Multiple Modes of Active Center Communication in Thiamin Diphosphate-Dependent Enzymes

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ABSTRACT

Detection of interaction between cofactors at the active centers of homodimeric and homotetrameric enzymes is usually elusive by steady-state kinetic approaches and requires protein variants where such interactions are diminished or exaggerated. In this Account, evidence for active-center interactions will be presented for the following thiamin diphosphate-dependent enzymes: yeast pyruvate decarboxylase, benzoylformate decarboxylase, and examples from the 2-oxoacid dehydrogenase multienzyme complex class. The dissymmetry of active sites is especially evident in the X-ray structures of these enzymes with substrate/substrate analogues bound. Perturbations that reveal active center communication include use of chromophoric substrates and substitutions of active center residues on putative pathways.

Introduction

It has long been known that the binding of thiamin diphosphate (ThDP) to its cognate enzymes[1,2] follows kinetic hysteresis, as exemplified by studies of Sable and co-workers on transketolase (TK), a ThDP enzyme with an α_2 quaternary structure.[3] Khailova and co-workers suggested active center communication between the two ThDP molecules at the active center of the first E1 subunit of pigeon breast pyruvate dehydrogenase complex, an enzyme with an $\alpha_2\beta_2$ quaternary structure.[4] The deductions in these cases relied on kinetic experiments. Once X-ray structures of ThDP enzymes started to appear in the early 1990s, it became clearer whether any dissymmetry existed in the active centers. While not seen in the structure of the wild-type pyruvate decarboxylases from two closely related yeast strains (YPDC),[5] the active centers became distinct in the presence of the substrate activator surrogate pyruvamide, which occupied half of the active centers in this tetrameric α_4 quaternary structure enzyme, prompting the authors to dub the two conformations "open" and "closed".[6] Indeed, distances between key residues were found to vary significantly in the two types of active centers. More recently, the publication reporting the structure of the recombinant E1 subunit from human sources suggested active center interactions in that enzyme, but without defining a particular pathway for such interactions.[7] Also, just in 2004, Perham's group proposed not only a pathway, but also a mechanism of information transfer via a chain of acidic amino acids (aspartate and glutamate) from one ThDP to the second in the $\alpha_2\beta_2$ quaternary structure E1 from *Bacillus stearothermophilus*[8] and also provided supporting experimental evidence for the hypothesis. With the examples outlined below, we suggest that the evidence for active center communication in ThDP enzymes is solid and warrants close scrutiny on other ThDP enzymes as well.

Case Study of Benzoylformate Decarboxylase

The enzyme benzoylformate decarboxylase (BFD) is a homotetramer; its structure has been solved to high resolution in a complex with ThDP[9] and in a ternary complex with ThDP and the competitive inhibitor (*R*)-mandelate.[10] BFD carries out the following reaction:

$$C_6H_5C(=O)COO^- + H_2O \rightarrow C_6H_5CHO + OH^- + CO_2 \quad (1)$$

As shown in Scheme 1, the accepted mechanism for ThDP-catalyzed nonoxidative decarboxylations[11] of pyruvic or benzoylformic acid invokes nucleophilic attack by the C2-carbanion (ylide/carbene) conjugate base of the thiazolium ring at the ketone leading to the C2α-lactylThDP (LThDP) or the C2α-mandelylThDP (MThDP), the first ThDP-bound intermediate. Next, by virtue of the electron sink created at a position β to the carboxylate, the LThDP or MThDP is poised to undergo decarboxylation to the C2α-carbanion/enamine, the second ThDP-bound intermediate. The conjugated enamine is then protonated at the C2α position leading to the C2α-hydroxyethylThDP (HEThDP) and the C2α-hydroxybenzylThDP (HBThDP), respectively, the third ThDP-bound intermediate, from which the aldehyde product is then eliminated.

Building on our previous experience with YPDC and conjugated pyruvate analogues,[12] we queried BFD whether *p*-nitrobenzoylformic acid [*p*-nitro-$C_6H_5C(=O)COOH$, NBFA] would serve as alternate substrate and whether the

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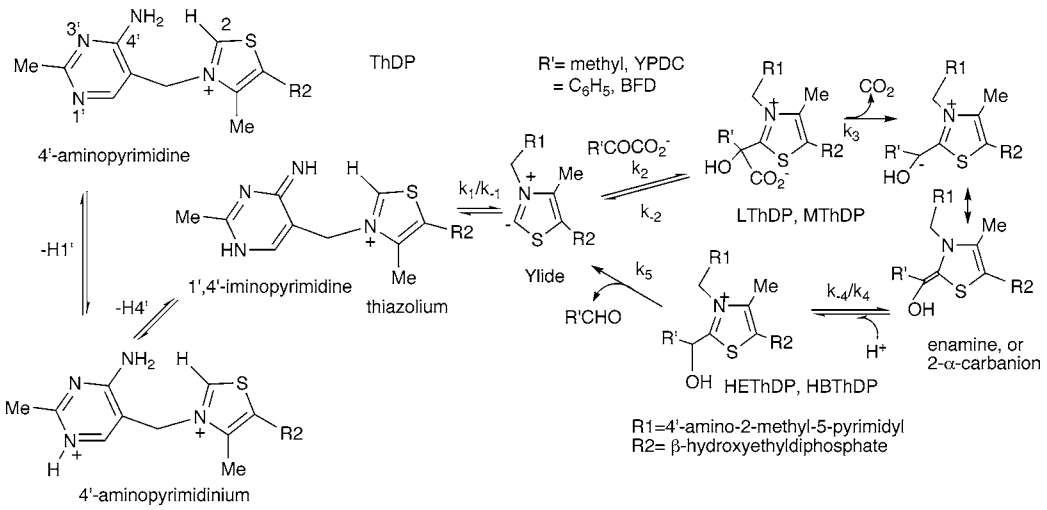
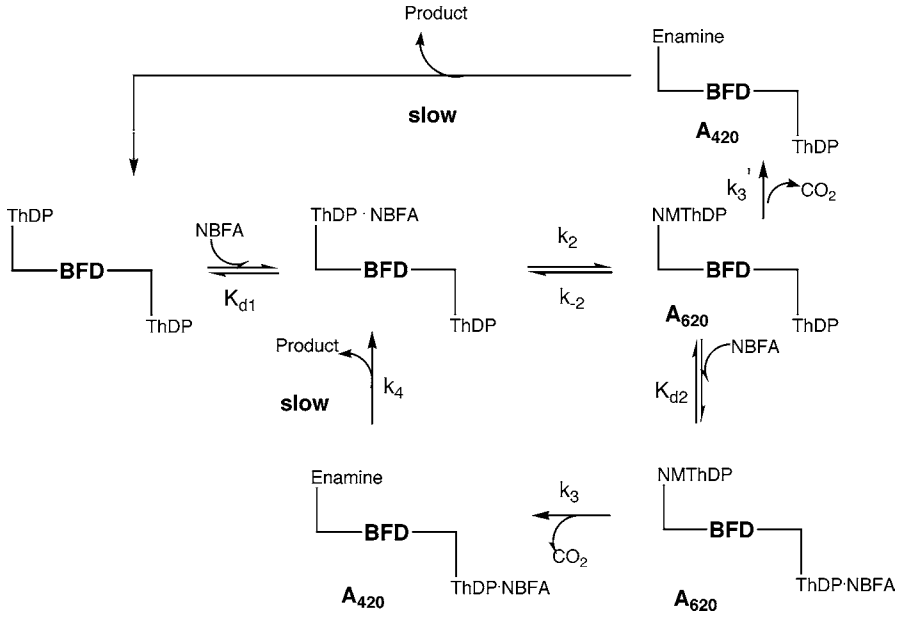
Scheme 1

Mechanisms of pyruvate decarboxylase (YPDC) and benzoylformate decarboxylase (BFD)



Scheme 2

Decarboxylation of NBFA by BFD requiring two active sites



enamine intermediate would appear at a longer λ_{max} than the substrate itself. On mixing BFD and NBFA on a stopped-flow instrument, two transient absorptions were apparent, one near 410−420 nm (I2) and the other very broad one centered at 620 nm (I1). A time slice at the two wavelengths indicated that the transient I1 was formed on the ms time scale and was depleted at the same rate as the rate of formation of I2. On the basis of model studies, the transient near 410 nm was assigned to the enamine, while the faster-forming I1 must correspond to either a Michaelis complex or the MThDP.[13] It would be difficult to imagine conditions under which formation of the Michaelis complex is on the relatively slow time scale observed on the stopped-flow; hence, we assigned the transient at 620 nm with its very broad feature to a charge-transfer transition involving the *p*-nitroMThDP (NMThDP).[13]

Transient-state kinetic analysis was applied to the two relaxations, whose assignment to individual reaction steps requires determination of the concentration dependence of the two relaxations. Surprisingly, both relaxations displayed dependence on NBFA concentrations: the rate of formation of NMThDP may show dependence on substrate concentration, but if the rate of decarboxylation does, then this suggests some unexpected complexity in the mechanism. Consistent with the kinetic observations, the mechanism shown in Scheme 2 was suggested which invokes "alternating of active sites in a functional dimer"

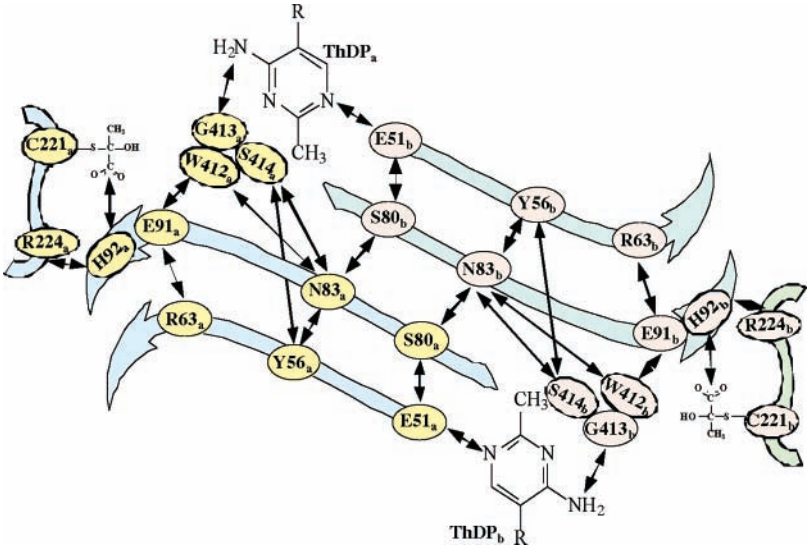


FIGURE 1. Substrate activation pathway and active center communication on YPDC.

in this homotetrameric enzyme. According to this scheme, with the alternating sites mechanism, the decarboxylation step (k_3) is assisted by substrate, and without it, the substrate concentration has no influence (k_3'). As one progresses through the steps, prior to decarboxylation of NMThDP in the first site, the second ThDP site must be occupied by substrate NBFA as a Michaelis complex; hence, the two ThDP sites must interchange their roles during the catalytic cycle. The substrate-dependent kinetics for the k_3 step was present even with the active-center H70A BFD variant, where the first transient was not observable.[13]

Case Study of Yeast Pyruvate Decarboxylase

Wild-Type YPDC Is a Substrate-Activated Enzyme. The YPDC is a tetramer of ca. 60 000 Mr monomers that represents a "dimer of dimers" structure.[5] The YPDC displays strong positive cooperativity vis-à-vis pyruvate.[14] An important contribution showed that pyruvamide [$CH_3C(=O)CONH_2$, an analogue that cannot undergo decarboxylation] can function as a substrate-activator surrogate at high concentrations, converting a sigmodial Michaelis–Menten curve to a hyperbolic one.[15] Site-directed mutagenesis work from this laboratory suggested that the substrate activation takes place at C221 of the β domain: most likely, C221 forms a hemithioketal with the substrate, and the carboxylate anion of this adduct forms an ion pair with H92 on the α domain.[2] This ion pair mimics the interactions at the analogous positions in space in pyruvate decarboxylase from *Zymomonas mobilis* and BFD, two enzymes which are not subject to substrate activation. A pathway for information transfer from H92 to E91 to W412 and then to the active site was then mapped (Figure 1). It was also suggested that enzyme that is devoid of substrate activation can still function; hence, substrate activation is not absolutely required (depending on external conditions such as pH).[16]

Structure of YPDC in the Absence and Presence of Pyruvamide. The initial structure determinations of YPDC

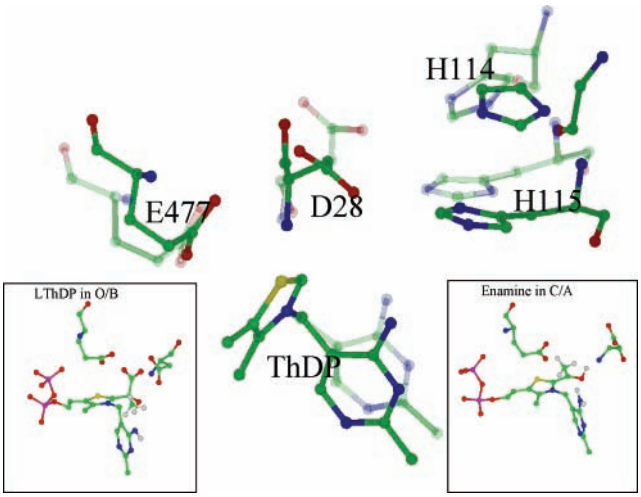


FIGURE 2. Different relative positions of active center residues around ThDP in YPDC.ThDP.pyruvamide complex.[6] The LThDP and enamine were constructed into this structure in the lower insets.

were carried out in the absence of substrate or activator and showed no asymmetry of the active centers surrounding ThDP.[5] The groups of Schneider and König succeeded in crystallizing YPDC in the presence of pyruvamide,[7] and this enzyme form exhibited significant active center asymmetry (Figure 2) with the active center of one subunit/functional dimer being occupied by pyruvamide, and pyruvamide also being found near a loop spanning residues 290–300 on the β domain. There were significant differences in distances between corresponding active center residues in subunits occupied by, or devoid of, pyruvamide. Two monomers, depicted A and B, form a tight dimer and the two dimers form a loosely bound tetramer. In the presence of pyruvamide, one monomer in each dimer exhibited a "closed" conformation (subunit A/C), where the active site was essentially screened from the surroundings by two interacting loops (residues 104–113 and 290–304) from neighboring subunits of the dimer, and these loops created a lid over the active site. At the same time, the second active site (subunit B/O) was accessible to solvent. The two dimers displayed a remark-

able degree of symmetry to one another, interacting through the interfaces created by the subunits in the same conformations.

Requirement for YPDC Dimer as a Minimal Catalytic Unit. Detailed kinetic studies of the D28A and E477Q variants (active center residues form different domains and different subunits) brought to light two features that were difficult to explain without invoking a functional dimer:[17] (1) Under some conditions, the Hill coefficient was >2.0, and (2) with the D28A and D28N variants, severe substrate inhibition was also in evidence.[18] We therefore developed a new kinetic model for YPDC with a dimer as a minimal catalytic unit.[19] The suggested kinetic model takes advantage of the asymmetry in the tetramer with the tight dimer (hereafter called "functional dimer") being the minimal catalytic unit, possessing a total of four pyruvate binding sites, two regulatory and two active sites. The phenomenological model presented could explain the kinetic results on some YPDC variants, which required the presence of enzyme species with three substrate molecules bound.

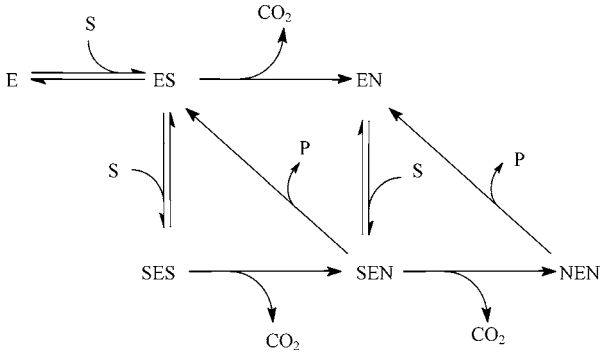
The signal transduction from the regulatory site to the active site of YPDC discussed above[2] very likely links two sites of the same subunit. The presence of the third binding site for pyruvate infers the existence of additional signal transduction pathways connecting two different subunits. The two active center acid−base residues D28 and E477 appear to be important both for catalysis and for the postulated signal transduction pathway.

The proposed mechanism is based on the principles exploited in an internal combustion engine, adapted from studies of enzymes which sustain some functional locomotion. As mentioned above, the same type of mechanism was used to rationalize the transient kinetic behavior of BFD,[13] where substrate binding in the second active site of BFD was required for the decarboxylation of the ThDP−NBFA covalent intermediate in the first site.

According to the mechanism suggested for YPDC, decarboxylation in one active site may supply the energy for a second active site to bind substrate or to release product, making the sequential operation a prerequisite for normal function.[19] If the enzyme were to utilize alternating sites, it must ensure that the environment of the active center is optimal for the designated function at each point in time. Two active centers of the functional homodimer of YPDC have different conformations in the presence of the pyruvamide (Figure 2), but the differences could be more dramatic in the presence of the true ThDP-bound covalent adducts in Scheme 1. We suggested that the state of protonation of D28 changes during the catalytic cycle and the charge at D28 reflects the functional state of the active site. We tentatively concluded that D28 and E477 might be in the signal transduction pathway, which keeps the work of alternating sites in sequence.

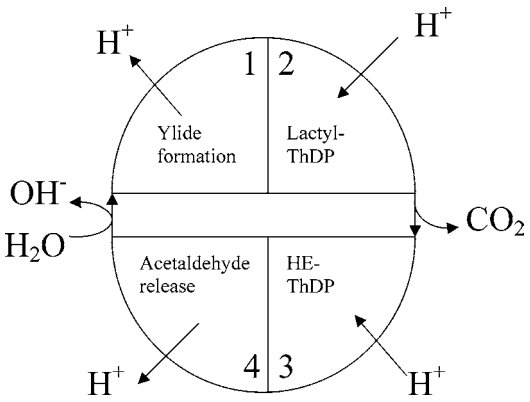
We supposed that both active centers catalyze the entire catalytic cycle, with a mandatory phase shift between two active sites of the functional dimer: one active site of the functional dimer is catalyzing the pre-decarboxylation phase, whereas the second subunit at the same point in time is catalyzing the post-decarboxylation phase of the reaction. Decarboxylation renders the reaction irreversible and could provide a possible source of energy for the endothermic parts of the reaction or for the shift of an unfavorable equilibrium to enhance the overall throughput of the enzyme. Alternation of sites through a conformational change would ensure the physical coupling of the two parts of the reaction, pre-decarboxylation and post-decarboxylation, as illustrated in Scheme 3. In this scheme, E represents activated enzyme (i.e., substrate already bound at the regulatory site) with the apparent dissociation constant K_a of the regulatory site, and S is free substrate. ES and EN represent one of the pre-decarboxylation (enzyme−substrate noncovalent complex or LThDP) and post-decarboxylation (enamine or HEThDP, or enzyme−product noncovalent complex) intermediates, respectively. The positioning of the letter S or N to the right or the left side of the symbol for the enzyme reflects involvement of two different monomers of the functional dimer. The release of product is only possible after substrate binding and possibly LThDP formation in the neighboring subunit. The intermediate EN is formed in an irreversible step from decarboxylation of ES. The product release is irreversible because of reduction of acetaldehyde (P) in the coupled reaction (alcohol dehydrogenase/NADH). If either of the two irreversible steps is obstructed, the accumulation of pre- or post-decarboxylation intermediates would result. While Scheme 3 does not include catalysis by nonactivated enzyme, this would be a logical extension of the model.

Scheme 3. YPDC Alternating Active Sites in a Functional Dimer



Evidence Supporting Alternating Sites Reactivity. Mechanistically, formation of LThDP from pyruvate and ThDP and the release of acetaldehyde from HEThDP represent reversal of the same reaction, the transfer of a proton to or from the C2α oxygen. These reactions are preceded by proton abstraction from C2 of the thiazolium ring and are separated by proton donation to the C2α of the enamine to form HEThDP. The latter two reactions also represent the reverse of mechanistically similar reactions. Therefore, the sequence of reactions carried out by YPDC can be viewed as two reactions in tandem, where, in the second repeat, the reverse reactions are utilized. The four parts of the catalytic cycle are shown in Scheme 4, where Parts 1 and 3 represent reversal of one reaction and Parts 2 and 4 represent reversal of another reaction.

Scheme 4. Alternating Occupancy of Active Sites in YPDC



On the basis of the structural data from many ThDP enzymes and our own experiments, we proposed a model where the N4′-amino group of ThDP (or its imino tautomer[20]) may participate in virtually all of the proton transfer reactions, probably assisting E477 and D28, in the first half of the reaction. With the V conformation enforced by several factors,[21] it is difficult to displace the amino group by several angstroms. However, in the YPDC structure with pyruvamide,[6] the beginning of a modest shift is already present (Figure 2). In the closed active centers (in semitransparent colors), the distance between N4′ and C2 in ThDP is decreased to 3.09 Å compared to 3.5 Å in the open active site (shown in solid colors), whereas the distance between these two atoms is the same in both active sites of nonactivated YPDC, suggesting plausibility of conformational differences in the two active centers. The closed active site of the A/C subunit conformation can accommodate proton transfer from C2 to N4′, but the site is too tight to accommodate the covalently bound pyruvate of LThDP. Therefore, we suggest that the A/C conformation of the closed active site provides a working model for formation of both the ylide and HEThDP, where the N4′ atom comes within close contact with the C2 or C2α atom to donate or accept a proton (inset B in Figure 2). In the open active site of the B/O subunit conformation, the N4′ atom is further from C2α and provides a model for participation of N4′ in the protonation or deprotonation of the C2α-hydroxyl group in LThDP formation (inset A in Figure 2) or acetaldehyde release.

Comparing the results of C2H/D exchange studies on ThDP in wild-type YPDC and the D28A and E477Q variants, both in the absence and presence of pyruvamide, also hinted at the participation of both D28 and E477 in the signal transduction pathway, leading from the regulatory or perhaps inhibitory site for pyruvamide to the active center.[17]

It was also concluded that the D28 residue may have multiple roles, in all of which its ability to lose a proton is utilized. The residue D28 protects the post-decarboxylation intermediates from attack by pyruvate that could result in acetolactate formation (a caboligase-type reaction observed with many ThDP decarboxylases as a side reaction). A different role for D28 would involve protection of the vacant second active site of the enzyme species with pre-decarboxylation intermediates in the first active site (depicted by ES in Scheme 3). We also reasoned that most residues among D28, H114, H115, and E477 are essential for signal transduction from one active site to another. Were decarboxylation in one active site a prerequisite for the release of product from the second site, then disruption of the communication between the active sites would be expected to compromise the product release phase to a much greater degree than the leading phase. If the chain of the signal is broken, the decarboxylation in one active site results in suboptimal changes at the second site, thereby leading to accumulation of post-decarboxylation intermediates.[19]

It was suggested that the active centers in the functional dimer are not acting independently of one another, and the pre- and post-decarboxylation phases of the reaction are tightly coupled. On the basis of this coupling, the alternating sites model could explain: (a) Equal rates of pre- and post-decarboxylation in WT YPDC, since two parts of the reaction are synchronized; (b) apparent participation of all active center acid−base groups in both pre- and post-decarboxylation steps, suggested by the steady-state kinetics reported earlier, according to which both V/K and V-type kinetic terms are affected by substitutions at D28, H114, H115, and E477.[17]

Recently, we found that the pyruvate analogue methyl acetylphosphonate, when bound to the YPDC, occupies half of the catalytic centers as a Michaelis complex and the other half as an adduct with ThDP, an analogue of LThDP (see reaction and circular dichroism spectrum in Figure 3).[22]

Evidence for a Functional Dimer Being Sufficient for Activity. A study reported that YPDC tetramers could reversibly dissociate into active dimers under alkaline conditions and also proposed a new method for urea-induced dissociation of YPDC tetramers to dimers.[23] With experiments inspired by that study, we demonstrated that two very low activity variants D28A and E477Q could reassociate and complement each other to fully active tetramers with nearly the predicted activity after hydroxide-induced dissociation.[24]

When the E477Q and D28A (or D28N) variants of YPDC were mixed in different mole fractions, but at a constant total concentration, and were subjected first to pH increase to 8.6, then reassociation at pH 6.0, a much higher activity (50-fold at a 1:1 molar ratio) resulted than with the individual variants. However, when the same variants were mixed in different molar ratios in the presence of 0.5 M urea and were then diluted at least 12-fold in buffer without urea, no regain of activity was apparent.[24]

Potentially, two types of tetramers with distinct features might result from the reassociation of dimers created under these different conditions (Scheme 5), but only the dimers that were formed by dissociation of the active centers along plane A could reassociate to tetramers with the dramatic activity increase found. The monomer of each variant would contribute one substituted residue and one wild-type residue to the hybrid dimer. According to

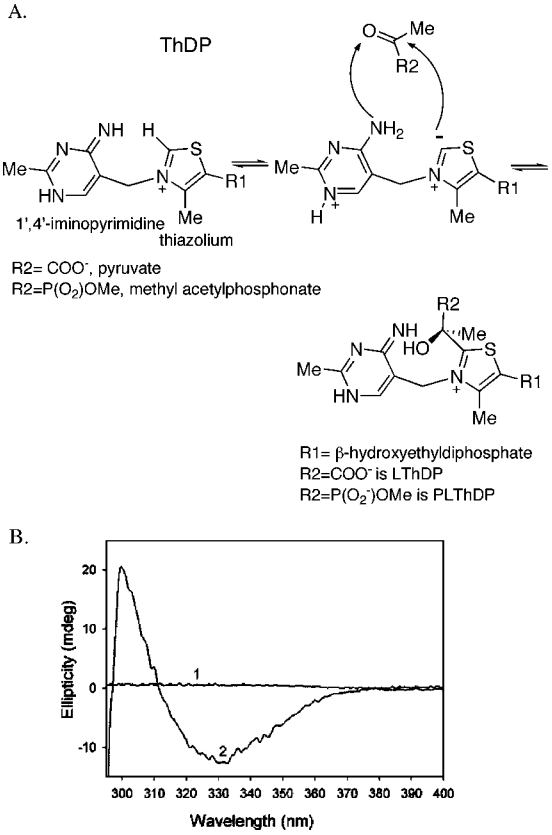


FIGURE 3. (A) Mechanism of formation of LThDP and its stable phosphonolactylThDP analogue. (B) Circular dichroism spectrum of E91D YPDC with sodium methylacetylphosphonate. The spectrum shows the formation of the adduct of NaMAP with ThDP (PLThDP) via the 1′,4,-imino tautomer at 300 nm and of the Michaelis complex at 330 nm.

the alternating sites mechanism for YPDC discussed above,[19] two active sites in the functional dimer act in an antiphase manner during the reaction, with each active site eventually completing the full catalytic cycle; however, at any instant, the pre-decarboxylation phase of the reaction is taking place in one monomer and the post-decarboxylation phase in the other. Also, decarboxylation in one active center is believed to promote product release in the second one. In the hybrid tetramers, one active site of the functional dimer has substitutions at both D28 and E477. Both amino acids are important in the post-decarboxylation phase of the reaction, and D28 is also believed to participate in the decarboxylation step. Therefore, loss of catalysis in one active center of the functional dimer should inhibit catalysis in the second one, consistent with our observations. The data predict that interactions between functional dimers are less important than those between subunits of the dimer, yet interdimer interactions have an effect on the stability of enzyme−substrate complex.

Case Study of the E1 Subunit of 2-Oxo Acid Dehydrogenase Multienzyme Complexes

The E1 or 2-oxoacid dehydrogenase subunit of these complexes displays a variety of quarternary structures and uses ThDP as cofactor as shown for the pyruvate enzyme in Scheme 6.

Pyruvate Dehydrogenase-E1 Subunit from *Escherichia coli.* *Structural Information on Binding ThDP and a "Transition-State" Analogue.* The *E. coli* PDHc-E1 is a homodimer of mass 99 474 Da each. The structure of PDHc-E1 in complex with ThDP was determined at 1.85 Å[25] and in complex with a transition-state analogue thiamin 2-thiazolone diphosphate (ThTDP, a C=O bond in place of C2H) at 2.1 Å resolution.[26] The two molecules of ThDP are bound at the subunit−subunit interfaces and form two active sites and have an almost identical arrangement of cofactor and residues, indicating no structural asymmetry. With ThTDP bound to the PDHc-E1, there is structural reorganization in the active sites, leading to an increase of the number of hydrogen bonds and more tight binding of ThTDP as compared with ThDP, but also with both sites occupied with ThTDP.[26] In neither structure is there electron density defined for residues 1−55, 401−413, and 541−557; nonetheless, it was shown that the residue H407 is important for interaction of the E1 and E2 subunits.[27] The active sites appear to be in the open form in both structures, with no obvious asymmetric distortions of individual subunits. In the recently completed structure of apo-PDHc-E1, again no disorder−order transition of the loops upon binding of ThDP was observed;[28] they are disordered in apo-PDHc E1 as in the other two structures.

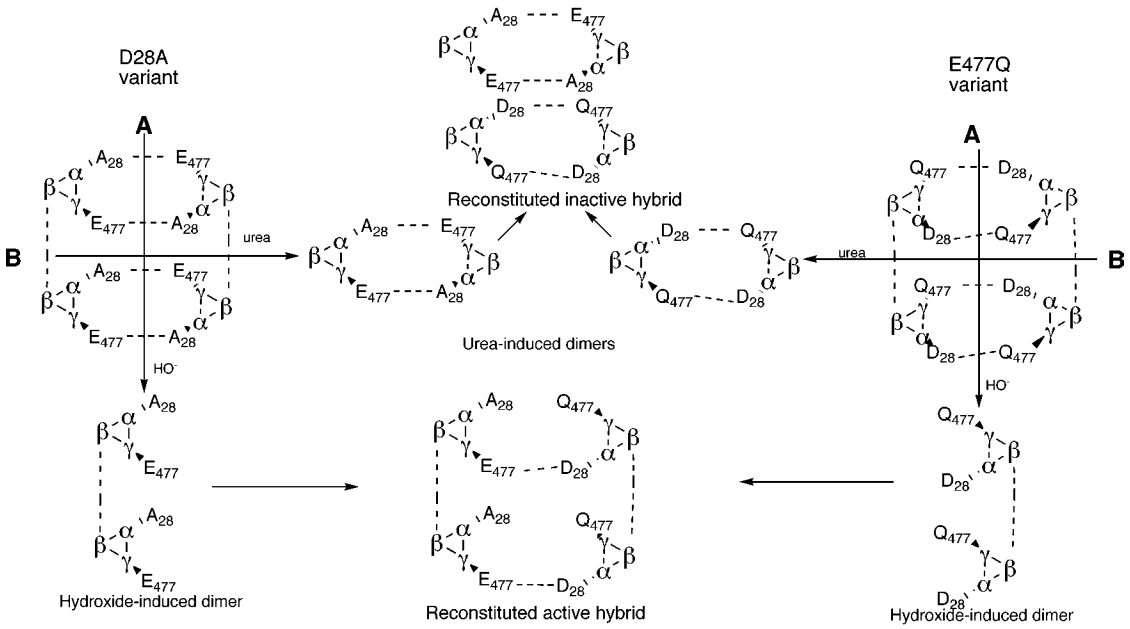
Kinetic and Spectroscopic Evidence Supports Communication between Two Active Centers. The binding of ThDP to *E. coli* PDHc-E1 exhibited weak positive cooperativity; however, a weak negative cooperativity was observed with the Y177A and Y177F active center substitutions.[29] According to progress curve analysis of inhibition (Y177A) by ThTDP and fluorescence studies (Y177A and Y177F) of binding by the same inhibitor, ThTDP binds in one of two active centers.[29]

The "half-of-the-sites" type reactivity as reflected by occupancy of only one of two active centers was most evident for E636-substituted variants:[30] (1) Binding of ThDP to the E636A variant suggested negative cooperativity; (2) while the progress curve analysis for the overall PDHc reaction exhibited an activation lag phase on ThDP binding to the E636A and E636Q variants (as with parental PDHc-E1), analysis of the lag phase demonstrated ThDP binding in one active site; (3) the magnitude of the CD signal reporting formation of 1′,4′-iminopyrimidine tautomer of phosphonolactylthiamin diphosphate (PLThDP, a stable analogue of the first covalent intermediate LThDP, see Figure 3 for reaction) to E636 substituted variants also revealed binding in one active center. These results and the similar kinetic behavior of the E636- and Y177-substituted PDHc-E1 variants, along with the observation that in the crystal structure of PDHc-E1.ThDP complex, the E636 (subunit B) forms a hydrogen bond with Y177 (subunit A), suggested that both residues participate in subunit−subunit communication.

Analysis of ThDP-bound covalent intermediates trapped during catalysis using ^{1}H NMR revealed communication

Scheme 5

Urea and hydroxide-induced dimer formation and reconstitution to tetramers (α, β and γ denote domains)

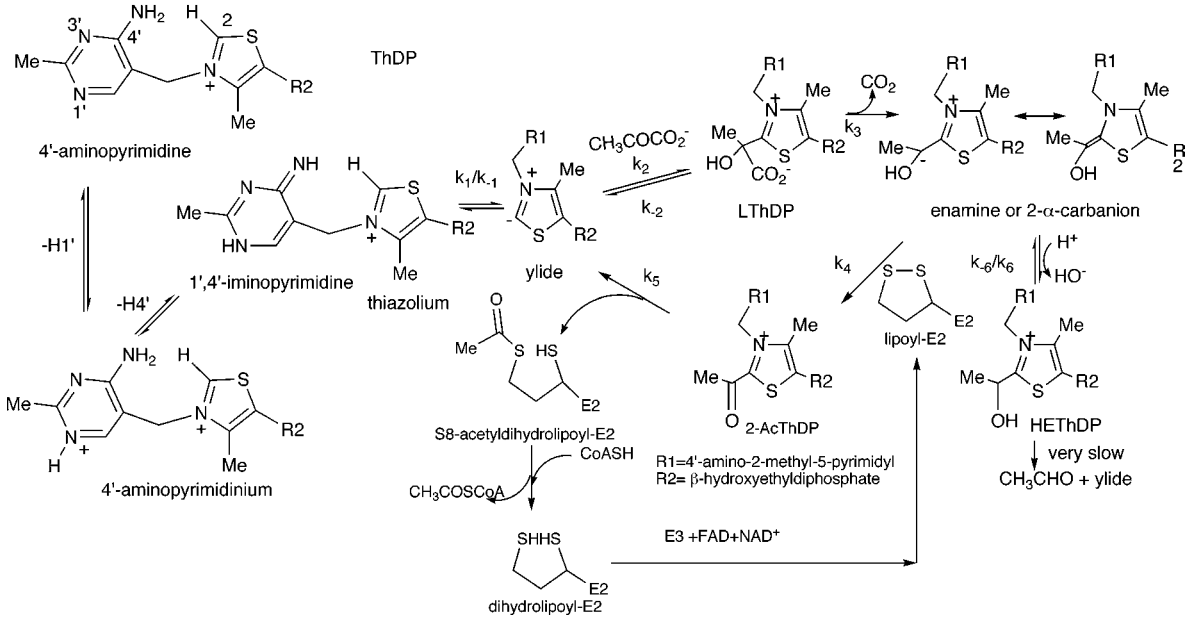


Reconstitution of urea-induced dimers.
Formation of tetramer from either two urea dimers of D28A or of E477Q individually, or of a hybrid tetramer created by mixing one D28A and one E477Q urea dimers, leads to inactive (low activity) species.

Reconstitution of alkaline-induced dimers.
Formation of tetramer from two hydroxide-induced dimers of D28A or of E477Q individually will lead to low activity species. However, formation of a hybrid tetramer created by mixing one D28A with one E477Q hydroxide-induced dimer would lead to active species; the activity could be reduced by symmetry considerations.

Scheme 6

Mechanism of pyruvate dehydrogenase complex-E1 subunit



between active sites.[30] After 30 s of reaction time at 30 °C and in the presence of saturating concentrations of pyruvate, 50% of the active sites were occupied with HEThDP (resulting from protonation of the enamine on acid quench) and 50% with ThDP. With the E636A variant, only half of the active sites were occupied with intermediates: 14% with HEThDP, 6% with acetolactyl-ThDP, and 30% with ThDP, while the second half of the active sites were vacant, suggesting disruption of communication between two active centers by the E636 substitution. In contrast, for the E571A and E571D variants (this highly conserved residue is hydrogen bonded to N1′ atom of ThDP), acid quench showed 80% of active sites being occupied with HEThDP, again indicating disruption of

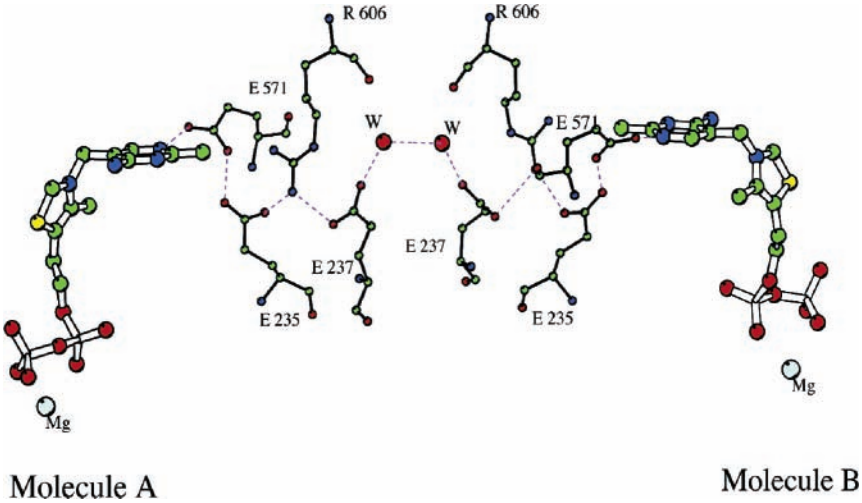


FIGURE 4. Putative active center communication pathway in *E. coli* PDHc-E1, fashioned after the proposal of Frank et al.[8]

communication between the active centers, but in this case each active center could act independently (unpublished results).

Recently, inspired by a report by Frank et al.,[8] we suggested a communication pathway between two ThDPs for *E. coli* PDHc-E1 including the residues E571, R606 (B subunit) and E235, E237 (A subunit) and a molecule of water (Figure 4).[31] While the residue E571 is on this pathway, neither E636 nor Y177 is, indicating that there are additional pathways for active center communication.

Further evidence for occupation of only half of the active centers by HEThDP was also apparent from a study of the catalysis of D→H exchange from HEThDP-d_4 by PDHc-E1.[32]

Pyruvate Dehydrogenase from *Bacillus stearothermophilus* (E1bs). This enzyme has been the subject of studies by Perham's group. Active center communication was suggested to take place via a "proton wire" in a tunnel comprising four aspartate and six glutamate (acidic) residues over a total distance of about 20 Å.[8] On the basis of crystal structure and limited proteolysis experiments, conformational asymmetry of two active sites was identified: two loops (inner, α203−212, and outer, α275−293), which were ordered in one α subunit and were disordered in the other α subunit. Limited tryptic digestion experiments demonstrated that only the outer loop in one α subunit is subject to proteolysis. The mechanism suggested involves activation of the first ThDP by binding in the active center, while activation of the second ThDP is coupled with the binding and decarboxylation of pyruvate in the first active site. The proton removed from the first ThDP is shuttling to the second active site through the tunnel comprised of acidic amino acid residues. The activation of ThDP is accompanied by a disorder−order transition of two active site loops, which keep the active sites in the closed form.

Branched-Chain 2-Oxo Acid Dehydrogenase from *Thermus thermophilus HB8*. The structure of this enzyme (BCOA E1tth) with $\alpha_2\beta_2$ assembly was solved for two heterodimers I and II.[33] Although the structures of heterodimer I and II were similar, the disordered region identified in apo E1tth was more extensive in heterodimer I (amino acids 274−291α) than in heterodimer II (amino acids 279−291α).[33] On ThDP binding, the disordered loops containing residues Y206α−S218α (loop I) and S274α−W291α (loop II) become ordered and shield ThDP from the solvent. In the structure of BCOA E1tth with ThDP and the substrate analogue 4-methylpentanoate, the active center of heterodimer I was filled with substrate analogue and was in the closed form. At the same time, the heterodimer II contained no substrate analogue and was in the open form, indicating structural asymmetry of the two active sites. The authors did not propose a pathway for communication between two active sites; instead, they suggested that the differences are due to the lower crystal packing effect on heterodimer I than on heterodimer II.[33]

Human-Branched Chain 2-Oxo Acid Dehydrogenase. This enzyme (E1bh, $\alpha_2\beta_2$ structure) is inactivated by phosphorylation by a kinase and is reactivated by a phosphatase. Phosphorylation of S292α and S302α in one α subunit abolishes the activity of the entire branched chain α-keto acid dehydrogenase complex (BCKD).[34] In the crystal structure of E1bh, these two serine residues are located on the phosphorylation loop, which is disordered in apo E1bh. Binding of ThDP to the apo E1bh induced a disorder to order transition of the conserved phosphorylation loop carrying two phosphorylation sites at S292 and S302[34] and protects the E1bh from inactivation by phosphorylation. The ordered conformation of the phosphorylation loop is important for the recognition of the E2 lipoyl-bearing domain. It is unclear how phosphorylation of S292 and S302 on one α subunit of one heterodimer would abolish the activity of the entire complex.

Conclusions. There appears to be more than one pathway between the active centers on at least two ThDP enzymes: (1) on YPDC, there is one involving D28 and E477 and the putative pathway in Figure 1, analogous to the proposal by Frank et al.[8] with the difference that no acidic amino acids other than the conserved E51 participate; (2) on the *E. coli* PDHc-E1, there is a pathway shown

in Figure 4, as suggested by Frank et al., and there appears to be one including Y177 and E636. Elucidation of such pathways promises to provide useful insight to the mechanism of active center communication in ThDP enzymes.

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