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Activation of thiamine diphosphate in pyruvate decarboxylase from *Zymomonas mobilis*

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Abstract Replacement of tryptophan 392 located in the active site cavity of pyruvate decarboxylase (PDC; EC 4.1.1.1) from *Zymomonas mobilis* by methionine or glutamine yields enzymes with smaller catalytic constants of 8.5 s^{-1} and 3.6 s^{-1} at 4°C, compared to that of the wild-type enzyme (17 s^{-1}). The rate constants of the H/D exchange at the C2 of the coenzyme thiamine diphosphate have been determined to be 130 s^{-1} for the wild-type enzyme, 56 s^{-1} for the methionine and 30 s^{-1} for the glutamine mutant, respectively. A group with a pK_a of about 5 has been identified to be essential for C2 deprotonation of the enzyme-bound thiamine diphosphate from the pH dependence of the H/D exchange.

Key words: Thiamine diphosphate; Pyruvate decarboxylase

1. Introduction

The thiamine diphosphate (ThDP)-dependent enzyme pyruvate decarboxylase (PDC) catalyzes the conversion of pyruvate to acetaldehyde and CO_2. The enzyme from *Zymomonas mobilis* tightly binds the coenzyme at pH values below 8.0 forming a 240 kDa tetrameric enzyme, which contains four ThDP/Mg^{2+}. In order to react with the substrate, the C2-H of ThDP, showing a pK_a of 17–20 [1–5] in the free coenzyme, must be activated by the protein component. A key function for this activation is the interaction of a conserved glutamate [6–11] with the N1′ atom of the coenzyme, resulting in an increased basicity of its 4′-amino group facilitating the deprotonation of the C2 [12]. The allosteric yeast PDC requires the reaction with an activator for the catalytic activity and also a fast deprotonation of the C2 [12]. The pyruvate oxidase from *Lactobacillus plantarum* requires the additional binding of the second coenzyme FAD [13]. These results hint at a sensitivity of the proton translocation mechanism as a result of structural changes induced by the binding of ligands.

In this work we investigated (i) whether a mutation at a side chain, which cannot directly interact with the coenzyme but is able to stimulate the activity of the enzyme, influences the deprotonation rate of the C2 of the coenzyme, and (ii) whether the reduced activity results from a rate-limiting deprotonation. It is known from the literature that a mutation at W392 in PDC from *Z. mobilis* results in a reduced enzymatic activity and, in some cases, in a reduced stability of the cofactor binding [14,15], although this side chain is at a distance of about 9 Å from the C2 of ThDP [16]. The W392M and the W392E mutants were selected for the H/D exchange experiments from the mutants already described, because of the significant differences in their enzymatic activities and the stable cofactor binding, which is a prerequisite for the determination of the deprotonation rate by H/D exchange experiments.

(iii) Additionally, we expected evidence concerning the deprotonation mechanism of the C2 of the coenzyme from the pH dependence of the deprotonation rate.

2. Materials and methods

2.1. Preparation of the mutant enzymes

Mutants of PDC from *Z. mobilis* were produced by PCR as described elsewhere [15] using pPDC-His_6 as a template.

Protein expression was performed as described in [15] using a Ni^{2+}-NTA-agarose matrix (Qiagen). The column was equilibrated with 50 mM MES/KOH, pH 6.8. The same buffer was used to remove unbound proteins in a first washing step. Subsequently, weakly bound proteins were eluted with 50 mM MES/20 mM imidazole, pH 7.0. The elution of the His-tagged enzyme was achieved by 250 mM imidazole in 50 mM MES, pH 7.6. The eluted enzyme was free of unspecifically bound ThDP. Imidazole was removed by a desalting step on a PD 10 column (Pharmacia, Biotech), which was equilibrated with 10 mM MES/KOH, pH 6.5.

2.2. H/D exchange experiments

The exchange reactions were initiated by dilution of a sample containing 10 mg/ml of the wild-type enzyme or 7 mg/ml of the mutant enzymes in 0.1 M sodium phosphate buffer (pH 6.0 for investigations of the mutant enzymes, or pH 4.2–6.0 for the determination of the pH dependence of the exchange rates) with D_2O at a 1:1 ratio in a quenched-flow apparatus (Model RQF-3, Kin Tek Althouse, USA). All pH values refer to the respective pH meter reading. The sample solutions for the determination of the pH dependence of the H/D exchange contained an additional 0.4 M ammonium sulfate to stabilize the enzyme at low pH values. The exchange reactions were stopped by addition of DCl and trichloroacetic acid to final concentrations of 0.1 M and 5%, respectively. This procedure rapidly and completely denatures and precipitates the protein and the coenzyme is released. All reactions were carried out at 4°C. After separation of the denatured protein by centrifugation, the ^{1}H-NMR spectra of the supernatant containing the ThDP only were recorded in a 5 mm NMR tube on a Bruker ARX 500-MHz NMR spectrometer.

3. Results and discussion

The H/D exchange of the enzyme-bound ThDP was determined by measuring the time-dependent decay of the C2-H signal (9.68 ppm) of ThDP resulting from the C2-H/D exchange of the coenzyme in the enzyme (Fig. 1). The C6′-H (8.01 ppm) was used as a non-exchanging internal standard for quantification. As expected, the time-dependent decay in

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Abbreviations: PDC, pyruvate decarboxylase; ThDP, thiamine diphosphate

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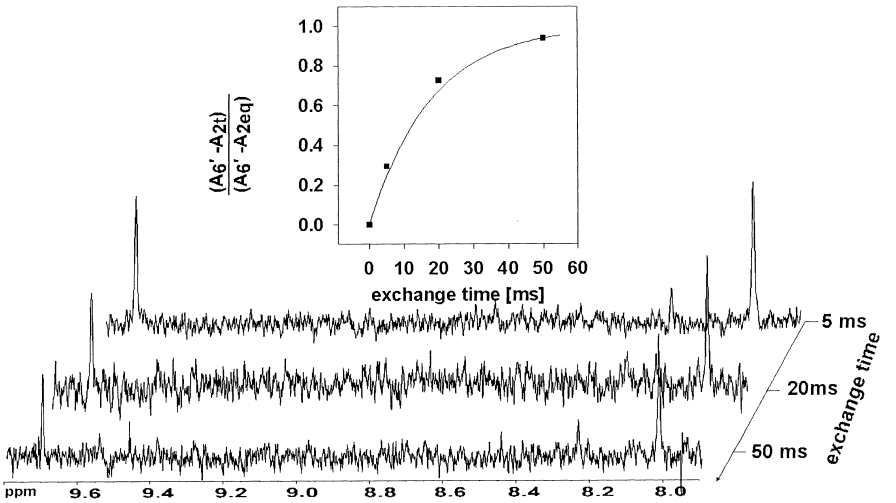


Fig. 1. Kinetics of H/D exchange of the ThDP C2-H in the W392M mutant of PDC from *Z. mobilis*. The ^{1}H-NMR spectra are extensions showing the ThDP signals C2-H (9.68 ppm) and C6′-H (8.01 ppm), the latter serving as a non-exchanging standard for quantification. Inset: Fit of the decay in relative integral intensities of the C2-H signal to a pseudo first-order reaction, where A_{2t} is the integral of the signal of the C2 proton at time t, A_{2eq} after complete exchange in 50% H_2O/50% D_2O, and $A_{6'}$ that of the C6′ proton.

the integral intensity of the C2-H signal can be fitted to a pseudo first-order reaction (inset of Fig. 1). The mutations at the side chain W392 influence not only the k_{cat} values, but also the H/D exchange rates of the C2 of ThDP (Table 1). The rate constant of the H/D exchange is above the catalytic constant of each active site in all mutants investigated and cannot be involved in rate-limiting steps of the overall reaction. Therefore, the good correlation in the tendency of the k_{cat} values and the H/D exchange rates is surprising (Table 1, column 3). We assume that the proton relay system is not only involved in the C2 deprotonation, but also in the partial rate-limiting acetaldehyde release [17], which requires the deprotonation of the hydroxyl group of the reaction intermediate 2-hydroxyethyl-ThDP (Scheme 1). This assumption is supported by the fact that an inverse ^{15}N isotope effect in k_{cat} has been found for the 4′-NH_2 group of the enzyme-bound ThDP [18]. Providing the efficiency of the proton relay system in the acetaldehyde release is dependent on the same prerequisites as in the deprotonation step of the C2, the observed correlation between the rate constants of the H/D exchange and the catalytic constants of the mutants and wild-type enzyme can be expected.

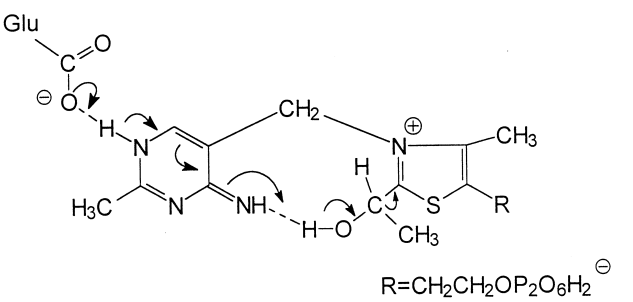
Investigations on yeast PDC, transketolase and their mutants and also on enzyme complexes containing ThDP analogues have shown an interaction of a conserved glutamate with the N1′ of the pyrimidine ring to be essential for a fast deprotonation of the C2 of the enzyme-bound ThDP [12]. PDC from *Z. mobilis* contains this glutamate at position 50 [19]. The mutation of the conserved glutamate 50 to aspartate yields an enzyme that has an activity less than 3% of the wild-type enzyme [19], showing the distance of the N1′ atom to the carboxyl group at this position to be important for catalysis. The differences in the deprotonation rate of the C2 of the coenzyme in the wild-type enzyme and the W392E mutant enzyme correlate with a difference in the free energy of activation of about 4 kJ/mol only. The different rate constants of the H/D exchange of the mutant enzymes may be discussed in terms of small structural changes influencing the interaction of the coenzyme with the protein component and, therefore, the efficiency of the proton relay system in ThDP catalysis.

In some cases, the mutations at position 392 influence the interaction of the coenzyme with the protein component in such a extent way that a reduced stability of the coenzyme binding results. Thus, the coenzyme dissociates from the holoenzyme in both mutant enzymes PDCW392G and PDCW392H with a half-life of 10 min and 2 min, respectively, in the absence of excess ThDP (data not shown).

In contrast to PDC from yeast, *Z. mobilis* enzyme has a sufficient stability in the presence of 0.2 M ammonium sulfate even at pH values below 4.5, allowing the determination of the H/D exchange at the C2 of ThDP over a wide pH range. In view of the role of the glutamate 50-N1′ interaction in C2 deprotonation, it is of interest to examine the pH dependence of the H/D exchange at the C2 of the enzyme-bound ThDP. As shown in Fig. 2, the rate constant of the H/D exchange increases with increasing pH and reaches a constant value at a pH of about 5–6. This result indicates that a group with a pK_a

Table 1
Pseudo first-order rate constants of the C2-H/D exchange in enzyme-bound ThDP ($k_{H/D}$) and catalytic constants (k_{cat}) for each active site of the PDC variants at pH 6.0 and 4°C

Sample	$k_{H/D}$ (s^{-1})	k_{cat} (s^{-1})	$k_{H/D}/k_{cat}$
Wild-type	130 ± 20	17	7.6 ± 1.2
W392M	56 ± 11	8.5	6.5 ± 1.3
W392E	30 ± 5	3.6	8.3 ± 1.4



Scheme 1.

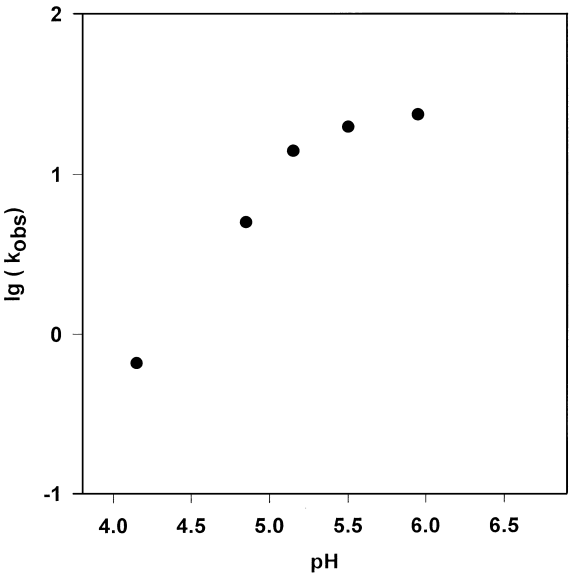


Fig. 2. Effect of pH on the rate constant of the H/D exchange of C2-H (k_{obs}) in PDC from *Z. mobilis*. Values were determined in 0.1 M sodium phosphate buffer containing 0.2 M ammonium sulfate at 4°C.

of about 5 in the dissociated form is very likely involved in the deprotonation of the C2. The conserved glutamate as well as the N1′ of ThDP may be good candidates for this function.

Based on structural data [7,11], it has been proposed that in the enzyme the aminopyrimidine ring exists in the iminotautomeric form (Scheme 2). Starting from this state of the enzyme-bound ThDP, a deprotonation of the N1′ atom by glutamate may enhance the basicity of the 4′-nitrogen, enabling the deprotonation of the C2 of ThDP. The determined pK_a of about 5 and the corresponding pK_a of 4.93 of the N1′ in ThDP [20] may facilitate a proton transfer between the N1′ of the enzyme-bound ThDP and the γ-carboxylate of Glu-50. In addition, it has been shown by quantum mechanics calculations [21] that the deprotonation of the iminotautomeric form of the coenzyme produces a species showing a proton affinity comparable with that of the 2-carbanion of the coenzyme. Therefore, the deprotonated iminotautomeric form would be able to deprotonate the C2 of ThDP.

In order to obtain further insight into this proton translocation mechanism ^{15}N-NMR experiments with ^{15}N-labelled ThDP are in progress.

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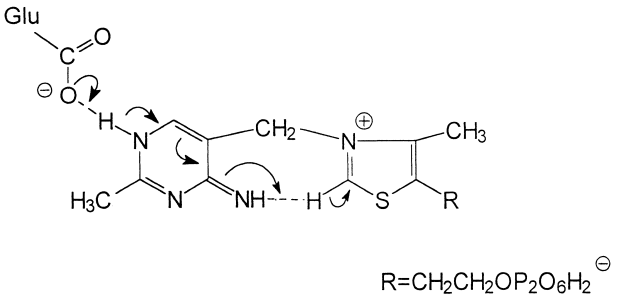

Scheme 2.

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