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ORIGINAL PAPER

Density-functional study on the equilibria in the ThDP activation

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Abstract The equilibria among the various ionization and tautomeric states involved in the activation of ThDP is addressed using high level density functional theory calculations, X3LYP/6-311++G(d,p)//X3LYP(PB)/6-31++G(d,p). This study provides the first theoretically derived thermodynamic data for the internal equilibria in the activation of ThDP. The role of the medium polarity on the geometry and thermodynamics of the diverse equilibria of ThDP is addressed. The media chosen are cyclohexane and water, as paradigms of apolar and polar media. The results suggest that all ionization and tautomeric states are accessible during the catalytic cycle, even in the absence of substrate, being APH^+ the form required to interconvert the AP and IP tautomers; and the generation of the ylide proceeds via the formation of the IP form. Additionally, the calculated $\Delta G°$ values allow to calculate all the equilibrium constants, including the pK_{C2} for the thiazolium C2 atom whose ionization is believed to initiate the catalytic cycle.

Keywords Activation · DFT · ThDP · Thermodynamics

Introduction

Enzymes are proteins which catalyze a quite diversity of biochemical reactions. Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and speed up only a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell.

Thiamin diphosphate (ThDP) is a coenzyme that assists in the catalysis of carbon-carbon bond-forming and bond-breaking reactions adjacent to a carbonyl group [1–3]. A large and diverse collection of enzymes require ThDP, including pyruvate decarboxylase (PDC) which is involved in the formation of alcohol in anaerobic fermentation; transketolase (TK) which transfer a two-carbon unit from ketose to an aldose; pyruvate dehydrogenase (PDH) which links the glycolytic pathway to citric acid; acetohydroxyacid synthase (AHAS) which is involved in the biosynthetic pathway of branched-chain amino acids (valine, isoleucine and leucine) in plants and microorganisms.

While ThDP enzymes catalyze a large variety of chemical reactions, the activation of ThDP is common for all of these enzymes, Scheme 1. The C2 atom of the coenzyme, which is located between the nitrogen and sulfur atoms in the thiazolium ring, acts as the nucleophile by attacking the carbonyl carbon of the different substrates. This reaction is strictly dependent on the generation of the carbanion, ylide, at the C2 atom. The deprotonation of the C2 atom is a key reaction in all thiamine diphosphate-dependent enzymes [4].

Despite the number of articles devoted to ThDP catalysis there are some aspects that still remain unknown or controversial on its activation, especially those related to the values of the equilibrium constants for the various ionization and tautomeric equilibria.

This study provides the first theoretically derived data for the internal thermodynamic equilibria involved in the generation of the carbanion at the C2 atom. All the equilibrium constants are determined using high level density functional theory calculations.

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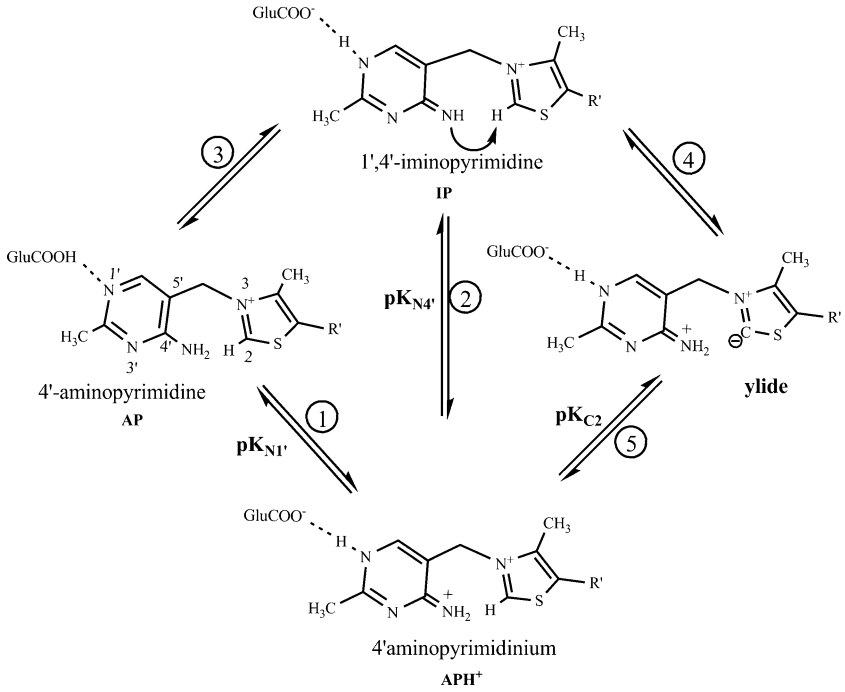
Scheme 1 Equilibria in the biosynthetic pathway leading to the ylide formation



Computational methods

All quantum chemical calculations in this study were performed using the Jaguar 7.0 suite of programs [5]. In order to reduce cpu time the system has been reduced by ignoring the diphosphate group, since its primary function is to anchor the cofactor and it is not involved in the catalytic mechanism, and the conserved side chain of glutamic acid, interacting with the N1′atom, has been replaced by acetic acid. The geometries of all structures were optimized in solution using the same level of theory: the basis set 6-31++G(d,p) was used with the X3LYP functional, which gives very good geometries for hydrogen-bonded complexes. All structures were optimized without any constraint. Implicit solvation effects are modeled using the Poisson-Boltzmann (PB) model as implemented in Jaguar. The media chosen are cyclohexane and water as paradigms of apolar and polar media, respectively, in order to stress the effect of the enzymatic environment since a likely dielectric constant in the interior of the protein might be 2–4, as it is a rather apolar environment [6]. For this reason we have used cyclohexane as solvent to model the apolar interior of the enzyme. All computations were done considering the highly conserved glutamate interacting with the N1′atom of the 4-aminopyrimidine ring, as a simple way of considering the apoenzymatic environment. The free energies were computed at the X3LYP/6-311++G(d,p)//X3LYP(PB)/6-31++G(d,p) level of theory.

Results and discussion

Geometry

The results of geometry optimization are shown in Table 1. For all structures, the geometry optimization leads to conformations in which the N1′atom is within optimal hydrogen bonding distance of the conserved glutamate side chain, 1.75 Å. On the other hand, it is possible to observe a strong effect of solvation on the geometries of the 4′-aminopyrimidine (AP) and 4′-aminopyrimidinium (APH^+) structures, quite the opposite of earlier reported results [6]. Thus, in cyclohexane, the AP tautomer reaches a V conformation with torsion angles ϕ_T and ϕ_P of −73.5° and 94.8°, respectively, in agreement with the values reported from high-resolution crystallographic experiments [7, 8]. In water, instead, AP adopts a nearly S-type conformation with torsion angles ϕ_T and ϕ_P of −97.4° and −172.6°, respectively, in agreement with the reported torsion angles for this conformation ϕ_T=±100° and ϕ_P=±150° [6] . However, it is well known that in all X-ray crystal structures reported to date, ThDP is found in the V conformation [7, 8]. Therefore, according to the results and the experimental evidence we can say that the enzymatic environment is best simulated with a solvent of low dielectric constant like cyclohexane.

For APH^+ the effect of solvation is practically the same than that for AP, and the geometric parameters and conformations remain nearly the same for the respective solvents.

Table 1 Geometric and partial atomic charges in cyclohexane and water for different forms of ThDP

	AP		APH^+		IP		Ylide	
	cyclohexane	water	cyclohexane	water	cyclohexane	water	cyclohexane	water
ϕ_T	−73.5	−97.4	−74.7	−92.7	−81.4	−88.3	−86.6	−88.8
ϕ_P	94.8	−172.6	87.7	−177.1	80.1	80.1	73.7	69.3
$R_{N4'\text{-}HC2}$ (Å)	2.86	5.22	2.62	5.13	2.27	2.57	–	–
$R_{N4'\text{-}C2}$ (Å)	3.62	5.25	3.41	5.20	3.03	3.18	2.99	2.95
q (N1′)	−0.69	−0.99	−0.43	−0.54	−0.65	−0.73	−0.36	−0.43
q (N4′)	−0.91	−1.03	−0.79	−0.98	−1.02	−1.12	−0.85	−0.88
q (C2)	−0.19	−0.23	−0.20	−0.23	−0.17	−0.14	−0.54	−0.63

On the other hand, in the 1′,4′-iminopyrimidine (IP) form the dihedral angles practically do not show a dependence on the polarity of the medium. The calculated values are (−81.4, 80.1) and (−88.3, 80.1) in cyclohexane and water, respectively. This allows to reach a conformation with minimal distance N4′-C2, 3.03 and 3.18 Å, respectively, in agreement with X-ray results which show that N4′and C2 are only 3.5 Å apart or less [9]. This optimal spatial orientation would be responsible for the acceleration of C2 deprotonation as consequence of intramolecular proton transfer between these two atoms [10, 11].

For the ylide form, the ϕ_T torsion angle values are −86.6 and −88.8 in cyclohexane and water, respectively. The ϕ_P angle, on the other hand, reaches values of 73.7 and 69.3, respectively. This conformation allows to set the N4′and C2 atoms at a distance of about 2.9 Å.

Partial atomic charges

The variation of the atomic partial charges on selected atoms for the different forms of ThDP is shown in Table 2. It is possible to observe the change of the negative charge on the N4′ atom from −0.79 to −1.02, when going from APH^+ to IP, accounting for the increase in basicity of the 4′-amino group, in conjunction with the decrease of the negative charge on the C2 atom from −0.20 to −0.17. These changes in the partial atomic charges would allow the intramolecular proton transfer from the C2 atom to the N4′ atom to form the ylide. After the proton transfer, the negative charge on the C2 atom reaches the highest value, -0.54, allowing later the nucleophilic attack on the α-carbonyl of pyruvate to form the α-lactyl-TDP adduct (LTDP).

Thermodynamics

The standard free energy changes, ΔG°, of the diverse equilibria are shown in Table 2.

Equilibrium 1

The first equilibrium involves the proton transfer from the glutamic acid side chain to N1′:

$$AP + Glu - COOH \longleftrightarrow APH^+ + Glu - COO^-$$

The free energy change, ΔG°, shown in Table 2 may be considered as the sum of two free energy changes corresponding to two concerted molecular processes, the deprotonation of glutamic acid and protonation of N1′atom. In order to evaluate pK_1 is necessary to determine the free energy change corresponding to the protonation of the

Table 2 Standard free energy of reaction in cyclohexane and water at 298 K

Solvent	Reaction	ΔG^0 (kcal/mol)
cyclohexane	$GluCOOH + AP \longleftrightarrow GluCOO^- + APH^+$	−1.1
water		+3.0
cyclohexane	$APH^+ + GluCOO^- \longleftrightarrow IP + GluCOOH$	+1.0
water		−0.2
cyclohexane	$AP \longleftrightarrow IP$	$-8.3x10^{-2}$
water		+2.8
cyclohexane	$IP \longleftrightarrow ylide$	+1.0
water		+0.1
cyclohexane	$APH^+ \longleftrightarrow ylide + H^+$	+11.6
water		+6.1

N1′atom, solely. In order to do this, it is necessary to considerer the following chemical equations:

$$AP + Glu - COOH \longleftrightarrow APH^+ + Glu - COO^- \quad (R1)$$

$$Glu - COO^- + H^+ \longleftrightarrow Glu - COOH \quad (R2)$$

$$AP + H^+ \longleftrightarrow APH^+ \quad (R3)$$

The free energy change corresponding to the ionization of glutamic acid depends on the medium, thus in aqueous solution the model value of pK_a is 4.5, whereas in the protein environment depends on the type of enzyme in particular. In this study we use the estimated value of 7, which corresponds nearly to an average value for ThDP dependent enzymes, as predicted by Propka software [12]. Using these values, the resulting ΔG° for the equation (R3) is −3.2 and −10.7 (kcal mol^{-1}) in water and cyclohexane, respectively. These values show that the protonation of the N1′atom is highly thermodynamically favored. The calculated values of pK_{N1}', the inverse of equation (R3), are 2.31 and 7.81, in water and cyclohexane, respectively. The extremely low value in water cannot be supported from a chemical point of view since it is in the characteristic range of weak acids and do not reflect the well-known basicity of amines.

The value of 7.81 in cyclohexane instead is in the range typical pK_a values of amines, and on the other hand it is in agreement with the accepted value of about 7 for the pK_a of glutamates in the enzyme environment, as predicted by Propka software [12].

Equilibrium 2

Equilibrium 2 involves the transfer of a proton from the N4′atom to a amino acid side chain, for instance Glu473 in pyruvate decarboxylase (PDC):

$$APH^+ + GluCOO^- \longleftrightarrow IP + GluCOOH$$

The value of the standard free energy change, shown in Table 2 includes the values corresponding to the deprotonation of N1′atom and the protonation of glutamate. In order to determine pK_4 we combine the following chemical equations:

$$APH^+ + Glu - COO^- \longleftrightarrow IP + Glu - COOH \quad (R4)$$

$$Glu - COOH \longleftrightarrow Glu - COO^- + H^+ \quad (R5)$$

$$APH^+ \longleftrightarrow IP + H^+ \quad (R6)$$

The resulting values of ΔG° for the reaction (R7) are +6.0 and + 10.6 (kcal mol^{-1}) in water and cyclohexane, respectively. Following a procedure analogous to that of equilibrium 1, we can evaluate pK_{N4}'. The resulting values are 4.35 and 7.72, in water and cyclohexane, respectively.

The values of ΔG° indicate that this reaction does not proceed under standard conditions. However, the spontaneity of a process at constant T and P, is given by ΔG and not by ΔG°. Both, are related by the well-known equation: $\Delta G = \Delta G^0 + RT \ln Q$, where Q is the ratio of the activities of the products to the activities of the reactants. Considering that the concentrations of the different forms of ThDP in the enzyme is quite low, the activity coefficients must not be very different from unity, and in consequence the activities should be replaced by concentrations. In this case Q takes the form $[IP][H^+]/[APH^+]$; where the concentration of hydrogen ions is about 10^{-7} at physiological conditions. Therefore, the reaction becomes thermodynamicly favored because the cancellation of the positive value of ΔG° by the contribution of $[H^+]$ to ΔG.

Equilibrium 3

The third equilibrium involves the tautomeric equilibrium between AP and IP. The standard free energy change for this reaction can be calculated by combining the chemical equations (R3) and (R6). The resulting values are +2.8 and -8.3×10^{-2} (kcal mol^{-1}) for water and cyclohexane, respectively; implying the following values for the tautomerization constants: 8×10^{-3} and 1.1, respectively. The value in cyclohexane is in agreement with the suggested value of about 1 for this constant [10].

Equilibrium 4

The fourth equilibrium is the transformation of IP into the ylide. The values of ΔG° for the reaction are +0.1 and +1.0 (kcal mol) in water and cyclohexane, respectively; implying equilibrium constants of 0.80 and 0.18, respectively. These values are in agreement with the suggested value in the range of 1–10 for the ratio [IP]/[ylide] [10].

Equilibrium 5

The fifth equilibrium involves the deprotonation of the C2 atom of APH^+ to form the ylide. The standard free energy change for this reaction can be obtained by combination of the following chemical equations, whose ΔG° are known.

$$IP \longleftrightarrow ylide \quad (R7)$$

$$GluCOOH \longleftrightarrow GluCOO^- + H^+ \quad (R8)$$

$$APH^+ + GluCOO^- \longleftrightarrow IP + GluCOOH \quad (R9)$$

$$APH^+ \longleftrightarrow ylide + H^+ \quad (R10)$$

The resulting equation (R10) is the reaction of equilibrium 5. The obtained ΔG° values are +6.1 and 11.6 (kcal mol^{-1}) in water and cyclohexane, respectively. These values suggest the formation of the ylide does not go by the direct transformation from APH^+, but via the IP species: $APH^+ \rightarrow IP \rightarrow$ ylide, as suggested in literature. The corresponding pK′s values for the deprotonation of the C2 atom are 4.45 and 8.48 respectively. The value in water does not fit with the weakly acidic nature of the thiazolium C2H group. The value in cyclohexane, instead, is in agreement with the value between 8 and 9 reported by Nemeria et al. [10] for the deprotonation of the C2 atom.

Conclusions

This study provides the first theoretically derived data for the internal thermodynamic equilibria involved in the activation of ThDP, i.e., the generation of the carbanion at the C2 atom of the thiazolium ring. The results show a strong influence of the polarity of the medium on both the geometry and thermodynamics. The geometric and thermodynamic results in aqueous solution cannot be supported from a chemical point of view, moreover they do not correlate with the experimental results. However, when the enzymatic environment is modeled with a solvent of low dielectric constant, like cyclohexane, the results correlate well both qualitatively and quantitatively to the empirical evidence. The results also suggest that all ionization and tautomeric states of ThDP are thermodynamically accessible even in the absence of substrate, being APH^+ the form required to interconvert the IP and AP tautomers. On the other hand, the generation of the ylide proceeds via the formation of the IP species, as consequence of the increase in the negative partial charge, basicity, on the N4′-atom in conjunction with a decrease in the negative partial charge on the C2-atom, allowing its deprotonation. The calculated values of pK_a′s for these key stages are 7.8, 7.7 and 8.5 for pK_{N1}′, pK_{N4}′ and pK_{C2}, respectively; supporting the suggestion of Tittmann et al. [11] concerning that the deprotonation and protonation of the C2 atom is accomplished by a fast proton shuttle enabled by a closely matched pK_a values. The calculated equilibrium constants for the remaining two equilibria are: $[IP]/[AP] = 1.2 \text{and} [IP]/[\text{ylide}] = 5.6$. These values are in agreement with those suggested by Nemeria et al. [10] of about 1 for equilibrium 3, and values in the range 1–10 for equilibrium 4.

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