

The Enamine Intermediate May Not Be Universal to Thiamine Catalysis**

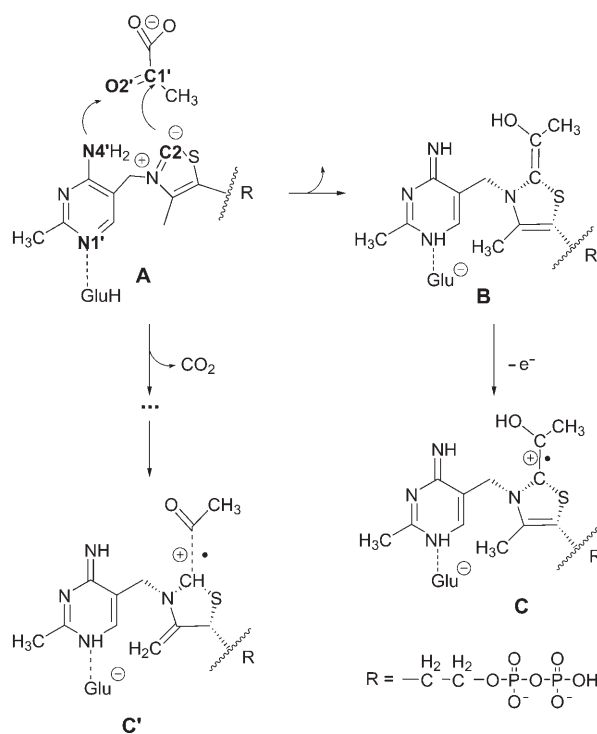
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Pyruvate:ferredoxin oxidoreductases (PFORs)^[1,2] are thiamine diphosphate (ThDP) dependent enzymes.^[3,4] They are responsible for the catabolism of pyruvate ($\text{CH}_3\text{C}(\text{O})\text{CO}_2^-$) by oxidative decarboxylation, whereby the electron carrier ferredoxin (Fd) is reduced, and acetyl-coenzyme A (acetyl-CoA) is formed [Eq. (1)].



Numerous studies on this family of enzymes have led to the following conclusions with respect to the catalytic mechanism (Scheme 1):

- 1) The so-called “V” configuration of the ThDP cofactor imposed by the protein matrix is essential for C2 of the thiazole ring to interact with the N4' atom of the aminopyrimidine group.^[5]
- 2) A conserved glutamate residue is involved in the activation of the ThDP cofactor through a hydrogen-bonding interaction between the carboxylate group of the glutamate residue and the N1' atom of ThDP. The protonation of N1' induces the tautomerization of the pyrimidine ring and thus the transformation of the amino substituent into an imino group. This change increases the basicity of the 4'-position. The proton may then be extracted from the C2 atom by the N4' atom of the ThDP iminopyrimidine prior to substrate binding.^[6,7]
- 3) The ylide intermediate attacks the pyruvate to form a 2- α -carbanion in resonance with the so-called hydroxyethylidene-ThDP (HE-ThDP) or enamine intermediate.
- 4) In the specific case of PFORs, a stable radical species is formed through the transfer of an electron from ThDP to



Scheme 1. Decarboxylation of pyruvate by ThDP in the “V” conformation starting from the ylide in the presence of pyruvate (state A). Upper scheme: Formation of the HE-ThDP (or enamine) intermediate (state B) followed by one-electron oxidation to the HE-ThDP π radical (state C).^[1,5,26] Lower scheme: Formation of a σ/n -type cation radical (state C') by one-electron oxidation with no enamine intermediate.^[11] Atoms mentioned in the manuscript are shown in bold type.

an iron sulfur cluster.^[8] The resulting acetyl-ThDP radical is very stable, except in the presence of CoA, when it decomposes to give acetyl-CoA.

The structure of the native form of PFOR from *Desulfovibrio africanus* (Da) was solved with unreacted pyruvate in the active site.^[9] The same research group later published the structure of the radical intermediate.^[10] Comparison of both X-ray models showed that during the reaction the thiazole ring moves substantially towards the pyruvate, whereas the positions of the acetyl group and CO_2 practically coincide with the initial substrate position. Furthermore, the thiazole ring of the radical species is distorted, which implies a loss of aromaticity, and the bond between the acetyl group and ThDP is long, which suggests that the intermediate is a σ/n -type cation radical. These observations do not agree with a conventional HE-ThDP π radical delocalized over a planar thiazolium ring, but do not exclude the possibility that the

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nonradical form of the enamine is an intermediate in the reaction.^[10]

The presence of an enamine intermediate in PFOR was brought into question recently by a kinetic crystallographic study, which showed that the pyruvate–ThDP distance was not short enough to allow the formation of a double bond between the acetyl group and the thiazole ring in any of the intermediates detected in the experiment.^[11] By contrast, a second recent study of the PFOR from *Moorella thermoacetica* (Mt) by electron paramagnetic resonance (EPR) spectroscopy supported the existence of a HE–ThDP π radical.^[12] The g values obtained from the EPR spectra correspond to spin density delocalized over the thiazolium ring, in agreement with density functional theory (DFT) calculations on models that mimic the active site. The authors concluded that the intermediacy of a σ/n -type cation radical, which would lead to the condensation of an acetyl radical (from PFOR) with a thyl radical (from CoA), is improbable, and that a more likely mechanism would involve attack of the HE–ThDP radical by the thiolate group of CoA, followed by one-electron oxidation to form acetyl-CoA.

The aim of the research described herein was to address the reaction mechanism of PFORs by computational methods. We used hybrid quantum-mechanical (QM)/molecular-mechanical (MM) potentials, which have provided insight into the mechanisms of numerous enzymatic reactions,^[13–15] in conjunction with the nudged elastic band (NEB) method for the determination of minimum-energy reaction pathways (MEPs). In the latter method, the pathway between the reactant and product states is represented as a set of n structures and then optimized until the MEP is attained.^[16] We employed these techniques, as implemented in the DYNAMO simulation program,^[17,18] to search for MEPs for the formation of enamine intermediates in DaPFOR and transketolase (TK) from *Saccharomyces cerevisiae*, another ThDP-dependent enzyme. TK transfers a ketol group from xylulose-5-phosphate to ribose-5-phosphate. It is generally accepted that this reaction involves the formation of an enamine intermediate between ThDP and a donor substrate. β -Hydroxypyruvate can act as a donor substrate for TK, whereby its nonoxidative decarboxylation leads to the formation of an HE–ThDP intermediate, as has been confirmed crystallographically.^[19] The existence of an X-ray model of an enamine intermediate in TK, and the similarities between the substrates in the X-ray studies of PFOR and TK, prompted us to carry out a comparative theoretical study. To determine whether the formation of the enamine in the DaPFOR active site was plausible energetically, we used the same protocol to calculate the energy barriers for the reaction of TK with β -hydroxypyruvate to form the enamine intermediate, and for the equivalent reaction of DaPFOR with pyruvate.

Models of PFOR complexed with (pyruvate+ThDP), (pyruvate+ThDP ylide), and (enamine+CO₂) were constructed from the available X-ray crystal data^[9,10] (see the Supporting Information). The system was partitioned into QM and MM regions. In the active site of PFOR, the ThDP cofactor, the side chain of residues N996 and E64, and the pyruvate (or the enamine+CO₂) were treated with the

PDDG/PM3 semiempirical method,^[20] whereas the rest of the protein matrix was described by the OPLS MM force field.^[21] Owing to the central role of the interaction between N1' of ThDP and the carboxylate group of E64, we placed the proton on either N1' (model named E64) or on one of the carboxylate oxygen atoms of E64 (model named E64P). The six models were geometry optimized on the hybrid QM/MM potential surface. Whereas the optimized structures of the (pyruvate+ThDP) and (pyruvate+ThDP ylide) complexes are similar to the native X-ray crystal structure of the active site of PFOR, some structural rearrangements were observed in the active site of the (enamine+CO₂) complex, as we started our calculations from the substantially different radical X-ray crystal structure (see Figure 1 a).

These optimized structures were the starting points for determining MEPs for the formation of the carbanionic form of ThDP from ThDP in the presence of pyruvate and for the formation of the enamine intermediate from the ylide. The NEB algorithm implemented in DYNAMO^[18] searches for the MEP from a set of structures interpolated between reactant and product structures, which are held fixed (see the Supporting Information for details of the method). Depending on the protonation state of E64, the ylide forms at no significant energy cost, with an activation energy that ranges from 4 to 30 kJ mol^{−1}. NEB pathways from the ylide to the enamine on the PDDG/MM potential-energy surface are shown in Figure 1 b for different E64 models. The activation energy, which depends on the protonation model, ranges from 150 to 180 kJ mol^{−1}. These values were corrected by higher level QM methods (see the Supporting Information), and the corrections confirmed the barrier heights for formation of the product. The high barriers arise from steric hindrance that occurs as the thiazole moiety and pyruvate approach one another when the double bond between the acetyl group and ThDP is formed. This movement of pyruvate is not observed in the crystal structures (see Figure 1 a).^[11]

By using the protocol applied to PFOR, we searched for MEPs for the formation of the enamine intermediate in the active site of TK. In contrast to the results obtained for DaPFOR, the superposition of the X-ray crystal structures of native TK and the corresponding enamine intermediate reveals no significant differences between the two structures.^[19] The β -hydroxyacetyl moiety moves towards the thiazole ring, which remains essentially in its initial position (Figure 2). As there was no available X-ray crystal structure of the native structure with the β -hydroxypyruvate substrate, we manually docked the substrate in the active site of TK. Site-directed mutagenesis experiments have shown that several histidine residues that are in direct contact with the enamine and the ThDP cofactor^[19] are important for catalysis.^[22] Accordingly, we tested different combinations of protonation states (see the Supporting Information for details) and found that the activation energy for enamine formation is highly dependent on the protonation states of the histidine residues. The activation energy of models in which histidine residues (103, 263, 481b), (69, 103, 263, 481b), (30, 69, 481b), and (69, 103, 263) are doubly protonated ranges from 10 to 80 kJ mol^{−1}. The formation of the enamine double bond in TK is clearly favored with respect to the equivalent

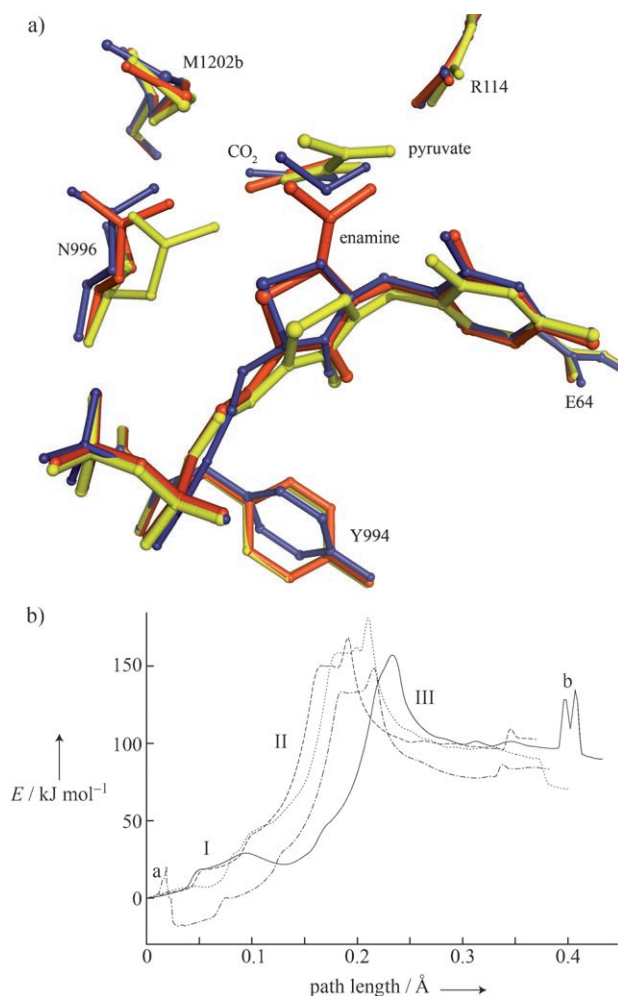


Figure 1. a) Active site of PFOR: Superposition of the optimized structure of the pyruvate+ThDP ylide/E64P model (in green) with the optimized structure of the enamine+CO₂/E64 model (in red) and the X-ray crystal structure of the radical PFOR^[10] (in blue). b) Energy profiles of the minimum-energy pathways to form the enamine intermediate in PFOR for ylide/enamine models E64P/E64 (see the Supporting Information), E64P/E64P, E64/E64, and E64/E64P, as represented by solid, dashed, dotted, and dash-dotted lines, respectively. The small energy barriers a and b correspond to the protonation (deprotonation) of E64. For all pathways, I) N996 moves away from the thiazole sulfur atom towards the sulfur atom of M1202b, II) the C2 atom of the thiazole ring and the C1' atom of pyruvate come closer to one another, and III) the enamine double bond is formed, and decarboxylation occurs, while (de)protonation of E64 takes place with no significant energy cost.

process in PFOR and requires minimal structural rearrangements in the active site (see the Supporting Information). Interestingly, the final protonation of the enamine requires a significant amount of energy, which suggests that the proton does not come from the N4' atom of ThDP but from a histidine side chain. In the X-ray crystal structure of the TK enamine, the C1'–C2 and C1'–O2' distances, both characteristic of double bonds (1.27 and 1.25 Å, respectively), cast doubt on the nature of these bonds.^[19]

Taken together, our results suggest that the enamine is not an intermediate in *Da*PFOR catalysis, as already hinted at by

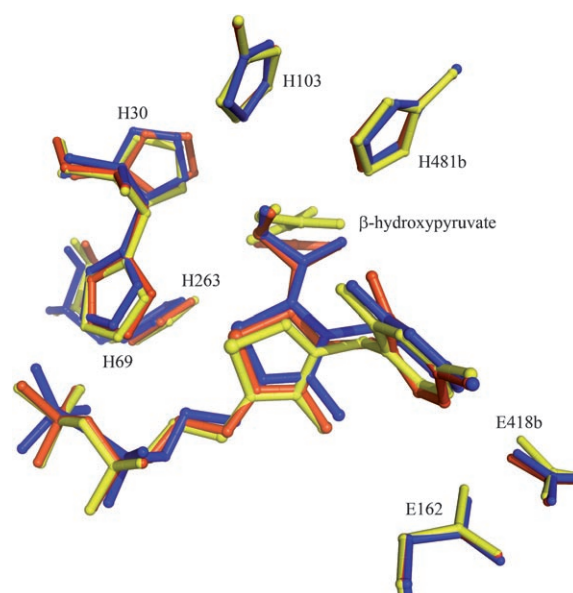


Figure 2. Active site of transketolase: Superposition of the optimized structure of the β-hydroxypyruvate+ThDP ylide/E418bP model (in green) with the optimized structure of the enamine+CO₂/E418b model (in red) and the X-ray crystal structure of the enamine^[19] (in blue).

experiment.^[11] On the other hand, they suggest that enamine formation is favored in the reaction of TK with β-hydroxypyruvate, in agreement with the corresponding X-ray crystal structure.^[19] However, as a recent EPR study of *Mt*PFOR^[12] implied the presence of a π radical HE–ThDP, there is still controversy concerning the reaction mechanism of PFORs. Although DFT calculations on model active sites indicated a spin delocalization on the thiazole ring, these results need to be confirmed in models in which the pyrimidine moiety of the ThDP is included, as well as the protein matrix. Furthermore, the determination of the crystallographic structure of *Mt*PFOR could reveal some differences to *Da*PFOR and help explain the contradictory results. Indeed, there are some evident differences between *Mt*PFOR and *Da*PFOR with respect to the strength of ThDP binding, which is much stronger in *Da*PFOR,^[12,23] and their catalytic activity: The reduction of a Fe/S cluster by CoA was observed in *Mt*PFOR^[24] but not in *Da*PFOR.^[25] Finally, the controversial X-ray crystal structure of the radical species in *Da*PFOR still needs to be rationalized. MEP searches are under way to try to evaluate the energy cost for the formation of a σ/n-type acetyl cation radical in *Da*PFOR versus the formation of a π-type HE–ThDP radical delocalized on the thiazole ring.

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