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REVIEWS

Vitamin B_1: Metabolism and Functions

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Abstract—The review highlights metabolism and biological functions of vitamin B_1 (thiamine). It considers thiamine transport systems in various organisms enzymes of its biosynthesis and degradation, as well as molecular basis of thiamine-dependent hereditary pathologies. A special attention is paid to discussion of the role of thiamine triphosphate and adenylated thiamine triphosphate, a new thiamine derivative recently discovered in living cells.

Key words: vitamin B1, thiamine, thiamine phosphates, transport, metabolism, biological role.

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INTRODUCTION

Vitamin B_1 (thiamine) is an essential dietary factor; its deficit causes polyneuritis in animals, development of cardiovascular and neurological disorders in humans, beriberi (a thiamine deficiency disease) and Wernicke-Korsakoff syndrome [1]. The history of thiamine research continues for more than 80 years since its isolation in the crystal form [2]. During this period huge progress was achieved in identification of molecular mechanisms involved into realization of catalytic function of thiamine diphosphate (ThDP) in intracellular processes. Now more than 25 ThDP-dependent enzymes (www.expasy.ch) are known. Nevertheless, it appears that we still do not have comprehensive picture, which would reflect all aspects of biological activity of thiamine. Recently obtained data give possibility for a new look at this vitamin and its role in the cell. This review deals with the present state of the art in the field of studies of thiamine metabolism and its functions.

1. THE SYSTEM OF THIAMINE METABOLISM

In most living objects vitamin B_1 exists in the free (unphosphorylated) form as well as in the form of three phosphate esters, thiamine monophosphate (ThMP), ThD, and thiamine triphosphate (ThTP) [3]; these forms together with corresponding enzymes constitute the system of thiamine metabolism. Recently, a new component, adenosine ThTP (AThTP) has been identified [4]. Thiamine and thiamine phosphates were found in all investigated animal tissues, bacteria, protozoa, plants and fungi.

Figure 1 schematically shows the system of thiamine metabolism in a neuronal cell. Historically most experimental data on vitamin B_1 metabolism were obtained using preparations derived from nervous tissues. However, one may assume this scheme in general outline may be also applicable for other animal cells, because its elements are present in all investigated organs and tissues, i.e. the system of thiamine metabolism appears to be universal.

2. TRANSPORT OF THIAMINE INTO CELLS

Vitamin B_1 is synthesized by bacteria, other microorganisms and plants [5–7]. Animals cannot synthesize thiamine and therefore they must obtain this vitamin with food.

At physiological concentrations (which are below 2 μM in human and rat gut lumen) thiamine uptake by epithelial cells involves a saturable protein carrier [8, 9]. This electroneutral carrier-mediated process does not depend on Na^+ and K^+ ions and it occurs via thiamine/H^+ antiport mechanism [10]. At higher (>2 μM) concentrations thiamine enters enterocytes mainly via passive diffusion [8]. These properties are also typical for thiamine transport systems in other mammalian organs and tissues [11–14]. After transfer inside cell thiamine molecule undergoes rapid phosphorylation to ThDP catalyzed by thiamine pyrophosphokinase (TPK; EC 2.7.6.2); it appears that phosphorylation is a "driving force" of the whole process [15, 16].

Penetration of thiamine and ThMP through the blood brain barrier involves active transport mechanism, which includes saturable and unsaturable components [17]. ThMP uptake by cells of other tissues may also employ reduced folate carrier [18].

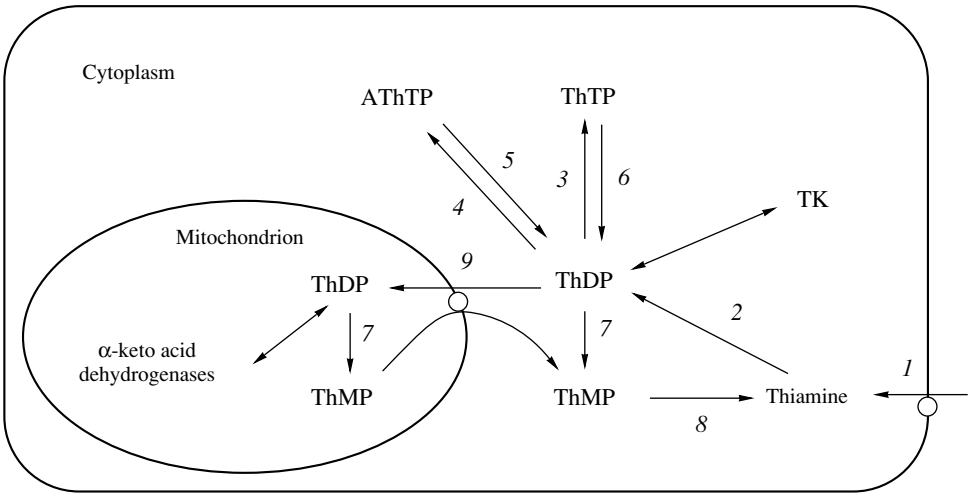

Fig. 1. The scheme illustrating metabolism of phosphorylated thiamine derivatives in a nervous system cell: *1*—protein carrier; *2*—thiamine pyrophosphokinase; *3*—ThDP kinase (adenylate kinase); *4*—ThDP adenylyl transferase; *5*—AThTPase; *6*—ThTPase; *7*—ThDPase (NDPase); 8—ThMPase; *9*—mitochondrial transporter; TK—transketolase.

cDNAs of protein carriers involved in thiamine transport into human and mouse organs and tissues have rather recently been cloned [19–21]. The human thiamine transporters, SLC19A2 (ThTr1), SLC19A3 (ThTr2), as well as their murine homologue Slc19a2, belong to the folate transporter family [19, 20, 22, 23]. The gene encoding *SLC19A2* is located in the region q23.3; it consists of 6 exons and 5 introns and encodes a protein of 497 amino acid residues; this protein presumably contains 12 transmembrane domains [20, 24]. Mutations in SLC19A2 cause a rare autosomal recessive disease known as thiamine responsive megaloblastic anaemia (TRMA), also known as Roger's syndrome; this disease is accompanied by diabetes mellitus and deafness [24, 25]. Being maintained on the thiamine deficient diet, mice with targeted disruption of *Slc19a2* gene develop all TRMA symptoms [26].

ThTr1 and ThTr2 are not involved into thiamine uptake by placental cells: in the human trophoblast-derived cell line BeWo transport process is characterized by several characteristic features, suggesting the involvement of serotonin transporter (SERT) into this process [27].

Many organisms, which can synthesize vitamin B_1 de novo, possess peripheral systems for its transport. For example, in the presence of thiamine in a cultivation medium yeast cells uptake of this vitamin via the active transport system with pH optimum of 4.5 and K_m value of 0.18 μM [28]. The gene encoding *S. cerevisiae* thiamine transporter *THI10*, has been identified on XII chromosome; it contains an open reading frame of 1794 base pairs encoding a protein with molecular mass of 66.903 kDa. Expression of this protein carrier is regulated at mRNA level by intracellular ThDP concentration [29].

In bacteria (*E. coli, S. typhimurium*) translocation of thiamine and its phosphate esters through the inner membrane involves ATP-binding cassette transporter (ABC-transporter) encoded by *thiBPQ* operone [30, 31].

3. ENZYMES INVOLVED INTO BIOSYNTHESIS OF PHOSPHATE ESTERS OF THIAMINE

3.1. Biosynthesis of ThMP

Formation of ThMP from 2-methyl-4-amino-5-hydroxymethyl pyrimidine diphosphate and 4-methyl-5-β-hydroxyethylthiazole monophosphate catalyzed by thiamine phosphate pyrophosphorylase (ThMP-PPase) (EC 2.5.1.3) is the penultimate step of thiamine biosynthesis in microorganisms and plants [7]. In *S. cerevisiae* yeast, *THI6* gene (encoding ThMP-PPase) is identified on XVI chromosome; it encodes a polypeptide of 540 residues [32]. ThMP-PPase molecule consists of 8 identical subunits [33]. *S. cerevisiae* ThMP-PPase is a bifunctional enzyme catalyzing the synthesis of both ThMP and its precursor, 4-methyl-5-β-hydroxyethylthiazole monophosphate. However, in *E. coli* and other bacteria these reactions involve separate enzymes because *thiM* and *thiE* genes encoding hydroxyethylthiazole kinase (EC 2.7.1.50) and ThMP-PPase are located at various loci [34, 35].

Besides the above considered ThMP-PPase reaction, which is typical for all organisms capable of de novo synthesis of vitamin B_1 in *E. coli* and *S. typhimurium* cells ThMP may be formed via direct phosphorylation of thiamine [36, 37]. This is intermediate reaction of ThDP biosynthesis during cultivation of these bacteria in the presence of exogenous sources of thiamine. Such a pathway was not found in *P. denitrificans* [38] and *B. subtilis* [39]. *E. coli* thiamine kinase (EC 2.7.1.89) is encoded by *thiK* locus [40].

It is generally accepted that in mammalian tissues ThMP is exclusively formed during ThDP hydrolysis;

no specialized pathways or enzymes of ThMP biosynthesis have been found in animal cells. It remains unclear whether ThMP just represents an intermediate of thiamine ester metabolism or it plays some specific biochemical function.

3.2. Biosynthesis of ThDP

In eukaryotes and at least in some prokaryotes (e.g. in *P. denitrificans*) biosynthesis of ThDP occurs via the following reaction catalyzed by TPK: thiamine + ATP ⟷ ThDP + AMP [38, 41, 42].

According to physicochemical analysis mammalian TPK is a homodimer of 50–64 kDa composed of two identical subunits [42–44]. cDNAs of human and murine enzyme have been rather recently cloned and expressed in *E. coli* cells [45–47]. ORF of both cDNAs encode proteins of 27 kDa, which share 89% identity. Human *TPK* gene is identified in the region 7q34 [46, 47]. Tertiary structure of murine TPK was determined by the method of X-ray crystallography [48].

In *S. cerevisiae* yeast, TPK is the product of *THI80* gene, which encodes a polypeptide of 36.616 kDa [49]. Gel-filtration has demonstrated that molecular mass of TPK expressed in *E. coli* and fused (via its N-terminal residue) with 3 amino acid residues of pTrc99A vector is 72 kDa; this indicated the yeast enzyme exists as homodimer. Spatial structure of *S. cerevisiae* TPK has been determined by X-ray analysis with resolution of 1.8 Å [50]. TPK subunit is composed of two domains, one of which represents Rossman fold with 4 α-helices at each side of 6-strand antiparallel β-sheet. The second domain contains 4-strand and 6-strand antiparallel β-sheets forming a sandwich-like structure. Active site is located in the cleft at the dimer interface.

It has been already mentioned above that TPK catalyzes biosynthesis of ThDP in *P. denitrificans* cells [38]. As in the case of yeasts and mammals the active form of this enzyme is a dimer with molecular mass of 44 kDa [51]. This form may aggregate with formation of tetramer, characterized by low activity. Similar tendency for association is also typical for *S. carlsbergensis* TPK, which may represent several pH-dependent oligomeric forms existing in dynamic equilibrium [52].

In gut bacteria, *E. coli* and *S. typhimurium* de novo synthesis of ThDP involves an alternative pathway including ThMP phosphorylation without initial hydrolysis to free thiamine [37, 53]. Thiamine monophosphate kinase (EC 2.7.4.16) is a product of *thiL* locus, which encodes a protein of 35 kDa [37, 40].

TPK is characterized by broad specificity with respect to both substrates [42, 54, 55]. Usually K_m values for thiamine and ATP are in the micromolar (0.1–10 μM) and millimolar (0.38–5.9 mM) range of concentrations respectively [42–44, 51, 54, 56]; this corresponds to physiological intracellular concentrations of these compounds. TPK reaction exhibits an absolute dependence on bivalent metal cations and the complex Me-NTP is a true substrate for this enzyme. Site-directed mutagenesis studies have shown that the residues Asp-71, Asp-73, Gln-96, Thr-99, Asp-100, Arg-131, and Asp-133 [57] are essential for catalysis by human recombinant TPK.

In rat liver, enterocytes, and erythrocytes TPK has exclusive cytosol localization [15, 58]. Studies of TPK distribution in subcellular fractions of *Euglena gracilis* have revealed 9.2% of enzyme activity in chloroplasts, 15.7% in mitochondria, and 65.7% in cytosol [59].

ThDP synthesized de novo plays a central role in metabolism of thiamine phosphate esters in eukaryotic cells (Fig. 1). The major proportion of ThDP is transported into mitochondria, where it is included into pyruvate and ketoglutarate dehydrogenase complexes (PDHC and KGDHC) as well as dehydrogenase complex of branched chain α-keto acids. The other proportion of ThDP is bound to cytosolic transketolase (TK; EC 2.2.1.1). According to some estimations, in nervous tissue 90–95% of total intracellular pool of ThDP is included into ThDP-dependent enzymes; this protein bound coenzyme forms a pool, characterized by turnover rate of 6–20 h [60–62]. However, in hepatocytes free ThDP represents up to 60% of total intracellular pool [63].

Transport of ThDP into rat liver mitochondria is mediated by a protein carrier with K_m value of 20 μM [64]. In mitochondrial matrix free ThDP may be hydrolyzed by ThDPase and forming ThMP is transported back to cytosole, where it undergoes further cleavage to thiamine, a TPK substrate. It is possible that transport of ThMP from mitochondria occurs as ThMP/ThDP exchange [65].

Using model knockout mice Lindhurst et al. [66] identified mitochondrial ThDP carrier as Slc25a19 protein, an ortholog of human SLC25A19 transporter also known as DNC; earlier it was thought to be responsible for deoxynucleotide transport into mitochondria [67]. Null-mutant mice exhibited symptoms of Amish lethal microcephaly (MCPHA), autosomal recessive disorder characterized by defects in the development of central nervous system as well as 10–100-fold increase of α-ketoglutarate level (α-ketoglutaric aciduria) [68].

Amino acid sequence of SLC25A19 shares 28% identity with Tpc1p, mitochondrial carrier in *S. cerevisiae* cells [69]. Tpc1p is an inner membrane monomeric protein with molecular mass of 35.5 kDa; in addition to ThDP and ThMP it can also transport nucleotides although with lower effectiveness. In dependence of physiological conditions Tpc1p may operate via mechanism of uniport or antiport (carrying exchange of mitochondrial ThMP for cytosolic ThDP).

In bacteria genetic control of regulation of ThDP biosynthesis can be realized via the riboswitch mechanism. It was shown that mRNAs of genes encoding enzymes involved into the thiamine pathway contain a conservative nucleotide region at 5'-UTR (*thi* box domain), which can specifically bind thiamine deriva-

tives [70]. This results in rearrangements of secondary structure of mRNA molecule in the Shine-Dalgarno sequence (unpairing of this sequence is crucial for effective translation in prokaryotes). For example, such mechanism is used for regulation of expression of *E. coli* proteins encoded by *thiC* and *thiM* and affinity of the *thi* box riboswitch for ThDP (K_d = 100–600 nM) is more than three orders of magnitude higher than for ThMP or thiamine [71]. The ThDP binding sensor domains have also been found in eukaryotic mRNA [72].

Crystal structure of the complexes ThDP-riboswitch was determined for several mRNA [73–75]. In the case of *E. coli thiM*, corresponding site of mRNA is folded as two subdomains. One of them forms an intercalation "pocket" for 4-amino-5-hydroxymethyl-2-pyrimidine ring of ThDP, whereas the other one forms a wider "pocket" for bivalent metal ion and water molecules involved into binding of a pyrophosphate tail. mRNA molecule does not recognize a thiazole ring [74].

3.3. Biosynthesis of ThTP

There is contradictory (and sometimes even incompatible) information on mechanisms of ThTP biosynthesis and so situation with solution of this problem appears to be very complicated.

Original mechanism for ThTP biosynthesis was proposed in 1964 by Eckert and Möbus [76]. These authors experimented with extracts from pig spinal cord. Enzyme catalyzing ThDP phosphorylation in the reaction ThDP + ATP ⟷ ThTP + ADP was denominated as ThDP-kinase (ThDP: ATP phosphotransferase; EC 2.7.4.15). Studying distribution of ThDP kinase in subcellular fractions from rat brain Itokawa and Cooper [77] concluded that this enzyme has mitochondrial localization.

In 1977 Ruenwongsa and Cooper [78] found ThDP kinase activity in cytosolic fraction of rat liver rather than in subcellular particles; it was found that the second substarte was protein bound rather than free ThDP. Rather strange value was reported for the amount of ThTP of 21.2 nmol/mg of protein, which was synthesized by supernatant. In this connection it should be noted that total content of ThDP in rat liver varies in the range of 0.117–0.155 nmol/mg of protein and about one third of this ThDP is bound to proteins [79].

Results of purification of ThDP kinase from acetone powder of bovine brain mitochondrial fraction were published in 1982–1983 [80, 81]. Enzyme catalyzed the reaction protein-ThDP + ATP ⟷ protein-ThTP + ADP and exhibited absolute dependence on bivalent metal ions and a low molecular weight cofactor, presented in filtered cytosolic fraction of rat liver. Protein-bound ThDP, a substrate for determination of enzyme activity, was also isolated from rat liver cytosol.

For identification of the macromolecular substrate of ThDP kinase Voskoboev and Chernikevich [82] performed a special study and demonstrated that TK is the only protein, which binds ThDP in hyaloplasm of rat liver cells and at least in rat liver this enzyme cannot serve as the substrate for ThTP biosynthesis.

Preparations of ThDP kinase utilizing free ThDP as substrate were obtained from rat liver cytosol [83] and *S. carlsbergensis* beer yeasts [84]. Both proteins exhibited kinetic properties typical for allosteric enzymes. During gel filtration the yeast enzyme was eluted as two peaks corresponding to molecular masses of 162 and 81 kDa; this may indicate that it belongs to associating-dissociating enzymatic systems. According to electrophoresis in polyacrylamide gel molecular masses of subunits of yeast ThDP kinase were 12.5 and 14 kDa.

In 1985 Koyama et al. [85] reported about partial (150-fold) purification of ThDP kinase from cytosolic fraction of guinea pig brain. The enzyme exhibited absolute dependence on the presence of Mg^{2+} ions and a low molecular weight thermostable cofactor, which was later identified as creatine [86]. It was shown that ThDP kinase is localized in cytosolic fraction and is widely distributed in organs and tissues and the highest activity is observed in skeletal muscles.

Enzyme exhibited low apparent affinity for ThDP (K_m of 1.11 mM) and high affinity for ATP (K_m of 10 μM) [85]. However, subsequent studies have shown that removal of creatine kinase during purification of cytosolic ThDP kinase from pig muscle also removes the need of creatine as cofactor for ThTP synthesis. Electrophoretically homogenous protein (purified 68.2 folds) catalyzed the reaction ThDP + ADP ⟷ ThTP + AMP. Thus, the ThTP synthesizing enzyme was identified as adenylate kinase isoenzyme 1 (AK1) (EC 2.7.4.3).

Miyoshi et al. [87] and Shioda et al. [88] believe that the main part of ThTP is synthesized in muscles by AK1. However, experiments on AK knockout chimeric mice question this viewpoint because organs and tissues of both control and *AK1* knockout mice were indistinguishable by ThTP content [89]. Experiments on AK-thermosensitive *E. coli* cells (CV2 strain) cultivated at 37°C in minimal medium in the presence of glucose also have shown that the amount of ThTP synthesized by bacteria was 0.25 nmol/mg regardless total inactivation of AK [90]. Taking into consideration these results involvement of AK1 into biosynthesis of ThTP in vivo appears to be very questionable.

4. ENZYMES HYDROLYZING PHOSPHATE ESTERS OF THIAMINE

4.1. Hydrolysis of ThTP

At the beginning of 1970th it was found that ThTP activity of homogenates of various rat organs and tissues is determined by additional effects of two phosphatases differing by pH optimuma and intracellular localization [91, 92]. In accordance with extractability into aqueous buffer solutions these enzymes were

denominated as soluble and membrane associated ThTPases.

Soluble cytosolic ThTPase (EC 3.6.1.28) was originally purified from bovine brain in 1992 [93]; nine years later properties of homogenous enzyme from kidneys were reported [94]. Bovine ThTPase is a Mg^{2+}-dependent enzyme exhibiting maximal activity in alkaline pH values (pH optimum of 8.9) and absolute specificity and high affinity towards substrate (K_m of 43–46 μM). Now, ThTPase of several animal species has been clones and enzyme has been overexpressed in *E. coli* cells [95, 96]. Enzymes from various species differ from each other by the number of amino acid residues in their polypeptide chain (from 218 to 229 in bovine and human enzymes, respectively) as well as by catalytic properties (K_m value varies within 16–126 μM). Three-dimensional structure of mouse ThTPase was determined by the method of NMR [97].

Soluble ThTPase is widely distributed in mammalian tissues, but it has not been found in representatives of other classes of living organisms [3, 95, 98]. ThTPase together with CyaB-like adenylate cyclase is included into the superfamily of proteins containing CYTH domain; their common evolution ancestor obviously was involved into metabolism of nucleotides and organic polyphosphates [99].

Analysis of amino acid sequence of soluble ThTPase indicates the presence in its structure potential sites for posttranslational modifications, including phosphorylation sites [95]. In vitro experiments have shown that human recombinant ThTPase is phosphorylated by casein kinase 2 [100]. Reversible phosphorylation is probably the main mechanism of regulation of activity of this enzyme and is the main cause for existence of multiple forms of this enzyme in bovine kidneys [101].

Recently a mitochondrial isoform of soluble ThTPase has been identified [102, 103]. This fact together with data on distribution of ThTP in intracellular structures [60] suggests existence of compartmentalization of ThTP metabolism.

In contrast to soluble enzyme ThTPase associated with membranes of animal cells exhibits maximal activity at neutral or weakly acid pH values [91, 104, 105]. According to data by Barchi and Braun [91] the enzyme from nuclear fraction of rat brain is activated by bivalent metal cations (such as Mg^{2+} or Ca^{2+}) and exhibits low apparent affinity for ThTP (K_m of 1.5 mM). Specificity of this phosphatase still requires further investigation because it has not even been solubilized from membranes.

A typical feature of membrane-bound ThTPases from rat muscles and an electric organ of the electric eel (*Electrophorus electricus*) consists in their high sensitivity to monovalent anions exhibiting marked activation effect [104, 106]. Identification of the products of the reaction catalyzed by membrane preparations from the electric organ suggests cascade hydrolysis of ThTP to thiamine [105]. *E. electricus* ThTPase solubilized by means of various detergents exhibited very low stability. Disintegration also markedly influenced its kinetic properties [107].

An enzyme catalyzed ThTP hydrolysis in extracts from parsley leaves was purified to homogeneity and identified as nonspecific acid phosphatase [108]. This is a heterodimer protein with molecular masses of its subunits of 62.9 and 53.5 kDa; its K_m value for ThTP is 49.8 μM, and pH optimum is within 4.0–4.5.

In *E. coli* cells ThTP hydrolysis presumably involves membrane-bound NTPase [109], but involvement of other nonspecific phosphatases localized in cytosol is also possible [3].

Besides the above-considered enzymes, myosin [110] and alkaline phosphatase [111] also exhibit ThTPase activity, however, good evidence exists that these proteins are not involved into regulation of intracellular metabolism of ThTP.

4.2. Hydrolysis of ThDP

Two types of ThDPase exhibiting a wide substrate specificity have been identified in rat tissues: these are defined as type B NDPase (brain) and type L NDPase (liver) [112].

Type B NDPase purified to homogeneity from rat brain membranes, is the enzyme of 75 kDa, which catalyzes hydrolysis of ThDP (K_m = 0.66 mM), GDP, UDP, CDP, and IDP [113]. In the presence of Mg^{2+} and with ThDP as a substrate the enzyme exhibited maximal activity at pH 6.0–6.5; ATP, ADP, and pyridoxal-5'-phosphate inhibited ThDPase activity in a competitive manner [112, 113].

Properties of type L NDPase were identical to bovine liver microsomal NDPase [114]. This protein with molecular mass of 130–140 kDa is composed of two identical subunits. At neutral pH values type L NDPase exhibited weak ThDPase activity (about 4% versus IDP [112]). ThDPase activity of this enzyme was maximal at pH of 8.8–9.0 and it was strongly increased by ATP.

Isoelectrofocusing (IEF) of partially purified preparations from rat brain revealed 9 isoforms of type B NDPase; their pI values varied from 5.4 to 7.1; type L enzyme gave only one band corresponding to pI of 4.6 [112].

Sano et al. [115] investigated localization of type B and type L NDPases in rat hepatocytes. During IEF ThDP activity of extract from Golgi apparatus was separated into 6 bands with pI values between 5.4 and 6.3. Partially purified enzyme exhibited properties typical for brain type B NDPase with respect to substrate specificity, pH optimum, and inhibition by ATP. At the same time IEF of a solubilized microsomal fraction followed by gel staining at pH 7.2 with IDP yielded one band of activity (pI of 4.6), which was not detected when ThDP was used as a substrate. These results indicate that type

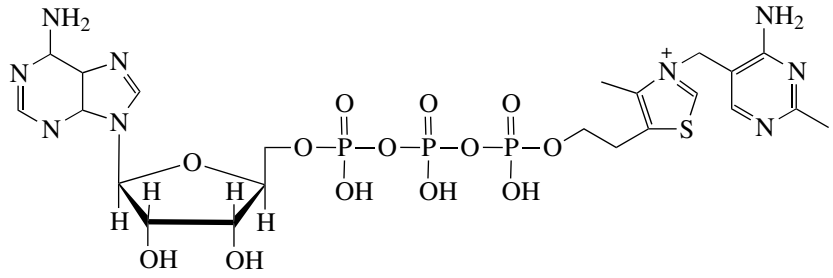

Fig. 2. Structural formula of AThTP.

B NDPase is localized in the Golgi apparatus, whereas type L enzyme is located in the endoplasmic reticulum. This conclusion is supported by results of histochemical studies [116].

However, the fact that ThDPase activity is rather uniformly distributed in brain subcellular fractions [117, 118] and is detected histochemically in various membrane structures [119] cannot be rationally explained provided that only type B and type L NDPases exist in the cells. Indeed, Cooper and Kini [118] described partial purification of ThDPase from rabbit brain microsomes; in contrast to type L NDPase this enzyme catalyzed hydrolysis of ADP. The other ThDPase soluble in 0.5 M NaCl was purified 1043-fold from sheep brain [120]. This enzyme as well as type B NDPase exhibits pH optimum at pH 6.0, but in contrast to the latter it is characterized by high ADPase activity. Using digitonin titration Barile et al. [65] have shown that the soluble enzyme exhibiting ThDPase activity is located in matrix of rat liver mitochondria.

4.3. Hydrolysis of ThMP

Hydrolysis of ThMP to thiamine is catalyzed by membrane-bound phosphatases, found in the brain and other mammalian organs [119, 121]. These enzymes obviously do not exhibit selectivity towards ThMP; at least no data, which would indicate existence of specific ThMPase, are known.

According to data of ultracytochemical staining of rat brain sections at pH 9.2 ThMPase activity is localized in neuronal plasmalemma, synaptic membranes and vesicles, pinocytic vesicles, apical and basal membranes of capillary endothelium [122]. At pH 5.5 ThMPase is visualized in the plasma membrane of central endings of sensory neurons of dorsal root ganglia contouring *substantia gelatinosa* over the whole length of the spinal cord [119]. This specific localization of ThMPase is identical to the localization of fluoride-resistant nonlysosomal acid phosphatase [123]. In addition there is a positive reaction in cisternae of the endoplasmic reticulum and reticular part of the perikaryon Golgi apparatus, from which enzyme traffic into axonal endings occurs [119, 123]. Fluoride-resistant "acidic" ThMPase is not detected cytochemically in brain structures. Consequently, "acidic" ThMPase activity detected in a fraction of gial cell but not neurons isolated from rat brain [124] should be attributed to the action of another phosphatase.

5. ADENOSINE ThTP

Recently a new thiamine derivative, adenosine thiamine triphosphate (AThTP), has been identified in biological objects [4] (Fig. 2). In bacterial and mammalian cells the biosynthesis of AThTP is carried out from ADP (ATP) and ThDP by a soluble enzyme, which exhibits an absolute dependence on bivalent metal cations (Mn^{2+} or Mg^{2+}) and the presence of a low molecular weight activator [125]. The K_m value of ThDP adenylyl transferase for ThDP is 7.1 mM and $S_{0.5}$ for ADP is 0.08 mM. Catabolism of AThTP is catalyzed by membrane-bound hydrolase, which cleaves it to ThDP and AMP [4]. The fact that *E. coli* cells synthesize large amounts of AThTP only under some conditions, for example, in the case of carbon starvation, and AThTP rapidly disappears when a suitable carbon source is added to a cultivation medium, may suggest involvement of this substance in some (yet unknown) regulatory process. It is also possible that AThTP is a form of storage of ThDP (and AMP) under conditions when decomposition processes prevail over a constructive metabolism.

6. BIOLOGICAL ROLE OF VITAMIN B_1

6.1. Coenzyme Function of ThDP

The coenzyme function of thiamine was discovered in 1937 when Lohmann and Schuster [126] found that ThDP is a cofactor of oxidative decarboxylation of pyruvate by yeast pyruvate decarboxylase (EC 4.1.1.1). Now 28 ThDP-dependent enzymes with individual code numbers are known (www.expasy.ch) and four of them play important roles in intermediate metabolism. These include pyruvate dehydrogenase (EC 1.2.4.1), α-ketoglutarate dehydrogenase (EC 1.2.4.2) and branched chain α-keto acids dehydrogenase (EC 1.2.4.4) (which are components of multienzyme dehydrogenase complexes localized in mitochondria of

eukaryotic cells and cytosol of prokaryotic cells), as well as cytosolic TK.

PDHC catalyzes oxidative decarboxylation of pyruvate resulting in formation of acetyl-CoA; this reaction is crucial for inclusion of products formed during specific catabolic pathways of sugars and amino acids into Krebs cycle. Besides pyruvate dehydrogenase (E1), PDHC contains two other catalytic components, dihydrolipoyl transacetylase (E2) (EC 2.3.1.12), and dihydrolipoyl dehydrogenase (E3) (EC 1.8.1.4). The reaction of acetyl-CoA formation is a key one for the whole metabolism and therefore PDHC activity is under strict control [127, 128]. Mutation in PDHC resulting in substitution of Arg-263 for Gly in E1 α-subunit is one of the causes of subacute necrotizing encephalopathy (Leigh's disease). Since gene encoding E1 α-subunit is localized on X-chromosome Leigh's disease associated with PDHC is a sex-linked disorder [129].

Structure-functional organization of mammalian PDHC has been studied in details. It is known that 60 subunits of E2 form its core, which is associated with 30 $\alpha_2\beta_2$-heterotetramer molecules of E1 and 12 α_2-homodimer molecules of E3. Active sites of all subunits of this complex are tightly "packed" to each other at contact sites and this provides effective running of sequentially catalyzed reactions [130, 131].

KGDHC, one of regulatory enzymes of Krebs cycle, and branched chain α-keto acids dehydrogenase complex, involved into catabolism of valine, leucine, and isoleucine, are composed in a similar manner, but differ in number of subunits. In addition, E1 component of KGDHC is a α_2-homodimer [127, 132, 133]. Impairments in activity of branched chain α-keto acid dehydrogenase complex (appeared due to various mutations in its catalytic components) are the cause of an autosomal recessive disease known as maple syrup urine disease (MSUD) [132]. Interestingly, at the thiamine-dependent form of MSUD mutations occur in E2 subunits rather than E1α or E1β subunits of this complex [134].

TK is a key enzyme of the nonoxidative part of the pentose phosphate pathway and photosynthesis; it catalyzes transfer of a two carbon groups from keto- to aldo-sugars. Metabolic role of TK consists in reversible linkage of glycolysis and pentose phosphate pathway. In photosynthetic organisms TK provides interaction between Calvine cycle with carbohydrate metabolism, on the one hand, and anabolic pathways leading to formation of nucleic acids, amino acids and their numerous derivatives, on the other hand [135]. In human genome, besides TK gene (*TKT*) two transketolase like genes (*TKTL1* and (*TKTL2*) have been recognized. According to one of hypotheses, increased expression of *TKTL1* is directly linked to carcinogenesis [136].

In 1999, a new ThDP-dependent enzyme, 2-hydroxyphytanoyl-CoA lyase, has been discovered in rat liver (this enzyme has not been included into enzyme classification yet). This peroxisomal enzyme catalyzes cleavage of C–C bond during α-oxidation of 3-methyl fatty acids [137].

1-Deoxy-D-xylulose-5-phosphate synthase is expressed in eubacterial cells, green algae, and plant chloroplasts (EC 2.2.1.7); this enzyme catalyzes formation of this product from pyruvate and glyceraldehyde-3-phosphate within the acetate/mevalonate-independent biosynthesis of isopentenyl diphosphate, a common metabolic precursor of all isoprenoids [138]. In addition, 1-deoxy-D-xylulose-5-phosphate is involved into biosynthesis of vitamins B_1 and B_6 [139, 140]. Such enzymes as sulfoacetaldehyde sulfo-lyase (EC 2.3.3.15) [141], phosphoketolase (EC 4.1.2.9) [142], benzoyl formate decarboxylase (EC 4.1.1.7) [143], acetolactate synthase (EC 2.2.1.6) [144], and pyruvate oxidase (EC 1.2.2.2) [145] exhibit specialized functions in metabolic pathways in certain groups of organisms (preferentially in bacteria).

Now cDNAs of almost all known ThDP-dependent enzymes have been cloned and three-dimensional structure for most of these enzymes has been obtained by means of X-ray analysis [146–156].

6.2. Noncoenzyme Function of Thiamine

6.2.1. Specific function of thiamine in nervous tissue. In 1938 Minz [157] found thiamine release into incubation medium during electric stimulation of the isolated bovine vagus nerve. This observation became a basis for hypothesis on a special role of vitamin B_1 in nerve conductance, which is not related with its metabolic role as a cofactor of ThDP-dependent enzymes [158]. Later this phenomenon appears to get new support: there was experimental evidence that thiamine release was coupled to hydrolysis of its phosphate esters [159, 160]. Simultaneously, it was also reported that in vitro pyrithiamine influenced the rate of action potential and repolarization time in the Ranvier's nodes of the isolated frog sciatic nerve [161]. In addition, electrophysiological experiments demonstrated that thiamine partially restored action potential in the UV-irradiated rabbit vagus nerve [162]. These facts represented a basis for notion on involvement of thiamine into regulation of permeability of excitable membranes for Na^+ ions.

Belief in the existence of specific function of vitamin B_1 in nervous tissue became stronger when Itokawa and Cooper [163, 164] published results of their experiments on thiamine release (accompanied by hydrolysis of its phosphate esters) from intact nerve fibers and membrane fragments induced by such neuroactive compounds as tetrodotoxin (TTX), acetylcholine (ACh) and ouabain. This hypothesis on involvement of thiamine in nerve impulse conductance is still discussed in the literature [165–168]. Although it looks attractive, its inadequateness seems evident. Some of the above-mentioned facts were not confirmed by other researchers, some other facts were explained outside

the framework of this hypothesis. For example, Fox [169] did not observe any restoration of action potential in the Ranvier's nodes of UV-irradiated frog nerve fibers. Berman and Fishman [170] did not observe thiamine release by rat brain cortex sections after electric stimulation, TTX, ACh, and ouabain treatment. In excitable neuroblastoma cells neither depolarization nor TTX influenced release of vitamin B_1 [171]. It should be noted that pyrithiamine and other antimetabolites influencing electric characteristics of isolated nerves and neuronal cells exhibit their effects only at millimolar concentrations [165, 172]. Under these conditions membrane conductivity may be altered for both Na^+ and K^+ ions. Taking into consideration that in nervous tissue concentration of free vitamin B_1 derivatives (unbound to proteins) is at least two orders of magnitude lower their physiological importance for nerve conductance seems illusive. Goldberg et al. [172] believed that thiamine antagonists stabilize axonal membrane nonspecifically. Other viewpoints on possible mechanism of antagonist effects on excitable membrane may be illustrated by results obtained by Matsuda et al. [173], which demonstrate specific inhibition of Na^+, K^+-ATPase by pyrithiamine.

Alternative hypothesis that vitamin B_1 is involved into excitation transmission in cholinergic synapses rather than impulse propagation was proposed by Eder et al. [174]; these authors found the effect of thiamine and oxythiamine on amplitude and duration of potential in electric plates of the *Torpedo marmorata* electric organ. It is important to indicate that the effect of thiamine was concentration dependent: relative low concentrations (1 mM) increased electric discharge, whereas 10 mM thiamine decreased discharge. Similar opposite effects were also observed during studies of impulse transmission in nerve-muscle junctions. For example, Romanenko [175] found that thiamine potentiated effect of ACh. According to Enomoto and Edwards [176] thiamine blocked nerve-muscle transmission. In this connection it is interesting to mention that high doses of thiamine were even proposed for anesthesia [177].

It is possible that these and other contradictions [178] may have simple and rational explanation. This may be easily explained by two reasons: ability of thiamine molecule to interact (like ACh) with ACh-receptor and properties of the receptor. Indeed, Waldenlind et al. [179] demonstrated that thiamine interacts with AC-recector isolated from *T. marmorata* (K_d of 30–50 μM). However, the major problem consists in specificity of such interaction. Detailed analysis of peripheral myorelaxants regardless their depolarizing (dithylinum) or curare-like action mechanism (d-tubocurarine, diplacinum, pavulon, anatruxonium) shows that two tertiary nitrogen atoms located at the distance of 1.4–1.5 nm are the only structural element required for their activity. These substances compete with ACh for receptor binding sites due to these atoms [180, 181]. It is possible that thiamine binding to ACh receptor is also determined by the presence of a cationic center (thiazole tertiary nitrogen) in its molecule. On the other hand, long-term exposure of ACh-receptor to high concentration of ACh causes its inactivation and it is not opened even in the presence of ACh [182]. Thus, one can expect that in dependence of their concentrations interaction of thiamine and its analogues with ACh-binding sites on the receptor may exhibit opposite effects. Taking into consideration this information it also seems unlikely that thiamine has any specific neurotransmitter function.

Besides specific noncoenzyme functions of thiamine in nervous tissue other possible mechanisms of thiamine involvement into intracellular processes are also under consideration. For example, it was demonstrated that thiamine molecule might serve as a peroxynitrite scavenger, protecting tyrosine residues of proteins against inactivation (nitration) [183]. Moreover, thiamine may release NO from its endogenous stores (such as S-nitrosoglutathione) or reduce NO to nitrites. All these features may explain vasoprotector effects of thiamine seen in diabetes mellitus and arterial thrombosis [184].

6.2.2. Biological role of ThTP. In 1969 Cooper et al. [185] suggested that ThTP is a neuroactive form of vitamin B_1. Developing this idea Schoffeniels [186] proposed a model in which ThTP plays a role of regulator of Na^+-channel of axonal membrane. However, as it has been discussed above, suggestions on the interrelationship between thiamine and Na^+-channel do not have serious background. Indeed, thiamine phosphates were found in partially purified preparation of Na^+-channel from the *E. electricus* electric organ [187], however, subsequent purification revealed that phosphorylated thiamine derivatives had distinct localization [171]. In this connection it is quite surprising that the textbook "Basic Neurochemistry: molecular, cellular and medical aspects" published in 1999 [188] considers involvement of ThTP in regulation of Na^+- channels as the generally accepted scientific fact. In reality there is the only experimental study demonstrating the effect of thiamine phosphates on electric characteristics of axonal membrane: in 1975 Fox and Duppel [189] demonstrated that 2 mM ThTP and 2 mM ThDP prevented exponential decrease of sodium and potassium currents in the Ranvier's nodes of the isolated sciatic nerve from *Rana esculenta* frog. The authors attributed this effect to stabilization of the membrane electric field by thiamine phosphate in resting state. However, it is important to indicate here that such high concentration of the compounds studied cannot be achieved under physiological conditions.

Using the method of microdialysis in vivo Yamashita et al. [190] found that in the presence of Ca^{2+} ions ThTP caused dopamine release in rat striatum. Again, a marked effect was observed at ThTP concentration exceeding 0.1 mM. Since dopamine release is a process mediated by Ca^{2+} flow into cell via potential dependent N type Ca^{2+}-channel and ThTP effect was observed

after inactivation of this channel by ω-conotoxin, it is clear that this effect may be attributed to nonspecific activation of ATP-binding P_2-purinergic receptor coupled to ionic channel permeable for Ca^{2+} ions.

In experiments with membrane vesicles (crude nuclear and synaptosomal fractions) from rat brain Bettendorff et al. [191, 192] revealed interrelationship between ThTP and chloride ion transport. The study of transport mechanism has shown that it involves potential-dependent anion channel [193, 194]. This channel exhibited unusually high conductance (350–400 pS) and low anion selectivity ($P_{Cl}/P_{gluconate}$ = 3) and long-term opened state; these properties are typical for maxi Cl^- channels, found in cells of various specialization. The fact that ThTP-induced activation of this channel was observed after 4 min and channel remained opened after substrate removal would suggest its phosphorylation. In this connection certain interest attracts reports on in vitro phosphorylation of rapsin (postsynaptic membrane protein of nurve-muscular junctions) by endogenous protein kinase, utilizing ThTP as a substrate [195].

Thus, there are indications on involvement of ThTP into activation of plasma membrane maxi Cl^- channel of nervous cells by phosphorylation. On the other hand, certain experimental evidence exists that in murine neuroblastoma C1300 cells phosphorylated maxi-Cl^- channel is closed and its activation is coupled to dephosphorylation by PP2A like phosphatase; in this context a role of ThTP as a possible modulator of activity of this channel has not been considered at all [196].

If ThTP activates maxi Cl^- channel whether it is the only function of this substance? It seems that definitely not. Wide distribution of ThTP in various organisms from bacteria to mammals indicates its basic role in cell biology unrelated to bioelectrogenesis [3]. Recently a dynamic mode of changes of intracellular concentration of ThTP in dependence of physiological state of organisms has been recognized in relatively simple objects such as *E. coli* cells and *Arabidopsis thaliana* leaves. For example, it is basically impossible to detect this substance in *E. coli* cells cultivated in LB-medium in the presence of oxygen. However a sharp increase in ThTP level was observed under conditions of energy deficit (during anaerobic cultivation) and also during transfer of cell culture from a nutrient rich cultivation medium into a minimal medium containing glucose or some other carbon sources [3, 197]. In *A. thaliana* leaves ThTP content increased upon drying and reached maximal values 2 h after plant removal from soil. Under these conditions stoma is closed due to loss of water and this results in hypoxia due to impairments in gas exchange [198]. After reseeding into soil followed by intensive watering and restoration of leave turgor ThTP concentration reached initial (basically zero) level. Results of these experiments represented a basis for hypothesis on involvement of ThTP in adaptation to the effects of stress factors [199]. This hypothesis raises several conceptual questions on peculiarity of ThTP metabolism in various phylogenic forms, evolution of enzymatic systems involved into its biosynthesis and degradation, regulatory mechanisms of control of its intracellular level and their improvement during historical process of species formation. One can hope that subsequent studies in these directions will give clear answer on the principal question on the molecular mechanisms underlying ThTP participation in vital processes.

CONCLUSIONS

Although phosphate esters of thiamine, ThMP, ThDP, and ThTP are universal components of various cell types, only the function of ThDP, acting as a cofactor for more than 25 enzymes is known to date. Biological role of ThMP and ThTP still remains a point for discussion.

Since the beginning of 1970th ThTP has been considered as a "carrier" of some special noncoenzyme function of thiamine in membranes of excitable tissues. However, results of recent studies have shown that stress factors cause the increase ThTP concentration in various cells; this suggests its basic role in vital cell processes possibly related to mechanisms of adaptation. Quite recently, a new thiamine derivative, AThTP, has been identified in living organisms. In bacteria it is synthesized in response to carbon starvation. These new data suggest existence of multiple functions of thiamine and indicate that our knowledge on its biological role is not exhaustive yet.

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