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The origin of the absorption band induced through the interaction between apotransketolase and thiamin diphosphate☆

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Abstract

It has long been known that formation of a catalytically active holotransketolase from the apoenzyme and coenzyme (thiamin diphosphate) is accompanied by the appearance of a new band, in both the absorption and CD spectra. Binding and subsequent conversion of the substrates bring about changes in this band's intensity. The observation of these changes allows the investigator to monitor the coenzyme-to-apoenzyme binding and the conversion of substrates during the transketolase reaction and thus to kinetically characterize its individual steps. The origin of the thiamin diphosphate induced absorption band has been postulated to be resulted from formation of a charge transfer complex or alternatively from an induced conformational transition of the enzyme. The latter brings aromatic amino acid residues into close proximity and generates the absorption. However, X-ray crystallographic and enzyme point mutation experiments cast doubts on both of these hypotheses. Here we show that the binding of thiamin diphosphate to the apotransketolase leads to the conversion of the 4′-amino tautomeric form of its aminopyrimidine ring into the $N^{1'}$H-imino tautomeric form. This imino form emerges as a result of the coenzyme's aminopyrymidine ring incorporation into the hydrophobic pocket of the transketolase active center and is stabilized through the interactions with Glu418 and Phe445 residues. The $N^{1'}$H-imino tautomeric form of thiamin diphosphate is thought to be the origin of the holotransketolase absorption band induced through the coenzyme binding.

Keywords: Thiamin diphosphate; Tautomeric forms; Transketolase; Induced absorption band; Circular dichroism

Transketolase from the yeast Saccharomyces cerevisiae (EC 2.2.1.1.) is a ThDP-dependent enzyme, a transferase, that catalyzes the reversible transfer of two carbon residues from keto- to aldo-substrates [1]. The enzyme has been studied in sufficient detail. Much X-ray data have been collected on the 3D structures of the apo- and holoenzyme [2–4] as well as on various transketolase complexes with ThDP analogs [5,6] and a holotransketolase complex with the acceptor substrate [7]. The combination of site-directed mutagenesis, X-ray structural studies, reaction kinetics, and CD spectroscopy data enabled the characterization of the amino acid residues involved in the interactions with ThDP, in substrate channel formation and in catalysis [4,8–12].

Transketolase is composed of two identical subunits and has two active sites [3,13,14]. ThDP binds at the interface between the subunits and interacts with residues from both subunits [3]. The comparison of the 3D structures of some thiamin diphosphate holoenzymes has shown that the enzyme-bound ThDP has a so-called "V conformation," which provides a direct contact between the amino group of the pyrimidine ring and the C^2–H bond of the thiazolium ring [15]. Since both subunits are involved in active site formation, the dimer may be considered as a catalytically competent unit.

The aminopyrimidine ring of ThDP is located in a hydrophobic pocket formed by the aromatic side chains, Phe442, Phe445, and Tyr448 (Fig. 1). The side chain of Phe445 forms stacking interaction with the aminopyrimidine ring. The aminopyrimidine ring forms several hydrogen bonds, the most significant is between the $N^{1'}$-atom of the aminopyrimidine ring and the side chain

☆ *Abbreviation:* ThDP, thiamin diphosphate.

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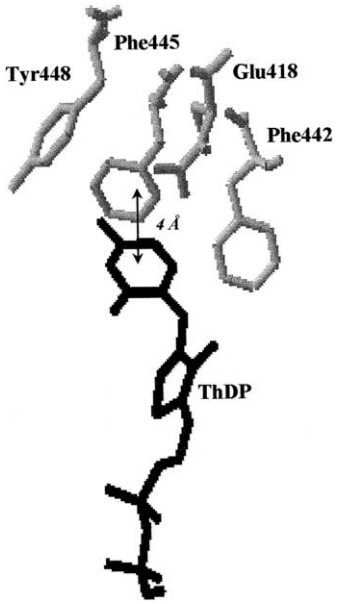


Fig. 1. The hydrophobic "pocket" of the aminopyrimidine ring of ThDP in holotransketolase (atomic coordinates are taken from Protein Data Bank, Trk).

of Glu418 [3,4]. An analogous interaction is observed with other ThDP-dependent enzymes [16–18] and plays an important role in the mechanism of thiamin catalysis.

The interaction of apotransketolase with ThDP is accompanied by the appearance of a new band in the absorption and CD spectra [19–22]. There is a linear correlation between the quantity of ThDP bound to the apoenzyme and the catalytic activity [23]. This optical effect is widely used to investigate the binding of ThDP to apotransketolase [23,24]. In addition, interaction of the substrates with holotransketolase and their subsequent conversion is accompanied by significant changes in the intensity of the new absorption band [19,21], and these changes are widely used for the characterization of the individual steps of the transketolase reaction [20,25–27].

Thus far, the origin of the new absorption band of holotransketolase has not yet been identified. Earlier, it was suggested that the emergence of the new absorption band of holotransketolase is the result of the charge transfer complex formation between ThDP and a tryptophan residue in the transketolase active center [19]. However, according to X-ray data, the distance between ThDP and the closest tryptophan residue is 19 Å (data taken from Protein Data Bank, ID = 1 Trk). When the 3D structure of holotransketolase was solved, the tryptophan function was attributed to the Phe445 residue which is in stacking interaction with the aminopyrimidine ring of ThDP [4,8] (Fig. 1). To verify this suggestion, Phe445 was replaced by isoleucine. This replacement maintains the hydrophobic environment around the pyrimidine ring, but the aromatic ring disappears. The CD spectrum of mutant F445I has shown that this replacement does not lead to the disappearance of the induced absorption band, but is accompanied by decrease of its intensity [28].

Another explanation was then offered. It is known that ThDP binding to the transketolase active site, followed by conformational changes in the protein molecule, leads to stabilization of two loops, which are flexible in apotransketolase and well ordered in holotransketolase [2]. As a result, the Trp391 residue from the one loop and the Tyr370 residue from the other are brought closer together while their aromatic rings become parallel to each other. Based on this finding, it was suggested that the new ThDP-induced absorption band is caused by stacking interaction between the aromatic rings of the two residues [28]. To ascertain if this is indeed the case, Trp391 was replaced by phenylalanine. The CD spectrum of the mutant holotransketolase thus obtained proved very similar to that of wild type holotransketolase (Wikner, C., and Meshalkina, L., unpublished data).

Thus none of the suggestions proposed to elucidate the origin of the induced absorption band that accompanies the ThDP–apotransketolase interaction and the catalytically active holoenzyme formation appears plausible.

By now, the idea about the participation of the imino tautomeric form of ThDP in catalysis is generally accepted [29–31]. This paper presents experimental data indicating that in the resting state of the holotransketolase, the ThDP aminopyrimidine ring exists in the imino tautomeric form, and that this tautomer is the source of the inducible absorbtion band of holotransketolase.

Materials and methods

Materials. Sephadex G-50 was purchased from Pharmacia (Sweden); ThDP, $CaCl_2$, and glycyl-glycine from Serva. Other chemicals were of the highest quality commercially available.

Transketolase purification. Transketolase was isolated from baker's yeast according to a method described earlier [32]. The crystalline enzyme was stored at 4 °C in a 50% saturated ammonium sulphate solution, pH 7.6. Transketolase (specific activity of 20 U/mg) was homogenous as judged by SDS–PAGE. The concentration of transketolase was determined using the absorbance coefficient $A^{1\%}_{280} = 14.5$ [33]. Prior to use, the transketolase solution was passed through a Sephadex G-50 column to remove ammonium sulphate.

Absorption and circular dichroism spectra. Absorption spectra were recorded at a protein concentration of 0.5 mg/ml in 50 mM glycyl-glycine, pH 7.6, at 25 °C using Aminco DW 2000 spectrophotometer (path length of 1 cm). Circular dichroism spectra were recorded at a protein concentration of 1 mg/ml in 50 mM glycyl-glycine, pH 7.6, at 25 °C using Mark V, "Jobin Ivon" (France) spectropolarimeter (path length of 1 cm), and RDM software with standard parameters for smoothing the spectra.

Absorption and circular dichroism spectra at difference pH values. 0.035–0.07 M HCI was added to the transketolase solution in 20 mM glycyl-glycine, pH 7.6, until the necessary pH value, then the spectrum

was recorded. The difference spectra of holotransketolase at different pH values were obtained by subtraction of the individual spectra of apotransletolase and ThDP recorded at the same pH values from the summary spectra.

Results and discussion

The interaction of apotransketolase with ThDP is accompanied by appearance of a new band in the absorption spectrum (the range 285–360 nm) and in the CD spectrum (the range 300–380 nm) lacking in the initial components (corresponding, Figs. 2A and B).

When free ThDP is placed into a medium less polar than water, a band appears in its spectrum, which was absent when the coenzyme was in aqueous solution (Fig. 3, curve 1). An analogous band in the near UV region of the spectrum was detected when thiamin was dissolved in dioxane [34]. It should be noted that in both cases the new absorption band appeared in the same region of the spectrum as the induced absorption band of holotransketolase (Fig. 2A).

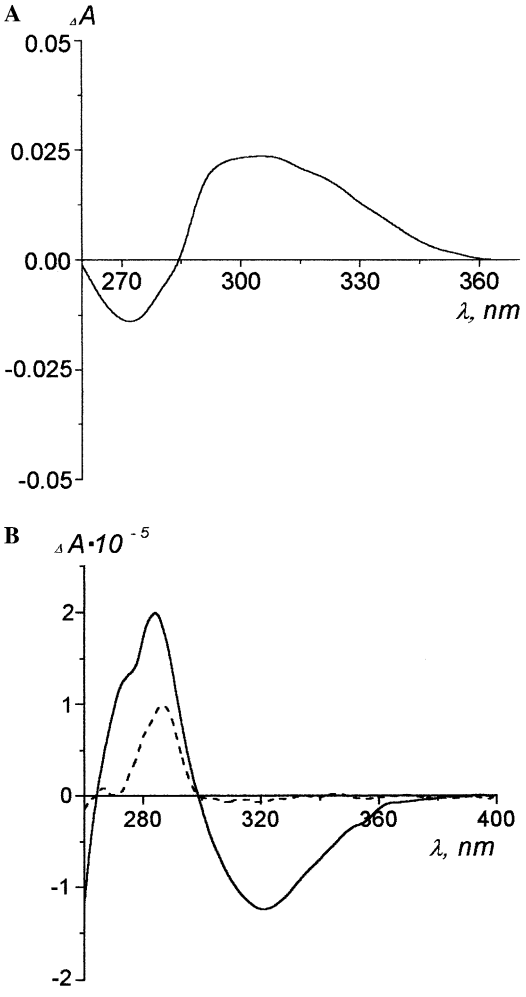


Fig. 2. Different absorption spectrum of holotransketolase with respect to apotransketolase and ThDP (A) and CD spectra of apotransketolase (dashed curve) and holotransketolase (solid curve) (B). Glycylglycine buffer, 50 mM, pH 7.6; $CaCl_2$, 2.5 mM; ThDP, 40 μM; TK, 3 μM (A) and 6 μM (B).

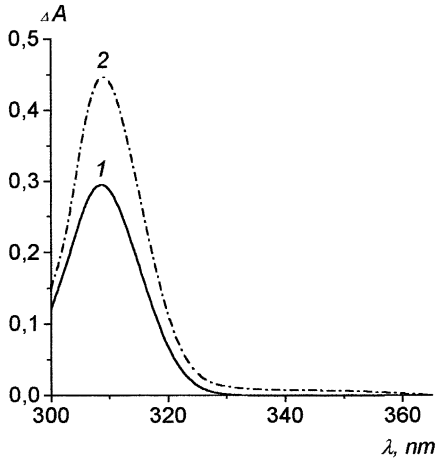


Fig. 3. Different absorption spectra of ThDP (2.5 mM) in ethanol solution (50%) in the absence (curve 1) and presence (curve 2) of phenylalanine (2.5 mM) with respect to ThDP in water and ThDP + phenylalanine in water, respectively.

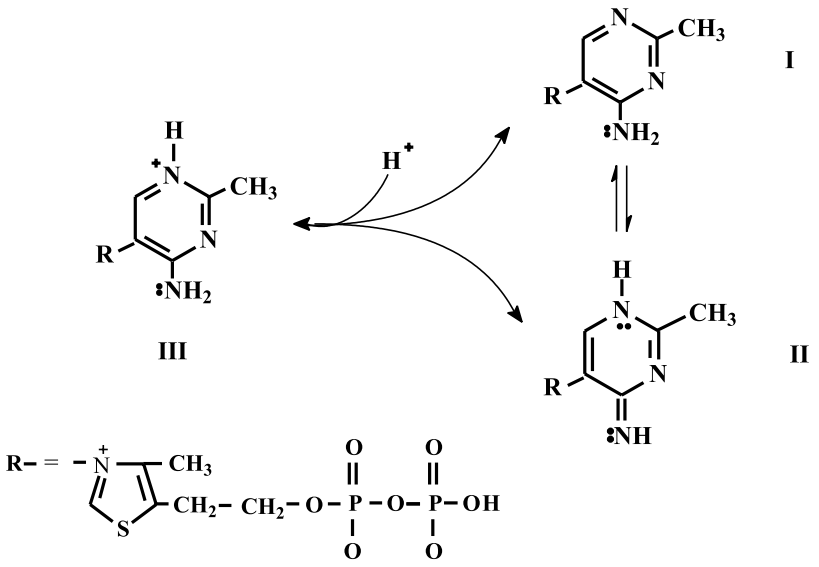
In the presence of phenylalanine, the intensity of the ThDP absorption band in the ethanol solution is increased (Fig. 3, curve 2), consistent with the data obtained with mutant transketolase: the replacement of Phe445 (located 4 Å away from the aminopyrimidine ring of ThDP, Fig. 1) by isoleucine leads to a significant decrease in the intensity of the same band in the CD spectra [28].

Given the hydrophobicity of the transketolase active center, the data on the induced absorption band of holotransketolase were interpreted as resulting from changed optical properties of the coenzyme following its incorporation into the hydrophobic pocket of the active center. Apparently the intensity of the induced absorption band increases as result of the interaction between the ThDP and the Phe445 residue.

What is the chemical species possessing this induced spectral band?

It is known, that the aminopyrimidine ring of ThDP (I in Scheme 1) can convert to the imino tautomer (II in Scheme 1) concomitant with hydrogen bond formation between the carboxyl of Glu418 and the $N^{1'}$-atom of the ThDP aminopyrimidine ring. Fast and easily reversible interconversion between amino and imino tautomeric forms of the ThDP during catalysis is well known [29,35].

In aqueous solution ThDP exists predominantly in the amino form (I in Scheme 1), having no absorption bands in the near UV region [36]. A hydrophobic environment promotes the formation of the imino form [29] (II in Scheme 1). In the active site of holotransketolase the aminopyrimidine ring of ThDP is surrounded by hydrophobic amino acids [3,4]. Therefore, we concluded



Scheme 1. Tautomeric and resonance forms of ThDP.

that appearance of the new absorption band 290–360 nm (Figs. 2A and B) results from ThDP transformation to the imino form (II in Scheme 1) after incorporation of its aminopyrimidine ring into the hydrophobic pocket of the active site. Then the amplitude of this band should depend on pH: decreasing of pH would shift the equilibrium from the imino form (II in Scheme 1) to the amino form (III in Scheme 1) with the consequent disappearance of the long-wave absorption. Indeed, as shown in Fig. 4, the intensity of the induced long-wave absorption band of the ThDP solution in ethanol decreases with the increasing of medium acidity. Similar pH dependence in pH region 5.9–3.0 has holoTK absorption (Fig. 5, solid curve) and CD band (Fig. 5, dashed curve): amplitude decreases with pH decreasing below 6.0. The only difference consists in the increase of the absorption in the 7.6–5.9 pH range. The reason for

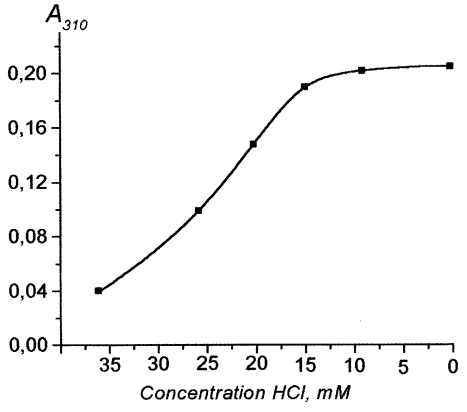


Fig. 4. Dependence of the intensity of the ThDP absorption band in the ethanol solution (50%) on the medium acidity. ThDP concentration was 2.5 mM.

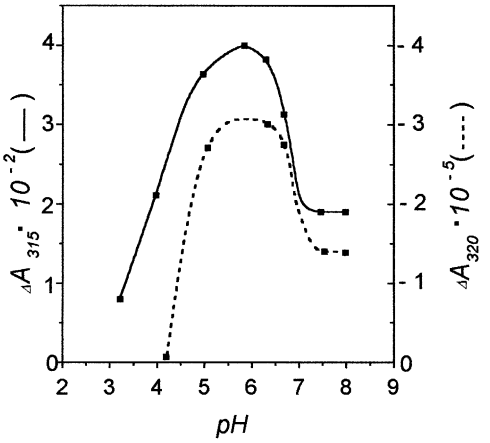


Fig. 5. Effect of pH on the intensity of holotransketolase absorption band, induced through ThDP binding, in the absorption spectrum (solid curve) and CD spectrum (dashed curve). All spectra were recorded in 20 mM glycyl-glycine buffer, pH 7.6, containing 2.5 mM $CaCl_2$. Concentration of transketolase—3 μM (solid curve) and 6 μM (dashed curve), concentration of ThDP—2.5 mM.

this currently is unknown and needs a further investigation. It is necessary to notice the stability of holotransketolase in the investigated pH range and no ThDP release from the holoenzyme during the whole experiment time (data not shown).

In the holoenzyme the imino form of ThDP is stabilized not only by its interaction with Phe445, but also by its interaction with the glutamic acid residue through the $N^{1'}$-atom of the aminopyrimidine ring [31,37]. In transketolase this residue is Glu418 [4,8]. Indeed, the interaction of the transketolase mutant (where the Glu418 residue is replaced by glutamine or alanine) with ThDP was not accompanied by the appearance of a new absorption band in CD spectrum [8].

Together, presented data allow us to formulate a general concept for the molecular origin of the induced optical band of transketolase–ThDP. During the interaction of ThDP with apotransketolase the aminopyrimidine ring of the coenzyme incorporates into the hydrophobic pocket of the enzyme active site, after which the 4′-amino tautomeric form of the aminopyrimidine ring converts to the $N^{1'}H$-imino tautomeric form, which is stabilized through the interaction with the Glu418 and Phe445 residues.

The investigation of a variety of catalytically inactive ThDP analogs has shown that their interaction with transketolase is either not accompanied by the appearance of the induced optical band or the character and intensity of optical changes differ from those occurring in the native holoenzyme [38]. There is one known exception: $N^{3'}$-pyridyl–ThDP, in which the $N^{1'}$ atom is replaced by carbon. The CD spectrum of holotransketolase, bound to $N^{3'}$-pyridyl–ThDP, does not differ from that of the native holoenzyme [39]. This is not surprising, since at certain conditions, the α-aminopyridine ring of this analogue, like the aminopyrimidine ring of ThDP, possess the ability to convert into the imino form [40–42]. The fact that ThDP after the replacement of $N^{1'}$ for carbon ($N^{3'}$-pyridyl–ThDP) has lost its catalytic activity when incorporated into the holoenzyme, can be explained by the lacking of the $N^{1'}$-atom, which is capable of forming a hydrogen bond with Glu418 residue—a necessary requirement for catalysis.

The participation of the $N^{1'}H$-imino tautomeric form of ThDP in catalysis is generally recognized for all thiamin diphosphate enzymes. In this paper, we have shown, that in the case of transketolase, this form arises at the step of the coenzyme binding to the apoprotein, prior to the act of catalysis. With other thiamin diphosphate enzymes, which do not have these spectral properties, the imino form appears probably during catalysis only.

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