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Function of the Aminopyrimidine Part in Thiamine Pyrophosphate Enzymes

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To answer the question on the mechanistic significance of the pyrimidine moiety of thiamine pyrophosphate (TPP), the two pyridine analogs of TPP (N_1-pyridyl-TPP and N_3-pyridyl-TPP), as well as 4′-deamino-TPP, have been resynthesized and incubated with the apoenzymes of pyruvate decarboxylase, pyruvate dehydrogenase complex, and transketolase. By comparison of activity and binding properties of the three TPP analogs it is shown that only N_1-pyridyl-TPP causes catalytic activity (between 65 and 100%) with all the enzymes tested. N_3-Pyridyl-TPP as well as 4′-deamino-TPP proved inactive generally. The binding experiments demonstrate that both analogs with the N_1-atom preserved in the structure (N_1-pyridyl-TPP and 4′-deamino-TPP) offer practically the same affinity as TPP to the three apoenzymes tested. A mechanism is proposed that explains the essential function of the amino group *and* the pyrimidine-N_1 in TPP catalysis.

INTRODUCTION

In 1967 we described for the first time the function of the aminopyrimidine component of thiamine pyrophosphate (TPP),[2] i.e., the function of the three nitrogens in the N_1 and N_3 positions and in the amino group, in the catalytic mechanism of pyruvate decarboxylase (PDC) (*1*). With the help of these "site-directed" substitutions of the coenzyme we could show that modifications of the 4′-amino group (elimination or substitutions), as well as elimination of the N_1 atom of the pyrimidine ring (**II**, Fig. 1), produce inactive TPP analogs after incubation with the apoenzyme of PDC.

To explain these findings three possible functions of the aminopyrimidine nitrogens in TPP enzymes had to be considered:

1. The essential nitrogens could be involved in the binding mechanism, thus

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[2] Abbreviations used: PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase complex; TK, transketolase; ADH, alcohol dehydrogenase; TPP, thiamine pyrophosphate.

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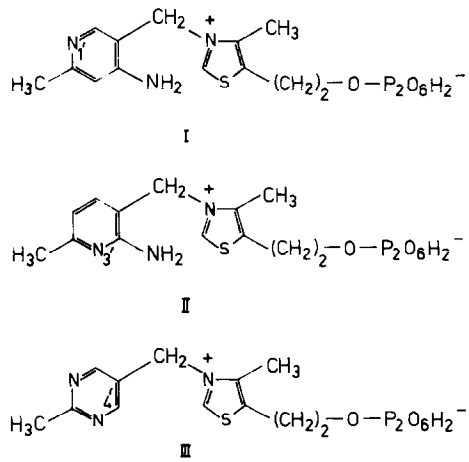

FIG. 1. Structures of the three analogs of thiamine pyrophosphate tested. (**I**) N_1-Pyridyl-TPP, (**II**) N_3-pyridyl-TPP; (**III**) 4′-deamino-TPP.

functioning in addition to the pyrophosphate–Mg^{2+} link (*2*) as a second anchor of the coenzyme.

2. The aminopyrimidine part could activate the catalytic function of the thiazolium moiety via inductive contributions.

3. The aminopyrimidine part of TPP could function as a second catalytic site, realizing, e.g., an essential proton transfer step via the 4′-amino group as discussed in previous papers (*3*).

The following experiments compare activity *and* binding properties of the three analogs—N_1-pyridyl-TPP, N_3-pyridyl-TPP, and 4′-deamino-TPP (Fig. 1, **I–III**)—after incubation with the apoenzymes of TK and PDH (characterized by a reversible binding of the cofactors) and PDC [showing irreversible binding behavior at the functional pH 6.0 (*4*)]. As a result of these experiments the functional role of the three nitrogens of the pyrimidine component in the mechanism of TPP enzymes will be reevaluated.

MATERIALS AND METHODS

Preparation and Activity Estimations of the Enzymes

Pyruvate decarboxylase. PDC was isolated and purified (40 U/mg) from dried brewer's yeast (*5*). The apoenzyme was prepared according to Sieber *et al.* (*6*). The regained catalytic activity of the apoenzyme incubated with the different TPP analogs was determined at 340 nm and 30°C using ADH and NADH as complementary enzyme systems. The test mixture contained 0.1 M sodium citrate buffer, pH 6.2, 10 U/ml ADH, 0.2 mM NADH, 40 mM pyruvate, and 1.5–2 μg/ml PDC.

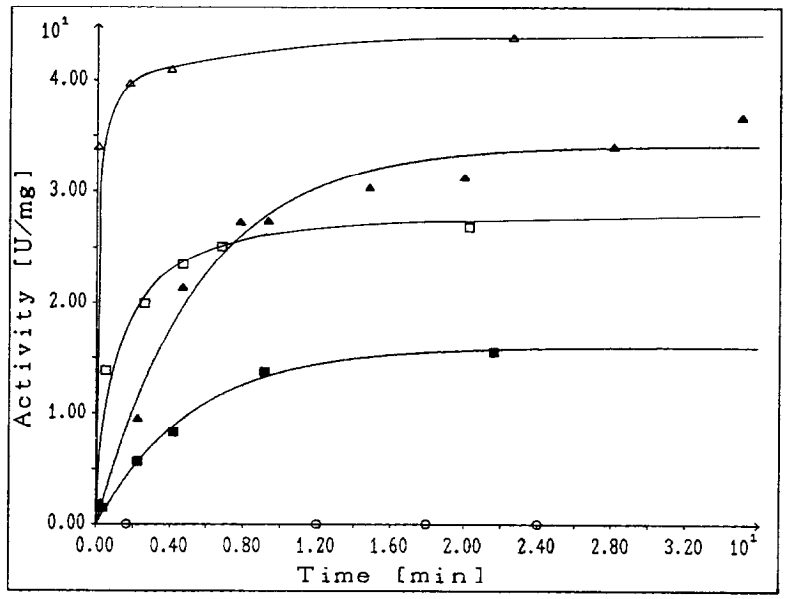

FIG. 2. Progress curves comparing the reconstitution rates of apo-PDC and II-holo-PDC with different TPP analogs. △, Apo-PDC (0.27 mg/ml) + TPP (4.9 mM); $k > 8.6$ min^{-1}; A_{max} = 44 U/mg. ○, Apo-PDC (0.27 mg/ml) + **II** (5.9 mM); k not detectable; A_{max} = 0.08 U/mg. □, Apo-PDC (0.18 mg/ml) + **I** (2.1 mM); $k \sim 1.4$ min^{-1}; A_{max} = 27 U/mg. ▲, II–PDC (0.18 mg/ml) + TPP (3.2 mM); $k = 0.18$ min^{-1}; A_{max} = 34 U/mg. ■, II–PDC (0.11 mg/ml) + **I** (2 mM); $k = 0.19$ min^{-1}; A_{max} = 16 U/mg. k and A_{max} were calculated by fitting the data points to first-order kinetics. Apo-PDC was incubated at 30°C in 0.5 M Hepes buffer, pH 6.8, containing 5 mM $MgSO_4$ and the corresponding TPP analog.

Former experiments (*4*) had shown that TPP binds to apo-PDC irreversibly at pH > 7. An estimation of binding constants was impossible for this reason. Therefore, the relative binding stabilities of the two pyridine analogs and TPP were characterized qualitatively by comparing the incorporation rates of TPP and N_1-pyridyl-TPP respectively with those of apo-PDC and the (inactive) holoenzyme that forms by incubation of apo-PDC with N_3-pyridyl-TPP as coenzyme (Fig. 2).

For reconstitution 0.1 ml apoenzyme was incubated at 30°C in 0.1 ml 1 M Hepes buffer, pH 6.8, containing 10 mM $MgSO_4$ and 11 mM TPP, or 4 mM N_1-pyridyl-TPP, or 11 mM N_3-pyridyl-TPP, respectively. Samples were taken from this mixture for measuring the catalytic activity of PDC regained after distinct time intervals. In the case of competition experiments we used mixtures of the analogs (concentrations indicated in Fig. 2) in 1 M Hepes buffer, pH 6.8.

Pyruvate dehydrogenase complex. PDH from *Escherichia coli* K12 (strain Ymel) was purified according to Bisswanger (*7*). The overall activity of the PDH was determined according to Schwartz *et al.* (*8*), following photometrically the reduction of NAD at 340 nm and 30°C. The test mixture contained in a 1-ml test volume 50 mM Tricine buffer, pH 7.6, 1 mM $MgSO_4$, 2.5 mM dithiothreitol, 2.5 mM NAD, 0.1 mM coenzyme A, 2 mM pyruvate, 1–2 μg of the purified enzyme preparation, and TPP, N_1-pyridyl-TPP, N_3-pyridyl-TPP, or 4′-deamino-TPP. Reaction was

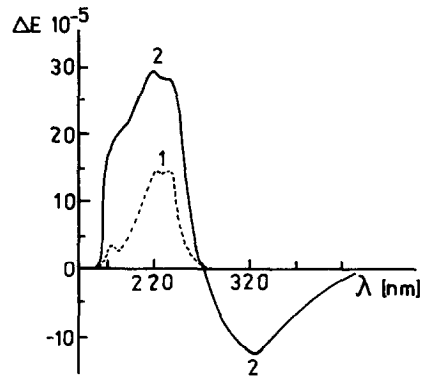

FIG. 3. CD spectra of apo-TK (1) and holo-TKs (2) formed by addition of TPP or N_1-pyridyl-TPP or N_3-pyridyl-TPP to the apoenzyme in 5 mM glyclyglycine buffer, pH 7.6. Concentrations: TK, 5.1 μM; $CaCl_2$, 2.5 mM; TPP, 30 μM; N_1-pyridyl-TPP, 30 μM; N_3-pyridyl-TPP, 17 μM.

started by addition of pyruvate. Binding constants (K_d) for TPP and N_1-pyridyl-TPP were estimated from Lineweaver–Burk-plots. K_i values of N_3-pyridyl-TPP and 4′-deamino-TPP were estimated according to Dixon (*9*).

Transketolase. Transketolase was isolated from dried baker's yeast according to Racker and co-workers (*10*) with the modifications described in (*11*). Apotransketolase exhibiting 4% residual activity in the absence of TPP was prepared according to the method of Kochetov and Isotova (*12*) by incubation of the holoenzyme in 1.6 M $(NH_4)_2SO_4$, pH 8.4, for 48 h and deionization via Sephadex G-50. Transketolase activity was measured by following the reduction of NAD spectrophometrically at 340 nm (*13*). The reaction mixture contained, in a final volume of 2 ml, 50 mM glycylglycine buffer, pH 7.6, 0.37 mM NAD, 25 mM sodium arsenate, 0.5 U glyceraldehyde-3-phosphate dehydrogenase, 2 mM dithiothreitol, 2.5 mM $CaCl_2$, 2–10 nM transketolase [specific activity 19 U/mg (*14*)], and TPP, or N_1-pyridyl-TPP, or N_3-pyridyl-TPP. Reaction was started by adding 10 mM pentose-5-phosphate mixture [as described by Gubler *et al.* (*15*)] to the reaction mixture.

As in the case of PDH, a reversible binding of TPP is observed in the case of TK. Moreover, the correct binding of the coenzyme reflects significantly in the CD spectra (*16*) (Fig. 3). CD spectra of the TK preparations were measured with a CD dichrograph Mark III (Jobin Ivon, France) with a path length of 1 cm and a sensitivity of 2×10^{-4} absorption units per 0.1 cm.

Binding constants (K_d) for TPP and N_1–pyridyl-TPP were estimated from Lineweaver–Burk plots. In the case of the inactive N_3-pyridyl-TPP the K_i value was estimated according to Dixon (*9*).

Substances

N_1-Pyridyl-TPP and N_3-pyridyl-TPP. The analogs were resynthesized with few modifications by the methods described earlier (*17*). The synthesis of N_1-pyridyl-TPP was modified in some steps, producing a considerably higher yield (*18*). All analogs were tested for identity and purity by ^{1}H-NMR spectroscopy.

TABLE 1

Activity and Binding Constants (K_d, K_i) of N_1-Pyridyl-TPP (**I**), N_3-Pyridyl-TPP (**II**), 4′-Deamino-TPP (**III**), and TPP as Cofactors of Pyruvate Decarboxylase (PDC), Pyruvate Dehydrogenase (PDH), and Transketolase (TK)

	I		II		III		TPP	
Apoenzyme of	V_{rel} (%)	K_d (M)	V_{rel} (%)	K_i (M)	V_{rel} (%)	K_i (M)	V_{rel} (%)	K_d (M)
PDC	65	—[a]	0	—[a]	0	—[a]	100	—[a]
PDH	70	3.6×10^{-6}	0	—[b]	0	1.0×10^{-6}[c]	100	1.0×10^{-6} 2.9×10^{-5}[c]
TK	100	8.0×10^{-7}	0	0.1×10^{-7}	0	2.6×10^{-7}	100	9.7×10^{-7}

Note. Activity data (%) refer to the activity of the corresponding apoenzymes (residual activities taken as 0%) after recombination with TPP under optimum conditions (see Materials and Methods).

[a] Irreversible binding (*6*).

[b] No measurable affinity after simultaneous incubation with TPP.

[c] In the absence of pyruvate.

Other chemicals. TPP, NAD, NADH, cysteine hydrochloride, and ADH were obtained from Serva, dithiothreitol and coenzyme A from Boehringer, potassium pyruvate and glycylglycine from Merck, and Bio-Gel P2 from Bio-Rad. All other reagents used were of the highest purification grade available.

RESULTS

The catalytic properties of N_1-pyridyl-TPP, N_3-pyridyl-TPP, and 4′-deamino-TPP, after binding to the different apoenzymes, are summarized in Table 1. The only compound producing between 65 and 100% activity after incorporation into the apoenzymes of PDC, PDH, and TK is N_1-pyridyl-TPP. Both N_3-pyridyl-TPP and 4′-deamino-TPP proved inactive in all the cases tested.

The binding behavior of the analogs is described in comparison to the native coenzyme TPP in Figs. 2 and 3, as well as in Table 1.

Pyruvate Decarboxylase

As a result of irreversible binding mechanism of TPP (*4*) a comparison of the binding properties via the usual binding constants (K_d, and K_i as in the cases of PDH and TK) was not possible. On the other hand, the retarded substitution rate of the enzyme-bound N_3-pyridyl-TPP analog by TPP (Fig. 2, ▲) suggests a competition with the native coenzyme for the binding sites of PDC. The substitution experiments with TPP (▲) and N_1-pyridyl-TPP (■) demonstrate a considerably weaker binding of the N_3-pyridyl-TPP analog. A dominant role of the N_1–protein interaction in the binding mechanism of PDC can be derived from these experiments.

Pyruvate Dehydrogenase

Activity and binding properties of the analogs with PDH are summarized in Table 1. Both the N_1-pyridyl-TPP analog and the deamino-TPP analog show practically the same affinity to the active site of the enzyme as compared with the native coenzyme. But, contrary to PDC, practically no affinity of N_3-pyridyl-TPP to the enzyme could be measured in the presence of TPP. This result underlines again the essential binding function of the N_1 atom of TPP in the cofactor binding mechanism.

Transketolase

Like PDH a reversible cofactor binding mechanism has been observed in the case of TK (*19*). Moreover, binding of TPP (i.e., the interaction of the thiazolium moiety with apo-TK) is also reflected in the CD- spectra (*16*). As shown in Fig. 3, no difference is found after substitution of TPP by the two pyridyl analogs (deamino-TPP was not measured).

The binding constants of the TPP analogs with apo-TK (Table 1) confirm the results of the CD experiments. Contrary to PDH and PDC, a stable fixation of all three cofactors (obviously via the same binding mechanism) has been found. This result demonstrates on the one hand the anchor function of the thiazolium pyrophosphate link in the binding mechanism of this enzyme. On the other hand, comparing the different catalytic properties with the identical molecular geometry as well as the identical position of the N atoms with respect to the CH_2 bridge, an essential function of the N_1 atom in the TK mechanism seems obvious.

DISCUSSION

The results of the binding experiments indicate that the main binding capacity of the aminopyrimidine system depends on the N_1 atom. Only those analogs containing N_1 [i.e., N_1-pyridyl-TPP (active) and 4′-deamino-TPP (inactive)] form stable bonds with all the enzymes tested. With the inactive N_3-pyridyl-TPP, stable binding is observed only in the case of TK. With apo-PDC a considerably reduced affinity has been found and with PDH no interaction with the protein could be detected.

Only analogs with a complete N_1-4′-amino system offer full binding capacity *and* activity with all the three enzymes tested. The following conclusions can be drawn from the experiments described in this paper:

1. Binding of TPP to the apoenzymes is realized evidently via the thiazolium pyrophosphate moiety and the N_1 atom of the pyrimidine part. In the case of TK, N_1 and N_3 can substitute for the binding function. PDC and PDH show a higher selectivity with respect to the position of the ring nitrogens.

2. Activation of the catalytic function of the thiazolium component via inductive contributions of the aminopyrimidine part can be excluded. First, the two chemically related pyridine analogs could hardly cause per se the completely different catalytic effects after incorporation into the apoenzymes of PDC, PDH, and TK.

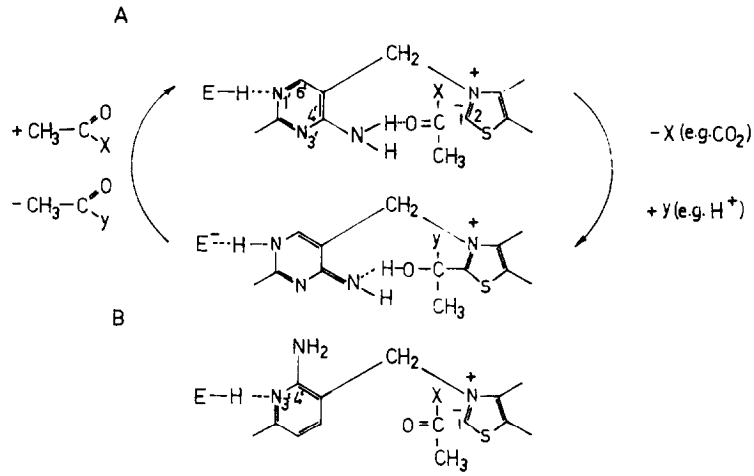

FIG. 4. (A) Proton relay mechanism explaining the essential function of a protein–N_1-interaction in the proton translocation steps of the TPP mechanism. (B) N_3-pyridyl-TPP, obeying the same cofactor binding mechanism like TPP or N_1-pyridyl-TPP.

In addition, this conclusion is confirmed by the insensitivity of the C_2-H acidity (NMR) after substitutions of the pyrimidine moiety as measured by Fierke and Jencks (*20*). As proposed for the first time by Breslow (*21*) H/D exchange rates at C_2 can be used as an indicator of the electronic state of the catalytic center of TPP. Mainly for this reason the amino group of TPP (which is not essential for cofactor binding as shown by the high affinity of 4′-deamino-TPP!) should function as a second and essential operator in the catalytic mechanism of TPP.

3. The affinity of N_1-pyridyl-TPP and 4′-deamino-TPP to the active sites of the three enzymes as well as the activity of only N_1-pyridyl-TPP suggests the following mechanism (Fig. 4): On the assumption that the 4′-amino group functions besides the C_2 position as an essential part of TPP, this mechanism will be influenced strongly by the protonation state of N_1 (*3*, *22*). As a result, N_1 protonation, e.g., by interaction with the protein, will influence the catalytic properties of the amino group (Fig. 4A). To understand the complete inactivity of the sterically and chemically related N_3-TPP analog we assume that the N_3 atom occupies in this case the N_1-binding site within the active center of the TPP enzymes (Fig. 4B). In this case the 4′-amino group would lose (by turning to the opposite 6′ position) its catalytic function.

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