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Alternating Sites Reactivity Is a Common Feature of Thiamin Diphosphate-Dependent Enzymes As Evidenced by Isothermal Titration Calorimetry Studies of Substrate Binding

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Supporting Information

ABSTRACT: Thiamin diphosphate (ThDP)-dependent enzymes play vital roles in cellular metabolism in all kingdoms of life. In previous kinetic and structural studies, a communication between the active centers in terms of a negative cooperativity had been suggested for some but not all ThDP enzymes, which typically operate as functional dimers. To further underline this hypothesis and to test its universality, we investigated the binding of substrate analogue methyl acetylphosphonate (MAP) to three different ThDP-dependent enzymes acting on substrate pyruvate, namely, the *Escherichia coli* E1 component of the pyruvate dehydrogenase complex, *E. coli* acetohydroxyacid synthase isoenzyme I, and the *Lactobacillus plantarum* pyruvate oxidase using isothermal titration calorimetry. The results unambiguously show for all three enzymes studied that only one active center of the functional dimers accomplishes covalent binding of the substrate analogue, supporting the proposed alternating sites reactivity as a common feature of all ThDP enzymes and resolving the recent controversy in the field.

Although thiamin diphosphate (ThDP)-dependent enzymes share a limited amino acid sequence similarity (usually <20% identical), the tertiary structure of all ThDP-binding domains is highly conserved.[1,2] All ThDP enzymes exist as either tetramers or dimers but function as a "dimer of active sites".[3] The first evidence of a structural nonequivalence of the two corresponding active sites was reported as early as 1992 for yeast transketolase.[4,5] Subsequent structural analysis of several other ThDP enzymes, including pyruvate decarboxylase, pyruvate dehydrogenase, and pyruvate:ferredoxin oxidoreductase, also revealed a structural asymmetry suggesting an alternating sites reactivity of the functional dimers.[6−8] As opposed to these findings, structural studies of branched-chain α-keto acid dehydrogenase in the resting state and of pyruvate oxidase, pyruvate decarboxylase, and transketolase in complex with covalent reaction intermediates suggested that the active sites operate independently.[9−13] Besides these structural indications, kinetic and spectroscopic evidence implicated a functional nonequivalence of the active sites for some ThDP enzymes.[14−17] The data revealed that the two ThDP molecules in the two active sites of the functional dimer can be found at different stages of activation (one active and one dormant site) and contain intermediates at different stages of the catalytic cycle.

In this study, we have used isothermal titration calorimetry (ITC) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy to test the hypothesis of alternating sites reactivity (ASR) and of a negative cooperativity between the active sites in the ThDP enzyme superfamily while analyzing the thermodynamics of substrate binding under true equilibrium conditions. Our analysis included enzymes for which previous structural and kinetic data seemed to support (pyruvate dehydrogenase and acetohydroxyacid synthase) or rule out (pyruvate oxidase) the notion of an ASR.[3,11,15,17] As both structural and kinetic data suggested an ASR for the pyruvate dehydrogenase multienzyme complex (PDHc),[15,18,19] we used the E1 component of the *Escherichia coli* complex (*Ec*PDHc-E1) for the establishment and validation of the ITC-based method. We subsequently adopted this method for analysis of the related bacterial acetohydroxyacid synthase isoenzyme I (*Ec*AHAS I), for which ASR has been proposed on the basis of spectroscopic data.[17] Finally, we studied pyruvate oxidase from *Lactobacillus plantarum* (*Lp*POX) as an example, for which no hints of an ASR had been reported so far. Although the three proteins catalyze the formation of different products, they all act on pyruvate as the substrate. This allowed us to exploit the pyruvate analogue methyl acetylphosphonate (MAP) for a thermodynamic analysis of substrate binding as MAP covalently binds to the enzyme-bound ThDP cofactor akin to physiological substrate pyruvate but is not further processed as opposed to the latter.[20] Thus, the reaction cycle stalls, and the stable predecarboxylation intermediate analogue phosphonolactyl-thiamin diphosphate (PLThDP) is formed on the enzyme, making possible a reliable thermodynamic analysis of substrate binding under true equilibrium conditions.

First, we analyzed the binding of MAP to all three enzymes using ITC, a powerful technique for quantitative determination of the binding affinity (K_d), enthalpy changes (ΔH), and, most importantly in the context of this study, the binding

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stoichiometry (N) of the interaction between the individual enzymes and MAP. The results of a typical experiment of the interaction between *Ec*PDH-E1 and MAP are shown in Figure 1A. The outcomes of the titration experiments with *Lp*POX and *Ec*AHAS I are provided in the Supporting Information.

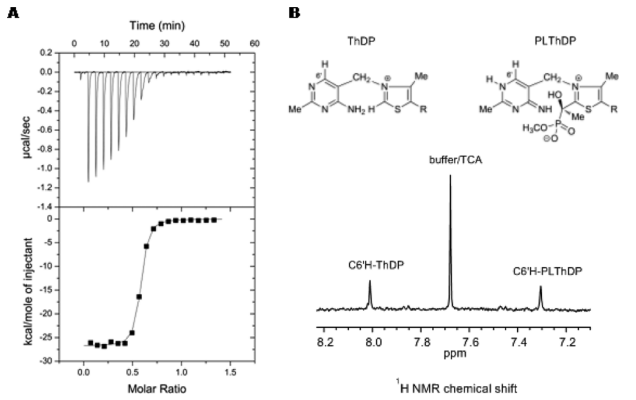

Figure 1. ITC and ^{1}H NMR spectroscopic analysis of the reaction of *Ec*PDH-E1 with MAP. (A) ITC data obtained after addition of 445 μM MAP to 63 μM *Ec*PDH-E1 (active site concentration) in 150 mM KH_2PO_4 (pH 7.0), 100 mM KCl, 1 mM ThDP, and 10 mM $MgCl_2$. The top panel displays the heats of each injection, while the bottom panel shows the integrated and concentration-normalized heats for each injection, corrected for control dilution heats. A nonlinear least-squares analysis of the data was conducted (—) with estimated parameters listed in Table 1. (B) ^{1}H NMR-based analysis of quench-isolated ThDP and PLThDP. The distribution of both for the reaction (5 min) of 187 μM *Ec*PDH-E1 with 1 mM MAP is shown. The C6′-H chemical shifts of ThDP (8.01 ppm) and PLThDP (7.31 ppm) were used for analysis.

As shown in Figure 1A and Figure 1 of the Supporting Information, the interactions between the substrate analogue and the enzymes are exothermic in nature, and the binding reaction is enthalpically driven in all three cases (Table 1). From these measurements, the dissociation constant (K_d), stoichiometry (N), and enthalpy (ΔH_0) for each binding process were determined. The results are listed in Table 1. The estimated binding stoichiometry in all three cases was in the range of 0.5 (*Ec*PDH-E1, 0.557 ± 0.002; *Ec*AHAS I, 0.534 ± 0.056; *Lp*POX, 0.486 ± 0.007). This finding leads us to conclude that only half of the active sites of the respective enzyme bound one MAP molecule. This result is fully consistent with the hypothesis of an ASR of ThDP-dependent enzymes. In a recent study of *Ec*PDH-E1, the authors reported on a temperature-dependent ASR behavior of this enzyme.[21] Results below 30 °C seemed to indicate a single binding site, while above 30 °C, two unsymmetrical active centers were proposed. According to analysis from that group, MAP binds in both active centers of *Ec*PDH-E1, which shall behave similarly at temperatures below 30 °C. When the temperature is increased to >30 °C, two active centers with different binding constants are inferred: a K_d of 0.03 μM for the first active site and a K_d of 3.0 μM for the second. In conflict with these data, we do observe strong negative cooperativity between both active centers and formation of the PLThDP conjugate in only one of the two active sites over the whole tempearature range tested (15−30 °C). This finding was reproducible for numerous independent batches of purified *Ec*PDH-E1. Unfortunately, we cannot resolve this apparent controversy because no primary ITC data are provided in the reference in question.[21]

To further underpin our ITC measurements, we performed one-dimensional ^{1}H NMR experiments to analyze the distribution of covalent intermediates and C2-unsubstituted ThDP after reaction of *Ec*PDH-E1 with MAP. The ^{1}H NMR chemical shifts of the C6′-H singlets of the aminopyrimidinium moiety can be used as a fingerprint region for discrimination of all the C2-derived covalent ThDP adducts.[22,23] The experiment was performed as described in the Supporting Information. The result is shown in Figure 1B. The spectrum conclusively shows an equal distribution of C2-unsubstituted ThDP (signal at 8.01 ppm) and the covalent predecarboxylation intermediate analogue PLThDP (signal at 7.31 ppm). The relative integrals of the C6′-H singlets of ThDP and PLThDP are identical. This implies that only one active site of the dimer catalyzed covalent addition of MAP whereas the other did not. Similar results are obtained in case of *Lp*POX and *Ec*AHAS I (Figure 2 of the Supporting Information). Our ITC and NMR data cannot rule out the possibility that the second active site accomplished noncovalent binding of MAP as the heat of this interaction might go undetected in the ITC experiments.

Previous NMR-based analysis of reaction intermediates in the homotetrameric *Lp*POX under steady-state turnover conditions seemed to imply that there is no ASR in this enzyme as we observed that ∼25% of the active sites contain C2-unsubstituted ThDP, ∼25% are occupied with hydroxyethyl-ThDP, and 50% are isolated as lactyl-ThDP.[11,24] When these data are reconsidered in light of the new findings, it seems likely that the two functional dimers of the *Lp*POX tetramer are communicating with one another in addition to the active center crosstalk of each dimer. The NMR analysis of AHAS I is less conclusive in this regard as this enzyme tends to accumulate only a small percentage of covalent intermediates under both steady-state and single-turnover conditions.[25]

Taken together, the ITC and NMR analysis of substrate binding reveals that in all studied ThDP-dependent enzymes only one active site of the functional protein dimer forms a covalent intermediate with pyruvate analogue MAP. Previously published data for the E1 component of human PDHc-E1

Table 1. Thermodynamic Parameters for Binding of MAP to Different ThDP-Dependent Enzymes Using ITC

	N (sites)	K_d (μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)
*Ec*PDH-E1	0.557 ± 0.002	0.13 ± 0.01	−26.74 ± 0.13	1.70
*Ec*AHAS I	0.534 ± 0.056	38.5 ± 7.7	−6.34 ± 0.89	0.03
*Lp*POX[a]	0.486 ± 0.007	5.6 ± 0.4	−14.21 ± 0.26	0.59

[a]In 50 mM MES, 100 μM ThDP, and 1 mM Mg^{2+} (pH 6.0). We observed a higher apparent K_d of 19.0 ± 0.9 μM in 200 mM phosphate, 100 μM ThDP, and 1 mM Mg^{2+} (pH 6.0) (N = 0.523 ± 0.009, ΔH = −12.08 ± 0.30 kcal/mol, and $-T\Delta S$ = 0.47 kcal/mol). As phosphate is a substrate of *Lp*POX, the higher K_d most likely reflects a direct competition between MAP and phosphate for binding to the active site. The specified errors are errors of the nonlinear least-squares fit of a representative experiment.

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produced the same result.[15] Although the PDHc-E1 of any so far published species contains two spatially well separated active sites with one ThDP molecule each, a direct communication pathway exists via a "proton wire" as suggested by Frank and co-workers.[18] In addition, the fact that phosphorylation at only one of the two active sites is sufficient to inactivate the entire enzyme further underpins our finding.[26] Our new results thus support the hypothesis that chemical events in the two active sites of the dimer are indeed synchronized by reversible proton transfer between the two ThDP molecules as a general feature of all ThDP enzymes. ITC turned out to be a valid and valuable method that even allows the detection of ASR in enzymes, for which an active site communication has been excluded so far on the basis of structural data as in the case of *Lp*POX. Reinspection of the structure of *Lp*POX in light of the new findings led to the identification of a potential proton wire that synchronizes action of the two active sites of the functional dimers akin to the mode proposed for PDHc-E1 (Figure 3 of the Supporting Information).[18] Still, there is an apparent discrepancy between functional and structural data because the latter often revealed accumulation of identical intermediates in all active sites. It thus seems likely that the superphysiological substrate or analogue concentrations (50−100 mM) typically used for soaking protein crystals prior to crystal structure analysis "override" the intrinsically encoded negative cooperativity between the active sites observed at physiological substrate concentrations (≤1 mM). Previous analysis of binding of MAP to related ThDP enzymes such as, e.g., *Ec*AHAS II highlighted the fact that the apparent K_d values for both active sites of the functional dimer differ by 3 orders of magnitude (K_d = 2.7 μM for the high-affinity active site, and K_d = 2.6 mM for the low-affinity active site).[17] Thus, after the addition of 50−100 mM MAP, all active sites will contain the covalent MAP−ThDP conjugate despite the fact that there is a chemical nonequivalence between the two active sites. In view of this ambiguity, thermodynamic analysis of substrate binding by ITC and NMR is recommeded as a straightforward and reliable tool for analysis of ASR in ThDP enzymes.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, materials and methods, Figures 1−3, and Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

K.S.-T. and D.M. contributed equally to this work. K.T. and D.M. conceived the work. K.S.-T., D.M., J.A., C.W., M.T., R.G. and K.T. conducted and analyzed experiments. All authors discussed the data, and K.S.-T. and K.T. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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