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Molecular Characterization of a Specific Thiamine Triphosphatase Widely Expressed in Mammalian Tissues*

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Thiamine triphosphate (ThTP) is found at low concentrations in most animal tissues, and recent data suggest that it may act as a phosphate donor for the phosphorylation of some proteins. In the mammalian brain, ThTP synthesis is rapid, but its steady-state concentration remains low, presumably because of rapid hydrolysis. In this report we purified a soluble thiamine triphosphatase (ThTPase; EC 3.6.1.28) from calf brain. The bovine ThTPase is a 24-kDa monomer, hydrolyzing ThTP with virtually absolute specificity. Partial sequence data obtained from the purified bovine enzyme by tandem mass spectrometry were used to search the GenBank™ data base. A significant identity was found with only one human sequence, the hypothetical 230-amino acid protein MGC2652. The coding regions from human and bovine brain mRNA were amplified by reverse transcription-PCR, cloned in *Escherichia coli*, and sequenced. The human open reading frame was expressed in *E. coli* as a GST fusion protein. Transformed bacteria had a high isopropyl-β-D-thiogalactopyranoside-inducible ThTPase activity. The recombinant ThTPase had properties similar to those of human brain ThTPase, and it was specific for ThTP. The mRNA was expressed in most human tissues but at relatively low levels. This is the first report of a molecular characterization of a specific ThTPase.

In most cells, the major form of thiamine (vitamin B_1) is thiamine diphosphate (ThDP),[1] a cofactor for pyruvate and 2-oxoglutarate dehydrogenases, as well as transketolase. However, most animal tissues also contain free thiamine, thiamine monophosphate (ThMP), and small amounts of thiamine triphosphate (ThTP) (1). ThTP is found in most animal cells, as well as in yeast and bacteria, but its physiological importance remains unclear. For several decades, ThTP was thought to play a specific function in excitable tissues (2, 3) but until recently, no compelling evidence could be found to support this hypothesis. In inside-out patches of neuroblastoma cells, ThTP activates a high conductance chloride channel, possibly through phosphorylation (4), but the role of this so-called maxi-chloride channel remains unknown. In *Torpedo* electric organ, [γ-^{32}P]ThTP was found to phosphorylate rapsyn (5), a protein required for the clustering of acetylcholine receptors at the neuromuscular junction (6). This phosphorylation was highly specific for ThTP compared with ATP, and, surprisingly, ThTP phosphorylated a histidyl residue (5). Two or three phosphorylated protein bands were also observed in membranes prepared from rodent brain, but they have not been identified so far. To our knowledge, this is the first description of a protein phosphorylation in mammalian tissues with a phosphate donor other than ATP, and this could be part of a novel signal transduction pathway. It is therefore of interest to study the metabolism of ThTP in animal cells.

The enzymatic mechanisms of ThTP synthesis are still poorly understood. Skeletal muscles sometimes contain unusually high amounts of ThTP because its synthesis can be catalyzed by adenylate kinase according to the reaction ADP + ThDP ↔ AMP + ThTP (7). Because ThDP is a very poor substrate for adenylate kinase, this mechanism can be of importance only in cell types where adenylate kinase is very abundant. In most tissues, ThTP is believed to be synthesized from ThDP according to the reaction ThDP + ATP ↔ ThTP + ADP, catalyzed by ThDP kinase, an enzyme that remains poorly characterized. Purification procedures from bovine brain (8), rat liver (9), and brewer's yeast (10) were described, but in each case, the material obtained had a very low specific activity, and no sequencing of the enzyme was attempted.

In contrast, the enzymes catalyzing ThTP hydrolysis have been studied in more detail. Animal tissues contain a membrane-associated as well as a soluble thiamine triphosphatase (ThTPase; EC 3.6.1.28). The membrane-bound ThTPase (11–13) has not been purified, and its specificity for ThTP remains uncertain. The soluble ThTPase first described by Hashitani and Cooper (14) in rat brain has been purified to homogeneity from bovine brain (15) and kidney (16). Bovine ThTPase has an alkaline pH optimum, a relatively low K_m (about 35 μM), and a virtually absolute specificity for ThTP (15, 16). Soluble ThT-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF432862 (human) and AF432863 (bovine).

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[1] The abbreviations used are: ThDP, thiamine diphosphate; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; ThMP, thiamine monophosphate; ThTP, thiamine triphosphate; ThTPase, thiamine triphosphatase; HPLC, high performance liquid chromatography; MS, mass spectroscopy.

TABLE I
Purification of soluble ThTPase from bovine brain

Fraction	Total activity[a]	Total protein	Specific activity	Purification	Yield
	μmol·min^{-1}	*mg*	*μmol·min^{-1}·mg^{-1}*	*-fold*	*%*
S1	230	99000	0.0023	1	100
Acid supernatant	170	32000	0.0053	2.3	74
Ammonium sulfate precipitate	146	4700	0.031	13.4	64
DEAE-Sephacel	56	140	0.41	176	26
Toyopearl HW-65F	26	4.8	5.4	2310	11
Sephadex G-75	22	0.60	37	15931	8.5
Blue-Sepharose	11	0.11	103	44474	4.8

[a] The ThTP concentration in the reaction medium was 10 μM.

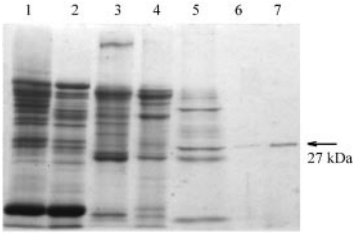

FIG. 1. **SDS-polyacrylamide gel electrophoresis after the different purification steps.** *Lane 1*, supernatant S1; *lane 2*, acidic supernatant; *lane 3*, ammonium sulfate precipitate; *lane 4*, DEAE-Sephacel; *lane 5*, Toyopearl HW 65F; *lane 6*, Sephadex G-75; *lane 7*, Blue-Sepharose Cl-4B.

Pase is found in most mammalian tissues studied so far (17).[2]

In this work, we report the purification of the soluble ThTPase from calf brain and its partial sequencing by tandem mass spectrometry. The partial sequence screened against known expressed sequence tags allowed us to obtain the complete bovine and human sequences. Both were cloned by reverse transcription-PCR, the human enzyme was functionally expressed in *Escherichia coli*, and its distribution in human tissue was investigated. After the high affinity thiamine transporter, whose mutation causes thiamine-responsive megaloblastic anaemia (18), and thiamine pyrophosphokinase (19), ThTPase is the third protein of thiamine metabolism to be characterized in mammals.

EXPERIMENTAL PROCEDURES

Chemicals—Chemicals, if not otherwise stated, were from Sigma or Merck Eurolab (Leuven, Belgium). ThTP was obtained from Wako Chemicals (Osaka, Japan). Trypsin was from Hoffman-La Roche Ltd. (Basel, Switzerland), and acetonitrile was obtained from J. T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ). The water used was of Milli-Q grade (Millipore Co., Bedford, MA).

Purification of Soluble ThTPase from Calf Brain—The procedure used was derived from methods previously described (14, 15). Briefly, 16 calf forebrains (4.8 kg) from a local slaughterhouse were homogenized (Polytron blender, 25,000 rpm for 5 min, 0 °C) in 2 volumes of Tris-Cl buffer (5 mM, pH 8.2) containing 1 mM Na_2EDTA. After gentle stirring at 0–4 °C for 30 min, the homogenate was centrifuged (30 min, 100,000 × *g*), and the supernatant (S1) was brought to pH 4.5 with acetic acid (14). After centrifugation (20,000 × *g*, 15 min), the acidic supernatant was neutralized to pH 7.8 with NaOH, and ammonium sulfate was added to 50% saturation. After centrifugation (15 min, 15,000 × *g*), the pellet was suspended in 5% of the initial volume of Tris-EDTA buffer (pH 7.8), dialyzed against the same buffer, and applied on a DEAE-Sephacel resin (Amersham Biosciences). Elution was carried out using a Tris-Cl gradient (20–500 mM, pH 7.8) containing 20% glycerol. The fractions containing ThTPase activity were dialyzed before chromatography on a Toyopearl HW 65F resin (Tosoh Corporation, Tokyo, Japan) as described earlier (15). After concentration of the fractions containing the highest specific activity (Centriplus 10, Amicon Inc., Beverly, MA), the enzyme was run on Sephadex G-75 and Blue-Sepharose Cl-4B (15). The purity of the preparation was tested by polyacrylamide (12%) gel electrophoresis in the presence of SDS according to Laemmli (20). Protein bands were visualized by silver or Coomassie Blue staining. The purification data are summarized in Table I.

Determination of ThTPase Activity—If not otherwise stated, the reaction medium contained 70 μl of Bis-Tris-propane buffer (50 mM, pH 8.7), 10 μl of $MgCl_2$ (50 mM), 10 μl of ThTP (100 μM), and 10 μl of the enzyme preparation at the appropriate dilution (20–10,000×). After incubation (10 min, 37 °C), the reaction was stopped by the addition of 500 μl of trichloroacetic acid. After extraction with 3 × 1.5 ml of diethyl ether, the ThDP formed was estimated by HPLC (21). To assess the substrate specificity of either the purified bovine enzyme or the GST-ThTPase, several potential substrates were tested for enzymatic hydrolysis. For 4-nitrophenyl phosphate, the absorbance of the released 4-nitrophenolate was read at 408 nm at pH 10. For ThDP, ThMP, ATP, GTP, CTP, and ITP, the inorganic phosphate released was measured (22). In all cases, the incubation was run at 37 °C for 100 min in the presence of 25 mM Tris buffer (pH 8.5), 5 mM $MgCl_2$, 4 mM substrate, and an enzyme concentration 20 times higher than for determination of ThTPase activity.

Amino Acid Sequencing of ThTPase by Mass Spectrometry—One of the purified protein fractions (about 12 μg/ml) obtained after Blue-Sepharose chromatography was concentrated by ultracentrifugation using a Microcon-YM10 centrifugal filter device (Millipore). The initial volume of 1 ml was concentrated to about 80 μl, and the buffer was replaced by digestion buffer (500 mM ammonium acetate, 20 mM $CaCl_2$). The protein was digested by adding 6 μl of a trypsin solution (0.1 μg/μl) reconstituted in 1 mM HCl. Acetonitrile (final concentration, 1% v/v) was added to accelerate digestion, which was performed for 12 h at 37 °C, pH 7.4. The tryptic peptides were fractionated and desalted by elution on a ZipTipC18 pipette tip (Millipore). The elution was carried out using a mixture of water/acetonitrile/acetic acid with successive volume ratios of 93/5/2, 88/10/2, 83/15/2, 73/25/2, 68/30/2, 58/40/2, 48/50/2, and 28/70/2. The fractions obtained were analyzed by nano-electrospray ionization MS/MS using a Q-TOF2 mass spectrometer (Micromass Co., Manchester, UK) as described by Shevchenko *et al*. (23). The selection of the analyzed ions and the adjustment of the collision energy were made manually. The obtained fragmentation data were analyzed using sequencing software, PepSeq (Micromass Co.). Data base search was performed with the sequences obtained to eliminate those resulting from trypsin autodigestion. The peptides were delivered to the mass spectrometer by silica capillaries purchased from Protana (MDS Proteomics, Odense, Denmark).

For the sequencing of the N- and C-terminal peptides, the enzyme was partially digested in the absence of acetonitrile for only 1 h at 37 °C. For the prediction of MS/MS fragmentation from peptide sequences and the comparison with mass spectra, BioLynx software (Micromass Co.) was used.

Cloning of Human and Bovine ThTPase—One microgram of human or bovine brain poly(A)$^+$ RNA (CLONTECH, Palo Alto, CA) was reverse transcribed into cDNA for 1 h at 37 °C by random priming using Moloney murine leukemia virus reverse transcriptase (Invitrogen) as described by the manufacturer. One-tenth of the reverse transcription reaction medium was submitted to 35 PCR cycles using *Pwo* polymerase (Roche Molecular Biochemicals). For human ThTPase amplification, primers HumF (5′-TCCTTGGGAACTCAGCAAACGT-3′) and HumR (5′-AGGAGTGGACTCCGTTAGACC-3′) were used. For bovine ThTPase, primers BovF (5′-ATGGCTCAGGGCCTGATTGAAG-3′) and BovR (5′-AGCGAGAGGAGTCACTGTGAG-3′) were used. Each PCR cycle consisted of denaturation at 94 °C for 30 s, hybridization at 63 °C for 30 s, and elongation at 72 °C for 60 s. The resulting PCR products were inserted into pCRII by TOPO cloning (Invitrogen) and sequenced using the T7 DNA sequencing kit (Amersham Biosciences).

Expression of ThTPase as a GST Fusion Protein in E. coli—The human ThTPase open reading frame was amplified from the cloned

[2] A. F. Makarchikov, unpublished results.

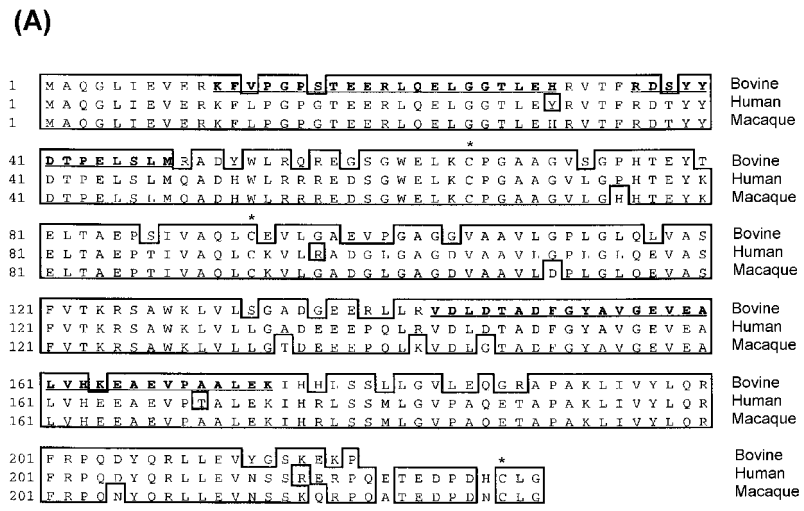

FIG. 2. **Alignment of ThTPase amino acid sequences deduced from bovine, human, and macaque (AB055296) cDNA and hydropathy plot of the human enzyme.** *A*, alignment was performed using the ClustalW program. The bovine and human sequences were obtained after sequencing of our PCR products and translation. Both corresponded to known expressed sequence tags. The sequences corresponding to the three bovine peptides obtained by tandem mass spectrometry are *underlined* and in *bold type*. The cysteyl residues are indicated by *asterisks*. *B*, hydropathy plot of human ThTPase by the method of Kyte and Doolittle (38). Two particularly charged sequences are indicated.

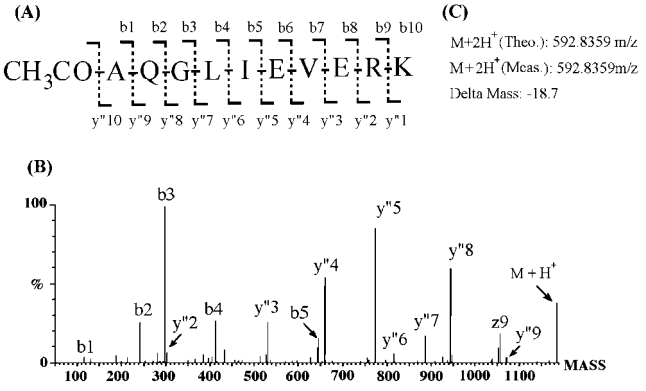

FIG. 3. **Determination of the N-terminal sequence of bovine ThTPase.** The N-terminal peptide (1–10) Ac-AQGLIEVERK was obtained by partial digestion of ThTPase with trypsin (see "Experimental Procedures") and subjected to collision-induced dissociation for generating a ladder of sequence ions. *A*, peptide ions fragment primarily at amide bonds. If the charge is retained on the N-terminal portion of the fragment, b-type ions are formed; however, if the charge is retained on the C-terminal portion, y-type ions are formed. *B*, the collision-induced dissociation mass spectrum of the N-terminal peptide, where *b1* corresponds to the acetylated N-terminal alanine. The same results can be obtained from the difference of the masses of the single protonated peptide ($M + H^+$) and the y″9 ions. The z9 ion corresponds to the y″9 peptide with deaminated glutamine. *C*, the accurate mass measurement of the double charged ion ($M + 2H^+$) had an error of about 20 ppm. Similar results were obtained for the Ac-AQGLIEVER peptide (not shown).

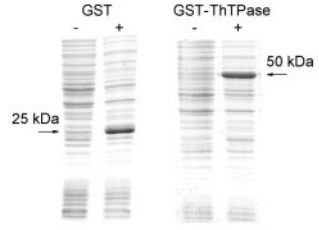

FIG. 4. **SDS-polyacrylamide gel (12%) electrophoresis of *E. coli* extracts transfected with pGEX containing the sequence of either GST-ThTPase or GST alone.** The bacteria were grown in 2XYT/ampicillin medium, and overexpression was induced by the addition of IPTG (+) 1.5 mg/ml. The *arrows* indicate the GST protein (25 kDa) or the GST-ThTPase fusion protein (50 kDa).

cDNA using forward (5′-GGATCCCCATGGCCCAGGGCTTGATTGA-3′) and reverse (5′-GCGGCCGCCTAGCCCAGGCAGTGGTCAG-3′) primers. The amplified product was then inserted into *Bam*HI/*Not*I sites of pGEX-5X-1 to produce a GST-ThTPase fusion protein. The *E. coli* strain BL 21 (Amersham Biosciences) was transformed with either the native or the recombinant plasmid and grown overnight on LB agar plates (1.5% (w/v) agar in LB broth) containing ampicillin (200 μg/ml). Individual bacterial colonies were grown under aerobial conditions at 37 °C in 2XYT/ampicillin medium at a density of about 5×10^9 cells/ml. Overexpression of GST or GST-ThTPase was induced by dilution of 100 μl of this bacterial culture in 1.6 ml of 2XYT/ampicillin medium in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) at 1.5 mg/ml. After 0, 1, 2, 3, or 4 h, the bacteria were collected by centrifugation (20,000 $\times$ *g*, 1 min) and suspended in 150 μl of 2XYT medium. Control experiments were made under the same conditions but without IPTG. 50 μl of the bacterial suspension were diluted with 50 μl of loading buffer (2×) and boiled for 1 min, and aliquots of 4 μl were submitted to SDS-PAGE electrophoresis on 12% gels. The rest of the bacterial suspension was incubated in the presence of 10% Triton X-100 for 30 min on ice and diluted 100–1000 times before determination of enzyme activity as described above.

Study of the Expression of ThTPase mRNA in Human Tissues—The entire cloned human ThTPase cDNA was used as a probe. It was labeled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA) using the Random Primers DNA labeling system (Invitrogen) and then purified on ProbeQuant G50 Micro columns (Amersham Biosciences). The human multiple tissue expression array (CLONTECH) was prehybridized for 1 h at 60 °C in ExpressHyb (CLONTECH). Hybridization was performed for 15 h at

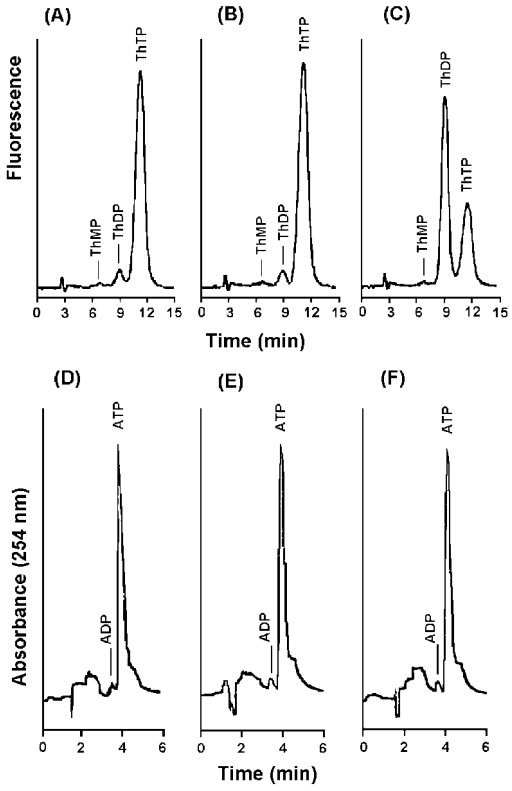

FIG. 5. **Chromatograms showing specific ThTPase activity in *E. coli* expressing GST and GST-ThTPase after a 4-h incubation with IPTG.** The bacteria were lysed in 10% Triton X-100 (30 min in ice) and diluted 1000 times in Tris-Cl buffer (20 mM, pH 7.5). The enzyme activity was measured under identical conditions (incubation for 10 min, 37 °C), either with 10 μM ThTP (*A–C*) or with 100 μM ATP (*D–F*) as substrate. *A* and *D*, control (no enzyme); *B* and *E*, extract from *E. coli* expressing GST; *C* and *F*, extract from *E. coli* expressing GST-ThTPase. ATP and ADP were determined by HPLC (39).

60 °C in the above solution containing 5×10^6 cpm/ml of the heat-denatured probe. The multiple tissue expression array was washed twice in 2× SSC, 0.1% SDS at 60 °C and twice in 0.1× SSC, 0.1% SDS at 55 °C as described by the manufacturer and then exposed in a PhosphorImager (Amersham Biosciences) for 36 h.

RESULTS

Purification, Properties, and Sequencing of Calf Brain ThTPase—The enzyme had to be purified about 45,000-fold before a homogenous preparation was obtained, suggesting that it is a relatively rare protein in the bovine brain. Our purification procedure gave 107 μg of ThTPase with a total yield of 4.8% (Table I). Analysis by SDS-PAGE revealed a single band with an apparent molecular mass of 27 kDa (Fig. 1). Mass spectrometry gave a molecular mass of 23,892 Da, a value lower than the one obtained from SDS-PAGE. The difference between the two values might be the consequence of the overall important negative charge of the protein, which can lead to decreased mobility during SDS-PAGE (24). Chromatography on Sephadex G-75 gave a molecular mass of 25 kDa (not shown), suggesting that the native protein is a monomer, in agreement with previous results (15).

The purified bovine enzyme obeyed Michaelis-Menten kinetics with a K_m of 39 ± 7 μM (substrate concentration, 0.01–1 mM) and a specific activity of 9 ± 2 μmol·s^{-1}·mg^{-1} (V_{max} at 37 °C). We can thus calculate that the catalytic constant (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) are 240 s^{-1} and 6×10^6 s^{-1}·M^{-1}, respectively.

In agreement with previous results (15–17), we found that the purified ThTPase was highly substrate-specific. A slight 4-nitrophenyl phosphatase activity was detected, but it was less than 1% of ThTPase activity. With ThDP, ThMP, and nucleoside 5′-triphosphates, no significant enzymatic hydrolysis could be detected by the very sensitive method used (22), indicating that if any enzymatic hydrolysis of those substrates occurs, it is less than 0.2% of ThTPase activity.

The sequence of several internal peptides (see Fig. 2 for their sequence) was obtained by electrospray ionization MS/MS analysis. Each peptide was compared with the sequences of the GenBank™ data base using the BLAST algorithm, and all gave a nearly perfect match (Fig. 2*A*) with two newly described hypothetical proteins, one in human called MGC2652 (NM_024328) and another in *Macaca fascicularis* (AB055296).

Cloning of Human and Bovine ThTPase—Using primers designed on the basis of the MGC2652 sequence, we were able to amplify a cDNA of the expected size from human brain poly(A)$^+$ RNA. The human protein is 230 amino acids long and has a predicted molecular mass of 25,550 Da. Its gene is located on the short arm of chromosome 14. The amino acid composition is characterized by a high percentage of negatively charged residues (17.4% of Glu and Asp) and a low content of Ile (1.7%) and Asn (0.4%).

The comparison of the sequence of the human cDNA with the bovine expressed sequence tags gave other matches (AW654551, BG690979, BF076311, and BF653287) that allowed the amplification of a cDNA encoding a 219-amino acid protein from bovine brain poly(A)$^+$ RNA. Its sequence perfectly matches the bovine peptide sequences obtained by mass spectrometry, except for the N-terminal methionine residue (see below).

The predicted average molecular mass of the bovine protein is 23,983 Da, a value slightly higher than the mass determined by mass spectrometry for the purified bovine enzyme (23,892 Da). Most of the difference can be accounted for if we assume that the N-terminal methionyl residue is cleaved and that the new N-terminal alanine is acetylated. This hypothesis was confirmed by comparing the predicted collision-induced dissociation mass spectra for two N-terminal peptides of the protein (Fig. 3). The C-terminal peptide (209–219, LLEVYGSKEKP) was obtained in a similar manner. In addition, considering that the bovine protein contains only two cysteyl residues, at positions 66 and 88, respectively (Fig. 2), the presence of a disulfide bridge would lead to the loss of two hydrogen atoms, giving a mass of 23,892 Da, exactly as determined. A difference of 2 Da is, however, within the error on the mass determination of the entire protein by mass spectrometry (100 ppm). On the other hand, modifiers of free thiols such as *p*-chloromercuribenzoate and Ellman's reagent (5,5′dithiobis-2-nitro-benzoic acid) inhibit the activity of the purified bovine ThTP (not shown), suggesting that the presence of free SH groups is essential for catalytic activity. In addition, we found that 2-mercaptoethanol does not change the electrophoretic mobility of the protein in polyacrylamide gels. The presence of a disulfide bridge therefore appears unlikely.

At the amino acid level, the bovine ThTPase has 80 and 79% identity with the human and the macaque enzyme, respectively (Fig. 2*A*). Analysis of the sequences using the PROSITE motif search revealed the presence of several potential phosphorylation sites present in the three sequences, among them two consensus sites (at positions 34 and 123) for protein kinase C and three consensus sites (at positions 34, 38, and 60) for casein kinase 2. The hydrophobicity plot of the human enzyme is typical of a soluble protein (Fig. 2*B*), with several highly polar or charged regions.

Interestingly, no homology, even partial, with any other known vertebrate protein was found. Partial short sequences with significant identity corresponding to hypothetical open

TABLE II
Kinetic parameters of ThTPase in the crude supernatant fraction of brain in several species

	V_{max}[a]	K_m[a]
	nmol·mg^{-1}·min^{-1}	*μM*
Human cerebellar cortex[b]	8.7 ± 0.9	126 ± 5
Calf cerebral cortex[c]	11.5 ± 3	32 ± 6
Rat cerebral cortex[d]	21 ± 4	23 ± 7

[a] The ThTP concentration varied between 10 and 200 μM. K_m and V_{max} were obtained by fitting the experimental data to the Michaelis-Menten equation using GraphPad Prism (GraphPad Software, San Diego, CA).
[b] Mean ± S.D. for five determinations on the postmortem (4 h postmortem delay) cerebellar cortex of a 59-year-old male deceased from bronchopneumonia (Department of Pathology, University of Liège).
[c] This study, means ± S.D. for three determinations on a pool of 16 calf brains.
[d] Mean ± S.D. for three animals.

reading frames were found in *E. coli* (AP002564 and AE000387), *Caenorhabditis elegans* (L23650), *Drosophila melanogaster* (AE003477 and AE003598), or *Saccharomyces cerevisiae* (NP_014781), but they do not seem to be related to each other.

Functional Expression of Human ThTPase in E. coli—The human ThTPase cDNA was overproduced in *E. coli* as a GST fusion protein in the presence of IPTG (Fig. 4). The fusion protein had a molecular mass of 50 kDa, which corresponds to an approximate molecular mass of 25 kDa for the ThTPase moiety, as expected from the amino acid sequence.

In *E. coli* transfected with GST, we found only a relatively low intrinsic ThTPase activity (120 ± 34 pmol·min^{-1}·mg of protein^{-1}, $n = 7$). In fact, bacteria do not appear to contain a specific ThTPase, but they do contain nonspecific phosphatases able to hydrolyze ThTP to some extent (25).[2] As shown in Fig. 5, the activity was increased over 1000-fold in noninduced GST-ThTPase recombinant bacteria, reaching 0.17 μmol·min^{-1}·mg^{-1}. After induction by IPTG, this activity still increased over 10-fold, reaching 2.1 μmol·min^{-1}·mg^{-1} after 4 h (Fig. 5*C*). No increase in ThTPase activity was observed after induction in bacteria transfected with GST alone (Fig. 5*B*). ThTP hydrolysis by recombinant GST-ThTPase resulted in the formation of ThDP only. If an unspecific phosphatase was present, ThDP would have been further hydrolyzed to ThMP, but this was not observed. Furthermore, when ATP (100 μM) replaced ThTP in the incubation medium under the same conditions (Fig. 5, *D–F*), no hydrolysis of ATP was observed. Although IPTG increased ThTPase activity over 10 times in GST-ThTPase recombinant bacteria, it did not increase to any significant amount the hydrolysis of 4-nitrophenyl phosphate, ThDP, ThMP, or nucleoside 5′-triphosphates. This suggests that the recombinant enzyme, like the native ThTPase, has little or no hydrolytic activity on substrates other than ThTP.

Comparison of GST-ThTPase with Genuine Human ThTPase—Because human ThTPase has not been studied in detail so far, we compared some properties of the GST-ThTPase with genuine ThTPase prepared from human brain. The activity of the GST-ThTPase fusion protein falls more abruptly at alkaline pH than with the enzyme prepared from human brain. Although both the human and the bovine enzyme have about the same pH optimum (around 8.5), the human enzyme has a broader pH spectrum, with a higher activity at neutral pH.

ThTPase from human cerebellar cortex had a K_m of 126 μM (Table II), a value three to four times higher than in crude extracts from bovine or rat brain. Notice that the K_m of the bovine enzyme for ThTP was not significantly different before (32 ± 6 μM) and after (39 ± 7 μM) purification. Human ThTPase was isolated from postmortem tissue, but we have not found any effect of the postmortem delay (≤15 h) on the V_{max} or the K_m of bovine enzyme. Actually, the enzyme was remarkably insensitive to chemical denaturation and proteolytic attack; although it was isolated from calf brain in the absence of protease inhibitors, it remained intact as was shown by sequencing. The properties of the recombinant GST-ThTPase were similar to those of the native human enzyme, with a K_m of 220 ± 23 μM ($n = 5$).

Expression of ThTPase mRNA in Human Tissue—ThTPase expression was profiled by dot blot hybridization on a mRNA multiple tissue expression array, using the entire cDNA as probe (Table III). The main conclusion to be drawn from this experiment is that ThTPase mRNA appears to be very widely expressed, but only at a low level, in agreement with the high purification factor needed to obtain a homogenous enzyme preparation from bovine brain (Table I). The highest hybridization signal was observed in uterus, testis, and prostate, followed by bladder, kidney, lung, and thyroid gland. Surprisingly, only small signals were found in different brain regions, with no detectable signal in the cerebellum. ThTPase mRNA was also poorly expressed in the digestive system, fetal tissues, and transformed human cell lines.

Qualitatively, these results agree with the measurements of ThTPase activities. The enzyme activity was detected in human brain (Ref. 26 and this study), but no regional distribution study was made. In rats, ThTPase activity was shown to exist in various tissues such as brain, heart, skeletal muscle, liver, lung, spleen, and kidney (17). In bovine tissues, specific ThTPase activity was highest in cerebral cortex and kidney, followed by liver, spleen, heart, and lung; the activity was much lower in skeletal muscle and lowest in small intestine.[3] Furthermore many partial and full-length expressed sequence tag sequences have been obtained for MGC2652 from various human tissues as recorded by the UniGene System (www.ncbi.nlm.nih.gov/UniGene/), confirming that ThTPase is widely expressed in mammalian tissues. However, this enzyme could not be found in nonmammalian tissues so far.[4] Likewise, its mRNA was not detected in brewer's yeast or in *E. coli*, confirming the absence of a specific ThTPase activity in these microorganisms (25).

DISCUSSION

The present study describes the first sequencing, cloning, and functional expression of a specific ThTPase. Our data show that this is a new kind of enzyme with no apparent sequence similarity to any other known protein. Another remarkable feature of the enzyme is its high specificity for ThTP (in agreement with published data; Refs. 15–17), together with a high catalytic efficiency.

ThTPase is widely expressed in mammalian tissues but at a rather low level. In human tissues, in particular, the mRNA was often barely detectable on the multiple tissue expression array. It should be noted, however, that a low level of expression does not necessarily mean that the protein has little biological importance. For instance, low amounts of mRNA were reported for mouse brain thiamine pyrophosphokinase (19), yet this enzyme is absolutely required for ThDP synthesis and hence for brain oxidative metabolism. In the case of ThTPase, the fact that it is a relatively rare protein in most tissues is compensated, at least to some extent, by its relatively high catalytic efficiency.

GST-ThTPase as well as the purified bovine ThTPase obeyed Michaelis-Menten kinetics. This is in contrast to one previous study, where bovine ThTPase displayed biphasic saturation

[3] A. F. Makarchikov, I. E. Gulyai, I. M. Rusina, and T. A. Luchko, unpublished results.
[4] A. F. Makarchikov and L. Bettendorff, unpublished results.

TABLE III
Dot blot analysis of the mRNA distribution of ThTPase in human tissues

A single + indicates that the signal was just detectable with the naked eye. Each additional + corresponds to approximately the doubling of the intensity of the signal.

Tissue	Score
Nervous system	
Whole brain	ND[a]
Cerebral cortex	+
Frontal lobe	+
Parietal lobe	++
Occipital lobe	++
Temporal lobe	+
Paracentral syrus of cerebral cortex	+
Pons	+
Cerebellum left	ND
Cerebellum right	ND
Corpus callosum	+
Amygdala	+
Caudate nucleus	+
Hippocampus	+
Medulla oblongata	+
Putamen	+
Nucleus accumbens	+
Thalamus	+
Transformed cell lines	
Leukemia, HL-60	ND
HeLa	+
Leukemia, K562	ND
Leukemia, MOLT-4	+
Burkitt's lymphoma, Raji	+
Burkitt's lymphoma, Daudi	+
Colorectal adeno carcinoma	+
Lung carcinoma, A549	+
Cardiovascular tissue	
Heart	+
Aorta	+
Atrium, left	+
Atrium, right	+
Ventricle, left	+
Ventricle, right	++
Interventricular septum	++
Apex of the heart	+
Lung	+++
Trachea	+
Digestive system	
Esophagus	+
Stomach	+
Duodenum	+
Jejunum	++
Ileum	ND
Ileocecum	ND
Appendix	ND
Colon, ascending	ND
Colon descending	+
Rectum	+
Pancreas	+
Salivary gland	++
Reproductive and urinary tracts	
Placenta	+
Uterus	++++
Ovary	ND
Prostate	++++
Testis	++++
Bladder	+++
Kidney	+++
Fetal tissue	
Brain	+
Heart	ND
Kidney	+
Liver	+
Spleen	+
Thymus	++
Lung	+
Other	
Thyroid gland	+++
Adrenal gland	++
Skeletal muscle	++
Liver	++
Immune system	
Spleen	+
Thymus	++
Leucocyte, peripheral	+
Bone marrow	++

[a] ND, not detectable.

with respect to ThTP (15). A more recent study by the same author (16) reports normal Michaelis-Menten behavior for the bovine kidney enzyme (K_m = 46 μM), as was also found in human and rat brain ThTPase (Table II).

We found no marked differences between bovine and human ThTPases as far as kinetic properties are concerned. Likewise, the recombinant human enzyme has essentially the same properties as the genuine ThTPase from human brain. Slight differences in K_m and activity at high pH between the recombinant GST-ThTPase and genuine human ThTPase might be due to the GST fusion moiety. It is important to point out that the GST fusion protein, like the purified bovine enzyme, is specific for ThTP.

Although ThTP is present in many animal tissues (1), the specific 24-kDa ThTPase has been found only in mammals so far. However, preliminary data suggest that other phosphohydrolases, able to hydrolyze ThTP, are present in nonmammalian tissues such as the quail brain.[4]

The physiological function of ThTP and the mechanisms regulating its cellular concentration in various cell types are matters that are still largely unexplored. In rat brain and cultured neuroblastoma cells, ThTP has a high turnover, but its steady-state levels remain low (27, 28). This suggests that intracellular ThTP concentration is highly regulated (29, 30). Previous results (30) suggested that in brain synaptoneurosomes, ThTP is rapidly synthesized but does not accumulate. Most of the ThTP was found to bind to an as yet unidentified protein, and it was hypothesized that any excess of free cytosolic ThTP was rapidly hydrolyzed. It seems thus reasonable to assume that the physiological role of ThTPase is to maintain a low cellular concentration of ThTP in most mammalian cells, a view compatible with its high catalytic efficiency.

Recent findings support the view that a physiological function of ThTP would be to specifically phosphorylate certain proteins (5). In view of the low intracellular ThTP concentrations ($\leq$1 μM) in most mammalian tissues, it is likely that protein phosphorylation by ThTP is restricted to specific circumstances in limited portions of a given tissue. This phosphorylation process might be triggered by a transient increase in cytosolic ThTP caused by down-regulation of ThTPase expression. So far, the mechanisms that may regulate ThTPase expression are completely unknown, but clues for testing the above hypothesis might be provided by producing a transgenic mouse deficient in ThTPase.

The mechanisms that control cellular ThTP levels may have pathophysiological implications. Although beriberi, the classical nutritional thiamine deficiency syndrome, has practically disappeared from developed countries, Wernicke-Korsakoff syndrome, associated with chronic alcoholism, remains a serious condition because it may lead to irreversible brain damage (31). Because brain tissue is strongly dependent on energy metabolism, it has been thought that decreased ThDP levels, and thus decreased pyruvate and 2-oxoglutarate dehydrogenase activities, account for all the nervous symptoms observed in thiamine deficiency. This hypothesis, however, cannot easily explain the selective vulnerability of certain brain regions (thalamus and mammillary bodies) typical of Wernicke-Korsakoff syndrome. Similar observations were made in animal models of thiamine deficiency (for review see Refs. 1 and 32). In thiamine deficiency models, all thiamine compounds are decreased (33–35), and it is thus difficult to estimate the contribution of each of these compounds to the symptoms or neuronal death observed. There is still no clear evidence for a region-specific decrease of ThDP levels, and it remains uncertain how it can be linked to selective vulnerability (36). In view of the observation that ThTP may participate in protein phosphoryl-

ation and possibly signaling, we cannot exclude the possibility that decreased ThTP levels contribute to the neuronal death observed in vulnerable brain regions during acute and chronic thiamine deficiency. With this in consideration, it may be significant that rats with a high sensitivity to alcohol have a lower ThTPase activity (37).

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