Kinetic Control of Thiamin Diphosphate Activation in Enzymes Studied by Proton−Nitrogen Correlated NMR Spectroscopy†

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ABSTRACT: Proton−nitrogen correlated NMR studies were performed on thiamin diphosphate, which has been specifically labeled with ^{15}N at the 4′-amino group. After reconstitution of the labeled coenzyme with the apoenzymes of both wild-type pyruvate decarboxylase from *Zymomonas mobilis* and the E50Q variant, a high-field shift of the ^{15}N signal of ∼4 ppm is observed at pH 5.9 when compared to that of the free coenzyme, indicating a higher electron density at the 4′-amino nitrogen in the enzyme-bound state. The pH dependence of the chemical shift of the ^{15}N signals in the $^{1}H-^{15}N$ heteronuclear single-quantum coherence NMR spectra reveals typical titration curves for the free as well as the reconstituted coenzyme with nearly identical chemical shift end points. The midpoints of the transitions are at pH 5.3 and 5.0 for the free and enzyme-bound coenzyme, respectively. We conclude that the tremendous rate acceleration of C2-H deprotonation in ThDP enzymes is mainly the result of the enforced *V* conformation of the cofactor in the active site being perfectly suited to allowing intramolecular acid−base catalysis.

Thiamin diphosphate (ThDP)[1] is an important coenzyme in biocatalysis, being involved in numerous metabolic pathways. While ThDP enzymes catalyze a large variety of chemical reactions, the first step in the catalytic cycle is common for all of these enzymes: 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 at the C2 atom. The deprotonation of the C2 atom is a key reaction in all thiamin diphosphate-dependent enzymes. As recently shown, a tremendous rate acceleration of the C2 deprotonation in the enzyme-bound state is accomplished by a proton shuttle consisting of the exocyclic 4′-amino group of the cofactor, which is within hydrogen bonding distance of the C2 atom, the N1′ atom, and a conserved glutamate positioned within hydrogen bonding distance of the N1′ atom (*1*).

To further characterize the key role of the 4′-amino group of ThDP for cofactor activation, we have studied here the influence of the chemical environment of the active site of pyruvate decarboxylase (EC 4.1.1.1) from *Zymomonas mobilis* on the electronic properties of the 4′-amino group by two-dimensional proton−nitrogen correlated NMR spectroscopy. Chemical shift analysis and its pH dependence indicate that the acceleration of C2 deprotonation by 5 orders of magnitude is not mainly of thermodynamic nature, caused by an increase in basicity of the 4′-amino group, but rather of kinetic nature, caused by an optimal spatial orientation of the activated 4′-amino group toward the C2 hydrogen enforced by the adopted *V* conformation of the cofactor in the active site.

MATERIALS AND METHODS

Protein Expression and Purification. The proteins were expressed and purified to homogeneity using established protocols (*2*). The apoenzymes of pyruvate decarboxylase and its variant were prepared as previously described (*3*, *4*). Transketolase was purified as described in ref *5*.

Synthesis of 4′-^{15}N-Labeled Thiamin Diphosphate. The 4′-^{15}N-labeled thiamin was synthesized according to the method of Todd and Bergel (*6*). The condensation of acetamidine with ethyl-α-ethoxymethylene-α-cyanoacetate yields the intermediate compound α-cyano-*β*-acetamidinoacrylate. Refluxing with phosphoryl chloride afforded 2-methyl-4-chloro-5-cyanopyrimidine. Nucleophilic substitution of chlorine for $^{15}NH_3$ was performed using $^{15}NH_4Cl$ (^{15}N content of 99.5%) according to ref *6* with slight modifications. The 2-methyl-4-[^{15}N]amino-5-cyanopyrimidine was subjected to catalytic hydrogenation using palladized charcoal in acetic acid saturated with hydrogen chloride at 0 °C, yielding 2-methyl-4-[^{15}N]amino-5-(aminomethyl)pyrimidine according to Nesbitt and Sykes (*7*). The thiazolium moiety was added to this labeled pyrimidine moiety as described by Todd and Bergel, yielding the 4′-^{15}N-labeled thiamin, the correct molecular mass of which was confirmed by mass spectrometry analysis. Phosphorylation was performed according to the method of

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[1] Abbreviations: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase; *Zm*PDC, PDC from *Z. mobilis*; TK, transketolase; HSQC, heteronuclear single-quantum coherence.

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Karrer and Viscontini (*8*). Diphosphorylated 4′-^{15}N-labeled thiamin (4′-[^{15}N]ThDP) was isolated by ion exchange chromatography on DEAE-Sephadex A25 (Amersham Pharmacia Biotech) using the acetate form and a linear acetic acid gradient (50 mM acetic acid).

Reconstitution of the Apoenzyme of Pyruvate Decarboxylase with the Labeled Coenzyme. The apoenzymes of pyruvate decarboxylase from *Z. mobilis* and its E50Q variant were reconstituted with 4′-$^{15}NH_2$-labeled thiamin diphosphate by incubating solutions of the respective apoenzymes (active site concentration of 1 mM) with 5 mM labeled coenzyme and 10 mM $MgSO_4$ in 200 mM MES (pH 6.6) for 100 min at 20 °C. The reconstituted protein solutions (1 mL) were concentrated to 200 μL using a Microsep (Pall Filtron Corp.) centrifugal concentrator (cutoff of 30 000 Da) and diluted with 600 μL of 50 mM MES (pH 6.0) containing 10 mM $MgSO_4$. The whole procedure of dilution and concentration was repeated five times to ensure removal of excess labeled cofactor. Finally, the concentrated solutions were mixed with 50 mM MES (pH 6.0) containing 10 mM $MgSO_4$, 20 mM $^{15}NH_4Cl$, and 20% D_2O, yielding a final active site concentration of 1.3 mM.

Apotransketolase (active site concentration of 1.2 mM) was reconstituted with an equimolar amount of the labeled cofactor in the presence of 5 mM Mg^{2+} in potassium phosphate buffer (pH 7.6). Under these conditions, approximately 98% of the cofactor is bound to the enzyme.

Proton−Nitrogen Correlated NMR Measurements. ^{1}H−^{15}N heteronuclear single-quantum coherence (HSQC) NMR spectra were acquired at 25 °C on a 500 MHz Varian Unity NMR spectrometer and a 500 MHz Bruker NMR spectrometer. Data were acquired with 128 and 512 complex points in the ^{15}N and ^{1}H dimensions, respectively. The spectral widths were 8333.3 Hz in the ^{1}H dimension and 2200 Hz in the ^{15}N dimension. The number of scans was adjusted to obtain sufficient signal intensity in the individual experiments. The pH was adjusted with 1 M bicine buffer (pH 8.5) or 1 M acetate buffer (pH 4.3) for the titration experiments. The reversibility of the titration was tested by back-titrating to the original pH and acquiring a spectrum again.

RESULTS AND DISCUSSION

To further investigate how the 4′-amino/imino group of enzyme-bound ThDP is capable of catalyzing the observed 10^5-fold rate acceleration of C2 deprotonation, ThDP was synthesized by total synthesis using $^{15}NH_4Cl$ to yield the cofactor specifically labeled with ^{15}N at the 4′-amino position ($^{15}NH_2$-ThDP). This labeling strategy enables the specific detection of this amino group of the cofactor when it is bound to pyruvate decarboxylase or transketolase using two-dimensional ^{1}H−^{15}N correlated NMR spectroscopy.

The ^{1}H−^{15}N HSQC spectrum of free $^{15}NH_2$-ThDP was compared to that after reconstitution of $^{15}NH_2$-ThDP with wild-type pyruvate decarboxylase from *Z. mobilis*, a homotetrameric enzyme with a molecular mass of 240 kDa.

First, we were surprised that the amino group of the cofactor could be detected well in the enzyme-bound state (Figure 1) given the large size and consequently overall large correlation time of the protein. From this experiment, it can be concluded that the 4′-amino group of ThDP must exhibit intrinsic mobility in the enzyme, resulting in T_2 transverse relaxation times larger than those estimated from the overall tumbling of the enzyme.

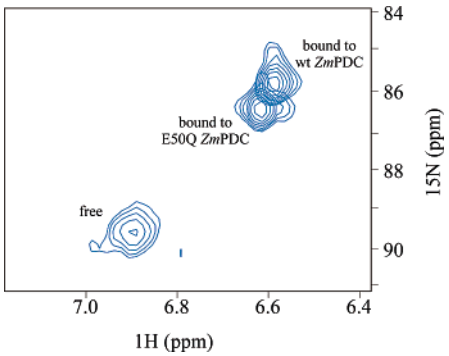

FIGURE 1: Proton−nitrogen correlated NMR spectra of free 4′-$^{15}NH_2$-labeled thiamine diphosphate (active site concentration of 17 mM, total acquisition time of 6 h and 21 min), 4′-$^{15}NH_2$-labeled thiamin diphosphate bound to wild-type pyruvate decarboxylase from *Z. mobilis* (active site concentration of 1.3 mM, total acquisition time of 1 h and 16 min), and 4′-$^{15}NH_2$-labeled thiamin diphosphate bound to the E50Q variant (active site concentration of 1.3 mM, total acquisition time of 1 h and 16 min) in 50 mM MES buffer at pH 5.9 and 25 °C.

Second, the ^{1}H−^{15}N HSQC spectra of both the free and enzyme-bound cofactor show the characteristic appearance of a second weak peak upfield in the ^{15}N dimension in all samples caused by the D_2O content (20%, v/v) of the solution. This upfield peak represents the amino species that has one proton and one deuteron attached. Consequently, two hydrogens are located at the amino nitrogen in the free and enzyme-bound cofactor in time average. It should be noted that the detection of these double peaks indicating the bonding of two hydrogens to the 4′-amino nitrogen does not rule out the existence of an imino-tautomeric form of thiamin diphosphate in equilibrium with the corresponding amino form. However, this result shows that the amino form is thermodynamically more stable when the substrate is absent. Circular dichroism spectroscopic studies on pyruvate dehydrogenase suggested that the equilibrium may shift to the 1′−4′ imino tautomer in later steps of catalysis (*9*).

Third, surprisingly, short acquisition times were required for the spectrum of $^{15}NH_2$-ThDP bound to the 240 kDa homotetramer of pyruvate decarboxylase in comparison to those of free $^{15}NH_2$-ThDP to obtain spectra with a comparable signal-to-noise ratio. Besides the high molecular mass, the active site concentration of the enzyme was more than 1 order of magnitude lower than the concentration of the sample of the free cofactor. The existence of the residual unbound cofactor could completely be ruled out because of the difference in chemical shifts (see below). The shorter accumulation time for the spectrum of enzyme-bound $^{15}NH_2$-ThDP can be explained by a proton exchange of the 4′-amino group with solvent in the enzyme-bound state that is slower than the exchange of the cofactor free in solution. In other words, the 4′-amino group appears to be partially shielded by the enzyme environment.

Since chemical shifts are very sensitive markers for changes in the electronic environment, potential differences in the environment between the amino group in free ThDP to those when bound to the enzyme can be detected. In addition, we aimed to determine the pK_a values from the pH dependence of the chemical shift. As shown in Figure 1, at pH 5.9 a high-field shift of ~4 ppm in the^{15}N dimension

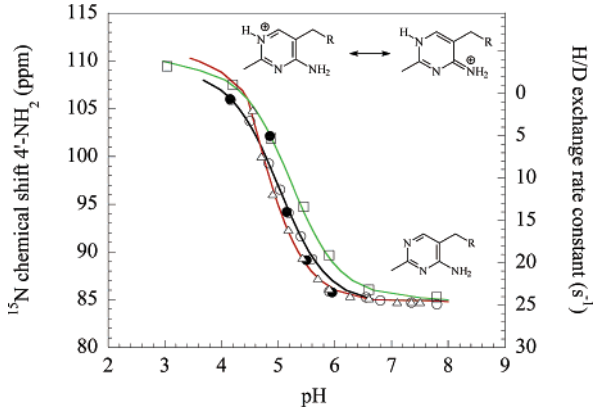

FIGURE 2: Effect of pH on the rate constant of C2-H deprotonation (●) of thiamin diphosphate in pyruvate decarboxylase from *Z. mobilis* (data taken from ref *11*) and on the chemical shift of the ^{15}N signal of the amino nitrogen of free 4′-$^{15}NH_2$-labeled thiamin diphosphate (□), 4′-$^{15}NH_2$-labeled thiamin diphosphate bound to wild-type pyruvate decarboxylase from *Z. mobilis* (○), and 4′-$^{15}NH_2$-labeled thiamin diphosphate bound to the variant E50Q (△). The lines represent fits (green for the free coenzyme and black and red for the coenzyme bound to the wild-type enzyme and the E50Q variant, respectively) according to the equation $-\log\{[(u - l)K_a]/(u - x) - K_a\} = \text{pH}$, where u and l represent the upper and lower limits of the chemical shift, respectively, and x represents the chemical shift measured at the respective pH.

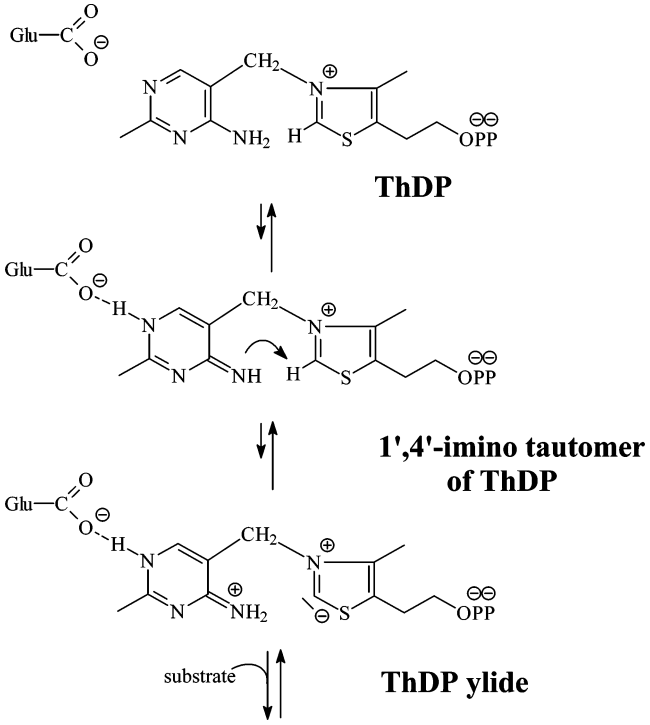

FIGURE 3: Proposed mechanism of cofactor activation in thiamin diphosphate enzymes.

was measured for the cofactor in the enzyme-bound state in comparison to that of free thiamin diphosphate, indicating a higher electron densitiy around the nitrogen in enzyme-bound ThDP. Abolishing the functionality of the proposed protein−cofactor proton shuttle in an E50Q variant results in a 1 ppm downfield shift of the amino nitrogen of the enzyme-bound cofactor relative to that of the wild-type enzyme.

Is the large rate acceleration of C2 deprotonation due to a shift of a pK_a value or, in other words, a higher basicity of the 4′-amino group? To address this question, we carried out pH titrations of free $^{15}NH_2$-ThDP, $^{15}NH_2$-ThDP bound to wild-type pyruvate decarboxylase, and $^{15}NH_2$-ThDP bound to the E50Q mutant. Chemical shifts were followed as a function of the adjusted pH values. As expected, the chemical shift of the amino nitrogen of the cofactor shows a pH dependence in both the free and enzyme-bound state (Figure 2). In all cases, typical titration curves could be observed, revealing nearly identical lower and upper limits of chemical shifts. This indicates a comparable electronic state of the amino nitrogen of the free and enzyme-bound cofactor in the N1′-protonated and deprotonated form, respectively. However, a distinct difference in the midpoints of the titration curves of the free and enzyme-bound coenzyme was observed, corresponding to a pK_a of ∼5.3 for free ThDP and pK_a values of 5.0 and 4.95 for ThDP bound to wild-type and E50Q pyruvate decarboxylase from *Z. mobilis*, respectively. The detected midpoint of 5.3 in free ThDP has been previously shown to be attributable to the protonation of the N1′ atom (*10*).

Strikingly, the rate constant of deprotonation at the C2 atom of the enzyme-bound coenzyme shows a pH dependence (*11*) similar to that of the ^{15}N signal of the amino group of the cofactor in the enzyme-bound state (Figure 2). The estimated pK_a of approximately 5 was attributed to the protonation of the N1′ atom of the coenzyme, as in the free cofactor (*11*).

The differences in the chemical shift indicating differences in the electron density of the 4′-amino nitrogen of ThDP between the free and enzyme-bound state (Figure 1) result from a small shift in the pK_a of N1′ protonation. However, at physiological pH, identical chemical shifts for the 4′-amino group in free and enzyme-bound ThDP are observed. Therefore, it can be concluded that the acceleration of C2 deprotonation in the enzyme-bound state is not mainly the result of a thermodynamically controlled process but of a kinetically controlled process. Binding of the cofactor into the active site leading to the formation of a proton shuttle among C2, the 4′-amino group, N1′, and the glutamate side chain does not result in a significant shift of the equilibrium toward the highly basic imino-tautomeric form in pyruvate decarboxylase (Figure 3). It rather decreases the activation barrier of deprotonation. These results are further consistent with the fact that a signal for the 4′-amino group was detectable after short acquisition times in the heteronuclear NMR experiment for such a large molecule. A predominant single-bond character allows for intrinsic mobility through rotation of the C−N bond. In contrast to pyruvate decarboxylase, no signal in ^{1}H−^{15}N HSQC NMR spectra could be detected for transketolase (MW = 140 000, active site concentration of 1.2 mM) when reconstituted with the ^{15}N-labeled cofactor at the working pH of 7.6 using NMR acquisition parameters identical to those described for pyruvate decarboxylase (data not shown). This points either to a faster proton exchange of the 4′-amino group of the cofactor with the solvent in transketolase when compared to that of *Zm*PDC or, alternatively, to a restricted intrinsic mobility of the 4′-amino group possibly caused by the formation of the 1′−4′-tautomeric form of the cofactor.

A kinetic control implies that sterical constraints and an efficient proton shuttle are the key factors for lowering the energy barrier for C2 deprotonation. All crystal structures

of thiamin diphosphate enzymes show that the cofactor adopts a conformation in the active sites where its 4′-amino group is within hydrogen bonding distance of the C2-H atom. In addition, the N1′ atom is within optimal hydrogen bonding distance of a conserved glutamate side chain (*12*−*17*).

As we have shown here, the electronic state of the 4′-amino group depends crucially on the N1′ protonation state. Strikingly, the determined pK_a of 5.3 for the N1′ protonation is in the range of typical pK_a values of glutamates in proteins, suggesting that deprotonation or protonation of the C2 is accomplished by a fast proton shuttle enabled by a closely matched pK_a comparable to that of serine/cysteine proteases (*18*). Like the Asp−His interaction in proteases, a low-barrier hydrogen bond between N1′ of ThDP and the glutamate side chain may account for the well-balanced "forward and reverse" acid−base catalysis in ThDP enzymes as all enzymes appear to use the protein−cofactor proton shuttle for *both* deprotonation and protonation reactions (*19*). Studies that address the potential involvement of a low-barrier hydrogen bond in ThDP enzymes are currently underway. Finally, kinetic control of the C2 deprotonation is in agreement with our previously performed experiments with ^{13}C2-labeled ThDP showing that the enzyme-bound cofactor does not generate a significant population of the C2 carbanion intermediate (*1*).

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