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Product inhibition of mammalian thiamine pyrophosphokinase is an important mechanism for maintaining thiamine diphosphate homeostasis

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ABSTRACT

Background: Thiamine diphosphate (ThDP), an indispensable cofactor for oxidative energy metabolism, is synthesized through the reaction thiamine + ATP ⇆ ThDP + AMP, catalyzed by thiamine pyrophosphokinase 1 (TPK1), a cytosolic dimeric enzyme. It was claimed that the equilibrium of the reaction is in favor of the formation of thiamine and ATP, at odds with thermodynamic calculations. Here we show that this discrepancy is due to feedback inhibition by the product ThDP.

Methods: We used a purified recombinant mouse TPK1 to study reaction kinetics in the forward (physiological) and for the first time also in the reverse direction.

Results: K_{eq} values reported previously are strongly underestimated, due to the fact the reaction in the forward direction rapidly slows down and reaches a pseudo-equilibrium as ThDP accumulates. We found that ThDP is a potent non-competitive inhibitor ($K_i \approx 0.4$ μM) of the forward reaction. In the reverse direction, a true equilibrium is reached with a K_{eq} of about 2×10^{-5}, strongly in favor of ThDP formation. In the reverse direction, we found a very low K_m for ThDP (0.05 μM), in agreement with a tight binding of ThDP to the enzyme.

General significance: Inhibition of TPK1 by ThDP explains why intracellular ThDP levels remain low after administration of even very high doses of thiamine. Understanding the consequences of this feedback inhibition is essential for developing reliable methods for measuring TPK activity in tissue extracts and for optimizing the therapeutic use of thiamine and its prodrugs with higher bioavailability under pathological conditions.

1. Introduction

Thiamine diphosphate (ThDP), the diphosphorylated derivative of thiamine (vitamin B1), is a coenzyme for several essential mammalian enzymes (transketolase, 2-hydroxyacyl-CoA lyase 1) and enzyme complexes (pyruvate, 2-oxoglutarate and 2-oxoacid dehydrogenase complexes) that play a key role in energy metabolism [1].

In animals, which are unable to synthesize thiamine, the vitamin is taken up by specific transporters [1]. Thiamine is then pyrophosphorylated to the coenzyme ThDP by cytosolic thiamine diphosphokinase (or pyrophosphokinase, TPK, EC 2.7.6.2) according to the reaction: thiamine + ATP ⇆ ThDP + AMP.

Bacteria, archaea, fungi and plants are able to synthesize thiamine *de novo*, with thiamine monophosphate (ThMP) being the first thiamine compound formed. ThMP is then hydrolyzed to thiamine by thiamine monophosphatases before diphosphorylation to ThDP by thiamine pyrophosphokinase (TPK) [2]. The only exceptions are enterobacteriaceae (including *E. coli*), which lack TPK and directly phosphorylate ThMP to ThDP by thiamine monophosphate kinase (EC 2.7.4.16).

The enzyme catalyzing ThDP synthesis was partially purified from yeast and characterized by Kazyro [3] and by Thomé-Beau et al. [4]. A TPK with very similar properties was also partially purified from rat

Abbreviations: ThDP, thiamine diphosphate; ThMP, thiamine monophosphate; TPK, thiamine pyrophosphokinase.

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liver [5] and from rat brain [6]. TPKs with slightly different properties were characterized from parsley leaves [7] and *P. denitrificans* [8].

TPKs from mammalian sources were studied with more detail by Gubler and coworkers [6,9]. They showed that the enzyme has a high affinity for thiamine ($K_m \approx 0.12$ μM). Since then, the enzyme has been extensively characterized. TPK is not specific for ATP, and significant activity is observed with other nucleoside triphosphates, sometimes UTP being the most effective [10–12]. However, as intracellular ATP concentrations are much higher than those of other nucleoside triphosphates, it is most probably the physiologically relevant substrate. There is an absolute requirement for Mg^{2+} as activator of TPK, only Co^{2+} is a good substitute [10].

Molecular cloning yielded the cDNA sequences of *S. cerevisiae* [13], mouse [14] and human [15] enzymes and predicted respectively a 319 amino acid protein (36.6 kDa) for *S. cerevisiae* and a 243 amino acid (27 kDa) protein for the mammalian enzymes. The three-dimensional structure was obtained for the *S. cerevisiae* [16], mouse [17,18] and *Candida albicans* [19] enzymes. In all cases the enzymes crystallize as homodimers.

There has been controversy concerning the mechanism underlying the pyrophosphorylation of thiamine by TPK1. Steady-state kinetic studies [4,9,10] suggest a ping-pong mechanism where Mg-ATP binds first and a pyrophosphorylated intermediate *E*-PP is formed. Subsequent to the release of AMP, thiamine binds to E-PP and the PP group is transferred to thiamine and ThDP is released. Alternatively, it has been proposed [11,20] that TPK1 obeys an ordered sequential mechanism where ATP binds first followed by thiamine, resulting in a catalytically competent ternary complex. More recent structural data [18,19] are consistent with the latter mechanisms as they suggest a one-step pyrophosphate transfer from ATP to thiamine. Crystal structures of TPK complexed with thiamine [17] or thiamine-PNP (a non-hydrolyzable analog of ThDP) [19] have been obtained, and data from the structure of mouse recombinant TPK [18] suggest that the formation of a ternary complex with thiamine and Mg-ATP is completely compatible with the structural evidence, while the possibility that thiamine would only bind to an *E*-PP intermediate seems unlikely.

On the other hand, mutational studies of human recombinant TPK [10] revealed the presence of 3 aspartate residues essential for catalysis. Structural data suggest that a Mg^{2+} ion is coordinated to these carboxylates and to the β-phosphoryl group of ATP in the enzyme-substrate complex [18]. Thus, Mg^{2+} appears to be indispensable for the binding of ATP and the transfer of the pyrophosphate group to thiamine at the active site.

While in animals there seems to be only one isoform of the enzyme, two biochemically redundant but differentially expressed cytosolic enzymes AtTPK1 and AtTPK2 have been identified in *A. thaliana* [21]. Mammalian TPKs are officially referred to as TPK1 [14], without implying a closer relation to AtTPK1 than to AtTPK2. Indeed, both Arabidopsis enzymes share 93% sequence similarity, but their sequence similarity with mammalian TPKs is low.

The *hTPK1* gene is located at 7q34, comprising at least eight exons and seven introns [22]. *hTPK1* mutations are rare and have been reported only fairly recently, but they are probably underdiagnosed [23,24]. These autosomal recessive disorders of early childhood can result from missense, splice-site and frameshift mutations [25–27]. TPK1 deficiency symptoms are severe, with generally fatal outcome. They result in Leigh disease-like phenotypes with acute encephalopathy, ataxia, dystonia, seizures and elevated lactate and oxoglutarate plasma and cerebrospinal concentrations. In some cases, when a significant enzyme activity remains, as reported for the TPK1 mutation affecting the dimerization of monomers, a thiamine therapy can give promising results [28], emphasizing the importance of an early diagnosis. Indeed, early age intervention is an important factor for improving the outcome after thiamine supplementation and whole blood ThDP measurements could be helpful for diagnosis and biochemical monitoring following thiamine supplementation [29,30].

In this study we reexamine several kinetic properties of TPK1 using the recombinant mouse enzyme. We especially focus on the feedback inhibition of the enzyme by its product ThDP. Actually, inhibition of TPK by ThDP was reported 40 years ago [11,20], but this was forgotten and the physiological significance of this property was never analyzed. Here, we show that product inhibition explains the erroneous estimation of the K_{eq} of the reaction reported in the past [9]. Studying for the first time the reaction in the reverse direction, we determined the true K_{eq} value and show that the equilibrium is strongly in favor of ThDP synthesis from thiamine and ATP. Finally, we emphasize the important role of feedback inhibition by ThDP in the regulation of thiamine and ThDP levels *in vivo*, especially in blood and brain after thiamine supplementation.

2. Materials and methods

2.1. Molecular cloning, expression and purification of mouse TPK1

2.1.1. Strain and plasmid

E. coli BL21(DE3) expression strain and the pET-28a(+) expression vector were obtained from Novagen. pET-28b(+) was used for cloning and protein production of mTPK1 protein.

2.1.2. Cloning of mTPK1

In contrast to Nosaka et al. [14], who made a GST fusion protein, we preferred a His-tagged protein (for sequence see Supplement Fig. S1). A double-stranded gBlocks fragment was ordered from Integrated DNA Technologies, BVBA (Leuven, Belgium). The gBlock was designed to contain a *Nco*I restriction site, 6 histidine codons, a TEV protease recognition site coding sequence, an *E. coli* codon optimized mTPK1 gene and a *Xho*I restriction site. The gBlock was suspended in water at a concentration of 10 ng/μl and inserted in pET28a(+) by restriction and ligation to create the pET18-mTPK1 expression plasmid. The ligation product was introduced by transformation into *E. coli* BL21(DE3) and the selection was performed on LB agar plates containing ampicillin (100 μg/ml).

2.1.3. mTPK1 expression and purification

E. coli BL21 (DE3) cells bearing pET18-mTPK1 were grown overnight in 10 ml of LB medium containing 100 μg/ml ampicillin, pelleted, grown in 250 ml of fresh medium for 2 h and induced for 4 h with isopropyl-*β*-D-thiogalactopyranoside at 0.5 mM. The cells were then pelleted by centrifugation, resuspended in 50 mL buffer A containing 50 mM phosphate-buffered saline (pH 8.0), 300 mM KCl and 10 mM imidazole and disrupted using EmulsiFlex C3 homogenizer (Aventin) on ice. Cell debris were removed by centrifugation at 20000 × *g* for 20 min. The clarified supernatant containing the mTPK1 protein was then filtered through a 0.2 μm filter and loaded onto a 5 ml Bio-Scale Mini Profinity immobilized metal affinity chromatography (IMAC) cartridge equilibrated in buffer A. The cartridge was washed with 10 column volumes of buffer A to remove contaminating proteins and mTPK1 was eluted from the column with elution buffer (buffer A containing 250 mM imidazole). The eluted fractions containing mTPK1 protein were pooled and filtered (0.22 μm).

The purity was tested by SDS-Page and UV spectroscopy (see Supplement Figs. S2 and S3). The protein concentration was estimated at 12.9 mg/ml by UV absorption at 280 nm. By using a chemical method [31] we obtained a value of 11.4 mg/ml. The value of 12.9 mg/ml was used throughout the study. Note that TPK is absent from *E. coli* and a contamination with an endogenous enzyme is thus excluded.

2.2. Assay of TPK enzymatic activity

Enzymatic activity was measured as previously described [32]. 500 mM stock solutions of ATP (A2383 Sigma-Aldrich) and AMP (01970 Fluka Biochemika) were prepared in water and adjusted to pH 7.0 with NaOH 15%.

For the forward reaction, the assay mixture (300 μl) contained Tris-HCl (0.1 M, pH 7.5), various concentrations of ATP, $MgSO_4$ (5886 Merck) and thiamine (T4625 Sigma-Aldrich). The enzyme concentration

was 0.19 μg/ml. In some cases, when the aim was not to work under initial rate conditions but to attain an equilibrium condition rapidly, a higher enzyme concentration was used (2.75 μg/ml or 11 μg/ml).

For the reverse reaction, which proceeds at a slower rate than the forward reaction, the assay mixture (300 μl) contained Tris-HCl (0.1 M, pH 7.5), various concentrations of AMP, $MgSO_4$, ThDP (Sigma-Aldrich C8754) and an enzyme concentration of 2.42 or 86 μg/ml was used.

The incubation was carried out for 20 min at 37 °C with the controls being incubated in the absence of enzyme. The reaction was stopped by addition of 60 μl trichloroacetic acid (60%). After centrifugation (10,000 *g*, 15 min), the acid was extracted with 3 × 1.5 ml diethylether and ThDP and thiamine were determined by HPLC [33]. In some cases the ATP formed in the reaction was determined by a HPLC method, using fluorescence detection after ethenylation with chloroacetaldehyde as previously published [34].

High sample concentrations of AMP (> 5 mM) interfere with the HPLC determination by considerably shifting retention times. Therefore, when higher AMP concentrations were used, the sample was diluted 10 times before injection on the HPLC.

2.3. Culture of Neuro2a cells

Mouse neuroblastoma cells (Neuro2a, ATCC n°: CCL-131) were grown in a custom-made DMEM medium dry-packed without thiamine (Life Technologies Limited, Paisley, Scotland, UK) and 10% fetal bovine serum (FBS, Gibco, Life Technologies Europe BV, Merelbeke, Belgium) as previously described [35,36] in a 5% CO_2 humidified atmosphere at 37 °C. FBS contains an initial concentration of 100 nM thiamine, hence the final thiamine concentration in the culture medium was 10 nM, which corresponds to the physiological concentration in human plasma. This concentration is sufficient for normal growth of the cells. The thiamine prodrug sulbutiamine (SuBT, 25 μM filter-sterilized (22 μm) solution in water) was added to Neuro2a cells and thiamine and ThDP concentrations were determined after various incubation times in 5% CO_2 humidified atmosphere at 37 °C.

2.4. Gel exclusion chromatography of recombinant mTPK1

100 μl of the TPK1 stock solution (12,9 mg/ml) were injected on a TSKgel® size exclusion column (G3000SW, 30 cm × 7.8 mm; Tosoh Corporation, Tokyo, Japan) at a flowrate of 1 ml/min in Tris/KCl buffer (Tris-Cl 50 mM + KCl 50 mM, pH 7.2). We collected 500 μl fractions for protein determination by UV absorbance at 280 nm and ThDP concentrations by HPLC. The molecular mass of the protein was estimated to be approximately 60 kDa as expected for the recombinant mTPK homodimer.

2.5. Statistical analysis

Data were analyzed and regression curves were generated using GraphPad Prism 9 for MacOS.

3. Results

3.1. Substrate dependence of the TPK1 forward reaction

3.1.1. K_m for thiamine

We measured the K_m for thiamine in the presence of ATP concentrations close to physiological (2 mM). As data reported with the recombinant human enzyme [10] suggest that a low concentration of free Mg^{2+} is required for optimal activity, our assay mixture contained Mg^{2+} in slight excess (2.5 mM) over ATP.

As shown in Fig. 1A, a hyperbolic saturation curve was obtained and non-linear regression to the Michaelis-Menten equation yielded a K_m value of 0.072 μM ($R^2 = 0.96$). The inset of Fig. 1A shows a Lineweaver-Burk plot of the same data, highlighting the linear relationship between $1/v_i$ and 1/[Thiamine].

3.1.2. Effect of increasing ATP concentrations

In their study using recombinant human TPK1, Onozuka and Nosaka [10] found Michaelian kinetics for ATP with K_m = 1.2 mM, provided that [Mg^{2+}] was in excess of [ATP]. This is much lower than the K_m values reported for TPK1 isolated from mammalian brain [6,9,32]. In order to explain these discrepancies, we performed a more accurate investigation of the initial reaction rate *versus* ATP concentration in the presence of different concentrations of the activator Mg^{2+}.

Data shown in Fig. 1B indicate that there are significant deviations from Michaelian kinetics. In the presence of low (2 mM) Mg^{2+}, there is no apparent deviation as long as [ATP] is lower than 2 mM but, at higher [ATP], there is a strong inhibition by an excess of the substrate. A plausible explanation is that the true substrate is the $MgATP^{2-}$ complex and that free ATP acts as a competitive inhibitor when it is in excess. At higher Mg^{2+} concentrations (10 and 20 mM) the v_i *vs* [ATP] plots have quite different shapes: the deviation from Michaelian kinetics is apparent when [ATP] > 2 mM and, now, there is an activation by excess substrate.

In order to estimate K_m and V_{max} and to visualize a possible deviation from linearity, the data obtained were represented as Lineweaver-Burk plots (Fig. 1C). These appear linear for ATP concentrations ranging from 0.1–1 mM. At higher concentrations of ATP (> 2 mM), we observe a very significant deviation from Michaelian kinetics. Extrapolation of the regression analysis to the x-axis allowed the estimation of apparent K_m values of 0.94 mM (2 mM Mg^{2+}), 1.1 mM (10 mM Mg^{2+}) and 1.33 mM (20 mM Mg^{2+}), in good agreement with each other. This suggests a $K_{m,app}$ of about 1 mM for ATP, close to the values reported by Onozuka and Nosaka (1.2 mM) and Voskoboyev and Ostrovsky (1.0 mM).

Activation by excess substrate is highlighted using a Hanes plot (gives a more prominent curvature than the Lineweaver-Burk plot of the same data) in the presence of 10 mM Mg^{2+} (Fig. 1D). Extrapolation of the data for low [ATP] yields a value of 1.2 mM for $K_{m,app}$. Extrapolation of the data for high [ATP] yields a value of 9 mM for $K_{m,app}$ under conditions of substrate activation.

The extrapolated V_{max} value at physiological ATP concentration (Fig. 1A, C, D) is around 60 nmol mg^{-1} min^{-1} or 1 nmol mg^{-1} s^{-1}, corresponding to $k_{cat} = 0.03\ s^{-1}$. This is in good agreement with the V_{max} (1.0 nmol mg^{-1} s^{-1}) previously published for the His-tagged human TPK [10]. Similar values were also reported for the natively purified mammalian enzyme [4,11]. It must be concluded that the catalytic power of TPK1 is low at physiological ATP concentrations.

3.1.3. Effects of Mg^{2+} ions

Mg^{2+} is an indispensable activator of TPK1, other bivalent cations being less effective [10]. Data from Fig. 1B show that maximal TPK1 activity is observed when Mg^{2+} is in excess over ATP. Under these conditions, most ATP is in the form of $MgATP^{2-}$ complex [37].

We tested the effect of Mg^{2+} at different concentrations of ATP (2, 5, 10 and 15 mM) on the enzyme activity. We plotted the % of the maximum activity (100%) for each ATP concentration as a function of [Mg^{2+}] (Fig. 2A). In each case, an optimum was obtained, followed by a decrease in enzyme activity. It is obvious that the optimum increased with increasing ATP concentration.

The question that arises is to know whether the activating effect of Mg^{2+} is only due to the formation of the complex with ATP, or whether there is a binding site that specifically binds free Mg^{2+}. In order to clarify this point, we plotted the optimum [Mg^{2+}]/[ATP] ratio (calculated from the data of Fig. 2A) as a function of [ATP] (Fig. 2B). The data show that at low [ATP] (< 5 mM), the optimum ratio is higher than 1, as expected from the previous data (Fig. 1B). However, at higher [ATP], the ratio becomes lower than 1, tending towards 0.6 under conditions of strong activation by excess substrate. This is in agreement with an earlier report [9] that the optimum [Mg^{2+}]/[ATP] ratio for TPK1 activity was 0.6 at high ATP concentrations.

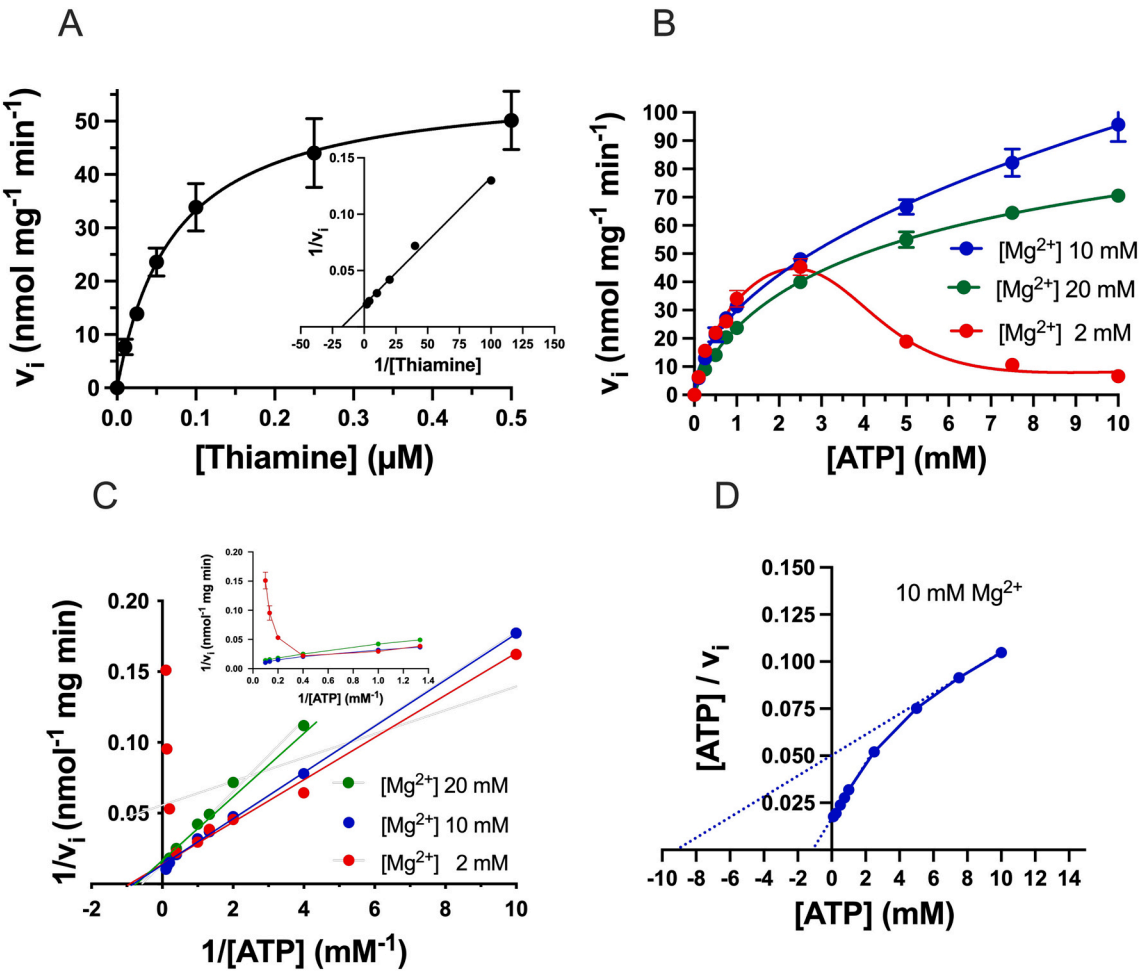

Fig. 1. Substrate dependence of recombinant mouse TPK activity for the forward reaction. (A) Dependence of ThDP synthesis on thiamine concentration (2 mM ATP, 2.5 mM Mg^{2+}). The data were fitted to the Michaelis-Menten equation using non-linear regression. The inset shows a Lineweaver-Burk plot of the same data and a linear regression line. (B) Dependence of ThDP synthesis on ATP concentration at three different Mg^{2+} concentrations (1 μM thiamine). (C) Lineweaver-Burk plots of the data from graph 1B. Linear regression lines were generated from the linear parts of the curves. The inset shows the same data for [ATP] > 0.75 mM only. (D) Hanes plot for the data obtained in the presence of 10 mM Mg^{2+}. The dotted lines represent the regression lines obtained for low [ATP] ≤ 2 mM (extrapolated $K_{m,app}$ ≈ 1.1 mM) and high [ATP] ≥ 5 mM (extrapolated $K_{m,app}$ ≈ 9 mM).

We then calculated the free [Mg^{2+}] for each combination of total Mg^{2+} and ATP concentrations from Fig. 2A and plotted v_i as a function of free [Mg^{2+}] in the presence of different concentrations of ATP (Figs. 2C-F). In each case, a free [Mg^{2+}] of 0.5–1 mM, close to cytosolic physiological concentrations [38], was required to reach full activity. The concentration of free Mg^{2+} corresponding to half-maximal activation (apparent K_a) was 0.1–0.25 mM. Unexpectedly, a significant inhibition by excess Mg^{2+} was observed.

The rather low value of K_a for Mg^{2+} is not unexpected in view of published structural data on the configuration of the mouse TKP1 active site [18]: in the enzyme-substrate complex, the cation is believed to be coordinated to a triad of aspartate residues (D71, 73 and 100) as well as to the β-phosphoryl group of ATP. This would result in a tight binding of Mg^{2+} at the active site in the presence of ATP.

3.2. TPK1 is inhibited at physiological ThDP concentrations

We measured the kinetics of the forward reaction by following the disappearance of thiamine and the appearance of ThDP as a function of time using a thiamine concentration of 3 μM (more than 10 times K_m) and an ATP concentration of 500 μM (lower than K_m) (Fig. 3A).

The rate of the reaction remains roughly linear for 15–20 min, and then it slows down. After two hours, the rate of ThDP formation becomes very slow, suggesting that an equilibrium is close, when about two thirds of the thiamine has been converted to ThDP. Extrapolation of the data suggests that, at equilibrium, the ThDP concentration would correspond to ≈ 2.1 μM. The apparent equilibrium constant was calculated using the simplified equation (Eq. (I)) used by Peterson et al., 1975 [9]. This assumes that at equilibrium the ThDP and AMP concentrations are equal and the ATP concentration, largely in excess with respect to the thiamine concentration, is considered equal to its initial value:

$$K_{eq} = \frac{[ThDP]_{eq}\,[AMP]_{eq}}{[ATP]_{eq}\left([Thiamine]_{init} - [ThDP]_{eq}\right)} = \frac{[ThDP]^2_{eq}}{[ATP]_{init}\left([Thiamine]_{init} - [ThDP]_{eq}\right)} \quad \text{(I)}$$

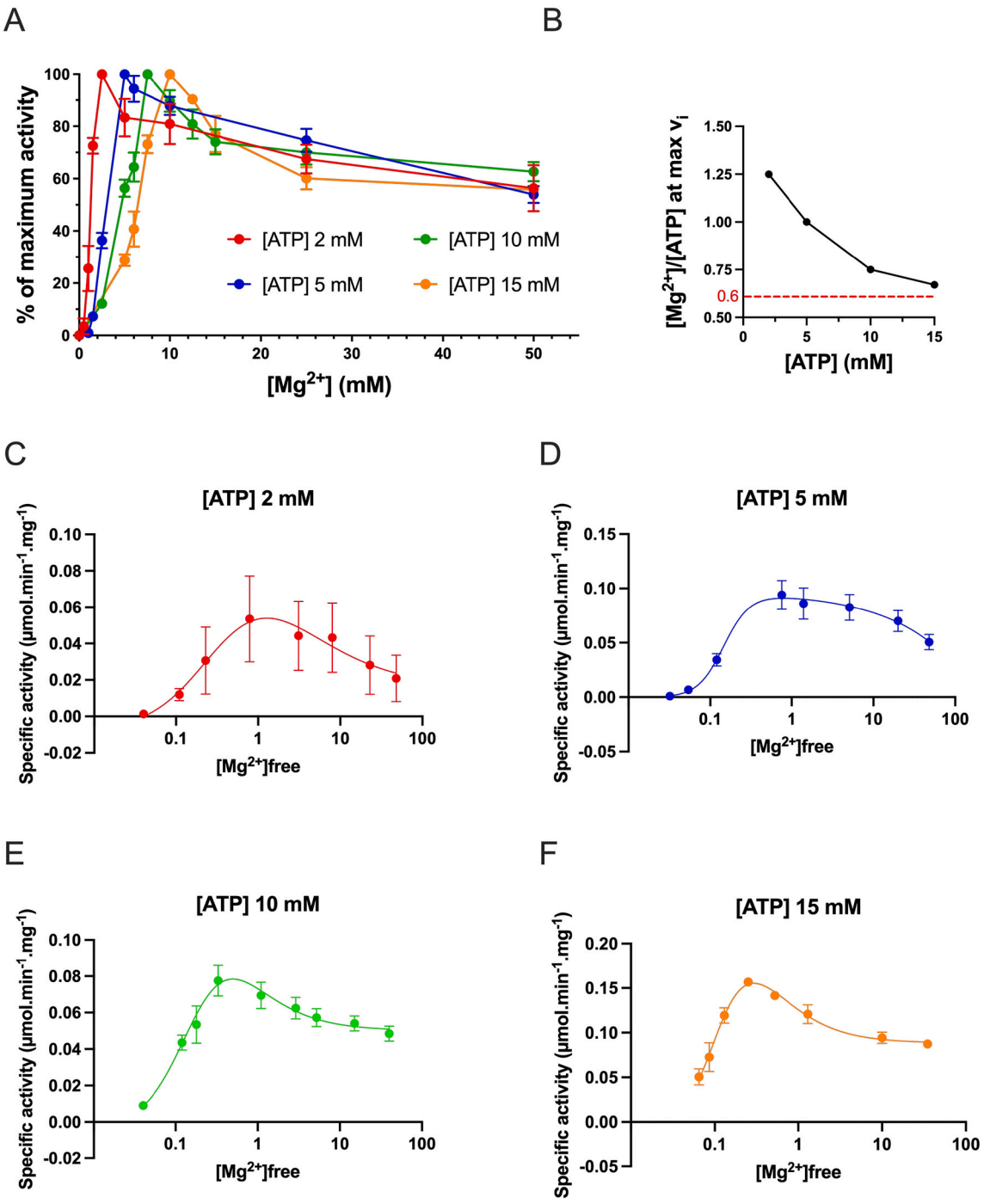

Fig. 2. Dependence of TPK1 activity in the forward direction on Mg^{2+}. (A) Effect of increasing $MgSO_4$ concentration on enzyme activity at different concentrations of ATP. In each case, the y axis shows the percentage of maximum activity (100%) obtained for each ATP concentration.
(B) Plot of the $[Mg^{2+}]$/[ATP] concentration ratio yielding maximal activity as a function of ATP concentration [data from (A)]. (C–F) Dependence of TPK1 activity on the free Mg^{2+} concentration for various concentrations of ATP. [39]. The data (total Mg^{2+} and ATP concentrations) were those used in Fig. 2A. We calculated free $[Mg^{2+}]$ with the Mg-ATP Calculator v1.3 from UC Davis (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/MgATP-TS.htm) using the constants from Schoenmakers' Chelator. All incubations were carried out at 37 °C and for 15 min. All data are expressed as mean ± SD (n = 3–10).

$$K_{eq} = \frac{[2.1\ 10^{-6}]^2}{[0.5\ 10^{-3}]\ ([3\ 10^{-6}] - [2.1\ 10^{-6}]} = 0.010 \ll 1$$

Such a low K_{eq} would mean that the equilibrium is strongly in favor of the formation of thiamine and ATP from ThDP and AMP. $K_{eq} \ll 1$ would be in agreement with the results of Peterson et al., 1975, who reported a $K_{eq} \approx 10^{-5}$ for the forward reaction [9]. However, as this reaction couples the hydrolysis of a phosphoanhydride (ATP → AMP + PP_i), $\Delta G^\circ \approx -45$ kJ/mol at 25 °C [40]) to the condensation of an aliphatic alcohol with pyrophosphate ($\Delta G^\circ \approx +14$ kJ/mol), the balance for the standard free energy change of the global reaction should be approximately −31 kJ/mol at 25 °C. Hence, according to the relation $\Delta G^\circ = -RT \ln K_{eq} = -8.3\ 10^{-3} \times 310 \ln K_{eq} = -31$ kJ/mol, we would expect a $K_{eq} \approx 1.7.10^5$, which is very different from the value estimated above.

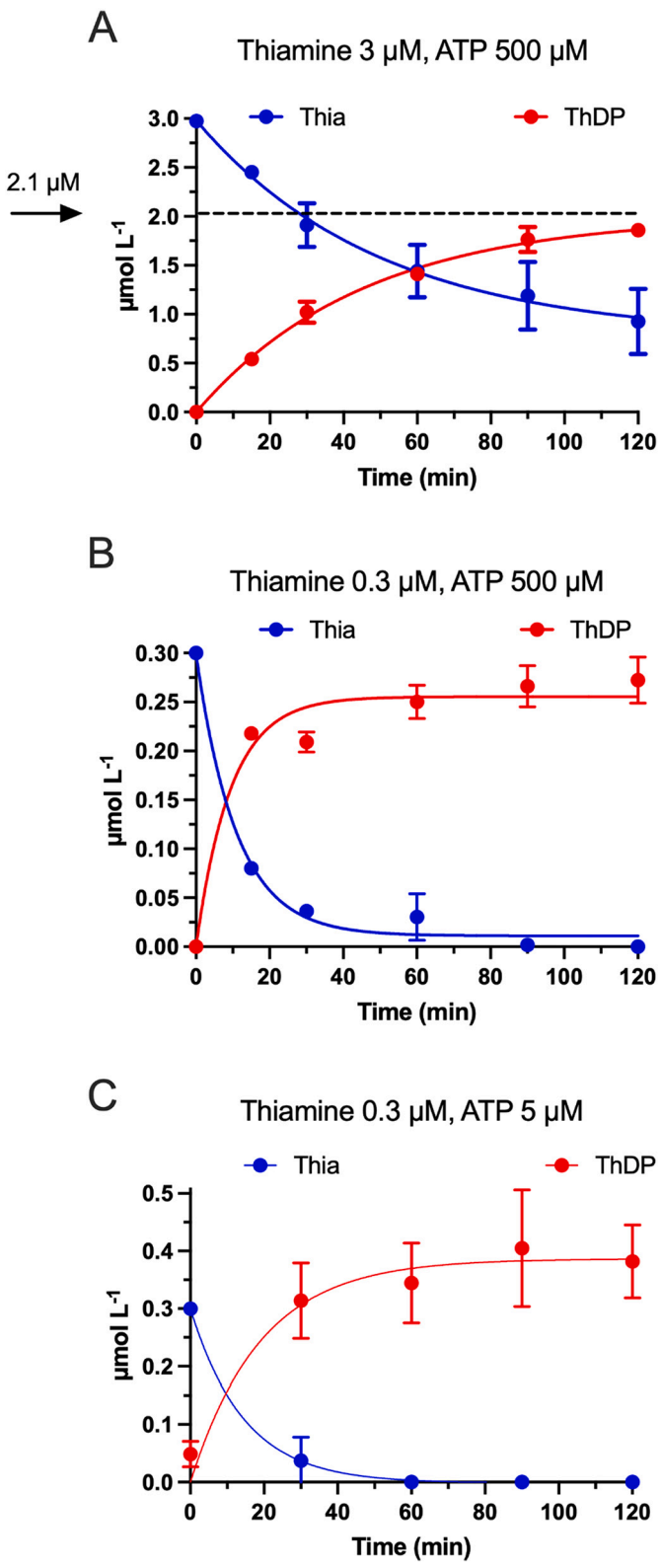

Fig. 3. Kinetics for the conversion of thiamine to ThDP by TPK1 for different thiamine and ATP concentrations. All other reagents were the same: 300 µM $MgSO_4$, 97 mM Tris-HCl (pH 7.5). The enzyme concentration was 0.00275 mg/ml in (A) and (B) and 0.011 mg/ml in (C). Note that in (C) a small amount of ThDP is already present at $t = 0$, corresponding to ThDP associated with the enzyme. The data are presented as mean ± SD (A: $n = 8$; B: $n = 3$; C: n = 3). The plateau was determined using a one-phase association model (Y = (Plateau)*(1-exp(—K.t)).

It is thus likely that the pseudo equilibrium reached in the experiment shown in Fig. 3A does not correspond to the true equilibrium of the reaction.

We then started the reaction with lower concentrations of the substrates. When the initial concentration of thiamine was 10 times lower (0.3 µM instead of 3 µM, Fig. 3B), the reaction was practically complete and thiamine was nearly undetectable after 60 min. It was thus impossible to estimate K_{eq} from these data. We therefore also decreased the ATP concentration to 5 µM (Fig. 3C). In spite of the very low initial concentrations of both substrates, the reaction was again practically complete. Decreasing the [ATP] by a factor 100 should have displaced the equilibrium towards the left, but this is obviously not the case. It can easily be calculated that, with a $K_{eq} = 0.01$, calculated from Fig. 3A, and [Thiamine] = 0.3 µM and [ATP] = 5 µM, only about 33% of the initial thiamine concentration should have been consumed (See also Supplement Table 1). This suggests that K_{eq} is much higher than 0.01.

It remains to be explained why the reaction is not complete under the conditions of Fig. 3A. An obvious possibility would be that the enzyme progressively loses activity. We checked this by preincubating the enzyme for 2 h at 37 °C before adding thiamine (Supplement Fig. S4). The initial rate was close to the one without preincubation (20 nmol/ l min^{-1} *vs* 33 nmol/l min^{-1}, Fig. 3A), indicating that denaturation is not the main reason for the slowing down of the reaction.

Another possibility is that the enzyme is progressively inhibited as a reaction product (AMP or ThDP) accumulates in the reaction medium (product inhibition). Inhibitory effects of AMP and ThDP have indeed been reported for TPK isolated from rat liver [11] and parsley leaves [20]. AMP was only a weak inhibitor with a $K_i > 20$ mM, but ThDP significantly inhibited the enzyme at submicromolar concentrations.

In order to check this hypothesis, we tested the effects of increasing ThDP concentrations for two different thiamine concentrations (0.25 µM and 1 µM) (Fig. 4). Because of the presence of ThDP at the start of the reaction, we were unable to measure the enzyme activities by quantification of the ThDP formed during the reaction. Thus, we could only measure the disappearance of thiamine. In order to remain under conditions of initial rate, the conditions of the experiment were such that after 20 min incubation, not more than 20% of the thiamine was converted to ThDP. For this reason, the difference between initial and final concentrations of thiamine was small, resulting in relatively high standard deviations. In order to minimize differences between experiments, the data were expressed in % of the maximum value ([ThDP] = 0 µM) in Fig. 4. We found an $IC_{50} = 0.40 \pm 0.07$ µM ($n = 4$) at 0.25 µM thiamine and an $IC_{50} = 0.35 \pm 0.28$ µM ($n = 6$) at 1 µM thiamine ($p = 0.39$, Mann-Whitney test), suggesting a non-competitive inhibition. Note that Voskoboyev and Ostrovsky [11] also reported non-competitive inhibition of the liver enzyme by ThDP.

The inhibitory constant is of the same order of magnitude as the concentration of ThDP produced during the forward reaction under the conditions shown in Fig. 3A. It can be estimated that the ThDP concentration reached after two hours is close to 2 µM, largely enough to almost completely inhibit the enzyme.

When analyzing our enzyme preparation, we found a significant amount of ThDP associated with the enzyme. This ThDP can only be a carry-over from the *E. coli* cells used to produce the enzyme. *E. coli* cells are very rich in ThDP, containing approximately 142 nmol g^{-1} wet weight [41], hence the intracellular concentration can be estimated to be >142 µM, which would completely saturate TPK. The fact that this ThDP remains associated with the enzyme during purification is another indication suggesting a high affinity of TPK for its product ThDP. According to our estimation, the preparation contained 0.14 mol of ThDP per mole of enzyme monomer. In order to confirm that this ThDP is indeed bound to TPK1, the preparation was injected on a gel exclusion column. The fractions were collected and, in each fraction, the ThDP concentration was estimated by HPLC and the protein concentration by absorption at 280 nm. Both profiles coincided and no free ThDP was detected (Supplement Fig. S5). The molecular mass of the eluted protein

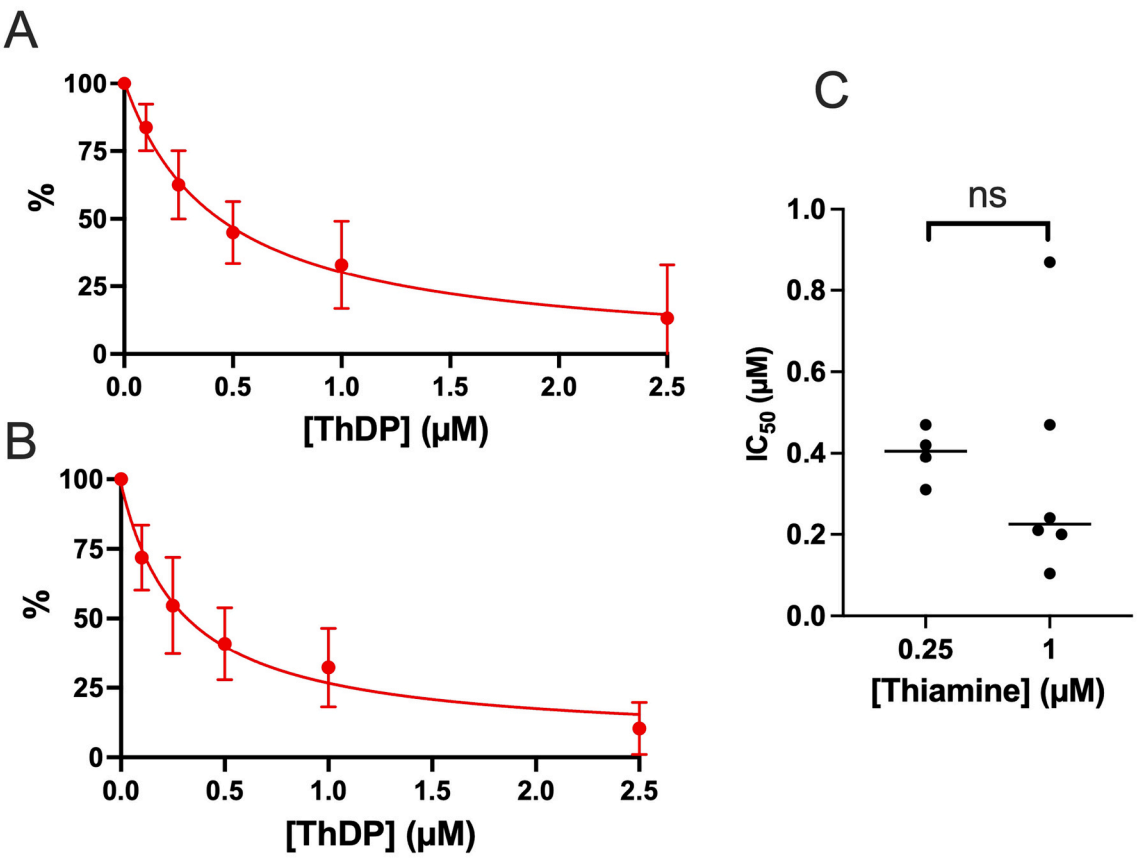

Fig. 4. Effect of increasing ThDP concentrations on TPK forward reaction activity (20 min at 37 °C) with a thiamine concentration of 0.25 μM (A) or 1 μM (B). The data are expressed as % of the maximum (0 μM ThDP) (mean ± SD, n = 4 (A), n = 6 (B)). (C) Distribution of the IC_{50} values calculated for each independent experiment (p = 0.39, Mann Whitney test). The ATP concentration was 2.5 mM and the Mg^{2+} concentration was 3 mM (free [Mg^{2+}] = 0.5 mM).

(60 kDa) is in agreement with a homodimeric structure as reported in the literature [16,17]. Such a tight binding of ThDP to TPK1 suggests that this enzyme can be considered a ThDP-binding protein.

3.3. Determination of the K_{eq} using the reverse reaction

To our knowledge, nobody ever reported data on the reverse reaction catalyzed by TPK. Moreover, in the discussion accompanying the paper by Voskoboyev and Ostrovsky, C.J. Gubler comments that "…nobody has been able to get the reaction to go in the opposite direction." [11]. This is all the more paradoxical, as Gubler and coworkers claimed that the equilibrium of the reaction is strongly in favor of ATP synthesis from ThDP and AMP [9].

Here, we show for the first time that the reverse reaction is possible and that it can proceed at a significant rate, provided there is a large concentration of AMP (≥ 5 mM) and a relatively high amount of enzyme is used. The activity was estimated by measuring the appearance of thiamine in the assay medium for two different concentrations of ThDP, 0.3 and 3.0 μM and a large excess of AMP (50 mM) (Fig. 5A). The reaction was roughly linear during 10 min, allowing a reasonably accurate determination of v_i. However, after longer times, the rate of reaction slowed down and a plateau was reached.

The initial rate is strongly dependent on AMP concentration between 5 and 50 μM (Fig. 5B), suggesting a low affinity ($K_m \approx 20$ mM). In contrast, the enzyme seems to have a very high affinity for ThDP: apparent K_m values were 0.043 ± 0.017 μM in the presence of 5 mM AMP and 0.053 ± 0.011 μM in the presence of 50 mM AMP (Fig. 5B). Thus, K_m values for ThDP are not significantly modified when the concentration of the co-substrate AMP is changed 10-fold.

Considering a maximum specific activity (V_{max} in Fig. 5B) of 5.3 nmol mg^{-1} min^{-1}, we can calculate a k_{cat} of 0.0026 s^{-1}, a value 10–20 times lower than for the forward reaction (Fig. 1D). This difference is not so high that TPK1 would be considered a "one-way" enzyme, but it is evident that the reverse reaction is unlikely to take place at a significant rate *in vivo*, particularly in view of the low physiological concentrations of AMP (< 1 mM) and Mg^{2+} (1 mM).

Mg^{2+} ions were absolutely required for activity (Fig. 5C) but, unexpectedly, the Mg^{2+} concentration required for half-maximum activity was relatively high: we calculated an EC_{50} (apparent K_a) of 7.1 ± 1.7 mM and 12 ± 5 mM in the presence of respectively, 5 or 50 mM AMP, about 50 times higher than the EC_{50} values for free Mg^{2+} found for the forward reaction (Fig. 2). Mg^{2+} forms only weak complexes with AMP [37], compared to ATP and ADP. O'Sullivan and Smithers (1979) indicate a stability constant of 40 for the MgAMP complex compared to 73,000 for $MgATP^{2-}$ and it was estimated to be 2400 for MgThDP [42]. Hence, under our conditions (5 mM AMP and 15 mM Mg^{2+}), about 87% of the Mg^{2+} should be free (corresponding to a free concentration of 13.3 mM).

These data suggest a high affinity of the enzyme for ThDP but a very low catalytic power in the reverse direction. It must however be kept in mind that at 2.42 μg/ml (Fig. 5A, B & C), and assuming a molecular mass of 29.5 kDa for the recombinant enzyme, the enzyme concentration is 0.08 μM, the same order of magnitude as the substrate concentration. Hence, an essential condition for the validity of the Michaelis-Menten equation ([E] ≪ [S]) is not verified.

Data from Fig. 5A show that the reaction rate rapidly slows down after 10–20 min and that a plateau is reached under the conditions used (0.3 or 3 μM ThDP, 50 mM AMP and 30 mM Mg^{2+}). When the plateau is reached at 0.3 μM ThDP, about one third of the ThDP is converted to thiamine. If the plateau corresponds to the equilibrium of the reaction,

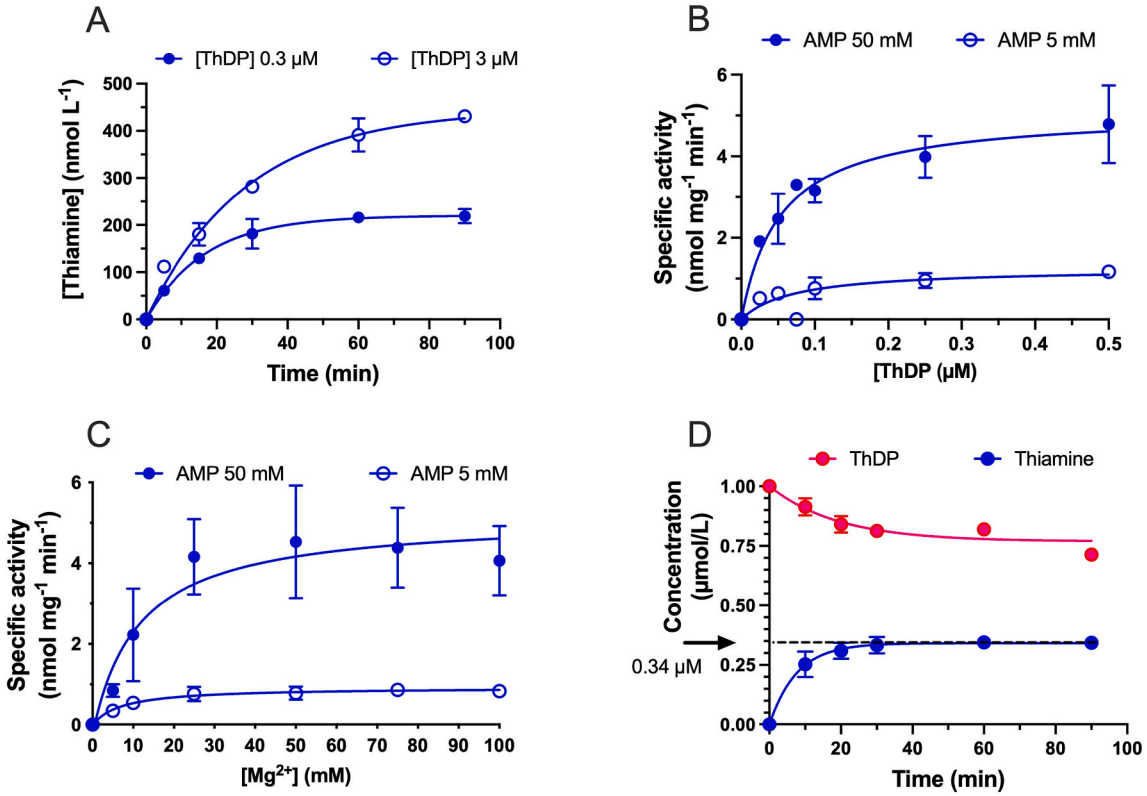

Fig. 5. Kinetic parameters for the mouse TPK reverse reaction ThDP + AMP → Thiamine + ATP at 37 °C. (A) Kinetics of thiamine synthesis at two concentrations of ThDP (0.3 and 3 µM). The AMP concentration was 50 mM and [Mg^{2+}] was 30 mM. (B) Dependence of the initial rate of the TPK reverse reaction on ThDP concentration at two different concentrations of AMP and at [Mg^{2+}] = 30 mM. (C) Dependence of the TPK reverse reaction on Mg^{2+} concentration at two different concentrations of AMP and at [ThDP] = 3 µM. (D) Kinetics of the reverse reaction catalyzed by TPK with ThDP (1 µM), AMP (5 mM), Mg^{2+} (15 mM). The enzyme concentrations were 2.42 µg/ml in A, B and C and 86 µg/ml in D. In panels A and D, the curves were obtained by a non-linear fit for one phase association: Y = (Plateau)(1-exp(—K.t)). In panel C, we used a [agonist] *vs* response model: Y = X.Top/(EC_{50} + X), while a Michaelis-Menten model was used in panel B. (Mean ± SD, n = 3–6).

[Thiamine]$_{eq}$ = 0.22 µM and K_{eq} can be calculated using the equation:

$$K_{eq,app} = \frac{[ATP]_{eq}[Th]_{eq}}{[AMP]_{eq}[ThDP]_{eq}} = \frac{\left([Th]_{eq}\right)^2}{[AMP]_{init}\left([ThDP]_{init} - [Th]_{eq}\right)} \quad \text{(II)}$$

$$= \frac{\left(0.22\ 10^{-6}\right)^2}{\left(50000\ 10^{-6}\right)\left(0.3\ 10^{-6} - 0.22\ 10^{-6}\right)} = 1.2{\cdot}10^{-5}$$

This suggests that the equilibrium of the reaction is strongly towards the side of the physiological products ThDP and AMP. Note that as the real substrate for the reverse reaction is most probably AMP and not a complex between AMP and Mg^{2+}, the latter simply being a cofactor, Mg^{2+} does not have to be considered for the determination of the equilibrium. As the equilibrium is already reached after 30 min, no denaturation of the enzyme should occur.

With the higher initial concentration of ThDP (3 µM, Fig. 5A), the plateau was not yet reached after 90 min and the equilibrium value is thus uncertain.

In order to get a more accurate estimation of K_{eq}, we repeated the experiment under different conditions (1 µM ThDP, 5 mM AMP and 15 mM Mg^{2+}) (Fig. 5D). In order to rapidly reach a plateau and thus minimize enzyme denaturation, we used a significantly higher enzyme concentration (86 µg/ml instead of 2.42 µg/ml). The plateau was reached after 30 min and maintained over a significant time with [Thiamine]$_{eq}$ = 0.34 µM (Fig. 5D). Using the above equation, we calculated a $K_{eq} = 3.5\ 10^{-5}$.

In order to exclude a mechanism where AMP would be a simple activator of ThDP hydrolysis by TPK1, we measured the synthesis of ATP for the reverse reaction under the following conditions: [ThDP] = 100 µM, [AMP] = 1 mM, Mg^{2+} = 10 mM and TPK1 = 0.86 mg/ml. A higher ThDP concentration was chosen so that enough ATP is synthesized to be detectable by our method. At equilibrium, the ATP concentration was 1.43 ± 0.22 µM and the thiamine concentration was 1.4 ± 0.06 µM (n = 3, p = 0.70, Mann-Whitney test) yielding a $K_{eq} = 2\ 10^{-5}$. Neither ATP nor thiamine was formed in the controls performed in the absence of enzyme.

The values of the K_{eq} obtained when the reverse reaction is carried out in the presence of various concentrations of substrates, activator and enzyme are shown in Table 1. The close agreement between K_{eq} values obtained under very different conditions suggests that true and relatively accurate values for equilibrium of the reaction have been obtained, in contrast with the uncertain values obtained when the reaction was studied in the forward direction.

With a $K_{eq} = 2\ 10^{-5}$, we can calculate a ΔG° of 27 kJ/mol for the reverse reaction and of −27 kJ/mol for the forward reaction. As shown above (paragraph 3.2), we expected a ΔG° of about −31 kJ/mol for the reaction: thiamine + ATP → ThDP + AMP. This value is close to the one found experimentally, not considering the difference in temperature as the experiments were done at 37 °C.

Table 1
K_{eq} values obtained under different experimental conditions for the reverse reaction ThDP + AMP ⇆ thiamine + ATP.

ThDP (µM)	AMP (mM)	Mg^{2+} (mM)	TPK1 (µg/ml)	K_{eq}
0.3	50	30	2.42	$1.2\ 10^{-5}$
1.0	5	15	86	$3.5\ 10^{-5}$
100	1	10	86	$2\ 10^{-5}$

Our data also show that, even in the absence of product inhibition, it would be virtually impossible to measure an equilibrium in the forward direction for this reaction. Indeed, even when we further decrease [ATP] to 5 μM for instance, at equilibrium, 99.999% of the thiamine would be consumed (Supplement Table S1), making the ThDP formed undetectable with our method.

3.4. Physiological significance of the feedback inhibition of TPK1 by its product ThDP

The strong inhibition of TPK1 by its product ThDP may give a plausible explanation for previously unexplained observations concerning the levels of thiamine and ThDP measured *in vivo*. We consistently observed that, when mammalian cells are incubated with thiamine or lipophilic thiamine prodrugs such as sulbutiamine or benfotiamine, the thiamine content rapidly increases inside the cells, while the ThDP content remains low [36,43,44]. Indeed, after a 6 h incubation of Neuro2a cells in the presence of 25 μM sulbutiamine, the ratio [Thiamine]/[ThDP] in the cells was about 1860/90 = 21(Fig. 6A). After longer times, the thiamine content seemed to decrease, probably as a result of thiamine exit caused by the outwardly directed concentration gradient [45], but it remained an order of magnitude above the ThDP content, which did not further increase during incubation. If product inhibition by ThDP is not considered, this result is difficult to understand with an equilibrium constant strongly in favor of ThDP synthesis. Moreover, as TPK1 is a cytoplasmic enzyme, most of the ThDP is transported into mitochondria, which would shift the equilibrium further towards ThDP synthesis. At the start, the ThDP content is higher than the thiamine content, but this is probably due to the fact that most of the ThDP is protein-bound and the free concentration is very low [46,47].

A similar observation was made in the blood of mice. After administration of the thiamine prodrug benfotiamine, thiamine concentrations rapidly increase in the blood (from 332 nmol/l to 33,000 nmol/l after 2 h, 100×), while ThDP remains comparatively low (increase from 662 nmol/l to 5200 nmol/l after 4 h, 8×) (Fig. 6B), though thiamine is rapidly transported into erythrocytes [48].

We previously determined that, in Neuro2a cells, an intracellular volume of 4.5 μl corresponds to 1 mg of protein [49]. Thus, we can estimate that the intracellular thiamine concentration after 4 h (Fig. 6A) is about 413 pmol/μl or 413 μM. The intracellular ThDP concentration would be at best 20 μM. This value is however largely overestimated as at least half of the ThDP is localized inside mitochondria, while thiamine is mainly cytosolic [46]. The intracellular ATP content was determined to be 14 nmol mg^{-1} of protein or 3.1 mM and the intracellular AMP concentration is approximately 0.2 mM [38]. With a $K_{eq,\ app}$ of $1/3.5\ 10^{-5} = 0.29\ 10^{5}$, we can write:

$$\frac{[ThDP]\,[AMP]}{[Thiamine]\,[ATP]} = \frac{[ThDP]}{0.413\ 10^{-3}}\ \frac{0.2\ 10^{-3}}{3.1\ 10^{-3}} = 0.29\ 10^{5} \qquad \text{(III)}$$

If we suppose that the thiamine and ThDP concentrations reach an equilibrium, we expect a $[ThDP]_{eq}$ of 185 M (Eq. (III)), instead of the $20\ 10^{-6}$ M observed. According to our data (Fig. 6A), the intracellular ThDP concentration attained, even if much lower than 20 μM, would be enough to inhibit the enzyme nearly completely.

It could be argued that the rate of ThDP synthesis is low, which would explain a [Thiamine]/[ThDP] ratio ≫ 1. The data from Fig. 6A suggest that the maximum intracellular ThDP concentration is already attained after 4 h. Thereafter, it does not increase significantly, though the thiamine concentration remains at least an order of magnitude higher. The rate of ThDP synthesis was about 70 pmol mg^{-1} per 2 h. The specific activity of our enzyme preparation was estimated to be 1.0 nmol $mg^{-1}\ s^{-1}$ for the forward reaction, which amounts to $1.0 \times 60 \times 60 \times 2 = 7200$ nmol mg^{-1} for two hours. As TPK1 represents about 0.05% of all proteins in mammalian tissues (based on the relative enrichment during purification [11,50]), we can estimate a specific intracellular activity of 8640/2000 = 4.32 nmol mg^{-1} for two hours, largely in excess of the 70 pmol mg^{-1} per 2 h required. Of course, the enzyme has a very low K_m for thiamine, meaning that it is saturated when excess thiamine is applied to the cells, but this should not prevent a chemical equilibrium to be reached.

Another possibility would be that ThDP does not accumulate because of rapid hydrolysis by thiamine diphosphatases. However, hydrolysis is slow in these cells and we previously estimated a half-life of 17 h for ThDP in the same neuroblastoma cells [46].

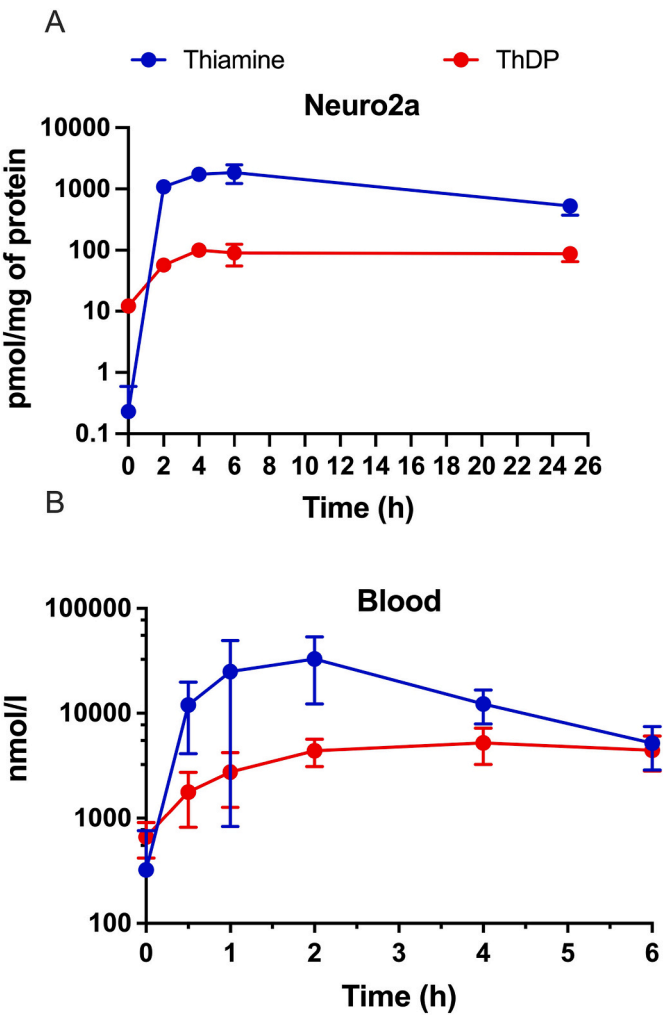

Fig. 6. Effect of the thiamine prodrugs sulbutiamine and benfotiamine on thiamine and ThDP levels in cultured Neuro2a cells and blood. (A) Effect of sulbutiamine on the content of thiamine and ThDP in Neuro2a cells. The cells were first grown for 2 weeks in a thiamine-poor medium (final concentration 10 nM) as previously described [36]. Sulbutiamine (25 μM) was added and thiamine and ThDP were determined by HPLC after 0, 2, 4, 6 and 25 h. (Mean ± SD, $n = 3$ different cultures; two-way ANOVA: row factor $p < 0.0001$, column factor $p < 0.0001$, interaction $p < 0.0001$; multiple paired *t*-test $p < 0.05$ for all comparisons) (B) Effect of benfotiamine on the content of thiamine and ThDP in mouse blood (Data from Volvert et al., 2008 [43]). Benfotiamine was dissolved in a 200 mM solution of hydroxypropyl-β-cyclodextrin and administered by gavage in a single dose (100 mg/kg). The animals were sacrificed at the indicated times and thiamine and ThDP were determined in blood by HPLC. (Mean ± SD, $n = 7$ animals; two-way ANOVA: row factor $p = 0.0004$, column factor $p = 0.0015$, interaction $p < 0.0001$; multiple paired t-test $p < 0.05$ for $t = 0.5$, 1, 2 and 4 h).

4. Discussion

Using for the first time the reverse reaction, we were able to estimate a K_{eq} for the TPK1 reaction and we show that this equilibrium strongly favors the synthesis of the coenzyme ThDP. This is in contrast to a previously published study [9] and we suggest that this discrepancy is due to inhibition of the enzyme by the reaction product ThDP. The K_i for this non-competitive inhibition is approximately 0.35–0.4 μM (in agreement with the K_i estimated by Mitsuda et al., 1975 [20] for the parsley leaf enzyme). Such a concentration can easily be reached in the cytoplasm and could account for the hitherto unexplained observation that ThDP rarely accumulates in the cytoplasm, even when thiamine concentrations are orders of magnitude higher [36,44]. Hence, ThDP controls its own synthesis as already suggested previously by Ostrovsky and coworkers [11].

Few enzymes are known to transfer a pyrophosphate group from a nucleoside triphosphate to an alcohol to form a phosphoester. Presently (as of August 31, 2021), the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology recognizes only five enzymes catalyzing the transfer of a diphosphate group (EC 2.7.6.x): ribose-phosphate diphosphokinase (EC 2.7.6.1), thiamine diphosphokinase (EC 2.7.6.2), 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase (EC 2.7.6.3), nucleotide diphosphokinase (EC 2.7.6.4), GTP diphosphokinase (EC 2.7.6.5). To our knowledge, except for TPK and, to some extent, for phosphoribosyl-diphosphate, none of these enzymes has been studied in detail yet. Ribose-phosphate diphosphokinase is involved in nucleotide biosynthesis, catalyzing the reaction ATP + D-ribose 5-phosphate ⇆ AMP + 5-phospho-α-D-ribose 1-diphosphate. Switzer et al. report a K_{eq} of 28.6 and a ΔG° of −2 kcal/mol (≈ −8.4 kJ/mol) [51], noting that 5-phospho-α-D-ribose 1-diphosphate has a particularly high glycosyl transfer potential. Therefore, we could expect a more negative ΔG° value and a $K_{eq} > 28.6$ for the reaction catalyzed by TPK, which is indeed observed.

In addition, we show that TPK1 tightly binds ThDP. *E. coli* cells contain very high amounts of ThDP but practically no unphosphorylated thiamine. This ThDP may bind to recombinant TPK1 produced within the cells and at least part of it remains bound during the purification, suggesting that TPK1 is a ThDP-binding protein. Indeed, the existence of thiamine and ThDP-binding proteins supposedly different from ThDP-dependent enzymes, has been reported in mammalian tissues [52].

TPK1 has a very low K_m for thiamine (< 0.1 μM), suggesting a high affinity for unphosphorylated thiamine, in agreement with previous published data (Table 2).

In contrast, previously published values of the K_m for ATP are highly variable (0.64–59 mM, Table 2). This is probably due to inadequate experimental conditions, especially the fact that the ATP concentration was maintained higher than the Mg^{2+} concentration [9], resulting in insufficient amounts of free Mg^{2+}. Indeed, previous studies reported an optimum $[Mg^{2+}]/[ATP]$ ratio of 0.6 at 37 °C and pH 7.5 [9,32]. With respect to ATP we observed a clear deviation from Michaelian kinetics. Hence, determination of the K_m for ATP is probably embroiled by the fact that, in addition to binding to the active site, ATP might bind to peripheral non-catalytic sites, as already suggested previously [20]. An alternative explanation is negative cooperativity [53]: binding of $MgATP^{2-}$ to one subunit of the enzyme would result in a decreased affinity of the second subunit for the substrate, so very high substrate concentrations would be necessary to reach V_{max}.

Another point that deserves attention is that ThDP inhibits the forward reaction, but not the reverse reaction when it is substrate. Following the same line is the observation of the much higher apparent K_a value for Mg^{2+} when the reaction is studied in the reverse direction compared with the forward direction. These differences may be related to the fact that other substrates are used: especially ATP is absent at the start of the reverse reaction. As pointed out above, in the forward direction, ATP seems to be required for tight binding of Mg^{2+} (in addition to the essential Asp residues) [57]. Binding of the co-substrate thiamine may also be necessary for an adequate configuration of the active site pocket. In the absence of ATP and thiamine, the configuration of the pocket may not be suitable for a tight binding of the cation, explaining the high apparent K_a value for Mg^{2+} when the activity is measured in the reverse direction.

These data suggest the existence of at least two conformations of the enzyme: one induced by ATP (or possibly thiamine) for the forward reaction, where ThDP is inhibitory and the affinity for Mg^{2+} is high and one for the reverse reaction where ThDP is not inhibitory and the affinity for Mg^{2+} is low.

The data presented in this study were obtained from the mouse recombinant TPK1 and we must wonder whether the conclusions are valid for the human enzyme. Sequence comparison revealed that the mouse and human sequences both contain 243 amino acids and share 89% identity [22]. The human recombinant enzyme was reported to have a specific activity of 3.1 nmol of TDP produced per min/mg of protein, which is comparable to the mouse enzyme [22]. The native hTPK1 had a low K_m of 0.14 ± 0.04 μM for thiamine [32]. Though not much kinetic data are available for the human enzyme it is most probable that inhibition of TPK1 by its product is a general property of not only mammalian enzymes but also the plant enzyme [20]. Indeed, several clinical studies have shown that administration of thiamine in patients leads to strong (often more than 10-fold) increases in blood and erythrocyte thiamine concentrations, while the effect on ThDP concentrations was much less marked [58–60].

Combined data from our present and previous work suggest that TPK1 is a complex enzyme, requiring further studies. This is particularly important in view of the discovery of an increasing number of mutations in the human enzyme leading to severe, often fatal, disorders due to low intracellular ThDP concentrations [23,27,30]. In some cases, the partial loss of enzyme activity in these mutations can be overcome by a high-dose thiamine administration. It is therefore important to understand the regulation of ThDP synthesis by TPK1, but also to have a reliable method for the determination of enzyme activities [29].

The results obtained in this study explain why thiamine precursors, which are very effective in increasing intracellular levels of thiamine, have only a limited effect on the intracellular concentrations of ThDP. But what could be the physiological advantage of this inhibition? On the one hand, it could prevent the accumulation of ThDP in cells and

Table 2
Published apparent K_m values of TPK from various sources for thiamine and ATP (n.d., not reported).

Reference	Thiamine	ATP	Enzyme source
	K_m (μM)	K_m (mM)	
Johnson and Gubler, 1968 [6]	0.12	20	Rat brain (partially purified)
Peterson et al., 1975 [9]	4.1	59	Pig brain (partially purified)
Mitsuda et al., 1975 [7]	0.15	0.8	Parsley leaves (purified)
Artsukevich, 1979 [12,54]	6	0.64	Rat liver (partially purified)
Molin and Frites, 1980 [55]	4.64	18.3	Soybean seedlings
Voskoboyev and Ostrovsky, 1982 [11]	6	1	Brewer's yeast (purified)
Bettendorff and Wins, 1994 [49]	n.d.	7	Rat neuroblastoma cells (cytosolic fraction)
Bettendorff et al., 1996 [32]	0.14 ± 0.04	14 ± 3	Human brain (cytosolic fraction)
Onozuka and Noska, 2003 [10]	0.21	1.2	Human recombinant enzyme
Ajjawi et al., 2007 [21]	0.96	n.d.	*A. thaliana* recombinant AtTPK1
Ajjawi et al., 2007 [21]	1.28	n.d.	*A. thaliana* recombinant AtTPK2
Rapala-Kozic et al., 2009 [56]	12.4	4.7 ± 2.8	*Zea mays* seedlings (purified)

possible toxic effects of this compound. However, there are currently no data suggesting a toxic effect of high ThDP. On the other hand, the inhibition of ThDP synthesis could regulate the distribution of thiamine in the various organs of the human body. The plasma concentration of thiamine is relatively low, especially in humans (10 nM, [61]) and, at physiological concentrations, thiamine is transported into the cells by a H^+/thiamine antiport [1]. Conversion to ThDP may be a driving force for thiamine entry [49]. We can hypothesize that, if ThDP would not inhibit its own synthesis, thiamine would continuously be pumped, for instance, into skeletal muscle and liver (organs representing a large mass), where it would accumulate as ThDP, causing thiamine deficiency in certain organs such as the brain and the heart, a life-threatening condition.

Author contributions

Sambon Margeaux: Investigation, Writing -review & editing. *Pavlova Oleksandra*: Investigation. *Alhama-Riba Judit*: Investigation. *Wins Pierre*: Analysis, Writing - review & editing. *Brans Alain*: Methodology Methodology. *Bettendorff Lucien*: Conceptualization, Analysis, Writing - original draft, review & editing review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2021.130071.

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