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Pig tissues express a catalytically inefficient 25-kDa thiamine triphosphatase: Insight in the catalytic mechanisms of this enzyme

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Abstract

Thiamine triphosphate (ThTP) is found in most organisms and may be an intracellular signal molecule produced in response to stress. We have recently cloned the cDNA coding for a highly specific mammalian 25-kDa thiamine triphosphatase. The enzyme was active in all mammalian species studied except pig, although the corresponding mRNA was present. In order to determine whether the very low ThTPase activity in pig tissues is due to the absence of the protein or to a lack of catalytic efficiency, we expressed human and pig ThTPase in *E. coli* as GST fusion proteins. The purified recombinant pig GST-ThTPase was found to be 2–3 orders of magnitude less active than human GST-ThTPase. Using site-directed mutagenesis, we show that, in particular, the change of Glu85 to lysine is responsible for decreased solubility and catalytic activity of the pig enzyme. Immunohistochemical studies revealed a distribution of the protein in pig brain very similar to the one reported in rodent brain. Thus, our results suggest that a 25-kDa protein homologous to hThTPase but practically devoid of enzyme activity is expressed in pig tissues. This raises the possibility that this protein may play a physiological role other than ThTP hydrolysis.
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Keywords: Thiamine triphosphate; Thiamine triphosphatase; Site-directed mutagenesis; Pig; Brain; Immunohistochemistry

1. Introduction

The triphosphorylated derivative of thiamine (thiamine triphosphate, ThTP) is found in most living organisms, though its cellular concentration is generally much lower (about 10- to 100-fold) than that of thiamine diphosphate (ThDP) [1]. In contrast to the well-known coenzyme role of the latter compound, the biological role of ThTP remains largely unknown.

However, recent data suggest that ThTP might act as an intracellular signal molecule that is produced under conditions of intracellular stress [1,2]. The mechanism of action of ThTP may involve the phosphorylation of specific target proteins [3].

Intracellular ThTP concentrations may be regulated at the level of synthesis, hydrolysis or both. While the mechanism of ThTP synthesis remains poorly characterized so far, a number of phosphohydrolases able to hydrolyze ThTP have been described. In bacteria, two distinct enzymes exhibit thiamine triphosphatase activity (ThTPase, EC 3.6.1.28) [4,5]. One of them, a 16-kDa peptide, also hydrolyzes nucleoside triphosphates, but its K_m for ThTP is particularly low (71 μM, [4]). In vertebrates, most tissues investigated contain a membrane-bound ThTPase [6–11]. As this

Abbreviations: BSA, bovine serum albumin; GST, glutathione *S*-transferase; hThTPase, human recombinant thiamine triphosphatase; IPTG, isopropyl-β-D-thiogalactopyranoside; pThTPase, pig recombinant thiamine triphosphatase; ThDP, thiamine diphosphate; ThTP, thiamine triphosphate; ThTPase, thiamine triphosphatase

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enzyme could not be purified, its specificity for ThTP is not established, but it is, at least, distinct from membrane-bound transport ATPases. In addition to the membrane-associated enzyme, mammalian tissues contain a soluble 25-kDa ThTPase [12–14]. The enzyme, first purified from bovine brain [14], is a 25-kDa monomer, exhibits nearly absolute specificity for ThTP, and has an alkaline pH optimum. In a recent study, we achieved the molecular characterization of this 25-kDa ThTPase [15]. No other sequence showing significant homology with this ThTPase was found in the human genome. Although a homologous gene sequence may be present in other classes of vertebrates, no ThTPase activity corresponding to a soluble low molecular weight protein could be found in animal species other than mammals [1]. In a recent study, Iyer and Aravind [16] suggested that the catalytic domains of 25-kDa ThTPase and bacterial CyaB-like adenylyl cyclases define the novel CYTH superfamily of domains that bind organic phosphates [16]. Members of the CYTH family can be traced back to the Last Universal Common Ancestor and appear to be implicated at the interface between nucleotide and polyphosphate metabolism. ThTPase may represent a relatively divergent acquisition of a new catalytic activity which, so far, appears to be restricted to mammalian tissues.

In this respect, it may seem surprising that the mammalian 25-kDa ThTPase has a high catalytic efficiency ($k_{cat}/K_m = 6 \times 10^6$ s^{-1} M^{-1} for the bovine enzyme [14] and 9.1×10^5 s^{-1} M^{-1} for the recombinant human ThTPase [15,17]). In rodent tissues, the specific activity was comparable to the activity found in bovine tissues. However, unexpectedly, the 25-kDa ThTPase activity was barely detectable in pig tissues [1]. It was not clear whether this was due to a very weak catalytic power or to low amounts of the protein—though the mRNA appeared to be normally expressed, at least in pig brain [1]. Using an antibody prepared against recombinant human ThTPase (hThTPase), we found an immunoreactive protein band from pig brain by Western blotting. This protein migrated at a slightly higher molecular weight than purified hThTPase and it was not excluded that our antibody might recognize a protein unrelated to ThTPase. In the present study, we cloned the pig 25-kDa ThTPase and expressed the recombinant protein in *E. coli*. Our results indeed show that the recombinant pig ThTPase (pThTPase) has only a very low catalytic efficiency, though the protein is well expressed in pig brain, with a localization similar to that found in rodent brain.

2. Materials and methods

2.1. Cloning of the cDNA for pig ThTPase in E. coli

Total RNA was isolated from domestic pig (*Sus scrofa*, local slaughterhouse) brain using Instapure (Eurogentec, Seraing, Belgium) and transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) and random primers. The sequence coding for ThTPase was then amplified by PCR using the forward pTTP2F (5′-ATGGCCCAGGGCTTGATT-GAGGTGGGAGCGA-3′) and the reverse pTTPR (5′-CTAGCCCAGTCTCACGTCCAGATC-3′) primers. The reaction medium was submitted to 40 PCR cycles using *Pfx* polymerase (Invitrogen) at a hybridization temperature of 45 °C. The PCR fragment was cloned into pCRII-TOPO (Invitrogen) and its sequence confirmed. To allow the expression of a GST-fused pThTPase, the open reading frame was amplified by 35 PCR cycle using *Pfx* and the primers pGex2F (5′-TTGGATCCCCATGGCCCAGGGC-TTGATTGAGGTGGAGCG A-3′) and pGexR (5′-GCGG-CCGCCTAGCCCAGTCTCACGTCCAGATC-3′). Each cycle consisted of 20 s at 94 °C for denaturation, 30 s at 45 °C for hybridization and 60 s at 72 °C for elongation. The fragment obtained was digested with *Bam*H1 and *Not*1 and cloned in pGEX-5X-1. The recombinant expression plasmid was then sequenced and used to transform the BL21 *E. coli* strain (Amersham Biosciences).

2.2. Expression of human and pig ThTPase in E. coli

Bacteria were grown overnight on LB agar plates (1.5% (w/v) agar) containing 200 μg/ml ampicillin, and individual colonies were resuspended in 2XYT/ampicillin medium and grown to a density of about 0.5×10^9 cells/ml. The overexpression of hThTPase [15] or pThTPase was induced by dilution of 100 μl of the bacterial culture in 1.6 ml of 2XYT/ampicillin medium in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma-Aldrich, St. Louis, MO, USA) at the concentrations indicated. After 3 or 4 h (37 °C or 25 °C, 250 rpm), the bacteria were harvested by centrifugation (3000×*g*, 10 min at 4 °C) and suspended in Tris–Cl (20 mM, pH 8.0) containing 1 mM EDTA. The suspension was frozen at −80 °C in 1 ml aliquots. For lysis, the bacterial suspension was thawed, sonicated (3 × 30 s on ice) and centrifuged (9000×*g*, 6 min). Either the total extract after sonication, the supernatant or the pellet was submitted to SDS-PAGE (8%). After electrophoresis, the proteins were detected by Coomassie blue staining.

2.3. Determination of ThTPase activity

ThTPase activity was measured as previously described [17]. The reaction medium contained 50 mM Na-TAPS (pH 8.5), 8 mM $MgCl_2$, 0.5 mM ThTP (synthesized as described [18]) and the enzyme preparation at the appropriate dilution (10- to 500-fold). The total volume was 0.1 ml. After incubation (20 min, 37 °C), the reaction was stopped by the addition of 1 ml of phosphate reagent [19].

2.4. Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuickChange method (Stratagene, La Jolla, CA, USA),

and the mutated plasmids were isolated using the QIAGEN (Valencia, CA, USA) Plasmid Midi Kit. The sequences of the mutagenic oligonucleotides are reported in Table 1. The mutations were confirmed by direct sequencing (GIGA, Liège, Belgium). The mutated proteins were expressed as GST fusion proteins for easy purification. Previous results suggested that the addition of the GST moiety to the N-terminal end of hThTPase has few effects on the catalytic properties of the enzyme [15,17]. Mutated enzymes were overexpressed in *E. coli* as described above and purified using the MagneGST™ Protein Purification System (Promega Corporation, Madison, WI, USA).

2.5. Immunohistochemical studies

Samples (about 1–2 cm^3) of brains from freshly slaughtered pigs (local slaughterhouse) were fixed for 1 h with 50 ml of 4% paraformaldehyde dissolved in 130 mM Na-phosphate buffer, pH 7.4, and immersed overnight in a 30% sucrose cryoprotectant solution in phosphate-buffered saline pH 7.4 (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). Then, tissues were frozen on dry ice and cut into 30-μm sections. Immunohistochemical studies were performed as previously described [20]. Controls were done by omitting the primary antibodies.

3. Results

3.1. Cloning and expression of recombinant pig ThTPase in E. coli

We have previously noted that soluble ThTPase activity is barely detectable in pig tissues [1]. In order to establish whether this lack of activity is due to a low catalytic power of the 25-kDa protein, pThTPase was cloned, sequenced and expressed as a GST fusion protein in *E. coli*. Fig. 1A shows the SDS-PAGE gels of total extracts of bacteria expressing either GST-pThTPase, GST-hThTPase or GST alone. In all three cases, similar amounts were expressed after induction by IPTG. As 25-kDa ThTPase is generally a soluble protein, it should be present in the supernatant of the bacterial extract (Fig. 1B). GST and GST-hThTPase were indeed found in the supernatant fraction after induction, but, unexpectedly, no significant amount of GST-pThTPase was found. We therefore hypothesized that GST-pThTPase

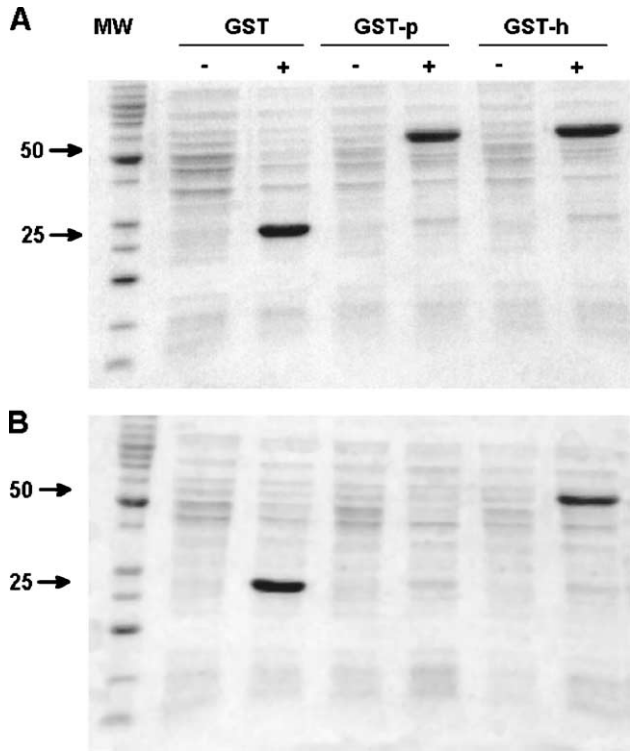

Fig. 1. Expression of GST pig (GST-p) and human (GST-h) ThTPases as GST fusion proteins in *E. coli* in the absence (−) and presence (+) of 1 mM IPTG. The bacteria were grown in 2XYT medium (A_{600}=0.8) and overexpression was induced with 1 mM IPTG. After 3 h, the bacteria were pelleted, suspended in Tris–HCl medium (pH 8.0) and submitted to 3 times 20 s sonication. (A) 10 μg of proteins from the total extract was submitted to SDS-PAGE (10%) and the gel stained by Coomassie blue. (B) The total extracts were centrifuged (10 min, 13,000×*g*) and 10 μg of protein from the supernatants was submitted to SDS-PAGE.

Table 1
Sequences of mutagenic oligonucleotides

Human ThTPase	
E63Q fwd	5′-GATAGTGGATGG**C**AGCTC AAATGTCCTG-3′
E63Q rev	5′-CAGGACATTTGAGCT**G**CCATCC ACTATC-3′
E78K fwd	5′-GGACCCCAC ACG**A**A**G**TATAAGGAACTC ACA GC-3′
E78K rev	5′-GCTGTGAGTTCCTTATA**C**T**T**CGTGTGGGGTCC-3′
E85K fwd	5′-GGAACTC ACAGCG**A**AACCTACAATTGTG G-3′
E85K rev	5′-CCACAATTGTAGGTT**T**CGC TGTGAGTTCC-3′
Pig ThTPase	
K85E fwd	5′-G GAGCTC ACAGCT**G**AGTCTGCAATTGTG-3′
K85E rev	5′-CACAAT TGC AGACT**C**AGC TGTGAGCTCC-3′

The modified bases are underlined and in bold characters.

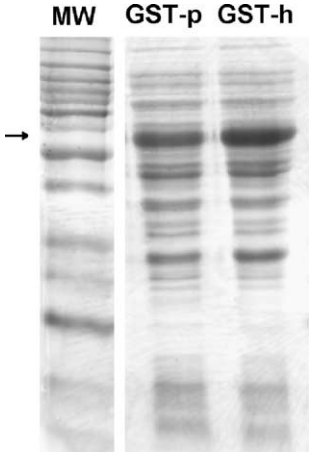

Fig. 2. GST-pThTPase and GST-hThTPase in supernatants of bacteria grown for 3 h at 25 °C in the presence of 1 mM IPTG. The arrow indicates the expected molecular weight of 50 kDa for the fusion protein.

agglomerates in inclusion bodies. We tested the effect of different concentrations of IPTG (with no effect) and different culture temperatures on the solubility of GST-pThTPase. Reducing the temperature from 37 °C to 25 °C resulted in the appearance of a significant amount of GST-pThTPase in the supernatant fraction (Fig. 2).

Quantification of both bands suggested that GST-hThTPase was about twice as abundant as GST-pThTPase in the supernatant fraction of *E. coli*. However, GST-pThTPase activity was only 0.048±0.004 μmol min^{-1} mg^{-1}, a value two orders of magnitude lower than in the supernatant of bacteria expressing GST-hThTPase (4.5±0.3 μmol min^{-1} mg^{-1}).

Before studying the catalytic activity and kinetic properties of GST-ThTPases overexpressed in *E. coli*, we must consider the possibility that the activity of bacterial ThTPases [4,5] might interfere with the measurements. We estimated this endogenous activity in the supernatant fraction of non-transformed control bacteria. Under optimum conditions (pH 6.8, $[Mg^{2+}]=5$ mM), ThTPase specific activity was still two orders of magnitude lower than the specific activity in supernatants from bacteria overexpressing GST-hThTPases (human, E63Q- or E78K-mutated). Endogenous bacterial ThTPase activity is thus negligible in these cases. The situation is however different in bacteria overexpressing pig ThTPase that is much less active (100-

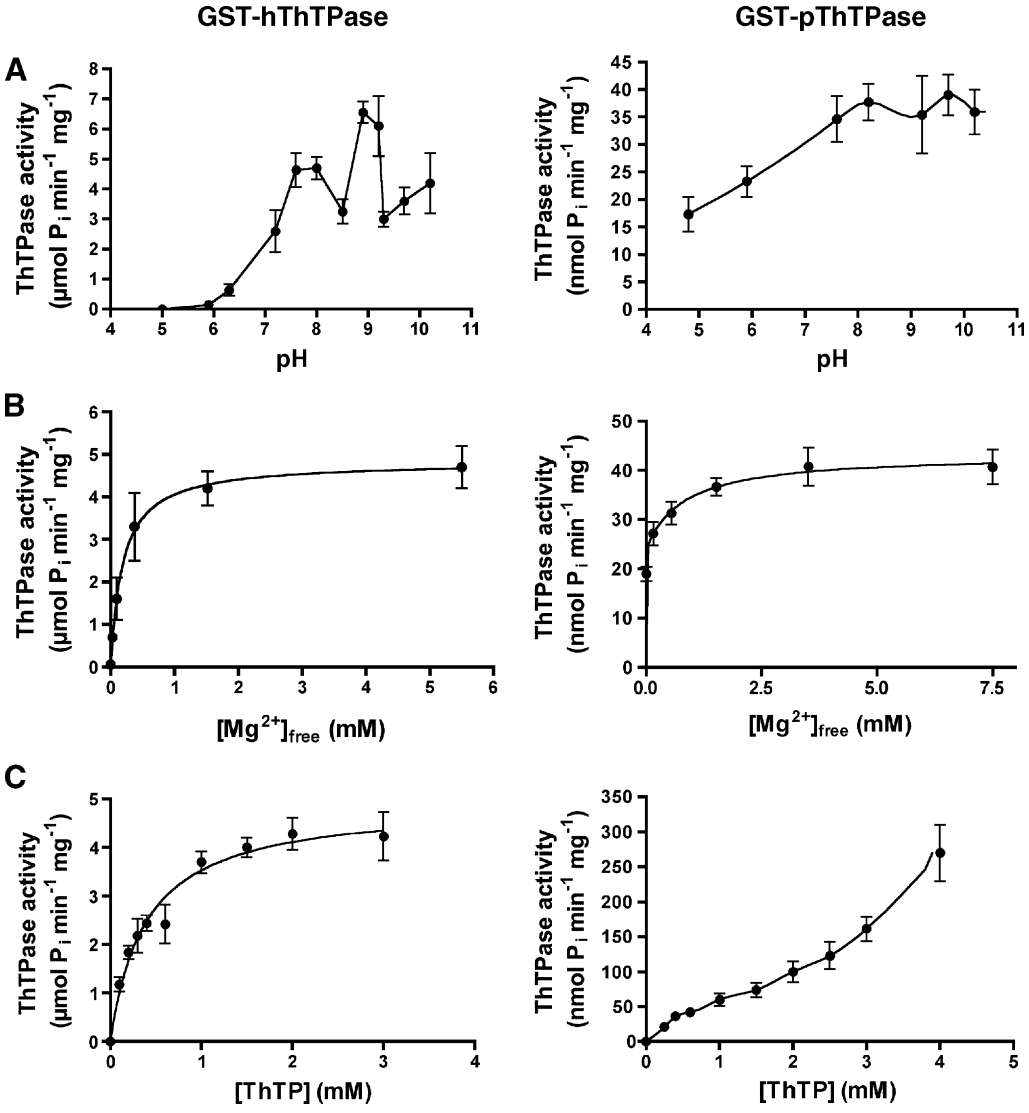

Fig. 3. Kinetic properties of recombinant GST-hThTPase and GST-pThTPase. (A) Effect of pH on ThTPase activity. The incubation medium contained ThTP (0.5 mM); $MgCl_2$ (5 mM); buffer (50 mM); and GST-ThTPase diluted in 50 mM NaCl containing 0.1% BSA. The buffers used were Na-acetate (pH 4.8), Na-MES (pH 5.9–6.3), Na-HEPES (pH 7.2–7.6), Na-TAPS (pH 8.0–8.9), Na-CHES (pH 9.2–9.7), and Na-CAPS (pH 10.2). (B) Effect of Mg^{2+} ions on ThTPase activity at pH 8.2. The incubation medium contained ThTP (0.5 mM); Na-TAPS buffer (50 mM, pH 8.2); EDTA (0.5 mM) when no $MgCl_2$ was present; GST-pThTPase diluted in 50 mM NaCl containing 0.1% BSA and various concentrations of $MgCl_2$. The concentrations of free Mg^{2+} were calculated from total concentrations of $MgCl_2$ and ThTP, using the value of 65 μM for the dissociation constant of the Mg-ThTP complex reported by [7]. (C) Effect of substrate concentration on ThTPase activity. The incubation medium contained $MgCl_2$ (5 mM); Na-TAPS (50 mM, pH 8.4); and GST-pThTPase diluted in 50 mM NaCl containing 0.1% BSA.

to 5000-fold, see below). It was thus necessary to purify the GST-pThTPase using the MagneGST protein purification system. The purified enzyme had a specific activity of 41 nmol min^{-1} mg^{-1} under usual assay conditions (37 °C, pH 8.2, 0.5 mM ThTP, 4 mM Mg^{2+}). For comparison, the specific activity of the pure untagged recombinant human ThTPase was around 225 μmol min^{-1} mg^{-1} under the same assay conditions [17]. This is about 5500-fold higher than the specific activity of the purified GST-pThTPase. However, the results obtained with bacterial supernatants (see above) suggest that the specific activity of the GST-hThTPase is only 100-fold higher than the activity of the GST-pThTPase. The discrepancy could be due to either a substantial loss of catalytic activity during the purification process or to an interference of the GST tag with enzyme activity. In any event, the results indicate that the catalytic activity of the pig ThTPase is at least two orders of magnitude lower than that of the human ThTPase.

Some properties of GST-pThTPase were found to be rather different from those of the recombinant hThTPase. The main results are displayed in Fig. 3. The optimum pH was found to be alkaline (as is the case for hThTPase), but the activity at pH 5 was nearly half the activity at pH 9 (Fig. 3A), while the activity of GST-hThTPase was nearly zero at pH 5.0 (see also [17]). In contrast to what was previously observed [14,17], the pH profile displayed two peaks. This complex shape was consistently observed when the ThTP concentration was ≥250 μM and is probably related to regulatory mechanisms in the presence of excess substrate. As shown in Fig. 3B, Mg^{2+} ions activate pThTPase with an EC_{50} value around 0.5 mM, but, in the complete absence of Mg^{2+}, the activity is still 40–50% of the maximum. In contrast, GST-hThTPase was practically inactive in the absence of Mg^{2+}. On the other hand, Zn^{2+} (10–50 μM), which is a strong inhibitor of hThTPase at pH 8 [17], activated GST-pThTPase by about 30% at the same pH and in the presence of Mg^{2+} (not shown). Finally, the effect of ThTP concentration on pThTPase activity (Fig. 3C) indicates non-Michaelian kinetics, with an important and strongly cooperative activation by excess substrate at [ThTP] > 1 mM. It is therefore difficult to estimate k_{cat} for pThTPase (as V_{max} is uncertain). But even if k_{cat} were relatively high, this would not invalidate the conclusion that the activity of pThTPase is extremely low in vivo, where ThTP concentration is much lower than 1 mM.

3.2. Site-directed mutagenesis of human ThTPase

We know the ThTPase sequences from six mammalian species (Fig. 4): rat (GenBank accession nos. AY065967), mouse (AF432864), macaque (AB055296), human (AF432862), bovine (AF432863) and pig (AY442334). In

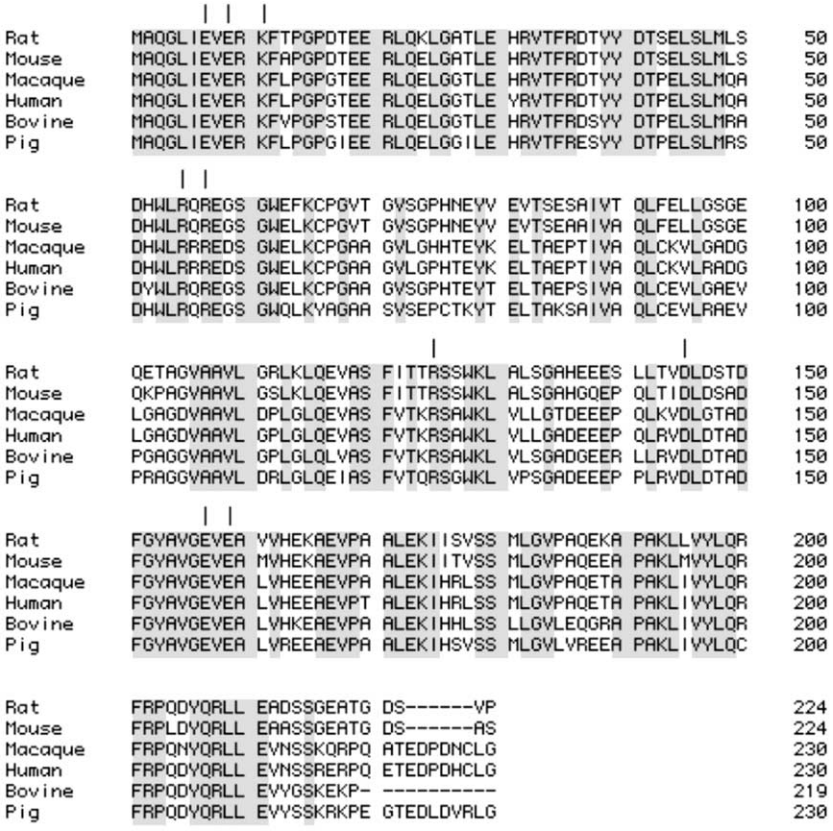

Fig. 4. Alignment of deduced 25-kDa ThTPase amino acid sequences for six mammalian species including pig. The predicted amino acids of the catalytic CYTH domain are highlighted by marks.

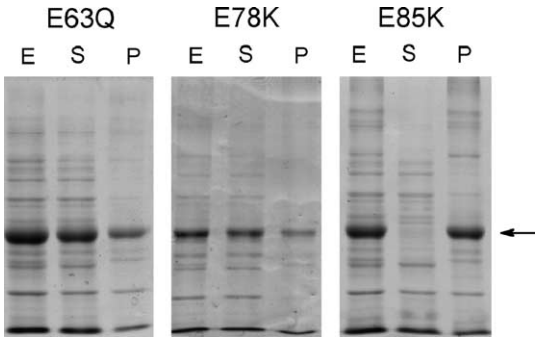

Fig. 5. Expression of human ThTPase carrying 3 different point mutations (E63Q, E78K, and E85K). The overexpression of the mutated GST fusion proteins was induced with 0.75 mM IPTG for 4 h at 25 °C. The presence of the fusion protein was checked by SDS-PAGE in the total extract (E) and after centrifugation (3000×*g*) in the supernatant (S) and in the pellet (P).

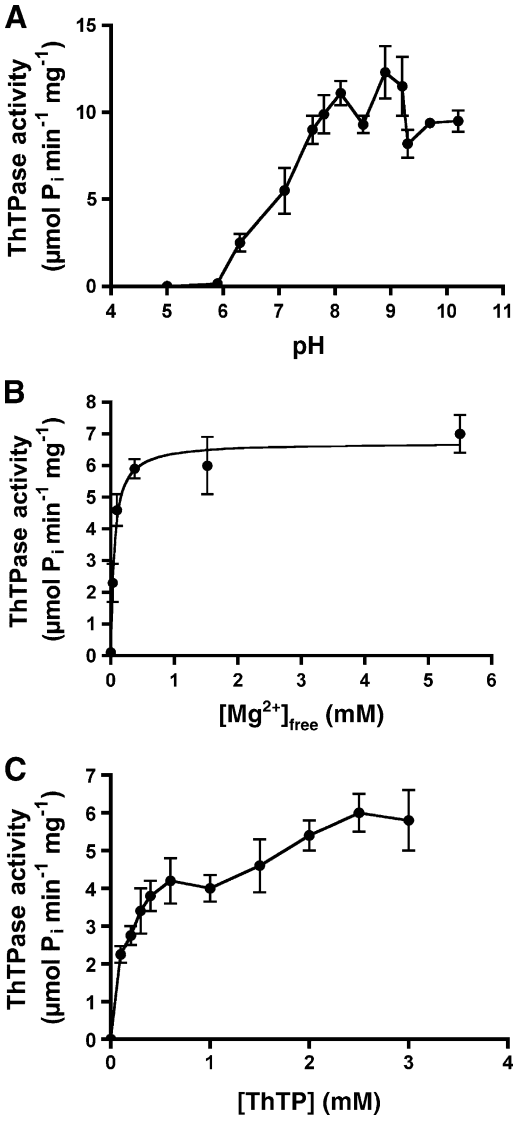

Fig. 6. Kinetic properties of the recombinant E78K-mutated GST-hThTPase. (A) Effect of pH on ThTPase activity (experimental conditions as in Fig. 3A). (B) Effect of Mg^{2+} ions on ThTpase activity at pH 8.5 (experimental conditions as in Fig. 3B). (C) Effect of substrate concentration on ThTPase activity (experimental conditions as in Fig. 3C).

contrast to the pig enzyme, the rat, mouse, human and bovine orthologs are highly active. The macaque ortholog was not tested for enzyme activity, but it shares 94.3% identity with the human ortholog and no drastic amino acid substitutions were observed. However, when comparing the pig sequence to the other sequences, we can see that 16, elsewhere conserved amino acids, are changed (T18I, T28I, D37E, E63Q, C66Y, P67A, G71S, G74E, H76C, E78K, E85K, V118I, L132P, H163R, Q187R, and R200C). Many of these modifications lie between positions 63 and 85. While some of the replacements could be neutral (D37E or V118I for instance), others consist in the replacement of charged amino acids by neutral or even oppositely charged residues (E63Q, E78K, and E85K).

The loss of these three Glu residues (which are conserved in other mammalian species) thus appears as a salient feature of the pig enzyme. The more positive charge of the pig enzyme may explain, at least partially, the reduced solubility. On the other hand, previous results obtained using Woodward's reagent K [17] suggested that carboxylic groups may play an important role in catalysis. Thus, the replacement of three Glu residues by Lys or Gln might explain the loss of catalytic power in the pig enzyme. In order to test this hypothesis, we first mutated separately the Glu63, 78 and 85 of the hThTPase to their pig counterpart.

Fig. 5 shows the expression of the mutated GST-hThTPases in bacterial supernatants and pellets. The E63Q- and E78K-mutated enzymes appeared predominantly in the supernatant fractions, suggesting that they are essentially soluble enzymes, like the non-mutated GST-hThTPase. In contrast, the E85K-mutated enzymes appeared in the pellet but was totally undetectable in the supernatant.

The E63Q mutation resulted in a slightly decreased specific activity of GST-ThTPase in the *E. coli* extract. On the other hand, the kinetic properties of the enzyme were not very different from those of the non-mutated hThTPase: The only notable differences were that the optimum pH was 9.4 rather than 8.5, that Woodward's reagents K was less effective as an irreversible inhibitor and that the apparent K_m was slightly increased (250 vs. 150 µM, data not shown). Thus, Glu63 does not appear to be important for

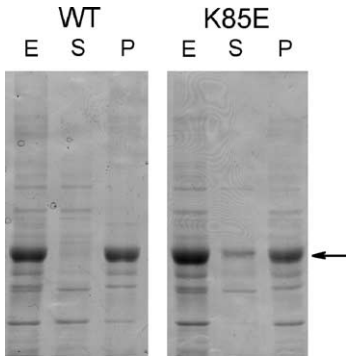

Fig. 7. Expression of wild-type (WT) and mutated (K85E) pig ThTPase in total bacterial extracts (E), supernatant (S) and pellet (P), as described in the legend to Fig. 5.

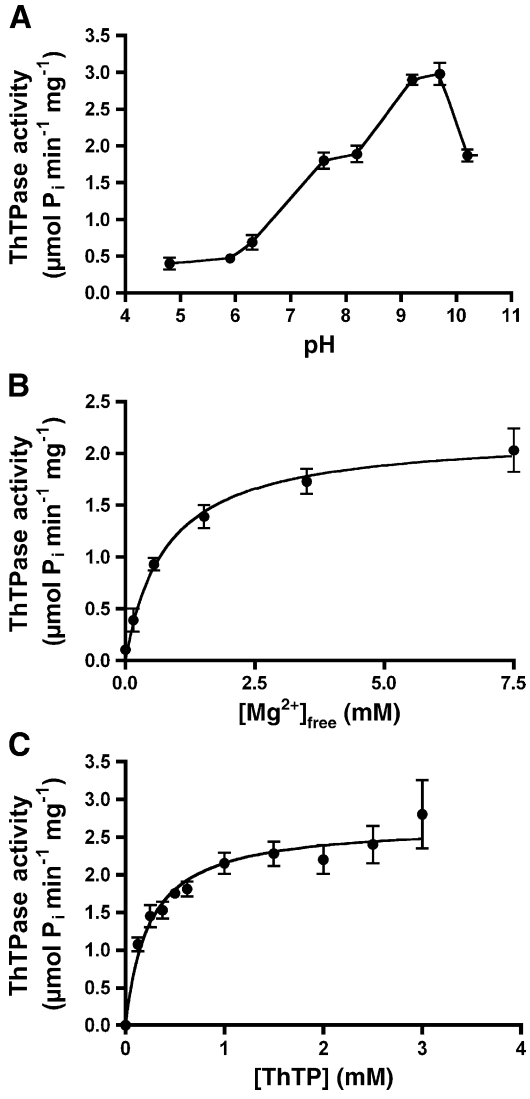

Fig. 8. Kinetic properties of the recombinant K85E-mutated GST-pThTPase. (A) Effect of pH on ThTPase activity (experimental conditions were as in Fig. 3A). (B) Effect of Mg^{2+} ions on ThTPase activity at pH 8.7. Experimental conditions were as in Fig. 3B except that TAPS buffer was adjusted at pH 8.7. (C) Effect of substrate concentration on ThTPase activity. The incubation medium contained $MgCl_2$ (4 mM); Na-TAPS buffer (50 mM, pH 8.2); enzyme (after purification by the MagneGST protein purification kit) diluted in 50 mM NaCl containing 0.1% BSA. The K_m value was obtained from simulation of the Michaelis–Menten equation (GraphPad Prism, GraphPad Software Inc., San Diego, CA) to the experimental data, omitting the points at [ThTP] > 2 mM yielding a K_m=185 µM; the x intercept for the Hanes plot gave 210 µM.

catalysis or ThTP binding. It also did not appear to be implicated in divalent cation binding, as the apparent dissociation constants for Mg^{2+}, Ca^{2+} and Zn^{2+} were not increased (data not shown).

The E78K mutation resulted in a few interesting modifications of kinetic properties, which are shown in Fig. 6. Compared to the wild-type GST-hThTPase, the specific activity was increased rather than decreased, and the apparent affinity for divalent cations was markedly increased. K_A for Mg^{2+} was 80 µM instead of 500 µM; Ca^{2+} inhibited by competition with Mg^{2+} and the estimated K_I was 30 µM instead of 150 µM. Zn^{2+} was strongly inhibitory at pH 8.5 (IC_{50}=7 µM). A lower K_A for Mg^{2+} was also observed for the GST-pThTPase (Fig. 3B), but in the latter case, there was no inhibition by Zn^{2+}. The plot of ThTPase activity vs. [ThTP] yielded a biphasic curve (Fig. 6C), showing activation by excess substrate. This phenomenon was also observed with GST-pThTPase (Fig. 3C). Thus, the E78K-mutated hThTPase appears to have a few properties resembling those of the pig ThTPase, though there is no decrease in catalytic power.

Finally, the properties of the E85K-mutated hThTPase could not be studied because this mutation led to a complete loss of solubility of the GST-ThTPase (Fig. 5). Thus, replacement of Glu85 by lysine may explain the poor solubility of GST-pThTPase compared to GST-hThTPase. The results suggest that the E85K mutation may induce an important structural modification of the enzyme.

3.3. Site-directed mutagenesis of pig ThTPase

As already pointed out, the overexpression of pThTPase in *E. coli* resulted in a poorly soluble protein. In order to check whether this is due to the E85K mutation, we reverse-mutated Lys85 of the pig enzyme to the Glu found in all the other species tested. Indeed, the solubility of pig K85E ThTPase increased (Fig. 7) and a significant enzyme activity could be measured. This confirms that the E85K mutation is an important factor for the reduced solubility of the GST-pThTPase.

We purified the K85E mutated GST-pThTPase and found that its activity, measured under the usual assay conditions, was about 40-fold higher than the activity of the non-mutated GST-pThTPase. Such an increase suggests that Glu85 may play a role in the catalytic mechanism, but it might also be explained by some structural change induced

Table 2
Effect of divalent cations on the activity of K85E-mutated GST-pThTPase at pH 8.2

Salt(s) added	Concentration	Activity (µmol P_i min^{-1} mg^{-1})
Na-EDTA	0.5 mM	0.09±0.04
$MgCl_2$	4 mM	1.70±0.20
+ $CaCl_2$	1 mM	0.99±0.11
+ $CaCl_2$	4 mM	0.59±0.13
+ $ZnSO_4$	40 µM	2.55±0.35
+ $ZnSO_4$	100 µM	1.90±0.40
$MnCl_2$	50 µM	3.30±0.20
$MnCl_2$	100 µM	3.45±0.15
$MnCl_2$	1 mM	3.08±0.15
$ZnSO_4$	40 µM	0.45±0.07
$ZnSO_4$	100 µM	0.63±0.06
$ZnSO_4$	200 µM	0.55±0.10

The incubation medium contained ThTP (0.5 mM), Na-TAPS buffer (50 mM, pH 8.2), enzyme diluted in 50 mM NaCl containing 0.1% BSA.

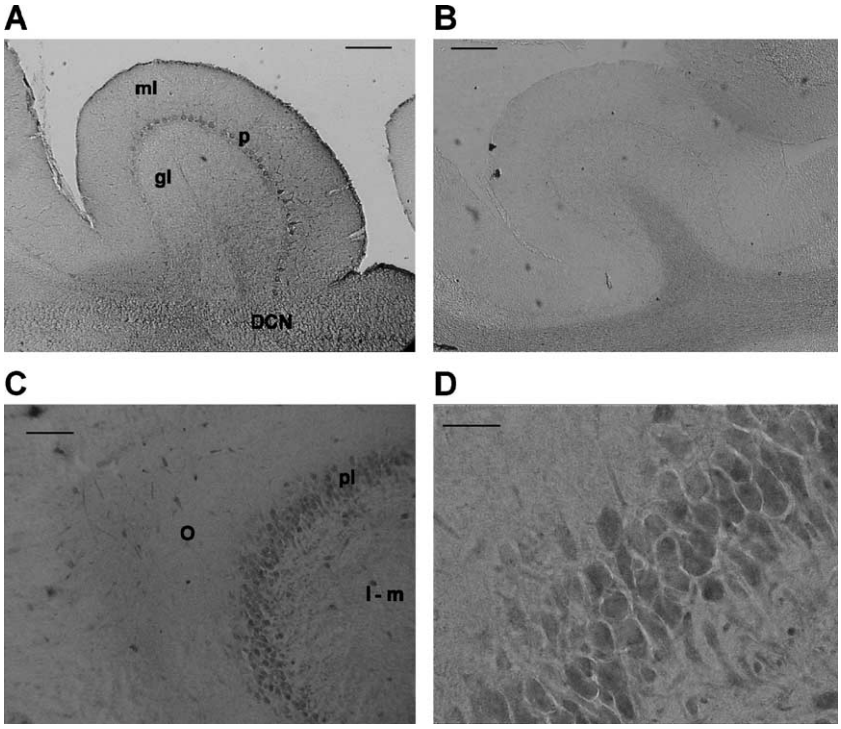

Fig. 9. Immunocytochemical studies on pig brain using an anti-ThTPase antibody. (A) Pig cerebellum showing intense anti-ThTPase immunoreactivity in Purkinje cells (p) and in some cells in the granule layer (gl) and in the deep cerebellar nuclei (DCN). (B) Control without primary antibody. (C) Hippocampus showing labeling of CA3 pyramidal cell layer (pl) as well as of some neurons in the stratum oriens (o) and in the stratum lacunosum-moleculare (l-m). (D) Enlargement of panel (C) showing labeling of pyramidal neurons. The scale bars represent 400 μm in panels A and B, 200 μm in panel C and 50 μm in panel D.

by the mutation. To clarify this point, we investigated the kinetic properties of the K85E-mutated enzyme, with the results shown in Fig. 8. In sharp contrast with data obtained with the non-mutated pThTPase (see Fig. 3C), we now observe essentially Michaelian kinetics (Fig. 8C). There is only a slight distortion that would suggest activation by excess substrate at [ThTP] > 2 mM. Importantly, the apparent K_m was about 200 μM, practically identical to the value found for GST-hThTPase (220 μM, [15]). This suggests that the K85E mutation of the pThTPase modifies the active site in such a manner that the interaction with the substrate becomes closer to what it is in the human enzyme. Fig. 8A shows the effect of pH on the K85E-mutated pThTPase. The optimum is slightly more alkaline than for hThTPase (9.4 vs. 8.9) and the activity at pH 5 is about 10% of the maximum (it is 45% in the case of pThTPase and close to 0% for hThTPase). Thus, the K85E-mutated enzyme seems to have some properties intermediate between pThTPase and hThTPase.

Finally, we investigated the effects of divalent cations (Fig. 8B, Table 2). The activation by Mg^{2+} was quite similar to that observed with the non-mutated hThTPase: The activity in the absence of Mg^{2+} was only 5% of the maximum and the apparent K_A value was 900 μM. Surprisingly, Mn^{2+}, at very low concentrations (0.1 mM), was a better activator than Mg^{2+} while similar concentrations of Zn^{2+} alone induced a smaller but significant activation (Table 2). In the presence of Mg^{2+} at pH 8, Zn^{2+} had an activating rather than inhibitory effect, while it is a strong inhibitor of hThTPase under the same conditions [17].

3.4. Immunohistochemical distribution of ThTPase in pig brain

We studied the localization of ThTPase in pig cerebellum and hippocampus by immunohistochemistry (Fig. 9). These structures were chosen as they seem to display a rather characteristic distribution pattern in rodent brain [20]. The distribution of pig brain ThTPase was quite similar to the distribution previously observed in rodents. The cytoplasm of Purkinje cells was strongly labeled, including their dendritic projections into the molecular layer. Many cells were also labeled in the granule layer and in deeper cerebellar nuclei. No labeling was observed in the absence of primary antibody. In the hippocampus, the pyramidal cell layer showed the strongest labeling, but many anti-ThTPase immunoreactive cells were also detected in the stratum oriens and the stratum lacunosum-moleculare.

4. Discussion

Soluble 25-kDa ThTPase activity was consistently found in most tissues of several mammalian species [1,12,14,15]. So it came as a surprise that no soluble activity was found in pig brain. In pig kidney and skeletal muscle, the activity was

so low that a characterization was impossible. An explanation may be either that the protein is expressed only at very low levels in pig tissues or that the pig enzyme is inactive due to the replacement of amino acid residues essential for enzyme activity or for correct folding of the protein.

Our previous studies demonstrated the presence of ThTPase mRNA in pig brain and kidney [1]. Our antibody recognized one single band in immunoblots from pig brain and two close bands in rat brain. The apparent molecular weight was slightly higher for the band detected in pig brain than for the recombinant human 25-kDa ThTPase. At that stage, it could not be excluded that the band recognized by our antibody could be another protein cross-reacting with the antibody. The immunohistochemical data presented in the present work make that hypothesis very unlikely.

We were unable to purify the enzyme, as no activity was recovered after any chromatographic step tested. Therefore, in order to characterize the enzymatic properties of pig ThTPase, we decided to produce recombinant pig ThTPase as a GST fusion protein in *E. coli*. Previous results [15] had shown that the addition of the GST moiety to the N-terminal of human ThTPase did not significantly alter its catalytic properties. It became immediately apparent that GST-pThTPase had a tendency to form inclusion bodies, which was not the case of GST-hThTPase. Decreasing the temperature from 37 to 25 °C strongly reduced this tendency. A comparison of the relative amounts of proteins and enzyme activities in the bacterial supernatant suggested that the enzymatic activity of GST-pThTPase was about two orders of magnitude lower than that of GST-hThTPase.

On the other hand, our immunohistochemical studies in pig brain show that the 25-kDa protein has a similar distribution in pig and in rodent brain. Taken together, those data suggest that the 25-kDa protein is expressed in pig tissues as in other mammalian species–though there may be quantitative differences–but that the pig protein has only a very low catalytic power.

Why is the pig 25-kDa protein nearly inactive as a ThTPase? The mammalian 25-kDa ThTPase has recently been shown to belong to a broad superfamily of catalytic domains (called the CYTH domain) that is present in all superkingdoms of life [16]. In most cases, the reaction(s) catalyzed by the CYTH domain is still unidentified. Only adenylyl cyclase activity in *Aeromonas hydrophila* and the 25-kDa ThTPase in mammals have been characterized. It has been suggested [16] that the CYTH domains utilize polyphosphates to synthesize different organo-phosphate derivatives, including nucleotides. They could also function as polyphosphoesterases hydrolyzing particular nucleoside polyphosphates. Thiamine triphosphatase activity seems to be a recent acquisition (in mammals) of a new catalytic activity. The catalytic core of the CYTH domain contains 6 conserved acidic residues—but the 3rd one is lacking in 25-kDa ThTPases. Those residues can probably coordinate two divalent metal cations. There are also four conserved basic residues, probably involved in the binding of acidic phosphate moieties of the substrates. As can be seen in the sequences shown in Fig. 4, those 9 residues (Glu7, 9, 157, and 159, Asp145, Lys11, Arg55, 58, and 125) are conserved in all mammalian species investigated, including the pig. We have indeed shown (Fig. 3B) that the pThTPase has an apparent affinity for Mg^{2+} slightly higher than that of hThTPase. No binding constant could be estimated accurately for ThTP (Fig. 3C), as a strongly cooperative activation by excess substrate complicates the picture. Further investigation will be necessary to clarify this point. Anyway, we must conclude that the loss of catalytic power of the pThTPase is due to mutations affecting residues other than those conserved in the CYTH domain. Our data using site-directed mutagenesis of hThTPase have shown that the replacement of Glu85 by Lys strongly decreases the solubility of the enzyme. Conversely, the replacement of Lys85 by Glu in pThTPase increases the solubility and increases the activity about 40-fold. Taken together, these results suggest that the mutation of Glu85 is, at least in part, responsible for the loss of catalytic activity of pThTPase and possibly some conformational modification resulting in decreased solubility.

Finally, the data presented in this study raise some questions about the physiological role of the 25-kDa ThTPase. It seemed reasonable to assume that this enzyme is involved in the control of intracellular ThTP concentration. If this hypothesis is correct, we would expect higher ThTP levels in pig tissues, compared with other species having a functional 25-kDa ThTPase. Indeed, previous studies have shown that pig skeletal muscle has an extremely high content of ThTP [21]. However, ThTP levels in pig brain are only slightly higher than in other mammalian species [1]. It is important to point out that, in brain, ThTP is mainly localized in particulate fractions, which are rich in membrane-associated ThTPase [6]. In skeletal muscle, ThTP is mainly localized in the soluble fraction [22]. 25-kDa ThTPase is an essentially cytosolic enzyme; thus, it may be expected that ThTP will accumulate in the cytosol of pig skeletal muscle, as the enzyme is practically inactive.

Obviously, pigs live quite well without 25-kDa ThTPase activity. Are there some compensatory effects, as is for instance observed in knock-out animals? In pig brain particulate fractions, we found a rather high activity of membrane-bound ThTPase, but it is not markedly higher than in rodent or bovine brain (A. Dafoun and L. Bettendorff, unpublished results).

Our results suggest that soluble ThTPase activity is not essential for survival and raises the question of an additional role of the 25-kDa ThTPase protein, such as ThTP binding for instance. The presence of a non-catalytic ThTP-binding site, previously suggested [23], might explain the phenomenon of activation by excess substrate observed with several forms of the 25-kDa ThTPase. The possibility that ThTP might also show specific interactions with CYTH domains other than mammalian ThTPase is an interesting topic for future investigations.

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References

[1] A.F. Makarchikov, B. Lakaye, I.E. Gulyai, J. Czerniecki, B. Coumans, P. Wins, T. Grisar, L. Bettendorff, Thiamine triphosphate and thiamine triphosphatase activities: from bacteria to mammals, Cell. Mol. Life Sci. 60 (2003) 1477–1488.

[2] B. Lakaye, B. Wirtzfeld, P. Wins, T. Grisar, L. Bettendorff, Thiamine triphosphate, a new signal required for optimal growth of *Escherichia coli* during amino acid starvation, J. Biol. Chem. 279 (2004) 17142–17147.

[3] H.O. Nghiêm, L. Bettendorff, J.P. Changeux, Specific phosphorylation of torpedo 43K rapsyn by endogenous kinase(s) with thiamine triphosphate as the phosphate donor, FASEB J. 14 (2000) 543–554.

[4] T. Nishimune, R. Hayashi, Hydrolysis and synthesis of thiamin triphosphate in bacteria, J. Nutr. Sci. Vitaminol. (Tokyo) 33 (1987) 113–127.

[5] T. Nishimune, S. Ito, M. Abe, M. Kimoto, R. Hayashi, Nucleoside-triphosphatase and hydrolysis of thiamin triphosphate in *Escherichia coli*, Biochim. Biophys. Acta 923 (1987) 74–82.

[6] R.L. Barchi, P.E. Braun, A membrane-associated thiamine triphosphatase from rat brain. Properties of the enzyme, J. Biol. Chem. 247 (1972) 7668–7673.

[7] R.L. Barchi, R.O. Viale, Membrane-associated thiamin triphosphatase: II. Activation by divalent cations, J. Biol. Chem. 251 (1976) 193–197.

[8] L. Bettendorff, C. Michel-Cahay, C. Grandfils, C. De Rycker, E. Schoffeniels, Thiamine triphosphate and membrane-associated thiamine phosphatases in the electric organ of *Electrophorus electricus*, J. Neurochem. 49 (1987) 495–502.

[9] L. Bettendorff, P. Wins, E. Schoffeniels, Thiamine triphosphatase from *Electrophorus* electric organ is anion-dependent and irreversibly inhibited by 4,4′-diisothiocyanostilbene- 2,2′disulfonic acid, Biochem. Biophys. Res. Commun. 154 (1988) 942–947.

[10] L. Bettendorff, C. Grandfils, P. Wins, E. Schoffeniels, Thiamine triphosphatase in the membranes of the main electric organ of *Electrophorus electricus*: substrate–enzyme interactions, J. Neurochem. 53 (1989) 738–746.

[11] L. Bettendorff, I. Longree, P. Wins, E. Schoffeniels, Solubilization of thiamine triphosphatase from the electric organ of *Electrophorus electricus*, Biochim. Biophys. Acta 1073 (1991) 69–76.

[12] Y. Hashitani, J.R. Cooper, The partial purification of thiamine triphosphatase from rat brain, J. Biol. Chem. 247 (1972) 2117–2119.

[13] H.K. Penttinen, L. Uotila, The relation of the soluble thiamine triphosphatase activity of various rat tissues to nonspecific phosphatases, Med. Biol. 59 (1981) 177–184.

[14] A.F. Makarchikov, I.P. Chernikevich, Purification and characterization of thiamine triphosphatase from bovine brain, Biochim. Biophys. Acta 1117 (1992) 326–332.

[15] B. Lakaye, A.F. Makarchikov, A. Fernandes Antunes, W. Zorzi, B. Coumans, E. De Pauw, P. Wins, T. Grisar, L. Bettendorff, Molecular characterization of a specific thiamine triphosphatase widely expressed in mammalian tissues, J. Biol. Chem. 277 (2002) 13771–13777.

[16] L.M. Iyer, L. Aravind, The catalytic domains of thiamine triphosphatase and CyaB-like adenylyl cyclase define a novel superfamily of domains that bind organic phosphates, BMC Genomics 3 (2002) 33.

[17] B. Lakaye, A.F. Makarchikov, P. Wins, I. Margineanu, S. Roland, L. Lins, R. Aichour, L. Lebeau, B. El Moualij, W. Zorzi, B. Coumans, T. Grisar, L. Bettendorff, Human recombinant thiamine triphosphatase: Purification, secondary structure and catalytic properties, Int. J. Biochem. Cell Biol. 36 (2004) 1348–1364.

[18] L. Bettendorff, H.O. Nghiem, P. Wins, B. Lakaye, A general method for the chemical synthesis of gamma-32P-labeled or unlabeled nucleoside 5(′)-triphosphates and thiamine triphosphate, Anal. Biochem. 322 (2003) 190–197.

[19] P.A. Lanzetta, L.J. Alvarez, P.S. Reinach, O.A. Candia, An improved assay for nanomole amounts of inorganic phosphate, Anal. Biochem. 100 (1979) 95–97.

[20] J. Czerniecki, G. Chanas, M. Verlaet, L. Bettendorff, A.F. Makarchikov, P. Leprince, P. Wins, T. Grisar, B. Lakaye, Neuronal localization of the 25-kDa specific thiamine triphosphatase in rodent brain, Neuroscience 125 (2004) 833–840.

[21] Y. Egi, S. Koyama, H. Shikata, K. Yamada, T. Kawasaki, Content of thiamin phosphate esters in mammalian tissues—An extremely high concentration of thiamin triphosphate in pig skeletal muscle, Biochem. Int. 12 (1986) 385–390.

[22] T. Matsuda, H. Tonomura, A. Baba, H. Iwata, Tissue difference in cellular localization of thiamine phosphate esters, Comp. Biochem. Physiol., B 94 (1989) 405–409.

[23] I.P. Chernikevich, A.F. Makarchikov, Cytosolic thiamine triphosphatase from bovine brain: 1. Regulation of enzyme activity, Ukr. Biokhim. Zh. 69 (1997) 41–50.