the aromatic thiazolium ring plane. This observation supports the predicted maximum overlap mechanism and stereoelectronic control of elimination [23] because a perpendicular orientation of the leaving group allows the positively charged thiazolium to function as an optimal electron sink for the incipient electron pair at C2alpha through facile conjugation with the thiazolium π-electrons. Furthermore, the corresponding leaving groups point outwards the active site such that diffusion of the reaction products via the funnel-shaped substrate channel can occur without steric hindrance after bond fission and product release.

The substrate carbonyl-derived C2alpha–OH moieties of X5P–ThDP and LThDP are bound in hydrogen-bonding distance (<3 Å) to the exocyclic amino/imino group of the cofactor pyrimidine supporting the proposed catalytic role of the aminopyrimidine as an intramolecular acid–base catalyst required for substrate binding (protonation of the substrate carbonyl) and product elimination (deprotonation of the C2alpha–OH of the adducts) [6,24–26].

In both adducts, the C2alpha atom is not coplanar with the thiazolium but rather substantially shifted above the aromatic ring plane. As a consequence, there is a significant out-of-plane distortion of the C2–C2alpha bond that connects the sp^2-hybridized C2 of ThDP with the sp^3-hybridized C2alpha, the former substrate carbonyl. In X5P–ThDP trapped at the active center of *Ec*TK, the torsion angle between the C2–C2alpha bond and the thiazolium ring plane (C5–S1–C2–C2alpha) was estimated to be ∼25–30°, because the high resolution (1.47 Å) and occupancy of all adduct atoms justified a relaxation of the normally applied in-plane restraints during crystallographic refinement [18]. The resolution of the POX–LThDP complex (1.97 Å) and thus the corresponding coordinate error were not as good as in case of TK, but even so, with the enforced restraint, the optimized position of C2alpha was ∼5–7° out-of-plane [22]. When refinement of the same *Lp*POX dataset was repeated with fully relaxed restraints similar to *Ec*TK, the C5–S1–C2–C2alpha torsion angle of LThDP increased up to 25° (Wille and Tittmann, unpublished). The experimentally observed out-of-plane distortion implies considerable strain in the tetrahedral adducts. A deviation from planarity of the C2–C2alpha bond was also reported for the pre-decarboxylation intermediate analog phosphono-LThDP bound to the E1 component of *E. coli* pyruvate dehydrogenase complex [27] and bound to *Lp*POX [22].

The driving force for formation of the strained, high-energy substrate–ThDP adducts appears to originate from multiple factors. At first, addition of the C2 carbanion to the substrate carbonyl is considered to be a thermodynamically favorable reaction with a substantial gain in enthalpy [12]. Further, if the C2–C2alpha bond of the adducts was to be coplanar with the thiazolium ring, the interatomic distance between C2alpha–OH and the 4′-amino/imino function of the pyrimidine would be less than 2.4 Å and thus enforce

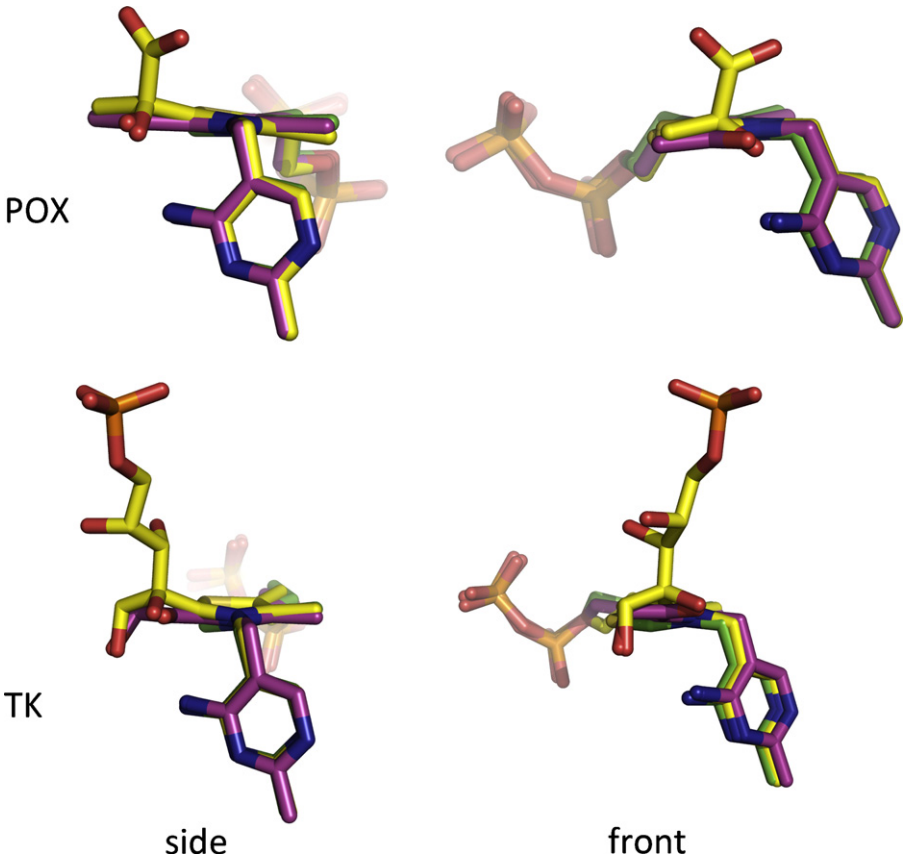

Fig. 3. Superposition of the resting state (in green), the tetrahedral substrate–ThDP adducts (yellow) and the post-elimination enamine intermediates (pink). Top row: ThDP, LThDP and HEThDP enamine all trapped at the active site of POX from *Lactobacillus plantarum*. Bottom row: ThDP, X5P–ThDP and DHEThDP all trapped at the active site of TK from *Escherichia coli*.

a strong repulsive interaction. There is also evidence in case of TK that productive interactions of protein active site side chains with the substrate leaving group moiety may help to restrain the substrate from moving deeper into the active site and binding closer to the cofactor [13,18]. Density functional theory analysis on simplified X5P–thiazolium models suggests that the thiazolium portion itself might also contribute to an out-of-plane distortion: after full optimization of the isolated X5P–thiazolium adduct (without the aminopyrimidine of ThDP and protein) still resulted in a pronounced deviation from planarity by 9° [18].

6. Strain relief as a driving force for product elimination

Structural analysis of the carbanion/enamine intermediates in TK and POX by cryocrystallography revealed the C2α atoms of the DHETHDP and HEThDP adducts to be sp^2-hybridized suggesting an enamine-type species in (*E*)-configuration with considerable delocalization of the electron pair formed upon elimination onto the thiazolium ring (Fig. 3) [19,22]. Noteworthy, the C2alpha atom in both intermediates is slightly out of the aromatic thiazolium ring plane. This distortion is more pronounced in the DHEThDP enamine bound to TK (torsion angle C5–S1–C2–C2alpha ∼10°) compared to HEThDP enamine trapped in POX (∼2°). Apparently, both enzymes are capable of forming these intermediates in strained structures, thus avoiding the "thermodynamic trap" that awaits every enzyme that generates strong bonds (e.g., C–C bonds) that must later be broken. In the present case, the built-in strain allows formation of the intermediates while keeping at hand the driving force to assure their later decomposition. The predominant stabilization of an enamine-type structure relegates a localized carbanion to minor status in the active site environment of the two enzymes and thus provides a rationale for the slow off-pathway protonation of C2alpha: a localized C2alpha carbanion as proposed to be on the pathway in ThDP-dependent decarboxylases would certainly be an extremely strong base and accept protons from any available proximal proton source.

A superposition of the resting state (green in Fig. 3), of the tetrahedral substrate–ThDP adduct (yellow) and of the enamine (pink) illustrates that angular strain is imposed on the initially formed adduct, which becomes partially relieved upon C2alpha–C2beta bond fission and formation of the enamine. Also, the structures reveal the cofactor to exhibit some flexibility: the thiazolium portion of the X5P–ThDP adduct is displaced by about 1 Å when compared to the resting state. The discrete energetic contributions of the observed angular strain for product elimination and the molecular origins of the apparent cofactor flexibility remain to be studied by theoretical analysis.

7. Structural basis for substrate specificity in POX and TK

Both LThDP in POX and X5P–ThDP in TK form multiple interactions with protein side chains and the aminopyrimidine part of the cofactors in terms of a three-center binding motif (substituent pocket, carbonyl/C2alpha–OH pocket and leaving group pocket) (Figs. 4 and 5).

The substrate-derived methyl substituent of LThDP in POX is bound in a very hydrophobic pocket constituted by the side chains Val394, Phe121, and Trp479 and the dimethylbenzene part of the FAD isoalloxazine, which points towards the ThDP thiazolium (Fig. 4). As detailed in an accompanying article of this special issue (Chipman et al., Origin of the Specificities of Acetohydroxyacid Synthases and Glyoxylate Carboligase) the Val residue in question is

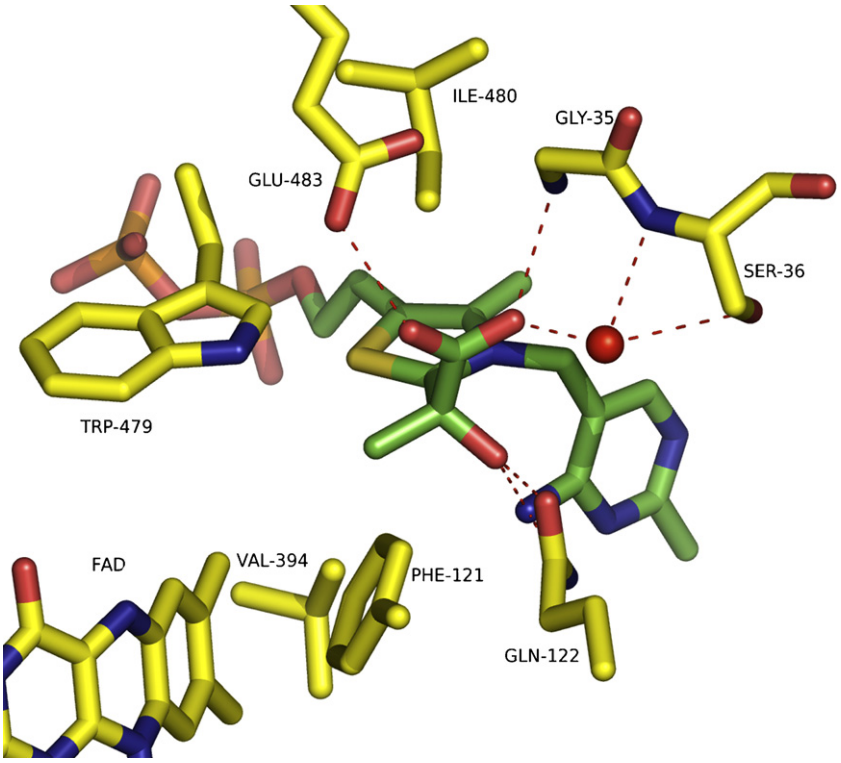

Fig. 4. Structure of LThDP at the active center of POX. The cofactor adduct and selected amino acids are shown in stick representation.

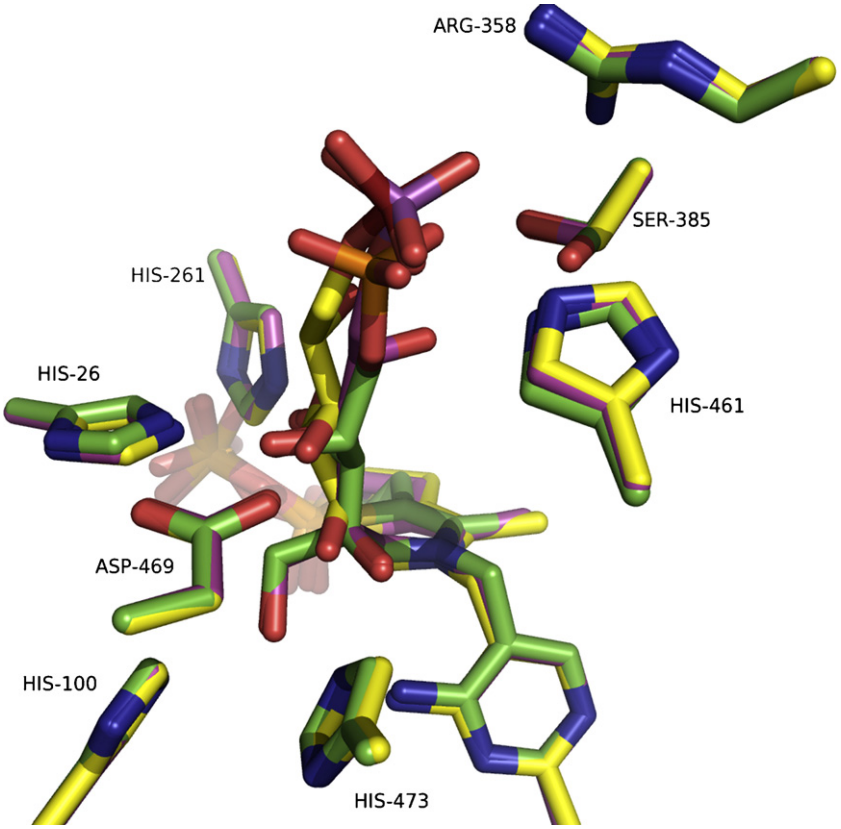

Fig. 5. Superposition of the active site of TK with the bound X5P–ThDP (green) and F6P–ThDP (pink) adducts and the non-covalently bound acceptor substrate R5P (yellow) in the linear form. The substrate–ThDP adducts X5P–ThDP and F6P–ThDP, acceptor R5P and selected amino acid residues are shown in stick representation. For clarity, the substrate-derived phosphorus atoms are colored differently.

conserved in many ThDP enzymes that act on pyruvate and its replacement ultimately leads to altered substrate specificities thus identifying this residue as a key determinant for specific recognition/binding of the methyl substitutent of pyruvate.

In TK, which converts sugar substrates with a hydroxymethyl substituent, two invariant His side chains (His100 and His473 in *Ec*TK) are perfectly positioned to develop a hydrogen-bonding interaction with the 1-OH group of the X5P–ThDP and F6P–ThDP adducts (Fig. 5). Mutagenesis studies on TK variants with exchanges of these two His residues confirm the proposed functional role for donor substrate recognition and alignment [28]. The structures of *Ec*TK in complex with X5P–ThDP and F6P–ThDP further suggest why TK readily turns over the commonly deployed artificial donor substrate β-hydroxypyruvate, whereas it fails to process the central metabolite pyruvate. Apparently, the selective recognition of the 1-OH group of the hydroxymethyl substituent by the two His residues is key to substrate discrimination.

The molecular architecture of the carbonyl/C2alpha–OH pocket in the two enzymes is very similar. The C2alpha–OH groups of LThDP and X5P–ThDP are held in place by interactions with the 4′-imino/amino group of the cofactor aminopyrimidine and either a Gln (POX) or His (TK) residue. Sequence comparison of TK from different species indicated that the His residues is not invariant and may be replaced by Gln as in the human enzyme thus qualifying this side chain to act as a hydrogen-bond acceptor rather than an acid–base catalyst [29].

As expected, the leaving group pockets of POX and TK greatly differ from each other. In POX, the carboxylate moiety of LThDP forms hydrogen-bonds with an acidic side chain (Glu483) and the backbone amides of Gly35 and Ser36 (Fig. 4). The X5P–ThDP and F6P–ThDP adducts bound to TK develop numerous hydrogen-bonding and electrostatic interactions with several His, an Asp, a Ser and Arg side chains (Fig. 5). In line with earlier mechanistic and crystallographic studies on TK from yeast, the Asp residue confers enantioselectivity of acceptor binding, whereas the Arg residues are clearly involved in binding the phosphate moiety of the substrates [30]. The close proximity of His261 and His26 to 3-OH of the donor–ThDP intermediates suggests a catalytic role of these side chains for deprotonation of this hydroxyl group as part of acceptor addition/elimination [28]. Remarkably, the catalytic His residues appear to be structurally conserved in many ThDP enzymes catalyzing a carboligation reaction, for which electrophilic activation of a weak base is mandatory [31]. In POX, acyl transfer to phosphate takes place in a complex radical mechanism presumably involving Glu483 and the cofactor itself as general acid/base catalysts.

A further remarkable feature of TK is its substrate 'promiscuity' that is its ability to act on sugar substrates of different chain length ranging from 3-carbon to 7-carbon skeletons. The superposition of the X5P–ThDP and F6P–ThDP structures and of the Michaelis complex with acceptor R5P all trapped at the active site of *Ec*TK indicates (Fig. 5) that the phosphate binding site of TK is perfectly arranged to ensure that the reactive 2-keto and 1-aldo groups of the donor and acceptor (termed the 'business ends' according to [13]) may easily reach either C2 of ThDP or C2alpha of the DHEThDP enamine. Although the spatial position of the phosphate is slightly different for X5P–ThDP on hand and F6P–ThDP and R5P on the other, there are no evident structural differences of interacting side chains.

8. Conclusions

The X-ray crystallographic studies on several reaction intermediates in POX and TK have provided exciting insights into the mode of action of the two enzymes and serve as a structural basis for conceiving general principles of enzymic thiamin catalysis. Carbonyl addition of the corresponding substrates to enzyme-bound ThDP yields a substrate–ThDP adduct with a conserved (*S*)-configuration at the C2alpha stereocenter. Elimination of the leaving groups is facilitated by a perpendicular orientation of the scissile C2alpha–C2beta bond to the thiazolium ring plane in terms of stereoelectronic control. In all tetrahedral substrate–ThDP adducts structurally characterized so far, a substantial deviation from planarity of the C2–C2alpha bond is observed. This angular strain is partially relieved after product elimination and formation of an enamine-type post-elimination intermediate. Enforced by the adopted *V* conformation of the cofactor and three-center substrate binding motif, the exocyclic 4′-amino/imino function of ThDP is perfectly positioned to act as an acid–base catalyst for substrate binding (protonation of O-carbonyl) and product release (deprotonation of C2alpha–OH). Substrate specificity is conferred by tailor-made substituent pockets, in which the different substrate substituents (POX: methyl group of pyruvate; TK: hydroxymethyl group of donor sugars) are accommodated.

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