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Activation of thiamin diphosphate in enzymes[1]

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Abstract

Activation of the coenzyme ThDP was studied by measuring the kinetics of deprotonation at the C2 carbon of thiamin diphosphate in the enzymes pyruvate decarboxylase, transketolase, pyruvate dehydrogenase complex, pyruvate oxidase, in site-specific mutant enzymes and in enzyme complexes containing coenzyme analogues by proton/deuterium exchange detected by ^{1}H-NMR spectroscopy. The respective deprotonation rate constant is above the catalytic constant in all enzymes investigated. The fast deprotonation requires the presence of an activator in pyruvate decarboxylase from yeast, showing the allosteric regulation of this enzyme to be accomplished by an increase in the C2-H dissociation rate of the enzyme-bound thiamin diphosphate. The data of the thiamin diphosphate analogues and of the mutant enzymes show the N1′ atom and the 4′-NH_2 group to be essential for the activation of the coenzyme and a conserved glutamate involved in the proton abstraction mechanism of the enzyme-bound thiamin diphosphate.

Keywords: Pyruvate decarboxylase; Pyruvate dehydrogenase multienzyme complex; Pyruvate oxidase; Regulation; Thiamin diphosphate; Transketolase

Contents

Abbreviations: DAThDP, 4′-desamino-ThDP; 5-dFAD, 5-carba-5-deaza-FAD; DTE, dithioerythreitol; N3ThDP, N1′-C substituted ThDP; PDC, pyruvate decarboxylase (EC 4.1.1.1); PDHc, *E. coli* pyruvate dehydrogenase multienzyme complex, consisting of E1, E2 and E3 proteins; POX, pyruvate oxidase from *L. plantarum* (EC 1.2.3.3); ThDP, thiamin diphosphate; TK, transketolase (EC 2.2.1.1)

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[1] Dedicated to Prof. Dr. Alfred Schellenberger on his 70th birthday.

1. Introduction

The deprotonation of the C2 atom of thiamin diphosphate (ThDP) is the key reaction in all ThDP dependent enzymes. For the reaction with different substrates, the C2-H of ThDP, showing a pK_a of 17–20 [1–5], must be activated by the enzyme environment. Different models have been discussed for this activation. In a first model, a stabilization of the C2-carbanion in the enzyme-bound state was proposed [2]. In contrast, a fast dissociation of the C2 proton in the enzyme-bound state of ThDP could also explain the rate of the enzyme mediated reaction [6,7]. In a third model, a concerted pathway for the addition step of the substrate has been discussed [8,9]. ^{13}C-NMR investigations on pyruvate decarboxylase (PDC) containing ^{13}C2 labeled ThDP exclude the existence of a C2-carbanion of ThDP in detectable amounts [10]. Therefore, in the enzyme catalyzed reaction, the addition of the carbonyl group of the substrate to the C2 of ThDP requires either a fast dissociation of the C2 proton or a concerted mechanism.

In order to address this question, the kinetics of the deprotonation of C2 of ThDP in the enzymes PDC, transketolase (TK), pyruvate oxidase (POX) and in the pyruvate dehydrogenase multienzyme complex (PDHc) have been analyzed by ^{1}H-NMR spectroscopy.

2. Materials and methods

2.1. Protein preparation

Yeast PDC was purified according to the method of Lu et al. [11]. Mutagenesis and expression of the PDC E51Q mutant was performed according to the method of Killenberg-Jabs et al. [12].

PDC from *Zymomonas mobilis* was purified from *Escherichia coli* SG 13009 prep 4 containing the plasmid of *Z. mobilis* PDC [13]. The cells were disintegrated in 50 mM MES/NaOH, pH 6.5 using a French Press (SLM Instruments). The buffer contained 0.1 mM ThDP and 5 mM $MgSO_4$ for enzyme stabilization. The crude extract was centrifuged in a Beckman L8-60M centrifuge at $125\,000 \times g$ for 45 min at 5°C. A 4-fold excess of disintegration buffer was added to the supernatant and a subsequent fractionated precipitation with ammonium sulfate in the range of 30% (w/v) and 42% (w/v) was performed. Afterwards, the solution was centrifuged at $125\,000 \times g$ and 5°C. The pellet of the second step of precipitation was resuspended in 10 ml of deionized water and dialyzed against 10 mM MES/NaOH, pH 6.5, containing 1 mM $MgSO_4$, 0.2 mM ThDP and 1 mM DTE. Anion-exchange chromatography using a FPLC system (Pharmacia) was carried out on a TMAE(S) (Merck) column (2.6 cm × 10 cm), equilibrated with 10 mM MES/NaOH, pH 6.5, containing 1 mM $MgSO_4$, 0.1 mM ThDP and 1 mM DTE. The dialysate was loaded on the column at a flow rate of 1 ml/min. The enzyme (120 U/mg) was eluted by a stepwise increase in ammonium sulfate concentration up to 40 mM.

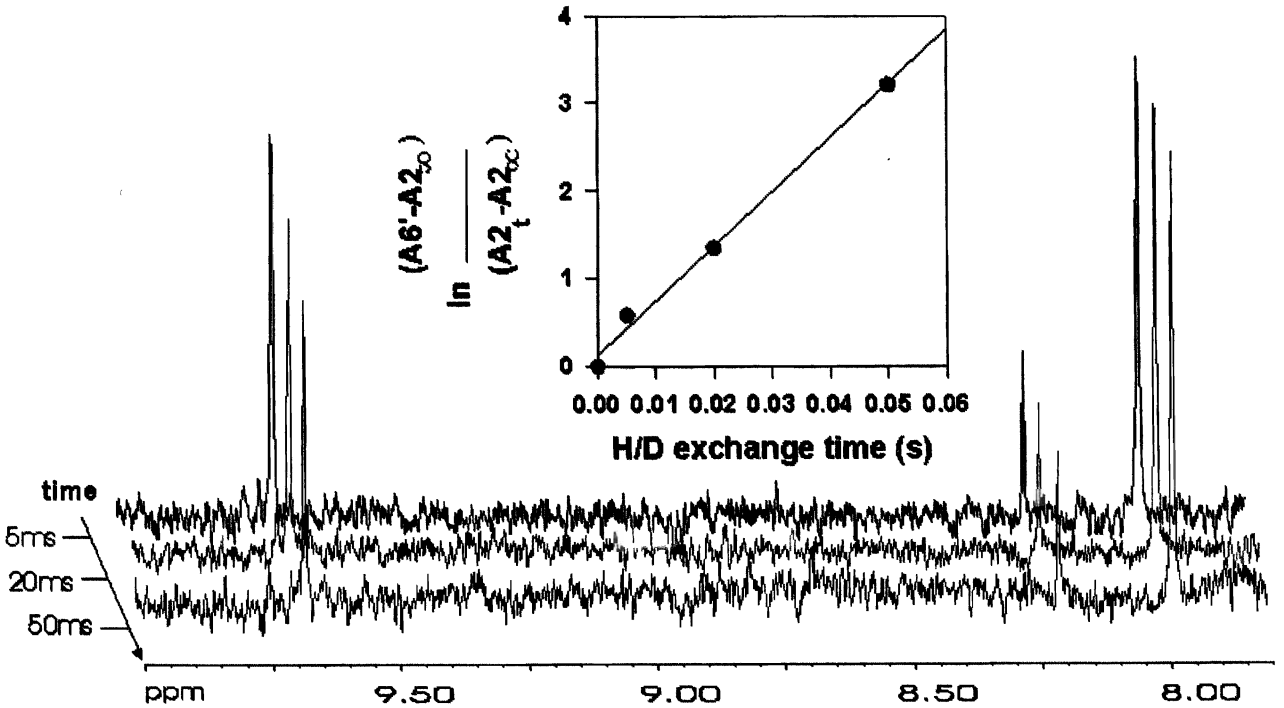

Fig. 1. Kinetics of H/D exchange of ThDP C2-H in TK. The ^{1}H-NMR spectra are expansions showing the ThDP signals C2-H (9.68 ppm) and C6′-H (8.01 ppm), the latter serving as a non-exchanging standard for quantification. Inset: fit of the decay in integral intensity of the C2-H signal to a pseudo-first-order reaction, where $A2_t$ is the integral of the signal of the C2 proton at time t, $A2_\infty$ is that of the C2 proton after complete exchange, and A6′ is that of the C6′ proton.

TK was purified according to the method of König et al. [14]. Mutagenesis and expression of the TK H418A and H481A mutants were carried out as described [15].

Highly purified POX from *L. plantarum* was a gift from Boehringer Mannheim. Apo-POX was prepared according to a method established by Strittmatter [16] and Sedewitz [17]. The binary apo-ThDP complex was prepared by incubation of apo-POX (5 mg/ml) with ThDP in a stoichiometric ratio in 0.2 M potassium phosphate buffer, 20% glycerol, pH 6.0, 1 mM $MnSO_4$ for 1 h at 10°C.

PDHc was isolated from the wild type strain Y mel of *E. coli* K12 and purified as described [18].

2.2. Coenzyme analogues

4′-Desamino-ThDP (DAThDP) was synthesized according to the method of Neef et al. [19], N1′-C substituted ThDP (N3ThDP) according to the method of Schellenberger et al. [20] and 5-carba-5-deaza-FAD (5-dFAD) according to the methods of Spencer et al. [21] and Manstein et al. [22].

2.3. NMR experiments

The kinetics of the H/D exchange of the C2-H of ThDP were measured by ^{1}H-NMR as described recently [10]. In order to obtain the exchange rate, the relative decay in the integral intensity of the C2-H signal at 9.68 ppm was fitted to a pseudo-first-order reaction (Fig. 1). The signal of the C6′ proton at 8.01 ppm was used as a non-exchanging internal standard. These signals do not interfere with the signals of FAD in POX and PDHc as shown in Fig. 2.

3. Results and discussion

3.1. Deprotonation rate of the C2 of ThDP in pyruvate decarboxylase

The H/D exchange rate of C2-H of the enzyme-bound ThDP is accelerated by three orders of magnitude compared with that of free ThDP under the same conditions (Table 1). However, this rate constant is still one order of magnitude too small to

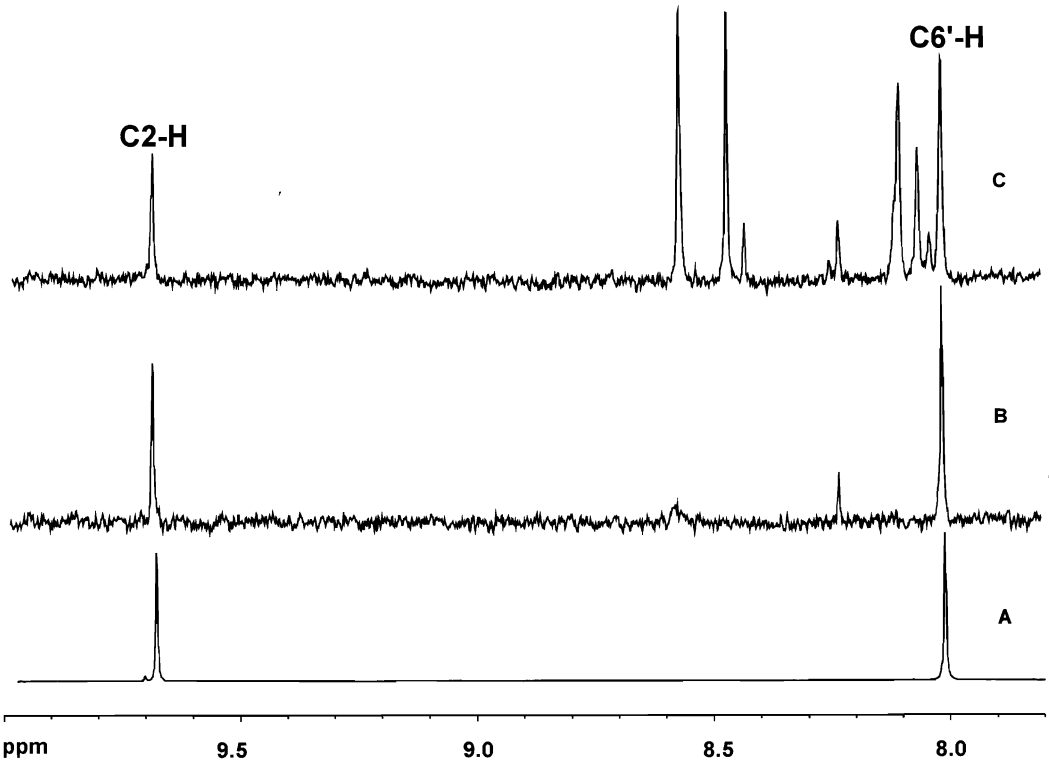

Fig. 2. ^{1}H-NMR spectra for the determination of exchange rates. Supernatants obtained upon centrifugation of the solutions from the quenched-flow experiments contained 2 mM ThDP (A), 6.5 mg/ml apo-POX-ThDP complex (B) or 9 mg/ml POX holoenzyme (C), respectively. The exchange times used were 5 min for ThDP (30% exchange) and for the apo-POX-ThDP complex (95% exchange) and 50 ms for the POX holoenzyme (100% exchange).

allow the enzyme catalysis to proceed at the observed catalytic constant of 10 s^{-1} at 4°C for each active site. We also measured the kinetics of H/D exchange for C2-H of ThDP in the pyruvamide activated PDC, because this k_{cat} value represents the rate constant in the activated state. The exchange reaction has already finished within the shortest mixing time of the quenched-flow device, resulting in a deprotonation rate at least three orders of magnitude higher than that of the non-activated enzyme (Table 1). The high exchange rate cannot be attributed to a pyruvate contamination of pyruvamide, because PDC was preincubated with pyruvamide before the H/D exchange was started. This shows that the C2-H dissociation is not rate limiting in activated yeast PDC. Therefore, the activation process in yeast PDC is

Table 1
Pseudo-first-order rate constants of deprotonation of C2 of thiamin diphosphate in free and pyruvate decarboxylase-bound coenzyme in 50 mM phosphate buffer, pH 6.0, at 4°C

Sample	Rate constant (s^{-1})
Free thiamin diphosphate	$(9.5 \pm 0.4) \times 10^{-4}$
Free 4′-desamino-thiamin diphosphate	$(1.2 \pm 0.1) \times 10^{-3}$
Yeast pyruvate decarboxylase (wild type)	$(9.7 \pm 0.9) \times 10^{-1}$
Yeast pyruvate decarboxylase (wild type, pyruvamide activated)	$> 6 \times 10^{2}$
Yeast pyruvate decarboxylase E51Q mutant	$(7.6 \pm 0.6) \times 10^{-2}$
Yeast pyruvate decarboxylase E51Q (pyruvamide activated)	1.7 ± 0.2
Z. mobilis pyruvate decarboxylase	$(1.1 \pm 0.2) \times 10^{2}$
Yeast pyruvate decarboxylase recombined with 4′-desamino-thiamin diphosphate	$(3.4 \pm 0.1) \times 10^{-5}$

accomplished by an increase in the H/D exchange rate of C2-H of the enzyme-bound ThDP. This model was substantiated by measuring the H/D exchange of C2-H of ThDP in PDC from *Z. mobilis*, an enzyme without substrate activation [23]. As expected, the H/D exchange rate in *Z. mobilis* PDC is above its k_{cat} of 17 s^{-1} at 4°C for each active site (Table 1) and is not changed in the presence of pyruvamide.

It must be noticed that the exchange rate reflects not only the deprotonation rate but also the required exchange of the base with a source of solvent protons. Therefore, the faster exchange in pyruvamide activated yeast PDC could be the result of either a higher C2-H dissociation rate compared to that of the non-activated yeast PDC, or a better solvent accessibility of the base involved in the C2-H proton abstraction.

The crystal structure of PDC from yeast [24,25] shows that the side chain of a glutamate is within a short distance of the N1′ nitrogen of the pyrimidine ring of ThDP, indicating the formation of a hydrogen bond.

Studies involving ThDP analogues in various ThDP dependent enzymes point to a requirement of the N1′ atom and 4′-NH_2 group for the catalytic activity [26–28]. On the basis of these findings, it has been proposed that the N1′ nitrogen enables the 4′-amino group to react in a proton translocation step [27–33].

In order to address this hypothesis, first this glutamate in PDC was altered to glutamine and, additionally, the 4′-amino group of the coenzyme was eliminated. The E51Q mutant enzyme binds ThDP as strongly as the wild type enzyme, as shown by characteristic changes in the near UV circular dichroism [12]. But the residual catalytic activity of the mutant was only 0.04% of that measured for the wild type enzyme. The slow dissociation rate of the C2-H of ThDP in the yeast PDC E51Q mutant (Table 1) suggests this glutamate to be indeed involved in the proton abstraction mechanism of the enzyme-bound ThDP. In addition, a comparatively small increase in the deprotonation rate by pyruvamide activation in the mutant PDC emphasizes that the signal transfer from the regulatory to the active site is probably mediated by E51. The structural events responsible for this step remain to be clarified.

PDC was recombined with DAThDP to unravel the function of the 4′-amino group of the coenzyme. This modification of the coenzyme results in an inactive enzyme and in a markedly decreased H/D exchange rate of C2-H of the analogue compared with the enzyme containing the natural coenzyme (Table 1). This refers to an essential function of the 4′-amino group in the deprotonation step.

3.2. *Deprotonation rate of the C2 of ThDP in transketolase from yeast*

We investigated whether the mechanism of ThDP activation is a common phenomenon in other ThDP dependent enzymes. H/D exchange experiments were performed in TK that has a different substrate and reaction specificity. As observed for PDC, mentioned above, neither the C2 deprotonation (Table 2) is rate limiting. The crystal structure of TK [34,35] shows that an important interaction is made by the N1′ of the pyrimidine ring, which is hydrogen bonded to the side chain of glutamate 418. The mutation of this glutamate to alanine results in an enzyme with only

Table 2
Pseudo-first-order rate constant of deprotonation of C2 of thiamin diphosphate in free and transketolase-bound coenzyme in 50 mM phosphate buffer, pH 7.0, at 4°C

Sample	Rate constant (s^{-1})
Free thiamin diphosphate	$(3.0 \pm 0.1) \times 10^{-3}$
Free 4′-desamino-thiamin diphosphate	$(3.2 \pm 0.1) \times 10^{-3}$
Free N1′C-substituted thiamin diphosphate	$(1.6 \pm 0.1) \times 10^{-4}$
Transketolase (wild type)	61 ± 2
Transketolase E418A mutant	$(3.7 \pm 0.1) \times 10^{-1}$
Transketolase H481A mutant	61 ± 2
Transketolase recombined with 4′-desamino-thiamin diphosphate	$(9.5 \pm 0.1) \times 10^{-5}$
Transketolase recombined with N1′-C-substituted thiamin diphosphate	$(1.6 \pm 0.2) \times 10^{-4}$

0.1% of the activity measured for the wild type enzyme and a slow H/D exchange rate (Table 2). In order to unravel the function of both the 4′-amino group and the N1′ of the coenzyme in TK, the apoenzyme was recombined with either the DAThDP or the N3ThDP analogue. Both modifications of ThDP result in inactive enzymes and in a markedly decreased H/D exchange rate of C2-H compared with the enzyme containing the natural coenzyme (Table 2). Structural changes of the corresponding holoenzyme complexes containing the analogues were not detectable by X-ray crystallography [36]. This establishes the essential function of both the 4′-amino group and the N1′ in the deprotonation step. It has been argued that neither the glutamate-N1′ interaction, nor the 4′-amino group contribute significantly to the activation of ThDP, but rather a base in the active site deprotonates the C2 [8,9]. The mutation of the closest base to C2 of ThDP in TK, H481A, does not alter the rate of deprotonation (Table 2). Therefore, a mechanism assuming histidine 481 as the base for C2 proton abstraction [34] can be ruled out.

Identical coenzyme binding and no structural changes of the protein component are detectable for the mutant enzymes and the complexes of TK with the coenzyme analogues by X-ray crystallography [36,37]. Therefore, in the case of TK it can be ruled out so far that the differences in the H/D exchange rates are a result of a different solvent accessibility of a base involved in the proton abstraction mechanism of ThDP.

3.3. *Deprotonation rate of the C2 of ThDP in the pyruvate dehydrogenase multienzyme complex from E. coli*

In the PDHc, the ThDP containing E1 component catalyzes the rate limiting step of the overall reaction [38,39] and, therefore, presents an ideal target for regulation. The H/D exchange experiments were carried out at a protein concentration of 20 mg PDHc/ml and in the presence of equimolar amounts of ThDP (100 μM) because of the reversible binding of ThDP to the complex. A small amount of free ThDP with a very slow exchange rate (3×10^{-3} s^{-1} at pH 7.0 and 4°C) exists in the reaction mixture under these conditions. Therefore, the ratio of the integral intensities of the C2-H signal and the C6′-H signal after completion of the exchange at C2 of the enzyme-bound ThDP in D_2O/H_2O at a 1:1 ratio differs from the expected value of 1:2. This difference was considered in the calculation of the pseudo-first-order rate constant of the C2-H deprotonation of ThDP in the PDHc (16 ± 5 s^{-1}). This value is above the catalytic constant of 2 s^{-1} at pH 7.0 and 4°C for each active site of the E1 component.

3.4. *Deprotonation rate of the C2 of ThDP in the phosphate dependent pyruvate oxidase from L. plantarum*

The POX holoenzyme catalyzes the oxidative decarboxylation of pyruvate under formation of acetylphosphate, CO_2 and H_2O_2 in the presence of oxygen and phosphate [40–42]. Each subunit of the homotetrameric POX binds one FAD and one ThDP in the presence of Mn^{2+} or Mg^{2+}. In the presence of these ions, either FAD or ThDP can form binary complexes with the apoenzyme [43]. The binary complexes, however, are enzymatically inactive in the native overall oxidation reaction. The deprotonation of the C2-H of ThDP is an important catalytic step in the POX reaction. The deprotonation of the C2-H of ThDP in the POX holoenzyme as well as in the binary apo-ThDP complex was investigated to study

Table 3
Pseudo-first-order rate constant of deprotonation of C2 of thiamin diphosphate in free and pyruvate oxidase-bound coenzyme at pH 6.0 and 4°C

Sample	Rate constant (s^{-1})
Free thiamin diphosphate in 50 mM phosphate buffer	$(9.5\pm0.4)\times10^{-4}$
Pyruvate oxidase holoenzyme in 50 mM phosphate buffer	314 ± 12
Pyruvate oxidase holoenzyme in buffer free solution at pH 6.0 and same ionic strength	20 ± 0.8
Pyruvate oxidase holoenzyme recombined with 5-carba-5-deaza-FAD in 50 mM phosphate buffer	8 ± 0.3
Apo-thiamin diphosphate complex in 50 mM phosphate buffer	$(1\pm0.05)\times10^{-2}$

the role of both coenzymes in this reaction. A POX holoenzyme where native FAD was substituted by the analogue 5-dFAD, incapable of carrying out rapid redox reactions was also investigated.

As shown in Table 3, the H/D exchange rate of the C2-H of ThDP in the binary apo-ThDP complex is very slow and would not allow a catalysis at the observed rate. FAD binding to this binary complex accelerates this rate by four orders of magnitude compared to that of free ThDP (Table 3). It exceeds the catalytic constant of 2 s^{-1} at 4°C of this enzyme. This fast H/D exchange in the native holoenzyme does not appear to be mediated by a direct interaction of the FAD with the C2-H of the enzyme-bound ThDP, but rather by interactions with functional groups of the protein that are operative only in the POX-FAD-ThDP ternary complex. In the crystal structure, the distance of the closest FAD atom to C2-H of ThDP is 11 Å [44,45]. Based on the structural homology of the ThDP binding site to other ThDP enzymes, it may be assumed that glutamate 59 is the residue mediating this activation in POX by interacting with the N1′ of ThDP. This would be in analogy to PDC and TK, where the same type of interaction occurs. This interpretation is consistent with the observation that in the ternary complex with 5-dFAD the rate of H/D exchange of ThDP is only marginally reduced compared to the native holoenzyme (Table 3).

Interestingly, the second substrate phosphate increases the rate of the H/D exchange of ThDP in the holoenzyme by further 16-fold compared to that measured in a phosphate free buffer (Table 3). This substantial increase in rate observed in the presence of phosphate cannot be interpreted in molecular details at present.

4. Conclusions

The data presented in this work provide a consistent model for the activation of ThDP in ThDP dependent enzymes. The directly determined high H/D exchange rates of the enzyme-bound ThDP in the presence of the substrates show that a concerted mechanism [8] does not have to be assumed to explain ThDP catalysis. In addition, the data show that the N1′ atom and the 4′-amino group of ThDP are essential for the activation of ThDP. A fast deprotonation of C2 requires an interaction of a conserved acidic group with the N1′ atom of the pyrimidine ring of ThDP, leading to tautomerization of the 4′-amino group, thereby enhancing the basicity of the amino nitrogen.

Acknowledgements

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