Molecular Mechanism of Allosteric Substrate Activation in a Thiamine Diphosphate-dependent Decarboxylase

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Thiamine diphosphate-dependent enzymes are involved in a wide variety of metabolic pathways. The molecular mechanism behind active site communication and substrate activation, observed in some of these enzymes, has since long been an area of debate. Here, we report the crystal structures of a phenylpyruvate decarboxylase in complex with its substrates and a covalent reaction intermediate analogue. These structures reveal the regulatory site and unveil the mechanism of allosteric substrate activation. This signal transduction relies on quaternary structural reorganizations, domain rotations, and a pathway of local conformational changes that are relayed from the regulatory site to the active site. The current findings thus uncover the molecular mechanism by which the binding of a substrate in the regulatory site is linked to the mounting of the catalytic machinery in the active site in this thiamine diphosphate-dependent enzyme.

Thiamine diphosphate (ThDP), the biologically active form of vitamin B1, is an essential cofactor for a wide variety of enzymes that mainly mediate the making and breaking of carbon-carbon bonds adjacent to a carbonyl group (Fig. 1) (1). Although ThDP-dependent enzymes are textbook examples of cofactor-aided catalysis, the fine details of their catalytic mechanism are still lacking (2). Moreover, two types of intramolecular signaling have been observed in ThDP-dependent enzymes. Whereas substrate activation results from communication between an allosteric regulatory site and the active site, the communication among active sites leads to alternating site reactivity (3–5). The molecular mechanism of substrate activation in ThDP-dependent enzymes has been an area of intensive research and debate ever since its first observation in 1967 (6).

Phenylpyruvate decarboxylase (PPDC) catalyzes the ThDP-mediated non-oxidative decarboxylation of phenyl- and indolepyruvate to phenyl- or indoleacetaldehyde and carbon dioxide (7). In the root-associated bacterium Azospirillum brasilense, AbPPDC catalyzes the second step in the conversion of phenylalanine and tryptophan into phenyl- and indole-acetic acid, respectively (8, 9). The latter compound is a plant hormone, which is responsible for the plant growth-promoting abilities of A. brasilense (10). AbPPDC shows high structural similarity to other 2-ketoacid decarboxylases from the pyruvate oxidase family (11), such as pyruvate decarboxylase (PDC) and indolepyruvate decarboxylase (IPDC) (12). Similar to several PDCs, the AbPPDC displays substrate activation with indolepyruvate and other substrates, characterized by sigmoidal vs versus [S] plots (7).

Here we present a series of crystal structures of the ThDP-dependent phenylpyruvate decarboxylase in complex with different substrates and an analogue of a covalent reaction intermediate. These provide new snapshots along the reaction coordinate and unveil the regulatory site and a detailed molecular mechanism for allosteric substrate activation.

EXPERIMENTAL PROCEDURES

Protein Expression, Purification, and Preparation of the Complexes—The wild-type AbPPDC was cloned, expressed, and purified as described previously (7, 12). During purification the enzyme activity was monitored with phenylpyruvate as substrate using the established coupled optical test with horse liver alcohol dehydrogenase and NADH (13).

Complexes of PPDC with the inhibitors 3-deaza-ThDP (PPDC-3dThDP) and 2-(1-hydroxyethyl)-3-deaza-ThDP (PPDC-2HE3dThDP) were subsequently obtained by incubating PPDC (purified without addition of ThDP) with 1 mM 3dThDP or 2HE3dThDP for 48 h at 4 °C. Complete exchange of ThDP with the analogues was confirmed by total loss of activity with phenylpyruvate as a substrate. The tertiary complexes with phenylpyruvic acid (PPDC-3dThDP-PPA) and 5-phenyl-2-oxovaleric acid (PPDC-3dThDP-POX) were then obtained.
by incubating the PPDC-3dThDP complex with 100 mM phenylpyruvate and 5 mM 5-phenyl-2-oxovaleric acid, respectively.

Crystallization and Data Collection—The PPDC-2HE3dThDP complex was crystallized by the hanging drop vapor diffusion method. Equal volumes of protein solution and precipitant containing 15% polyethylene glycol 4000 (w/v), 10% glycerol (v/v) in 100 mM Hepes buffer, pH 7.0, were mixed and equilibrated at 293 K. The crystals were transferred to a cryo-solution containing 20% polyethylene glycol 4000 (w/v), 25% glycerol (v/v) in 100 mM Hepes, pH 7.0, and transferred immediately to the cryostream. X-ray diffraction data were collected at 100 K to a resolution of 1.85 Å on beamline X11 (EMBL, DESY, Hamburg) using an x-ray wavelength of 0.8157 Å.

The PPDC-3dThDP, PPDC-3dThDP-PPA, and PPDC-3dThDP-POVA complexes were crystallized at 293 K using the hanging drop vapor diffusion method with 11% (w/v) polyethylene glycol 3350 and 0.2M di-ammonium tartrate, pH 6.5, as precipitant solution. Crystals were transferred to a cryo-solution containing 16% (w/v) polyethylene glycol 3350, 0.2 M di-ammonium tartrate, pH 6.5, 30% glycerol (with addition of PPA and POVA in case of the ternary complexes), and transferred immediately to the cryostream. X-ray diffraction data were collected at 100 K to a resolution of 1.85 Å on beamline X11 (EMBL, DESY, Hamburg) using an x-ray wavelength of 0.8157 Å.

Structure Determination and Refinement—Initial phases for all complexes were obtained by molecular replacement with the program PHASER (16) using a monomer of PPDC-ThDP (Protein Data Bank 2NXW) as a search model. The solutions were subjected to the simulated annealing procedure as implemented in CNS, and manual model building and inspection of electron density was performed in COOT (17). After several cycles of positional and temperature factor refinement using CNS combined with manual corrections, solvent molecules, cofactors, and alternative conformations were included in the models. Structure refinement was considered complete after crystallographic R-factor and free R-factor had converged, and the difference density was without interpretable features. The final models were checked with the Molprobity web server (18). Refinement statistics are summarized in Table 1. The structural superpositions were performed using the DALI server (19) and/or the program LSQMAN (20). Figures were prepared with PyMOL (21) and Molscript (22).

RESULTS

Substrate and Covalent Intermediate Complexes of PPDC—A standard procedure to obtain detailed structural information on the catalytic mechanism of an enzyme is to solve the structure of slow mutants with trapped substrates or covalent reaction intermediates. In a different approach we used unreactive analogues of the coenzyme to trap intermediates in a thiamine-dependent enzyme. 3dThDP is such an unreactive analogue of ThDP, in which the single nitrogen atom of the thiazolium ring is replaced by a carbon (23). Although an excellent steric mimic of the cofactor, 3dThDP electrostatically more closely resembles the overall neutral ylide form of thiamine, due to the absence of the positive charge on position 3. AbPPDC in which the naturally occurring ThDP is replaced by 3dThDP proves indeed to be enzymatically inert.
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The crystal structure of PPDC in complex with 3dThDP (PPDC-3dThDP) was solved to 3.2-Å resolution. This structure is nearly perfectly superimposable on the structure of the native PPDC holo-enzyme (PPDC-ThDP) that we previously solved, showing that replacement of ThDP with 3dThDP causes no structural rearrangements (12) (see supplemental materials for all structural details). Because PPDC-3dThDP is enzymatically inert we were able to trap the substrates PPA and POVA in its active site and to solve the structures of these ternary complexes to 2.15-Å (PPDC-3dThDP-PPA) and 1.9-Å (PPDC-3dThDP-POVA) resolution, respectively. Chemical synthesis also allows 3dThDP to be substituted on the C2 to obtain stable analogues of covalent reaction intermediates in the reaction cycle (24). Using such an analogue we solved the structure of PPDC in complex with 2-(1-hydroxyethyl)-3-deaza-ThDP (PPDC-2HE3dThDP) to 1.85-Å resolution to study the structure of the last covalent intermediate on the reaction coordinate (Fig. 1, intermediate 5).

All structures show clear electron density with full occupancy for all ligands bound to both active sites of the homodimer in the asymmetric unit (Fig. 2). In the PPDC-2HE3dThDP structure (Fig. 2b) the density for the 1-hydroxyethyl moiety of the intermediate analogue corresponds to a mixture of the R and S enantiomer at the Ca atom, consistent with the fact that a racemic mixture was used in the co-crystallization. Both enantiomers were modeled with half-occupancy. The overall structure of the active site is very similar in PPDC-3dThDP and PPDC-2HE3dThDP compared with the native holo-enzyme (Fig. 2, a and b). These three structures all exhibit an open active site with the active site loop spanning residues 104–120, completely disordered (see Versées et al. (12) for a detailed description of the PPDC-ThDP active site). In PPDC-3dThDP-PPA and PPDC-3dThDP-POVA, the binding of the substrates is accompanied by a complete structuring of the 104–120 loop. Upon substrate binding, this long loop folds over the active site bringing His112 and His113 into the active site pocket (Fig. 2, c and d). Concomitantly, a second active site loop spanning residues 280–294 of the neighboring subunit of the tight dimer is reorganized, wedging residues Asp282 and Thr283 deeper into the active site pocket. This structural transition causes a H-bond to be formed between His112 of the first loop and Asp282 of the second loop. The reorganization of the 280–294 loop also permits new interactions with the C-terminal helix (e.g. between the side chain of Gln336 and Arg214 and the main chain carbonyls of Asp282 and Ala287 and Ser288, respectively) allowing this helix to bend over the active site.

A Separate, Regulatory Substrate-binding Site—Apart from the substrate molecules bound in the active sites, the PPDC-3dThDP-PPA and PPDC-3dThDP-POVA structures unambiguously reveal a second site of bound substrate in each subunit. This site is located at a distance of 18 Å from the active site, at the interface of the PYR, R, and PP domains of each subunit. The substrate molecules in this second site are tightly bound by residues coming from the three domains (Fig. 3). In both structures, the carboxyl group of the substrate forms charged H-bonds with the Arg60 and Arg215 (bidentate) side chains and an additional H-bond to the main chain amide of Ala397. A third arginine residue, Arg214, is involved in a cation-π stacking interaction with the phenyl moiety of the substrate. The 2-keto oxygen of the substrate forms an H-bond with a protein-bound water molecule. Remarkably, this carbonyl oxygen makes very close (unfavorable) contacts with the main chain carbonyls of Met238 (2.9 Å), Arg240 (2.8 Å), and Leu395 (3.0 Å).

Tertiary Structure Rearrangements upon Substrate Binding—The asymmetric units of all PPDC structures contain a homodimer. In this dimer, each subunit adopts the archetypical pyruvate oxidase (POX) fold consisting of the PYR, R, and PP domains (25). The secondary complexes PPDC-ThDP, PPDC-3dThDP, and PPDC-2HE3dThDP are very similar in subunit architecture (see supplemental Table 1S for a full list of root mean square deviations upon superposition). Upon substrate binding in the active and regulatory sites, a change in this subunit architecture involving a domain rotation is observed in both ternary complexes (PPDC-3dThDP-PPA and PPDC-3dThDP-POVA). When the PYR and PP domains of PPDC-ThDP and either of the substrate complexes are superimposed, the R domains differ by a rotation of about 12° (Fig. 4). This domain rotation is accompanied by a large rearrangement of active site loop 280–294 of the R domain and the concomitant ordering of active site loop 104–120 of the PYR domain of the neighboring subunit causing the complete closure of the active sites in PPDC-3dThDP-PPA and PPDC-3dThDP-POVA.

Quaternary Structure Rearrangements upon Substrate Binding—PPDC adopts a homotetrameric assembly in solution (7). In all the crystal structures, the two tight dimers constituting the biological tetramers are related through a 2-fold crystallographic symmetry axis (7, 12). However, large differences exist between the tetramer architecture of the binary complexes (PPDC-ThDP, PPDC-3dThDP, and PPDC-2HE3dThDP) and the ternary complexes with substrates bound at the active and the regulatory sites (PPDC-3dThDP-PPA and PPDC-3dThDP-POVA). In the binary complexes, the non-perpendicular arrangement of non-crystallographic axes relating the monomers in the asymmetric unit and the crystallographic axis relating the two dimers results in an asymmetrical tetramer assembly, best described as an asymmetrical dimer of dimers (Fig. 5a). For the ternary complexes the non-crystallographic symmetry axes relating the two subunits in the dimer intersect with the crystallographic axis at an angle of 90°, resulting in a dimer of dimers with pseudo 222 symmetry (Fig. 5b). In going from the asymmetrical dimer of dimers observed for the binary complexes to the symmetrical dimer of dimers of the ternary complexes, one dimer has to be rotated by about 34°, vis à vis to the second dimer. This also has implications for the dimer-dimer interfaces. Whereas the AC and BD interfaces are different in the asymmetrical tetramers (see Fig. 5, for subunit nomenclature) these interfaces are the same for the symmetrical tetramers. A Cascade of Conformational Changes Links the Regulatory Substrate-binding Site to the Active Site—Comparison of the binary complexes (PPDC-ThDP, PPDC-3dThDP, and PPDC-
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2HE3dThDP) with the ternary complexes (PPDC-3dThDP-PPA and PPDC-3dThDP-POVA) uncovers a series of coupled local conformational changes running from the regulatory substrate-binding site to the active site (see Fig. 6). Binding of a substrate molecule in the regulatory site causes a change in side chain conformation of Arg214 leading to the formation of a cation-π interaction with the phenyl group of the substrate. The void left by the arginine side chain gets occupied by the side chain of Leu242. This rearrangement of Leu242, together with the steric repulsion caused by the binding of the 2-ketoacid moiety of the substrate and the loss of the H-bond between Arg214 and the main chain carbonyl of Arg240, induces a relocation of the entire region between Phe237 and Pro247. The changes in the Phe237–Pro247 segment lead to further structural rearrangements in two distinct branches both leading to the active site. In a first branch, changes at Met238 force the Tyr400 side chain to flip around with a concomitant displacement of its hydroxyl group by 9.8 Å. This conformational change in its turn forces a relocation of Leu109 of the neighboring subunit and the associated structuring of the entire 104–120 loop, bringing the two residues His112 and His113 into the active site. The second branch of the cascade is initiated by the steric clash of Phe237 with Phe285 located on the 280–294 active site loop. This causes a rearrangement of this entire loop, the most obvious change being a 13-Å displacement of the Phe285 side chain. Reorganization of the 280–294 loop (branch 2) liberates the necessary space for the 104–120 loop (branch 1) to enter the active site. Asp282 is also shifted into the active site in this process (a shift of 5 Å) and now forms a H-bond with His112. This cascade of transmitted conformational changes clearly provides a link between substrate binding in the regulatory substrate-binding site and structural changes in the active site.

Intermediate 1

Intermediate 2

Intermediate 3

Intermediate 4A

Intermediate 4B

Intermediate 5

Intermediate 6

R' = phenylpyruvate

indolepyruvate
we present the crystal structures of two other reaction intermediates. The use of the non-reactive cofactor analogue 3-deaza-ThDP allowed to solve structures in complex with the genuine substrates phenylpyruvic acid and 5-phenyl-2-oxovaleric acid to high resolution, providing images of the enzyme-substrate Michaelis complex for this class of enzymes (intermediate 2, see Fig. 1). Moreover, the structure of PPDC in complex with 2-(1-hydroxyethyl)-3-deaza-ThDP provides a structural model of the last tetrahedral 2-(1-hydroxyethyl)-ThDP intermediate on the reaction cycle (intermediate 5, see Fig. 1). Following our results, snapshots of five of six intermediates along the reaction cycle are now available, albeit from different but related ThDP-dependent enzymes (intermediates 1–5 on Fig. 1).

Assuming that the geometry of the intermediates is conserved among ThDP enzymes, educated modeling of the 2-(3-phenylactyl)-ThDP and 1-hydroxy-2-phenylethylamine intermediates in the active site of PPDC enables us to follow the decarboxylation reaction of phenylpyruvate step by step in a single active site (Fig. 7).

It is well established that the first step in the reaction cycle of the ThDP-dependent decarboxylases involves activation of the cofactor by abstracting the proton of the C2 of the thiazolium ring (29). Recent studies have shown that a conserved H-bond between a glutamate (Glu48 in PPDC) and the N1 amino/imino group in its imino form to act as a general base in the deprotonation of C2 (Fig. 7a).

Upon binding of the substrates PPA or POVA, the active site loops 104–120 and 280–294 and the C-terminal helix fold over the active site (Fig. 7b). These structures show the carbonyl group in its imino form to act as a general base in the deprotonation of C2 (Fig. 7a).

**DISCUSSION**

**Snapshots along the Catalytic Cycle of a ThDP-dependent Decarboxylase**—Multiple studies have been reported dealing with the complex kinetics of ThDP-dependent enzymes. However, a full interpretation of this wealth of data has always been hampered by a lack of structures visualizing the relevant reaction intermediates. Only recently structures of some of these intermediates have been solved: the lactyl-ThDP intermediate in the active site of pyruvate oxidase (intermediate 3, see Fig. 1); the planar enamine intermediate in the active site of pyruvate oxidase and transketolase (intermediate 4B, see Fig. 1) and the non-planar, more carbanion-like form of the latter intermediate in the active site of branched-chain 2-ketoacid dehydrogenase (intermediate 4A, see Fig. 1) (26–28). In the current paper we present the crystal structures of two other reaction intermediates. The use of the non-reactive cofactor analogue 3-deaza-ThDP allowed to solve structures in complex with the genuine substrates phenylpyruvic acid and 5-phenyl-2-oxovaleric acid to high resolution, providing images of the enzyme-substrate Michaelis complex for this class of enzymes (intermediate 2, see Fig. 1). Moreover, the structure of PPDC in complex with 2-(1-hydroxyethyl)-3-deaza-ThDP provides a structural model of the last tetrahedral 2-(1-hydroxyethyl)-ThDP intermediate on the reaction cycle (intermediate 5, see Fig. 1). Following our results, snapshots of five of six intermediates along the reaction cycle are now available, albeit from different but related ThDP-dependent enzymes (intermediates 1–5 on Fig. 1). Assuming that the geometry of the intermediates is conserved among ThDP enzymes, educated modeling of the 2-(3-phenylactyl)-ThDP and 1-hydroxy-2-phenylethylamine intermediates in the active site of PPDC enables us to follow the decarboxylation reaction of phenylpyruvate step by step in a single active site (Fig. 7).

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The next step in catalysis, the carbon-carbon bond formation between the C2 of ThDP and the carbonyl carbon of the substrate, requires a sterically oriented interaction of the substrate and a protonation of its carbonyl oxygen. Modeling of the 2-(3-phenylacyl)-ThDP intermediate in the active site of PPDC (starting from the structure of 2-lactyl-ThDP bound to the active site of Lactobacillus plantarum POX (26)) shows the C2α hydroxyl group within interaction distance to His113 and the N4′ cofactor, now in its acidic amino tautomer form (Fig. 7c).

Carbon-carbon bond cleavage in the substrate with release of CO₂ gives the following covalent α-carbanion/enamine intermediate, which was modeled as the enamine in the active site of PPDC (starting from the structure of the enamine bound to the active site of L. plantarum POX (26); Fig. 7d). Protonation of this intermediate at Ca will yield the last tetrahedral intermediate, 2-(1-hydroxy-2-phenylethyl)-ThDP. The protonation of this sp² hybridized enamine could in principle occur from either side (si or re face) of the prochiral center, thus determining the stereochemistry of the next intermediate. The structure of PPDC-2HE3dThDP does not resolve this issue because it has both enantiomers of the 2-hydroxyethyl moiety bound in its active site. This is probably a consequence of the presence of a small, non-physiological methyl side chain on 2HE3ThDP. Indeed, modeling of a phenyl group onto the β-methyl of 2HE3dThDP shows that only the R enantiomer of 2-(1-hydroxy-2-phenylethyl)-ThDP can be accommodated in the active site without inducing significant steric clashes with the protein (Fig. 7e). This provides indirect but strong evidence that addition of the proton to the Ca of the enamine occurs from the si face of the pro-chiral center, excluding the 4′-amino/imino group as the catalytic acid in this step. In PDC and IPDC a Glu-Asp-His catalytic triad has been implicated in protonation of the enamine (Glu468-Asp29-His115 in Enterobacter cloacae IPDC numbering) (35). However, in PPDC the glutamate residue of this triad is replaced by a leucine (Leu462) (12). In PPDC, Asp25 (equivalent to Asp29 in IPDC) is part of an Asp25-His112-Asp282 triad and could fulfill the role of the pro-chiral center, excluding the 4′-amino/imino group as the catalytic acid in this step. Alternatively the active site water molecule could fulfill the role of proton donor. This water molecule is located at 4 Å from the Ca of the product 2-(1-hydroxy-2-phenylethyl)-ThDP. A similar role has been proposed for the corresponding water molecule in oxalyl-CoA decarboxylase (33). In this scenario the Asp25-His112-Asp282 triad could be used to activate the water molecule by shuttling a proton into the active site. The latter proposal comes with a caveat, because activation of the water molecule requires an intact catalytic triad and hence fully structured active site loops. However, one would expect the active site loops to be open if CO₂ has diffused out from the active site. Activation of the water molecule in the active site by the Asp-His-Asp triad thus requires the decarboxylation and protonation of the carbanion/enamine to occur quasi simultaneously as recently proposed by Kluger (37). From this it would also follow that the carbanion/enamine intermediate maintains significant carbanion character, with tetrahedral geometry, throughout this process.
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In the last step of the reaction cycle the Co-hydroxyl group of 2-(1-hydroxy-2-phenylethyl)-ThDP is deprotonated with concomitant cleavage of the C2-Co covalent bond and release of the second product phenylacetaldehyde. The PPDC-2HE3dThDP structure shows the hydroxyl group within 2.5 Å from the N4’ imino group (Fig. 7e), confirming the current view that this group acts as a proton acceptor in the product release step (34).

In conclusion, these structures corroborate the central role of the N4’ amino/imino group of the cofactor in three of the four proton transfer steps of ThDP-catalyzed decarboxylation reactions (30, 31, 34). These pivotal protonation/deprotonation steps are promoted by a first proton relay involving the aminopyrimidine ring and Glu48. Protonation of the enamine/carbocation intermediate is mediated by a second Asp25-His112-Asp282 proton relay, either direct through Asp25 or via a tightly bound water molecule.

Allosteric Substrate Activation in PPDC—Nearly all pyruvate decarboxylases studied to date (with the exception of some bacterial PDC’s like the Z. mobilis PDC) are subjected to substrate activation, characterized by sigmoidal $v/\text{[S]}$ plots and time-dependent slow activation resulting in a marked lag phase in product formation. Ever since the first observation of homotropic allosteric activation in the PDC from wheat germ in 1967 (6), the elucidation of the molecular mechanism behind this phenomenon has been an active area of research. The currently accepted kinetic model for this cooperative behavior predicts that, only upon binding of substrate in a regulatory site, a slow activation process converts (nearly) inactive enzyme into the active form (1). Many different theories have been proposed throughout the years to explain the molecular mechanism behind this behavior (38, 39). In Saccharomyces cerevisiae PDC, the most commonly accepted mechanism for substrate activation involves a cysteine (Cys221) as the site of covalent binding of the regulatory substrate molecule (see supplemental “Results” and Fig. S1) (38, 40). A full understanding of any of these mechanisms is missing, however, mainly due to the lack of structures of substrate complexes with an occupied regulatory site. Here, we used the inactive cofactor analogue 3dThDP to obtain structures of PPDC in complex with its substrates PPA and POVA. Whereas PPDC shows clear positive cooperativity with POVA as a substrate (Hill coefficient of 1.93), the deviation from Michaelis-Menten kinetics is rather small for PPA (Hill coefficient of 1.26 when fitted on the Hill equation), and PPDC was reported to behave with Michaelis-Menten kinetics toward PPA (7). Each subunit reveals two separate sites with substrate bound, corresponding to the active site and a regulatory site. The regulatory site is located at 18 Å from the active site, at the interface of the three domains of the monomer (Fig. 3).

The biological tetramers of the binary complex PPDC-ThDP (a) and the ternary complex PPDC-3dThDP-POVA (b) are depicted with their AB homodimer in the same orientation. The transition from an asymmetrical dimer of dimers in substrate-free PPDC to a symmetrical dimer of dimers in substrate-bound PPDC is accompanied by a 34° rotation of the CD loops that fold over the active site. A H-bond between Asp282 and His112 is hereby formed completing the Phe237 and Leu242. These rearrangements are transmitted via Tyr400 and Phe285 to the 104–120 and 280–294 loops that fold over the active site. A H-bond between Asp372 and His112 is hereby formed completing the proposed Asp25-His112-Asp282 catalytic triad. This sterical relay mechanism hence provides a clear link between substrate binding in the regulatory site and catalytic competence in the active site.

In the last step of the reaction cycle the Co-hydroxyl group of 2-(1-hydroxy-2-phenylethyl)-ThDP is deprotonated with concomitant cleavage of the C2-Co covalent bond and release of the second product phenylacetaldehyde. The PPDC-2HE3dThDP structure shows the hydroxyl group within 2.5 Å from the N4’ imino group (Fig. 7e), confirming the current view that this group acts as a proton acceptor in the product release step (34).

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Comparison of the substrate-bound ternary PPDC complexes (PPDC-3dThDP-PPA and PPDC-3dThDP-POVA) with the binary complexes (PPDC-ThDP, PPDC-3dThDP, and...
PPDC-2HE3dThDP) reveals significant changes in the protein conformation that are linked to the binding of the substrate in the regulatory site. These structural changes are taking place on three different levels and convert the unactivated enzyme into the substrate-bound activated form. 1) Fig. 5 illustrates the quaternary structural transition upon binding of a substrate in the regulatory site of PPDC. A rotation of 34° of one dimer relative to the other converts the PPDC tetramer from an asymmetric dimer of dimers in the unactivated state to a fully symmetrical dimer of dimers in the substrate-bound activated state. Significant changes at the quaternary level were also observed in S. cerevisiae PDC upon binding of the synthetic activator pyruvamide (39). Remarkably, this enzyme undergoes the opposite transition: from a symmetrical tetramer in the unbound state to an asymmetrical tetramer in the pyruvamide-bound state. 2) Fig. 4 illustrates the changes occurring at the tertiary level. The subunits of the unactivated and activated enzymes differ by a 12° rotation of the R domain relative to the PYR and PP domains. This rotation can be triggered by local rearrangements in the R domain upon binding of a substrate.
molecule at the regulatory site located between the PYR, R, and PP domains. These rearrangements in the R domain involve a segment spanning residues Phe237 to Pro247 and the Arg214 side chain. In the unactivated state Arg214 is hydrogen bonded within the same domain to Arg540. In the substrate-bound enzymes Arg214 forms other H-bonds with the main chain carbonyls of Tyr61 and Arg60, located on the PYR domain. The rotation of the R domain is also accompanied by the movement of the active site loop 280–294 and the concomitant restructuring of the interacting 104–120 loop of the PYR domain of the neighboring subunit in a tight dimer. 3) Comparison of the activated and unactivated structures in Fig. 6 also reveals a signal transduction route that communicates structural changes at the regulatory site to the active site via a cascade of sterical clashes transmitted from one amino acid residue to the next. Substrate binding at the regulatory site changes the side chain conformation of Arg214 and triggers this cascade. The signal is then transmitted via Arg214, Phe237, Met238, and Tyr500 to two distinct active site loops spanning residues 104–120 and 280–294 (see “Results”). These loops fold over the active site hence providing catalytic residues His112 and His113 (41, 42). A new H-bond between Asp282 of loop 280–294 and His112 of loop 104–120 completes the Asp25-His112-Asp282 catalytic triad, which we propose to play a role in protonation of the enamine/carbanion intermediate.

These results thus provide the first pictures of a molecular mechanism underlying the kinetically observed allosteric substrate activation behavior of ThDP-dependent enzymes. Indeed, the observed structural changes between the activated ternary complexes and the unactivated binary complexes link the binding of a substrate molecule in a separate regulatory site with loop closures and concomitant alignment of the catalytic machinery in the active site. Once substrate is bound in the regulatory sites, multiple turnovers might occur in enzymatically competent active sites with facilitated, fast loop opening and closing, explaining the often observed slow time-dependent activation of ThDP-dependent enzymes. In such a hysteretic model for positive cooperativity the observed Hill coefficient will depend on the affinity of the regulatory site for the substrate (43). Very high affinity binding of a substrate in the regulatory site will pull the enzyme completely toward the activated form for all substrate concentrations used for obtaining the $v$ versus $[S]$ curve. This will yield nearly Michaelis-Menten kinetics as observed for PPA as a substrate. Lower affinity binding to the regulatory site will cause significant deviations from hyperbolicity as observed for POVA.

A Versatile PYR-R-PP Domain Interface—A clue to the evolution of the signal transduction pathway in PPDC comes from comparison with POX. POX is a homologous ThDP-dependent enzyme that, in the presence of phosphate, catalyzes the oxidative decarboxylation of pyruvate with formation of carbon dioxide, hydrogen peroxide, and acetylphosphate (26). The ThDP-dependent POX also uses a flavin adenine dinucleotide cofactor in its reaction mechanism (25). This FAD is bound in a deep cleft, with its ADP moiety located on the R domain and the FMN part at the interface of the PYR, R, and PP domains. Superposition reveals that the residues involved in the signal transduction pathway of PPDC coincide with the FMN binding groove in POX. More surprisingly, the activated form of PPDC provides a cavity almost large enough to accommodate the FMN moiety of FAD. In the unactivated form of PPDC the large conformational changes of the residues in the signal transduction pathway cause these residues to occupy this cavity. This observation suggests that the interface between the PYR, R, and PP domains provides a versatile scaffold adaptable to serve different purposes during evolution. In POX, this interface evolved toward a binding pocket for an additional cofactor. In PPDC this interface has evolved to a flexible region adapted to dynamical intramolecular signal transduction.

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Note Added in Proof—While this manuscript was under consideration for publication, a study on oxalyl-CoA decarboxylase was published that also used 3-deaza-ThDP to allow a crystal structure of an enzyme-substrate complex to be obtained (Berthold, C. L., Toyota, C. G., Moussatche, P., Wood, M. D., Leeper, F., Richards, N. G. J., and Lindqvist, Y. (2007) Structure 15, 853–861).
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