CMLS, Cell. Mol. Life Sci. 60 (2003) 001–12
1420-682X/03/010001-12
DOI 10.1007/s00018-003-2185-■

CMLS Cellular and Molecular Life Sciences

Research Article

Thiamine triphosphate and thiamine triphosphatase activities: from bacteria to mammals

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Received 7 March 2003; received after revision 11 April 2003; accepted 14 April 2003

Abstract. In most organisms, the main form of thiamine is the coenzyme thiamine diphosphate. Thiamine triphosphate (ThTP) is also found in low amounts in most vertebrate tissues and can phosphorylate certain proteins. Here we show that ThTP exists not only in vertebrates but is present in bacteria, fungi, plants and invertebrates. Unexpectedly, we found that in *Escherichia coli* as well as in *Arabidopsis thaliana*, ThTP was synthesized only under particular circumstances such as hypoxia (*E. coli*) or withering (*A. thaliana*). In mammalian tissues, ThTP concentrations are regulated by a specific thiamine triphosphatase that we have recently characterized. This enzyme was found only in mammals. In other organisms, ThTP can be hydrolyzed by unspecific phosphohydrolases. The occurrence of ThTP from prokaryotes to mammals suggests that it may have a basic role in cell metabolism or cell signaling. A decreased content may contribute to the symptoms observed during thiamine deficiency.

Key words. Thiamine triphosphate; thiamine triphosphatase; bacteria; plants; animals; stress; adenylyl cyclase; vitamin B1.

Thiamine (vitamin B1) is an essential molecule for all life forms, from bacteria to mammals, though only prokaryotes, fungi and plants retain the capacity for its synthesis. In humans, nutritional thiamine deficiency mainly affects the nervous system, causing either peripheral polyneuritis (beriberi) or irreversible lesions in the midbrain (Wenicke-Korsakoff syndrome) [1]. These lesions are thought to be caused by decreased levels of thiamine diphosphate (ThDP), a coenzyme for cytosolic transketolase, three mitochondrial enzyme complexes (pyruvate dehydrogenase, oxoglutarate dehydrogenase and branched-chain oxoacid dehydrogenase) and the recently characterized peroxysomal 2-hydroxyphytanoyl-CoA lyase (required for the shortening of 3-methyl-branched fatty acids by α-oxidation) [2]. ThDP is thus an essential cofactor, especially for oxidative metabolism, but the reason for the selective vulnerability of certain brain regions remains unexplained [3, 4].

Thiamine is transported by high-affinity carriers into eukaryote cells [5–7], where it is rapidly converted to ThDP by thiamine pyrophosphokinase (EC 2.7.6.2) [8, 9]. ThDP can then be further phosphorylated to thiamine triphosphate (ThTP), probably by an ATP:ThDP phosphotransferase (EC 2.7.4.15). Such an enzyme was purified and characterized from baker's yeast [10]. It was also

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partially purified from rat liver [11]. None of these enzymes were sequenced or cloned. It has also been proposed that, at least in skeletal muscle, adenylate kinase 1 (EC 2.7.4.3) might be responsible for ThTP synthesis according to the reaction ThDP + ADP ⇔ ThTP + AMP [12, 13]. This hypothesis cannot be generalized as we have recently shown that adenylate kinase 1 knockout mice have normal ThTP levels in all tissues tested, including skeletal muscle [14].

The biological role of ThTP remains unknown, but it specifically phosphorylates rapsyn [15], a protein essential for the clustering of acetylcholine receptors at the neuromuscular junction [16]. ThTP also phosphorylates proteins in rodent brain, but these proteins have not yet been identified [15]. Until now the only known compound able to phosphorylate proteins in eukaryote cells was ATP. Phosphorylation by ThTP may be part of a new cellular signaling pathway.

Though ThTP seems to have a relatively high turnover compared to ThDP [17, 18], its concentration in most mammalian cells remains low (0.1–1 μM), probably because it is continuously hydrolyzed. We have recently cloned and sequenced a specific 25-kDa ThTPase widely distributed in human tissues [19]. This soluble enzyme, which has a high catalytic efficiency ($k_{cat}/K_m = 6 \times 10^6$ $s^{-1}M^{-1}$) and a virtually absolute specificity for ThTP, could be responsible for the regulation of the intracellular ThTP concentration.

In view of the possible role played by ThTP in a new signaling pathway, we thought it important to assess the phylogenetic occurrence of ThTP and ThTPase. No comprehensive distribution study has ever been made and though some earlier studies report the existence of ThTP in some species including plants [20] and microorganisms [21], the results were not quantified. As we have already pointed out [22], many early results concerning ThTP must be taken with caution as modern analytical tools such as HPLC were not available. The present results demonstrate the existence of ThTP in all major taxonomic divisions, but in some organisms, such as *Escherichia coli* and *Arabidopsis thaliana*, ThTP is not detected unless the cells or tissue are submitted to special conditions. The specific 25-kDa ThTPase, however, seems to be restricted to mammals.

Materials and methods

Determination of thiamine and its phosphate esters in bacteria, fungi, plant and animal tissues

Animal tissues were homogenized in 5 vol of 12% trichloroacetic acid (TCA) in a glass-glass homogenizer, centrifuged (5000 g, 15 min) and the supernatant was treated with 3 × 1.5 ml diethyl ether to remove the acid [23]. Plant material and mushrooms were treated in the same way except that only 1 vol of 20% TCA was used for tissue homogenization.

The bacteria used were either non-transformed *E. coli* BL 21 or BL 21 transformed with pGEX-5X-1 encoding either gluthatione S-transferase (GST) or the GST-ThTPase fusion protein [19]. The bacteria were grown in an overnight culture (37 °C, 250 rpm) in 50–100 ml Luria-Bertani (LB) medium. Then the bacteria were centrifuged (5 min, 15,000 g) and suspended in the initial volume of fresh LB medium. Ten milliliters of this bacterial suspension was incubated for 4 h (250 rpm) in a 50-ml Erlenmeyer flask either under aerobic (cotton wool stopper) or anaerobic conditions (flasks were hermetically closed using three layers of laboratory film after perfusion of the medium with nitrogen). For transformed bacteria, ampicillin (200 μg/ml) was present in the culture medium. Then the bacteria were sedimented as above and the pellet was suspended in 12% TCA, homogenized in a glass-glass homogenizer and centrifuged (5 min, 15,000 g). The extract was treated with diethyl ether and thiamine compounds were determined by HPLC [23]. The pellet was dissolved in 0.8 N NaOH for protein determination by the method of Peterson [24].

Yeast cells (*Saccharomyces carlsbergensis*, sixth generation, gift from Interbrew Belgium) were collected by centrifugation at 5500 g for 15 min, washed six times with distilled water, and the extract was prepared and treated as indicated above.

The content of thiamine and its phosphate esters was determined as previously described using an HPLC method [23]. Prior to analysis, an 80-μl aliquot was oxidized with 50 μl of 4.3 mM potassium ferricyanide in 15% NaOH and a 20-μl sample was injected into the chromatographic system (System 522; Kontron Instruments, Milan, Italy). The separation was performed at a flow rate of 0.5 ml on a PRP-1 column (∅ 4.1 × 150 mm, Hamilton, Reno, Nev.) in 50 mM NaH_2PO_4 containing 25 mM tetra-n-butylammonium hydrogen sulfate and 4% tetrahydrofuran and adjusted at pH 8.5 with NaOH. Thiochrome derivatives were quantified using a fluorometric spectrometer (LS-4; Perkin-Elmer, Shelton, Conn.). In some cases, a 100-μl injection loop was used instead of the usual 20-μl loop in order to increase the sensitivity of the detection method.

Identification of ThTP

The authenticity of endogenous ThTP was checked by enzymatic hydrolysis as previously described [25]. To 70 μl of tissular TCA extract, treated with diethylether, we added 10 μl Bis-Tris-propane buffer (500 mM, pH 8.9), 10 μl $MgSO_4$ (50 mM) and 10 μl ThTPase. The ThTPase solution contained 50 mM Bis-Tris-Propane, 0.1% bovine serum albumin (BSA) and 1.3 μg/ml purified bovine ThTPase [19] (specific activity 1.7 μmol s^{-1} mg^{-1}). The mixture was incubated at 37 °C for 10 min and the reac-

tion was stopped by addition of 20 µl TCA (72%). After extraction of the TCA by 3 × 1.5 ml diethyl ether, the sample was analyzed by HPLC [23].

Determination of enzyme activities and protein concentration

Animal tissues were homogenized in a teflon-glass Potter-Elvehjem homogenizer in 5 vol of 50 mM Tris-HCl buffer, pH 7.5 containing 150 mM KCl, 0.2 mM EDTA and centrifuged at 15,000 g for 30 min to produce a crude supernatant. Bacteria (*E. coli* strain BL 21) were grown in LB medium to the density of the culture $A_{600} = 0.87$. The cells were collected by centrifugation (3000 g, 15 min), suspended in 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride and sonicated at 22 kHz by 20 cycles of 5 s each. The suspension was centrifuged at 105,000 g for 60 min to precipitate non-disrupted cells and membrane fragments.

ThTPase activity in the supernatants and eluates after gel filtration (Toyopearl HW-60 or Sephadex G-75) was determined as described previously [19, 26]. The standard reaction medium contained 50 mM Bis-Tris-propane buffer, pH 8.9, 5 mM $MgCl_2$, 1 mg ml^{-1} BSA, 10 or 100 µM ThTP and an aliquot of enzyme preparation. After incubation for 10–30 min at 37 °C, the reaction was stopped by addition of TCA (final concentration 10%). The acid was extracted with diethyl ether and the ThDP formed was estimated by HPLC [23] or by an enzymatic method [26].

ATPase and *p*-nitrophenylphosphatase (NPPase) assays were carried out by measuring inorganic phosphate (P_i) released during a 30–60 min incubation. The assay mixture consisted of 50 mM Tris-HCl, pH 8.9, 5 mM $MgCl_2$, 1 mM ATP or *p*-nitrophenylphosphate (NPP), 1 mg ml^{-1} BSA and an aliquot of enzyme preparation in a final volume of 0.5 ml. The amount of P_i formed was determined by the method of Sapru et al. [27].

The method of Peterson [24] or absorbance at 280 nm was used to determine the protein concentration.

Immunoblot studies

Human recombinant ThTPase, purified as described earlier [19], was used to raise anti-ThTPase antibodies in a chicken by four injections of 100 µg each (Eurogentec, Seraing, Belgium). Egg yolk IgY immunoglobulins, the chicken IgG homologue, were purified using the EGGstract IgY Purification system (Promega, Leiden, The Netherlands). Brain supernatant fractions and biotinylated low-range molecular-weight standards (Bio-Rad, Nazareth-EKE, Belgium) were subjected to SDS-polyacrylamide electrophoresis (15% polyacrylamide) and the proteins were transferred to PVDF Hybond-P membranes (Amersham Biosciences, Little Chalfont, UK) by semidry transfer at 25 V for 48 min. The membranes were then incubated in blocking buffer containing 3 % fat-free milk powder in Tris-buffered saline (TBS-T; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % Tween-20) for 1 h at 37 °C. After washing 2 × 10 min with TBS-T at room temperature, the membranes were incubated overnight (4 °C) with purified anti-ThTPase antibodies (0.04 µg/ml). The membranes were washed 3 × 10 min with TBS-T before incubation (1 h, 25 °C) with anti-chicken Biotin-SP-conjugated AffiniPure Donkey IgG $F(ab')_2$ fragment (Jackson ImmunoResearch Laboratories, West Grove, Pa.) at a working dilution of 1:20,000 in TBS-T. Then, the membranes were washed in TBS-T (4 × 10 min) and treated with 5 mU/ml streptavidin-conjugated peroxidase (Roche Diagnostics, Vivoorde, Belgium) for 1 h at 25 °C. After 3 × 10 ml in TBS-T, the membranes were stained with 1.5 mM 3,3′, 5,5′ tetramethyl-benzidine (Sigma-Aldrich, St. Louis, Mo.) in 50 mM acetate buffer (pH 6.0) containing 0.02% sodium perborate trihydrate (VWR, International, Leiven, Belgium) and 2 mM dioctyl sulfosuccinate (Sigma-Aldrich).

Molecular mass determinations

Molecular mass determinations were done at 4 °C on a Sephadex G-75 (∅ 2.5 × 36 cm) or Sephacryl S-200 (∅ 2.2 × 46 cm; for bovine tissues) column equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and calibrated with standard proteins. In both cases, the samples were chromatographed at a flow rate of 5 cm h^{-1} and ThTPase elution volumes (V_e) were estimated by activity assays. Molecular masses were calculated from the plots of $\log M_r$ versus $\log V_e/V_o$ ratio.

PCR detection of ThTPase mRNA in pig and quail brain

Total RNA was isolated from adult male pig brain and kidney (local slaughterhouse) or quail brain by Instapure reagent (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. RNA was reverse transcribed using M-MLV and oligo-dT primers. For PCR amplification by Acuprime polymerase (Amersham Biosciences), 2 µl of the synthetized cDNA was submitted, following the manufacturer's protocol, to 40 PCR cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s), and elongation (68 °C, 1 min) using the F1 (5′-ATG GCICARGGIYTIATHGARGTIGAR-3′) and R1 (5′-Y GIGGICCYGTGAAITYRAGGGTNGG-3′) degenerate primer pair combination. An aliquot of this PCR was reamplified under the same conditions using internal degenerated primers F2 (5′-GGIYTIATHGARGTIGARM-GIAARTTY-3′) and R2 (5′-RTCRTARTAIGTRTCICK-RAAIGTNAC-3′).

For specific pig ThTPase mRNA detection, brain and kidney samples were amplified for 40 cycles by incubation at 94 °C for 20 s, 62 °C for 30 s and 72 °C for 2 min using PTTPF (5′-CCAGCTCTGTGAGGTGCTGAG-3′) and

PUTR3 (5′-AGGAAGGATGGCTGAGGGT AGG-3′) primers.
PCR products were subcloned in pCRII by the TA cloning method (Invitrogen) and sequenced in both directions by the Sanger dideoxynucleotide chain termination method using the T7 DNA polymerase sequencing kit (Amersham Biosciences).

Cloning and expression of ygiF
Genomic DNA was isolated from *E. coli* DH5α bacteria using a standard protocol (Invitrogen). The ygiF coding sequence was amplified from 1 μg of genomic DNA using Acuprime polymerase and 35 PCR cycles of denaturation (94 °C, 20 s), annealing (64 °C, 30 s) and elongation (72 °C, 90s) using forward (5′-TTTGGATCCATGG CTCAGGAAATCGAATTAAAG-3′) and reverse (5′-TT TGCGGCCGCTTAACGTTTTCCGCTGTGCA ACC-3′) primers. The 1.3 kb PCR fragment was cloned with the TOPO-blunt end kit (Invitrogen). The plasmid containing the insert was sequenced as described above and then digested by *Bam*HI and *Not*I. The released fragment was purified on agarose gel and subcloned into pGEX-5X-1 (Amersham Biosciences) to produce the GST-ygiF fusion protein.

Results

Distribution of thiamine derivatives in various organisms and tissues
As can be seen from table 1, thiamine derivatives were present in all organisms tested, with the highest total thiamine concentration found in *E. coli* and the lowest in the leaves and roots of plants. ThDP was generally the most abundant derivative, accounting for 70 to over 90% of total thiamine. A relatively high proportion of non-phosphorylated thiamine was found in bean germs. This can most probably be explained by the abundance of thiamine storage proteins in seeds and germs [28, 29]. Such proteins are thought to retain thiamine in a dormant seed and release it during germination. We observed a particularly low proportion of non-phosphorylated thiamine compared with its total content in skeletal muscle, a situation that can possibly be related to the low AMP levels in resting skeletal muscle [30]. Indeed, following the law of mass action, the reaction thiamine + ATP ⇔ ThDP + AMP catalyzed by thiamine pyrophosphokinase is favored when AMP concentrations are low; that might be a depleting factor for free thiamine in skeletal muscle.
As shown in table 1, ThTP was detected in most organisms tested except in *E. coli* and in leaves and roots of higher plants. However, a small amount was found in bean germs and sphagnum leaves, suggesting that ThTP may be synthesized in plants as well. In most tissues, the ThTP content was found to be low, generally less than 1% of total thiamine. Some exceptions have been reported: ThTP accounts for over 50% of total thiamine in the *Electrophorus electricus* electric organ [31] as well as in pig [32] and chicken [13] skeletal muscle, a result reproduced here (table 1). We suggested that this is primarily due to either the absence or a low activity of specific ThTPase in these tissues [14], though other reasons could not be excluded. In mammals, the ThTP content is lower in brain than in skeletal muscle [14, 32], while in other vertebrates, the reverse seems to be true (table 1). The highest content of ThTP was found in pig and chicken skeletal muscle, slug foot muscle, total extract of the small crustacean *Artemia salina*, and chicken brain. Brewer's yeast and the mushroom *Tricholoma gambosa* also contained a high amount of ThTP.
Unlike results presented here, the existence of ThTP in bacteria was previously reported [33]. Using an HPLC method, these workers found about 3–7 nmol of ThTP per gram in *E. coli*. This discrepancy was subsequently shown to have a physiological basis: in certain circumstances, ThTP can be revealed in both bacteria and plants (see below).
The thiamine monophosphate (ThMP) content in different organisms was highly variable: from almost complete absence in the leaves and roots of plants to 2.4 nmol per gram wet weight in *A. salina*. There is so far no evidence that this compound plays a physiological role other than being the degradation product of ThDP or a precursor of thiamine in organisms able to synthesize it.

ThTP is synthesized only in response to cellular stress in *E. coli* and *A. thaliana*
We found no ThTP in fresh leaves of *A. thaliana* (table 1). But when the plants were retrieved from the soil, they lost turgor and started withering. Under these conditions and within 1–2 h, we observed the appearance of ThTP reaching up to 0.08 ± 0.02 nmol per gram wet weight (n=3). Though we do not know exactly what kind of factor might trigger the synthesis of ThTP under conditions of withering, these experiments clearly show that higher plants have the capacity to synthesize ThTP in response to an environmental stress.
This result prompted us to challenge another organism, namely *E. coli*, where at first we did not detect any ThTP (table 1, fig. 1A). The *E. coli* strain BL 21 was cultured in LB medium under standard aerobic conditions. After testing several experimental conditions, we found that when the bacteria were grown under anaerobic conditions, a relatively large amount of ThTP was synthesized (fig. 1B). Under these conditions, a ThTP concentration of 4.2 ± 0.9 nmol/g of wet weight (n = 4) was reached within 4 h of culture. When the experiment was repeated with bacteria expressing human ThTPase as a GST-fusion protein [19], no ThTP was detected (fig. 1C). These recom-

Table 1. Thiamine and its phosphate esters (nmol–g^{-1} of wet weight, mean ± SD) in various organisms and tissues.

Species	ThTP	ThDP	ThMP	Thiamine	Total
ANIMALS					
Homo sapiens					
Cerebral cortex (n = 3)	0.021 ± 0.005 (0.5)	2.9 ± 0.4 (71.1)	0.26 ± 0.13 (6.4)	0.9 ± 0.3 (22.0)	4.08
Baboon (*Papio papio*)					
Cerebral cortex (n = 3)	0.24 ± 0.10 (3.4)	5.5 ± 1.0 (77.2)	0.28 ± 0.07 (3.9)	1.1 ± 0.1 (15.4)	7.12
Rat (*Rattus norvegicus*)					
Cerebral cortex (n = 5)	0.07 ± 0.01 (1.1)	5.8 ± 0.5 (88.4)	0.27 ± 0.07 (4.1)	0.42 ± 0.09 (6.4)	6.56
Domestic pig (*Sus scrofa domesticus*)					
Cerebral cortex (n = 2)	0.15 ± 0.8 (3.8)	3.1 ± 0.7 (78.5)	0.30 ± 0.10 (7.6)	0.4 ± 0.1 (10.1)	3.95
Skeletal muscle (n = 2)	20 (64.3)	11 (35.4)	0.047 (0.15)	0.014 (0.045)	31.1
Domestic chicken (*Gallus gallus*)					
Brain (n = 1)	0.92 (8.4)	9.5 (86.4)	0.48 (4.4)	0.1 (0.9)	11.0
Skeletal muscle (pectoralis) (n = 1)	3.7 (70.7)	1.2 (23)	0.33 (6.3)	n. d.	5.2
Quail (*Coturnix coturnix japonica*)					
Brain (n = 3)	0.34 ± 002 (4.6)	6.8 ± 0.2 (91.6)	0.20 ± 0.02 (2.7)	0.08 ± 0.01 (1.1)	7.42
Skeletal muscle (pectoralis) (n = 3)	0.34 ± 0.02 (1.9)	16.7 ± 0.04 (91.1)	1.3 ± 0.1 (7.1)	n. d.	18.34
Trout (*Salmo trutta*)					
Brain (n = 3)	0.029 ± 0.002 (0.3)	7.8 ± 0.3 (93.4)	0.36 ± 0.02 (4.3)	0.16 ± 0.01 (1.9)	8.3
Muscle (axial) (n = 3)	0.013 ± 0.002 (0.4)	3.41 ± 0.14 (94.5)	0.16 ± 0.02 (4.4)	0.026 ± 0.003 (0.7)	3.61
Brine shrimp (*Artemia salina*)					
Total extract (n = 3)	0.35 ± 0.06 (6.0)	2.3 ± 0.4 (39.7)	2.4 ± 0.4 (41.4)	0.75 ± 0.2 (12.9)	5.8
Slug (*Arion rufus*)					
Foot muscle (n = 3)	1.8 ± 0.4 (10.0)	13.7 ± 1.2 (88.9)	0.34 ± 0.10 (1.9)	1.2 ± 0.3 (6.7)	18.0
Cerebral ganglion (n = 3)	0.88 ± 0.3 (9.1)	7.6 ± 1.8 (78.4)	0.5 ± 0.3 (5.2)	0.68 ± 0.15 (7)	9.7
FUNGI					
Brewer's yeast					
S. carlsbergensis (n = 3)	0.24 ± 0.1 (1.1)	20 ± 2 (88.9)	0.55 ± 0.03 (2.4)	1.7 ± 0.1 (7.6)	22.5
Tricholoma gambosa (n = 3)	0.025 ± 0.001 (1.8)	1.1 ± 0.2 (79.7)	0.09 ± 0.01 (6.5)	0.16 ± 0.1 (11.6)	1.38
PLANTS					
Parsley (*Petroselium crispum*)					
Leaves (n = 3)	n. d.	0.43 ± 0.02 (95.6)	0.005 ± 0.001 (1.1)	0.015 ± 0.002 (3.3)	0.45
Roots (n = 3)	n. d.	1.34 ± 0.02 (71.3)	n. d.	0.54 ± 0.01 (28.7)	1.88
Bean (*Phaeseolus vulgaris*)					
Germs (n = 3)	0.02 ± 0.01 (0.4)	1.82 ± 0.02 (37.9)	0.13 ± 0.01 (2.7)	2.8 ± 0.2 (58.3)	4.8
Arabidopsis thaliana					
Leaf (n = 3)	n. d.[a]	0.80 ± 0.04 (72.7)	n. d.	0.30 ± 0.01 (27.3)	1.10
Root (n = 3)	n. d.	0.083 ± 0.004 (92.2)	n. d.	0.007 ± 0.001 (7.8)	0.09
Sphagnum palustei					
Leaves (n = 3)	0.047 ± 0.005 (5.5)	0.54 ± 0.11 (62.8)	0.16 ± 0.4 (18.6)	0.11 ± 0.04 (12.7)	0.86
PROKARYOTES					
E. coli (n = 3)	n. d.[b]	142 ± 2 (93.4)	9.1 ± 0.2 (6.0)	0.94 ± 0.05 (0.6)	152

The percentage of total thiamine is indicated in paraentheses.
n, number of different individuals or samples (*E. coli*, *S. carlsbergensis*, *A. salina*). n. d., not detected.
[a] ThTP was found in withering plants.
[b] *E. coli* was cultured in LB medium under aerobic conditions.

binant bacteria have a high level of GST-ThTPase protein expression even in the absence of isopropyl-β-D-thiogalactoside [19], and endogenous ThTP was therefore continuously hydrolyzed.

To confirm the presence of authentic ThTP, the extracts of *A. thaliana* and *E. coli* were treated with purified bovine ThTPase as previously described [25]. In both cases, the ThTP peak disappeared (not shown), demonstrating that we were dealing with genuine ThTP.

ThTPase in various organisms and tissues

ThTP can be hydrolyzed by many phosphatases such as myosin ATPase [34], nucleoside triphosphatases [35] or acid and alkaline phosphatases [36], but until now only one soluble specific ThTPase has been characterized [19, 26]. This enzyme has a molecular mass of 25 kDa in humans and a virtually absolute specificity for ThTP. To study the occurrence of the enzyme in various tissues and organisms, we separated crude supernatants by gel chro-

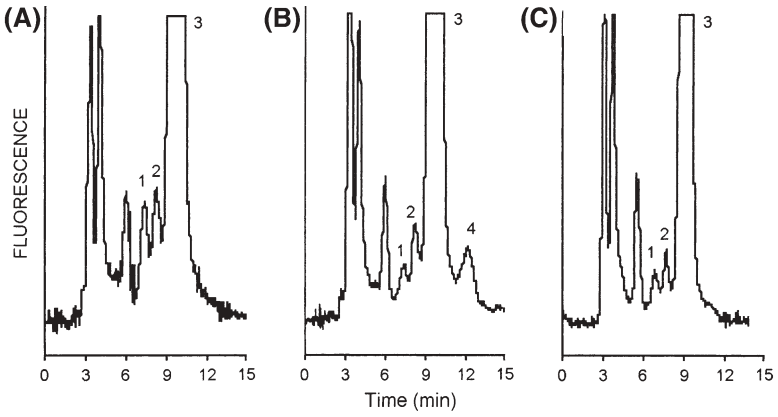

Figure 1. ThTP content in *E. coli* expressing GST (*A*, *B*) or GST-ThTPase (*C*). The bacteria were thawed and cultured overnight (37 °C, 250 rpm). They were then centrifuged (5 min, 15,000 g) and the pellet was suspended in the initial volume of fresh LB medium and cultured either aerobically (*A*) or anaerobically (*B*, *C*) for 4 h. The bacteria were sedimented (5 min, 15,000 g) and the pellets were resuspended in 250 µl of 12% TCA and centrifuged (5 min, 15,000 g). The supernatant was extracted with diethyl ether and thiamine compounds were determined by HPLC [23]. (1, ThMP; 2, thiamine; 3, ThDP; 4, ThTP).

Table 2. ThTPase activities and properties in various organisms and tissues.

		ThTPase activity (nmol min^{-1} per g^{-1} of wet weight)	Molecular mass by gel filtration (kDa)	K_m (µM)
Human	brain (cerebellum)	57	25.55[a]	126
Rat	brain	437 ± 10	32.2	16.0 ± 0.7
	liver	299 ± 4	29.7	27.8 ± 0.7
	kidney	189 ± 2	34.0	22.8 ± 0.7
	muscle (quadriceps)	52 ± 4	32.0	–
	embryo 10 days, total	72 ± 3	31.0	21.4 ± 1.3
	embryo 15 days, brain	253 ± 3	–	20.1 ± 2.6
	embryo 15 days, liver	128 ± 5	–	25.3 ± 1.6
Bovine	brain	309 ± 15	26.3	44.1 ± 1.1
	liver	354 ± 7	28.4	21.1 ± 1.2
	kidney	377 ± 20	28.3	45.1 ± 1.6
	spleen	275 ± 3	29.0	33.8 ± 1.6
	heart	217 ± 14	29.1	40.6 ± 0.3
	lung	333 ± 36	27.2	38.8 ± 1.8
	intestine (duodenum)	33 ± 3	31.7	44.6 ± 1.8
	muscle (trapezius)	169 ± 8	27.6	35.9 ± 1.0
	lymph node	220 ± 2	–	–
Pig	kidney	16.2	33.9	49.5
	skeletal muscle	12.5	–	–
	brain	n. d.	–	–
Quail	brain	0.6	> 80.0	–
	liver	2.4	–	–
	heart	0.6	–	–
	kidney	0.8	–	–
	muscle (pectoralis)	0.3	–	–
Chicken	brain	11.4	> 80.0	–
	muscle (pectoralis)	932	> 80.0	–
Trout	brain	0.52 ± 0.05	> 80.0	–
	muscle (axial)	0.29 ± 0.01	–	–
E. coli		0.14[b]	> 1000; 133	–
Parsley leaves		n. d.		
Yeast		n. d.		

The ThTP concentration was 100 µM, except for quail and trout tissues where 10 µM ThTP was used. The enzyme activities and the K_m values are expressed as mean ± SD (when applicable) for one to three experiments.

[a] As determined from the amino acid composition [19].

[b] nmol min^{-1} per mg^{-1} of protein.

n.d., not detected.

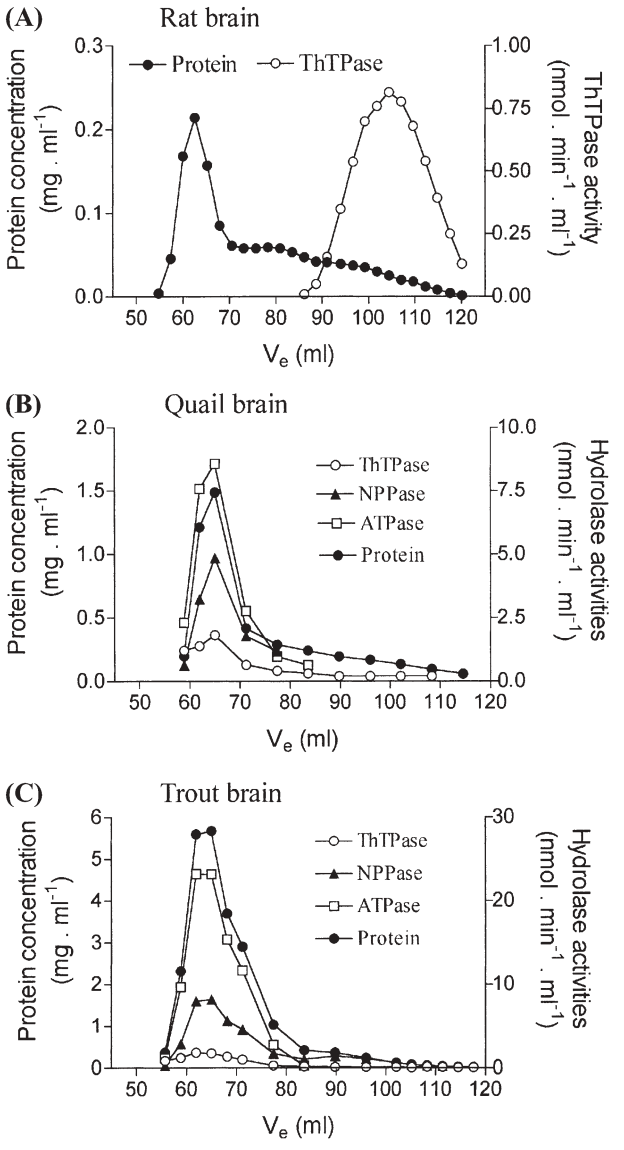

Figure 2. Gel filtration of supernatants from the brains of rat, quail and trout. In each case, the sample was run on a Sephadex G-75 column (∅ 2.5 × 35 cm) and protein content, ThTPase, ATPase and NPPase activities were determined. In rat brain, no measurable NPPase and ATPase activities were associated with ThTPase activity.

matography and assayed ThTPase activity in the elution profiles. As can be seen from table 2, in mammalian tissues, the activity peak corresponded to an expected molecular mass of about 30 kDa. This is somewhat higher than the molecular mass based on the amino acid composition but this discrepancy has already been observed previously [19, 26]. As an example, the elution pattern for rat brain is illustrated in figure 2A. All the ThTPase activities exhibited a pH optimum of 8.5–8.9 (not shown) and their K_m values ranged from 16 µM in rat brain to 126 µM in human brain. These data thus indicate that the specific 25-kDa ThTPase is widely distributed in mammalian tissues, though it is not a very abundant protein.

In rat brain and liver, ThTPase activity was higher in the adult compared to embryonic tissues. The activity was lowest in intestine, in agreement with the results of Penttinen and Uotila [36] who were unable to detect the enzyme in rat intestine.

Surprisingly, we were unable to detect any 25-kDa ThTPase activity in pig brain, though it was present in low amounts in the kidney (table 2). Because at that time there was no pig EST showing homology with ThTPase, we designed degenerate primers directed against conserved amino acid sequences of the human, macaque, bovine and mouse ThTPase. By performing nested PCR on pig brain cDNA, we were able to amplify a fragment with a sequence corresponding to ThTPase (fig. 3 A). With this approach, we could, however, not exclude amplification from traces of genomic DNA as the primers hybridize to sequences located in the first exon of the human and mouse gene. In the meantime, two pig EST sequences were published (GenBank accession numbers BG384847, BM481914). We performed a new analysis using non-degenerated primers hybridizing to sequences that span the intron. This allowed us to demonstrate unambiguously that pig brain and kidney expressed specific ThTPase mRNA and that an intron is also present in the pig gene (fig. 3 B). By combining our cloned sequences and the EST sequences, we were able to deduce the pig cDNA sequence and demonstrate that it encodes a 230-amino acid ThTPase (we expected the N-terminal sequence MAQ to be present in all ThTPases, although we did not clone it in pig) showing 77% identity with the human and bovine enzymes (fig. 3 C).

To see if the protein is present in pig brain, we used a chicken polyclonal antibody raised against human recombinant ThTPase (fig. 4). This antibody recognized two proteins in the 34- to 35-kDa range in rat brain, the fastest migrating at the same speed as purified recombinant human ThTPase. This band was absent from pig brain, suggesting that though the mRNA is expressed in pig brain, the active protein is probably absent. The 34.7-kDa protein is either unrelated to ThTPase or could represent a posttranslationally modified and enzymatically inactive ThTPase. This would explain the presence of mRNA in pig brain. As previously noticed [19, 26], ThTPase migrates at a significantly higher apparent molecular mass than expected from its primary sequence. This was attributed to its overall important negative charge [19].

We were unable to find any specific 25-kDa enzyme in non-mammalian tissues. Despite high ThTPase activity in chicken muscle, we observed no activity in the eluate after chromatography on a Sephadex G-75 column (not shown). Moreover, a wide band of protein precipitate was formed on the top of the column. Thus ThTP hydrolysis in chicken muscle extract is probably due to myosin which aggregated on the gel because of the sample dilution. Indeed, myosin was reported to be able to hydrolyze ThTP [34].

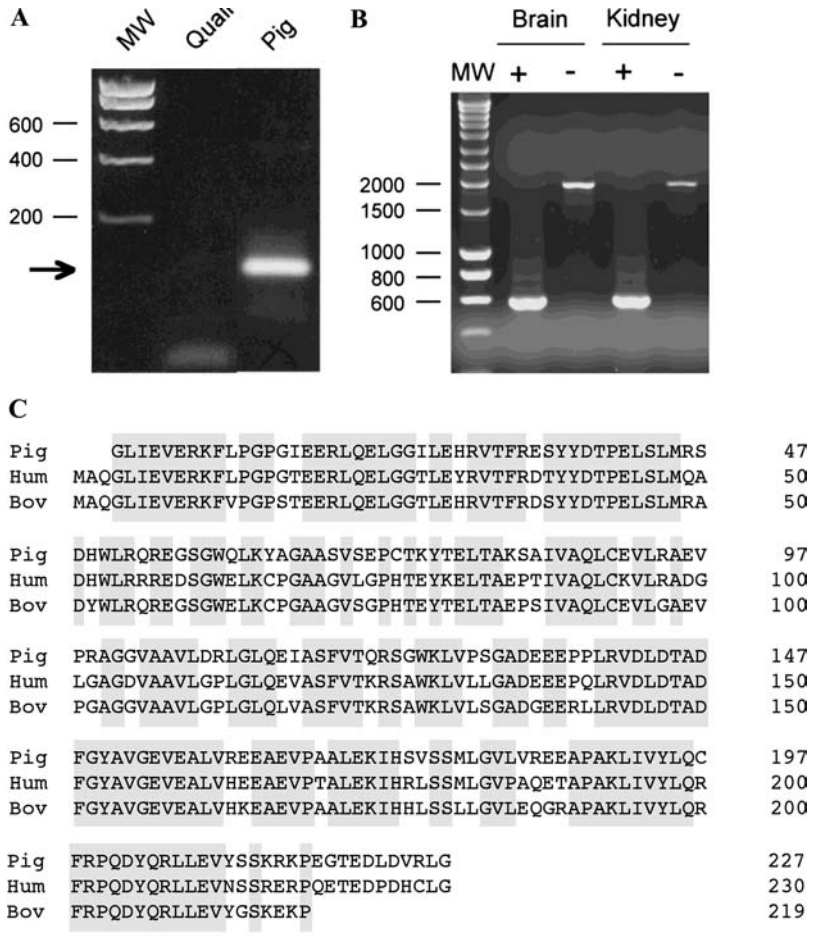

Figure 3. Search for ThTPase mRNA by RT-PCR in pig and quail brain and sequence comparison. (*A*) RT-PCR using degenerated primers directed against conserved amino acid sequences on quail and pig brain RNA. The arrow indicates the size of the expected fragment (120 bp). (*B*) RT-PCR using non-degenerated primers on pig brain and kidney RNA reverse transcribed in the presence (+) or absence (–) of M-MLV reverse transcriptase. (*C*) Multiple alignment of the deduced pig, human (Hum) and bovine (Bov) ThTPase. Identical residues in the three species are shaded. (*A*, *B*) MW, Smart Ladder molecular weight marker (Eurogentec).

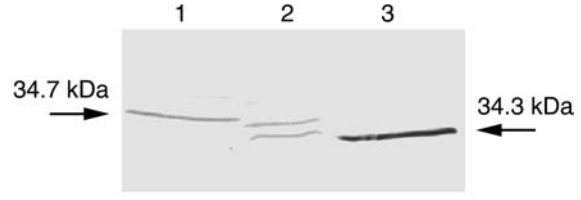

Figure 4. Expression of ThTPase in pig and rat brain. After SDS-PAGE (15% acrylamide), Western blots were performed as described in Materials and methods and ThTPase was revealed with purified chicken antibodies. Lane 1, pig brain supernatant (100 µg of protein); lane 2, rat brain supernatant (100 µg of protein); lane 3, purified recombinant human ThTPase (0.04 µg). The arrows indicate the molecular mass, extrapolated from the low-range biotinylated SDS-PAGE standards (BioRad).

A much lower ThTPase activity was found in fish and quail tissue extracts. To be noted here is that, taking into account a rather low K_m of the 25-kDa ThTPase compared to unspecific ThTPases, a 0.01 mM ThTP concentration was used in those experiments to decrease the activity of non-specific phosphatases. Figure 2 B, C shows that ThTPase activity of quail and trout brain was coeluted in the void volume together with NPPase and ATPase, suggesting that it is associated with proteins of molecular mass higher than 80 kDa. For both species, two pH optima were observed at pH 5–6 and 7.5 (not shown) but not at 8.9 as for specific mammalian ThTPase. On the other hand, no amplimer was obtained from quail brain by RT-PCR by nested PCR using degenerate primers against conserved sequences (fig. 3B). Taken together, those data suggest that the specific 25-kDa ThTPase does not exist in birds. Earlier work with *E. electricus* has shown that no soluble ThTPase with an alkaline pH optimum was pre-

sent in the electric organ of that fish [31]. Likewise, little activity was detected in trout brain and muscle (table 2) and this activity, coeluted with NPPase and ATPase activities, corresponded to high-molecular-mass proteins (fig. 2 C).

E. coli was previously shown to contain a membrane-bound non-specific ThTPase of 16 kDa [35]. A soluble ThTPase activity with a pH optimum of 7 was also reported in this bacterium, but its specificity was not proven and its molecular mass remains unknown. According to our experiments, there are several soluble enzymes with ThTPase activity in *E. coli*. In a crude supernatant, we found three pH optima: 6–6.5, 8.0 and 9.2 (fig. 5A). When the extract was applied on a Toyopearl HW-60 column, two different peaks of activity were eluted, one in the void volume (M > 1000 kDa) corresponding to pH optima 6.0 and 8.0 and one at 133 kDa with a pH optimum of 9.2 (fig. 5 B). The latter probably did not correspond to a specific ThTPase, as ATPase and NPPase activities of the peak were respectively 2700 and 40 times higher (not shown). No ThTPase activity was detected in the low-molecular-mass range, suggesting that the 25-kDa ThTPase does not exist in *E. coli*. In parsley leaves and yeast extracts we could not find any detectable ThTPase activity at alkaline pH (not shown).

Recently, ThTPase and CyaB-like adenylyl cyclases were shown to define a novel superfamily of domains (CYTH domain) that bind organic phosphates [37]. The CyaB protein from *Aeromonas hydrophila* displays adenylyl cyclase activity [38] at high temperature (65 °C) and alkaline pH (9.5). A homologous gene product was also found in hyperthermophilic archaebacteria, but until now no adenylyl cyclase was proven to be associated with it. An *E. coli* gene product related to this CYTH family, named ygiF (AP002564), shares 33% homology with human ThTPase over 95 residues and we raised the question whether this protein has ThTPase activity. To check this hypothesis, we cloned the open reading frame corresponding to ygiF and expressed the protein as a GST fusion protein. Figure 6 shows the induction of a protein band of the expected molecular mass (75 kDa for the fusion protein), but no ThTPase activity was detected. Arguably ThTPase activity could be lost after addition of the N-terminal GST moiety. This is however not very likely, as human ThTPase activity is not much influenced by the GST moiety [19].

These results are in agreement with the hypothesis that members of the CYTH family, which can be traced back to the last universal common ancestor, are involved at the interface between nucleotide and polyphosphate metabolism but with the exact function still unknown [37]. ThTPase activity seems to represent a relatively recent divergent acquisition of a new catalytic activity. Such might also be the case with acquisition of adenylyl cyclase activity by the *A. hydropila* cyaB protein.

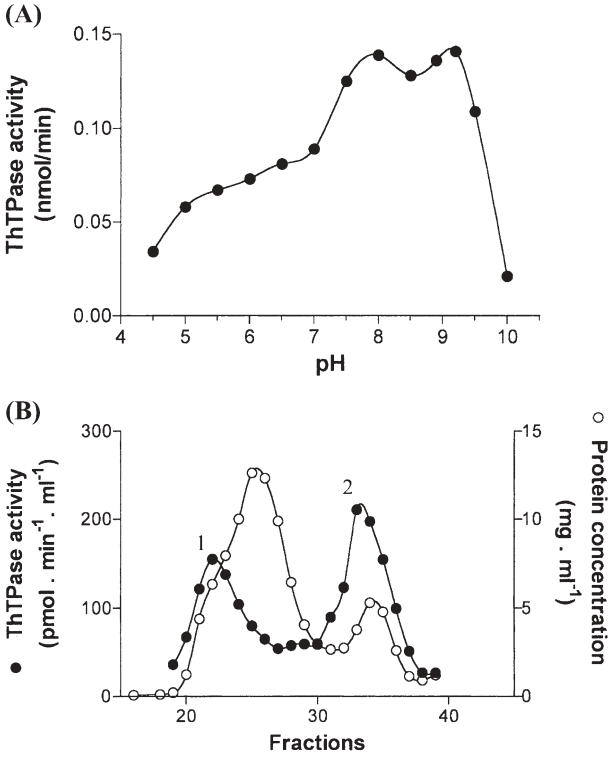

Figure 5. ThTPase activity in *E. coli*. (*A*) pH profile of ThTPase activity in a crude supernatant obtained after centrifugation (105,000 g, 60 min) of disrupted *E. coli*. (*B*) Chromatography of the *E. coli* supernatant on a Toyopearl HW-60 column. The molecular mass markers were: ferritin, 480 kDa; pyruvate kinase, 228 kDa; bovine serum albumin, 67 kDa and myoglobin, 17.8 kDa. 1, ThTPase peak corresponding to m > 1000 kDa (void volume of the column); 2, the peak corresponding to 133 kDa.

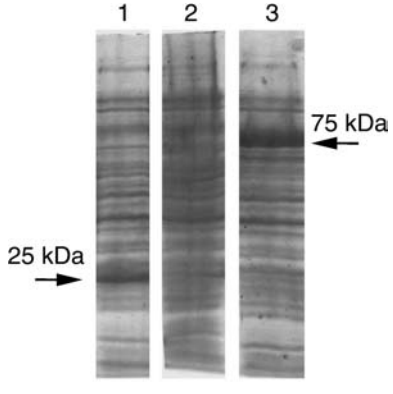

Figure 6. Expression of ygiF as a GST fusion protein in *E. coli*. Lane 1, GST with IPTG; lane 2, GST-ygiF without IPTG; lane 3, GST-ygiF with IPTG. The arrows indicate the expected location of GST (25 kDa) and GST-ygiF fusion protein (74.4 kDa).

Discussion

Thiamine is essential for all known organisms, but while prokaryotes, fungi, plants and probably some protists are able to synthesize it de novo, animals are not: it is thus a vitamin, and the coenzyme ThDP is generally the most abundant thiamine derivative.

In addition to ThMP and ThDP, the presence of a triphosphoric derivative of thiamine, ThTP, has been well documented in animal tissues [31, 32]. Early studies also reported the existence of ThTP in yeast and plant germs [20, 21, 39], but the results were not quantified and the authenticity of ThTP was not proven. A more recent report claims the absence of ThTP in phytoplankton and *A. salina*, but the authors did not even use a standard to determine the retention time of ThTP by their HPLC method [40]. Furthermore, the method used does not seem to be suitable for the detection of small ThTP peaks due to the long retention times. Thus a comprehensive distribution study of ThTP throughout the major kingdoms seemed long overdue.

Our study revealed the existence of ThTP, though mostly in small amounts compared to ThDP, in prokaryotes (*E. coli*), fungi (including yeast), plants (angiosperms and non-vascular plants) and animals (vertebrates and invertebrates). We did not have the opportunity to study archaebacteria and protists, but the presence of ThTP in all other major kingdoms suggests that this is a phylogenetically old compound and that its role is fundamental and independent of the mode of nutrition of the organism (heterotrophic, autotrophic or saprotrophic).

Indeed, modern systematics tends to recognize three domains at the highest taxonomic level: archea, bacteria and eucarya [41], though the division of prokaryotes in two separate domains has been challenged [42]. Eukaryotes can then be further subdivided in at least four kingdoms: animals, plants, fungi and protists. This division in animals, plants and fungi is justified from a nutritional point of view: animals are multicellular heterotrophs, while plants are multicellular autotrophs by photosynthesis and fungi are unicellular or multicellular heterotrophic saprotrophs.

The present study suggests that specific mechanisms for the synthesis of ThTP exist in bacteria and in the main groups of eucarya. Though ThTP is continuously synthesized [17, 18], it does not accumulate in most cells, indicating that it is rapidly hydrolyzed by phosphohydrolases. ThTPases were first described in rat brain [43, 44]. More recently, we purified [26] and sequenced [19] a soluble specific ThTPase from bovine brain. This 25-kDa enzyme has a high catalytic efficiency and absolute specificity for ThTP [19, 26, 36], suggesting that its role is a rapid and precise regulation of cytosolic ThTP content. The present results show that this specific soluble ThTPase is widely expressed in different types of mammalian tissues but not in non-mammalian organisms. However, the inability to detect ThTPase in pig brain, as shown by the absence of enzyme activity and immunoreactivity (absence of the apparent 34.3-kDa band), came as a surprise. At present, we can, however, not rule out that the apparent 34.7-kDa band revealed by our antibodies in pig and rat brain corresponds to a posttranslationally modified and enzymatically silent form of the enzyme.

We suppose that the 25-kDa ThTPase appeared in mammals by divergence from the CyaB superfamily of organic phosphate-binding proteins as a flexible instrument for the regulation of ThTP levels and, maybe, as an element of mammalian advanced adaptation mechanisms in the changing world. In non-mammalian organisms, ThTP is apparently hydrolyzed by other, much less specific, enzymes. No activity corresponding to the molecular mass of the specific ThTPase was detected in any non-mammalian species, though chicken muscle, quail and trout brain as well as *E. coli* contained phosphatases able to hydrolyze ThTP at alkaline pH. A different mechanism seems to be operating for maintaining low ThTP concentration under normal conditions in those organisms. Such a mechanism might regulate the expression and activity of ThTP-synthesizing enzyme(s) rather than the activity of hydrolases.

With a few exceptions, such as pig and chicken skeletal muscle as well as electric organ, ThTP levels are very low in most tissues. In *Torpedo marmorata* electric organ, ThTP phosphorylated rapsyn with a K_d of about 10 μM, but we can estimate that in many mammalian tissues, and especially in brain, its intracellular concentration is only about 0.1 μM provided it is homogeneously distributed in the cytosol. How ThTP might play a physiological role at such low intracellular concentration is difficult to conceive. Switching bacteria from aerobic to anaerobic conditions resulted in an increase of their ThTP content from nearly undetectable levels to 4.2 nmol per gram wet weight. This would correspond to an intracellular concentration of over 4 μM, compatible with a role as phosphate donor in kinase-catalyzed reactions. In *A. thaliana*, we also observed an important increase in ThTP levels in response to a physiological stress. A similar mechanism might operate in mammalian cells. ThTP levels are possibly low only in unchallenged cells, and in reaction to some kind of cellular stress, the ThTP concentration may increase, either by down-regulation of ThTPase activity, up-regulation of ThTP synthesis or both. ThTPase, with respect to ThTP, might play a role comparable to phoshodiesterase with respect to cAMP.

We have recently pointed out [22] that published values for ThTP levels in rat brain vary from 0.02 to 0.6 nmol per gram wet weight. Such big differences between different works may be explained if the ThTP content depends on the metabolic status of the brain.

Thus, our results favor the hypothesis that ThTP may have a rather limited role under normal physiological condi-

tions. However, in response to a physiological stress, which may depend on the particular cell type or organism, ThTP accumulates inside the cells where, possibly through the phosphorylation of some target proteins, it may induce a reaction to stress, favoring cell survival. Indeed, at least in neuroblastoma cells, thiamine deficiency has recently been shown to result in cellular stress leading to the loss of JNK1 activity rendering the cells more sensitive to apoptosis [45]. During thiamine deficiency, ThTP might not be available anymore, contributing to cell death.

Thiamine is an ancient molecule, probably already present in the RNA world. Thus, thiamine, like riboflavin and flavin mononucleotides, may directly interact with mRNA molecules for the control of gene expression at the level of transcription [45] or translation [46]. These completely new mechanisms emphasize our point of view that thiamine is far from having given away all its secrets.

Acknowledgements. We wish to thank Dr. P. Motte (Department of Plant Morphology, University of Liège) for the gift of *A. thaliana* plants. Human brain came from the Department of Pathology (University of Liège). Quails were a gift from Dr. J. Balthazart (Behavioral Neuroendocrinology, Center for Cellular and Molecular Neurobiology, University of Liège). The authors wish to thank the Fonds de la Recherche Fondamentale Collective (FRFC) for grant 2.4541.99 to L. Bettendorff and B. Lakaye. P. Wins is Research Associate and L. Bettendorff Senior Research Associate at the Fonds National de la Recherche Scientifique (FNRS). The stay of A. F. Makarchikov at the University of Liège was possible thanks to a postdoctoral grant by the FNRS. J. Czerniecki is a Marie-Curie fellow at the Marie Curie training site of the European Graduate School of Neuroscience (Maastricht, The Netherlands).

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