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Mini review

THE GENES AND ENZYMES INVOLVED IN THE BIOSYNTHESIS OF THIAMIN AND THIAMIN DIPHOSPHATE IN YEASTS [#]

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Abstract: Thiamin (vitamin B1) is an essential molecule for all living organisms. Its major biologically active derivative is thiamin diphosphate, which serves as a cofactor for several enzymes involved in carbohydrate and amino acid metabolism. Important new functions for thiamin and its phosphate esters have recently been suggested, e.g. in gene expression regulation by influencing mRNA structure, in DNA repair after UV illumination, and in the protection of some organelles against reactive oxygen species. Unlike higher animals, which rely on nutritional thiamin intake, yeasts can synthesize thiamin *de novo*. The biosynthesis pathways include the separate synthesis of two precursors, 4-amino -5-hydroxymethyl-2-methylpyrimidine diphosphate and 5-(2-hydroxyethyl)-4-methylthiazole phosphate, which are then condensed into thiamin monophosphate. Additionally, yeasts evolved salvage mechanisms to utilize thiamin and its dephosphorylated late precursors, 4-amino-5-hydroxymethyl-2-methylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole, from the environment. The current state of knowledge on the discrete steps of thiamin

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Abbreviations used: HET – 5-(2-hydroxyethyl)-4-methylthiazole; HMP – 4-amino-5-hydroxymethyl-2-methyl pyrimidine; TDP – thiamin diphosphate; TMP – thiamin monophosphate; TTP – thiamin triphosphate

biosynthesis in yeasts is far from satisfactory; many intermediates are postulated only by analogy to the much better understood biosynthesis process in bacteria. On the other hand, the genetic mechanisms regulating thiamin biosynthesis in yeasts are currently under extensive exploration. Only recently, the structures of some of the yeast enzymes involved in thiamin biosynthesis, such as thiamin diphosphokinase and thiazole synthase, were determined at the atomic resolution, and mechanistic proposals for the catalysis of particular biosynthetic steps started to emerge.

Key words: Thiamin biosynthesis, Thiamin diphosphate, Thiazole, Pyrimidine, *THI* genes, *Saccharomyces cerevisiae*

INTRODUCTION

Thiamin, the water-soluble vitamin B_1 discovered in 1911 by Casimir Funk and synthesized in 1935, occurs in all living cells. Microorganisms, fungi and plants are able to synthesize thiamin *de novo*, while animals including humans must take it up from the environment to sustain a proper metabolism [1].

The structural formula of thiamin is shown in Fig. 1. The molecule consists of two substituted aromatic moieties, 4-amino-2-methyl-5-pyrimidyl and 5-(2-hydroxyethyl)-4-methylthiazolium, connected by a methylene bridge. Besides free thiamin, i.e. the form with a free hydroxyl group, three thiamin phosphate esters – monophosphate (TMP), diphosphate (TDP) and triphosphate (TTP) – occur in the cells. Due to a rotation around the methylene bridge, thiamin and its derivatives can adopt several conformations. In solutions, an extended "F" conformation is preferred. The active centres of TDP-dependent enzymes force a stressed "V" conformation (Fig. 1).

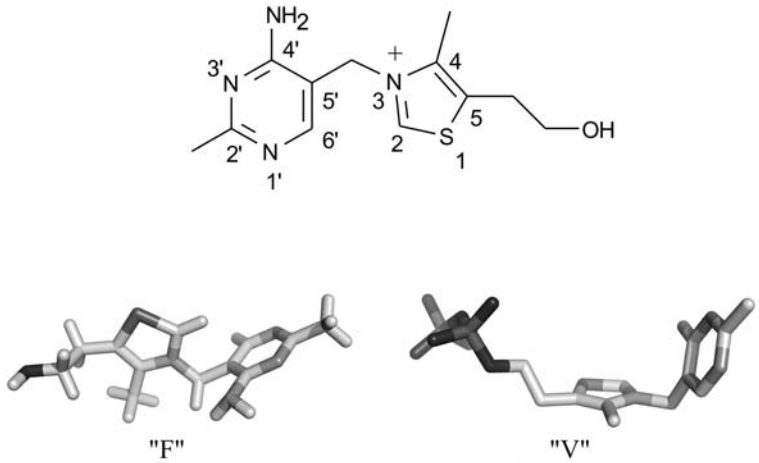

Fig. 1. The structural formula of thiamin (top), and stick diagrams of the two major thiamin conformations (bottom). Biological thiamin derivatives are esters at the hydroxyl group with one (TMP), two (TDP) or three (TTP) phosphate residues.

THE ROLES OF THIAMIN AND ITS PHOSPHATE DERIVATIVES

The main biologically active thiamin derivative is TDP. Its primary function, recognized early after its discovery, is its contribution to the universal metabolic pathways including glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle, where TDP serves as a cofactor of enzymes such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-ketoacid dehydrogenase, transketolase and pyruvate decarboxylase [1]. A regulatory activity of TDP has been reported, but the mechanisms of this regulation at the nucleic acid and protein levels is still poorly understood [2, 3]. Quite recently, TDP was shown to bind to mRNA domains called "riboswitches" and to affect mRNA structure, resulting in gene expression regulation, particularly of the genes involved in thiamin biosynthesis (for a review see: [4]). TTP plays a specific albeit not fully understood role in the physiology of the nervous system (for a review see: [5]). A recent report on the accumulation of TTP in *Escherichia coli* in response to amino acid starvation raises a hypothesis that TTP may play a more universal role as a signal molecule in prokaryotic and eukaryotic cells [6].

It has also recently been hypothesized that thiamin and its derivatives contribute to organism responses to various stress conditions, such as during UV illumination, when thiamin may be involved in the repair of DNA damage [7], or under conditions of oxidative stress and heat shock, when thiamin may increase mitochondrial stability [8]. Vitamin B_1 may activate plant disease resistance [9].

THIAMIN BIOSYNTHESIS PATHWAYS IN YEASTS

Like all thiamin-synthesizing organisms, yeasts first separately synthesize two precursors, 5-(2-hydroxyethyl)-4-methylthiazole phosphate (HET-P) and 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate (HMP-PP), which are then condensed into TMP (Fig. 2).

Our knowledge of the early steps of thiazole (HET-P) and pyrimidine (HMP-P) synthesis in yeasts [2, 3] is far from satisfactory. In the absence of experimental evidence, many intermediates are predicted only by extrapolations from the much more advanced characteristics of prokaryotic thiamin biosynthesis [for a review see: [10]). The substrates for yeast thiazole synthesis include cysteine as a sulfur donor, glycine, and D-pentulose-5-phosphate. The latter may be D-ribulose-5-phosphate or D-xylulose-5-phosphate, indicative of a link between thiamin biosynthesis and the pentose phosphate pathway [2]. Recently, a mechanism of thiazole synthesis was proposed in which NAD^+ serves as the early source of a five-carbon carbohydrate and the advanced intermediate is an ADP adduct of 5-(2-hydroxyethyl)-4-methylthiazole-2-carboxylic acid [11]. The final product of the thiazole synthesis pathway is HET-P. Yeasts also possess a salvage pathway through which the external 5-(2-hydroxyethyl)-4-methylthiazole (HET) is taken up and then phosphorylated to HET-P [12].

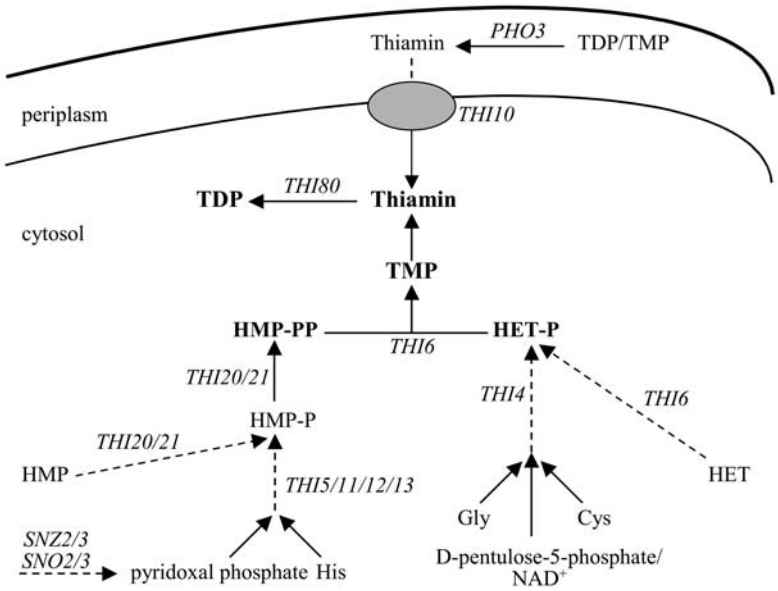

Fig. 2. The pathways of thiamin biosynthesis in yeasts. The major intermediates and products are written in bold, and the genes involved in italics. The dashed arrows mark processes whose steps have not yet been fully identified. Abbreviations are defined in the text. Adapted from [2, 3].

HMP-P is synthesized in yeast cells from histidine and pyridoxal-5-phosphate, the latter linking the thiamin and vitamin B_6 (pyridoxine) biosynthesis pathways [13]. Yeasts can also salvage HMP-P by an uptake of 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) from the environment followed by its phosphorylation [14]. The next phosphorylation step yields HMP-PP ready for condensation with HET-P to produce TMP.

Unlike many bacteria, yeasts cannot directly phosphorylate TMP to obtain the bioactive coenzyme, TDP. Therefore, TMP must first be dephosphorylated to free thiamin, which is then activated via one-step diphosphorylation [15]. TDP may also be produced from free thiamin taken up by the yeast cells from the environment [16]. The external thiamin phosphates which cannot be transported across the cell membrane are first dephosphorylated in the periplasm to be utilized by the yeast cells.

THE GENETIC CONTROL OF THIAMIN BIOSYNTHESIS IN YEASTS

The current list of the genes which control thiamin and TDP biosynthesis in *Saccharomyces cerevisiae* [3] contains at least 15 genes coding for the enzymes catalyzing discrete steps of the thiamin synthesis pathways, at least 3 genes whose products are involved in the uptake or cellular transport of thiamin and

thiamin phosphates, and 3 genes apparently involved in the genetic regulation of thiamin biosynthesis. While the functions of many yeast thiamin biosynthesis genes have been verified by genetic methods (e.g. by various mutant complementation tests), only a few gene products were characterized as the purified proteins (see below).

Only one gene, *THI4*, could be assigned to the *de novo* HET-P synthesis. The encoded THI4 protein probably catalyzes the formation of the adenosine diphospho-5-(2-ethyl)-4-methylthiazole-2-carboxylic acid intermediate [17]. Its orthologues in *Arabidopis thaliana* and *Neurospora crassa* play some not yet fully understood roles in the cellular responses to various stress factors [9, 18]. The salvage phosphorylation of HET is catalyzed by a bifunctional enzyme, TMP diphosphorylase/HET kinase, encoded by the *THI6* gene [12].

Four genes, *SNO2/SNO3* and *SNZ2/SNZ3*, are possibly involved in the synthesis of pyridoxal phosphate, the common precursor of other B_6 vitamers and HMP-P [13]. Later steps of HMP-P synthesis up till the final HMP-P phosphorylation are controlled by genes of the *THI5/THI11/THI12/THI13* family [19] and the *PET18* gene, but the exact enzymatic functions of their protein products have not yet been recognized. The members of another gene family, *THI20* and *THI21*, encode HMP-P kinases, which are actually trifunctional proteins, as they can also perform the salvage HMP phosphorylation, and contain an additional C-terminal domain with a thiamin degrading (thiaminase II) activity [14, 20]. Interestingly, the C-terminal domain exhibits a high sequence similarity to PET18 [14]. The function of the third member of the same gene family, *THI22*, is unknown at present [21].

The condensation of HET-P and HMP-PP into TMP is catalyzed by the bifunctional TMP diphosphorylase/HET kinase encoded by the *THI6* gene [12]. After the dephosphorylation of TMP by various, probably non-specific phosphatases, the free thiamin formed is converted to TDP by thiamin diphosphokinase (thiamin pyrophosphokinase), a product of the single *THI80* gene [15].

Two genes, *THI7* (*THI10*) and *PHO3*, encode the proteins involved in the uptake of thiamin and thiamin phosphates by yeast cells from the medium. The former is a thiamin transporter located in the plasma membrane [16]. PHO3 represents a periplasmic acid phosphatase with a high affinity to thiamin phosphates and with an additional capability to tightly bind free thiamin [22].

A *TPC1* gene encodes a mitochondrial membrane protein that transports TDP, synthesized *de novo* in the cytoplasm, into the mitochondria, where it can be incorporated into several essential TDP-dependent enzymes [23].

Our understanding of the genetic regulation of thiamin biosynthesis in yeast is at a very preliminary stage (for a recent review see: [3]), even though it has been known for four decades that the addition of thiamin to the medium represses thiamin synthesis and cellular uptake. In the presence of external thiamin, the expression of all structural genes is turned off except that of the thiamin diphosphokinase-encoding *THI80*, which is constitutively expressed at

a moderate level. The induction of the latter in response to thiamin starvation is the lowest (three-fold lower than the average) of all the *THI* genes. The control occurs at the transcriptional level, and TDP serves as an intracellular negative signal. To date, three positively acting proteins, products of the *THI2* (*PHO6*), *THI3* and *PDC2* genes, were found to regulate the expression of thiamin biosynthesis and transport genes [2, 24, 25]. The THI2 protein contains an N-terminal DNA-binding domain typical for many yeast transcriptional factors. THI3 is a TDP-binding protein (4-methyl-2-oxopentanoate decarboxylase), and PDC2, an isoform of TDP-dependent pyruvate decarboxylase, probably also contains a DNA-binding N-terminal region. In a recent model of yeast thiamin biosynthesis regulation [3], these three proteins form a ternary complex in the absence of TDP, and bind to putative upstream regulatory sequences of *THI* genes, inducing their transcription. At a sufficient concentration, TDP binds to THI3, the intracellular thiamin sensor, preventing the ternary protein complex formation, and thus, *THI* gene transcription cannot occur.

THE STRUCTURE AND ENZYMATIC PROPERTIES OF YEAST PROTEINS INVOLVED IN THIAMIN BIOSYNTHESIS

Of the thiamin biosynthesis enzymes in the baker's yeasts, only four are known in their purified forms, whether natural or recombinant. These include thiamin diphosphokinase (THI80), thiazole synthase (THI4), TMP diphosphorylase/HET kinase (THI6) and HMP/HMP-P kinase (THI20). The three-dimensional structures of the first two enzymes were reported on [17, 26]. Only the very basic enzymatic parameters are known for the HMP/HMP-P kinase [20].

Thiamin diphosphokinase (THI80)

Thiamin diphosphokinase (thiamin pyrophosphokinase, EC 2.7.6.2) is the universal thiamin-activating enzyme. Although the steady-state kinetic properties of the baker's yeast enzyme were extensively studied [27] and the crystal structure of the enzyme-thiamin complex at 1.8 Å resolution was recently published [26], the advanced mechanism of the catalyzed reaction has not yet been elaborated on. The overall structure of the yeast thiamin diphosphokinase is presented in Fig. 3.

The enzyme forms a homodimer within which each 37-kDa subunit is composed of two different domains. One domain consists of a twisted six-stranded parallel β-sheet with four α-helices attached at each side, and the other domain is a sandwich composed of one four-stranded and one six-stranded antiparallel β-sheets. Hence, the domains are respectively called the αβ- and β-sandwich domains. The αβαβαβ motif in the αβ domain is the typical Rossman fold, which commonly occurs in nucleotide binding proteins. Two domains are linked in an end-to-end fashion, resulting in an extended, mixed ten-stranded β-sheet within each subunit. The intersubunit interface within the dimer is formed by both domains, with the major contribution of two β-turns from the β-sandwich domain and the α-helix K from the αβ-domain.

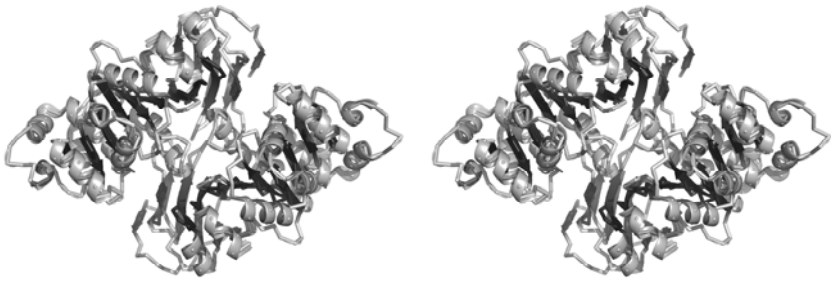

Fig. 3. A stereo ribbon diagram of the yeast thiamin diphosphokinase dimer. After [26]. The figure was generated with the PyMOL program (http://www.expasy.org) using the PDB file 1IG0.

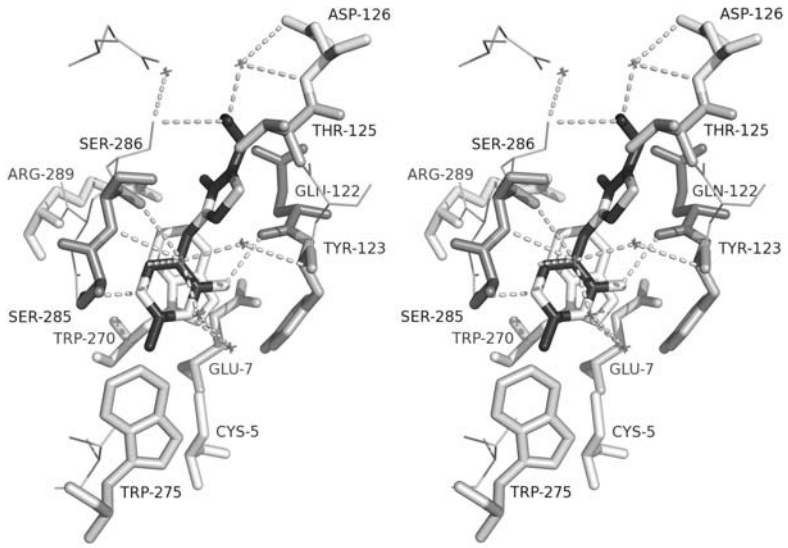

Fig. 4. A stereo stick representation of the arrangement of a bound thiamin molecule and amino acid residues in the active center of yeast thiamin diphosphokinase. The residues Ser286, Arg289, Ser285, Trp270, Trp275, Cys5 and Glu7 are from one subunit within a dimer, and the residues Tyr123, Gln122, Thr125 and Asp126 are provided by the other subunit. Further comments in the text. After [26]. The figure was generated with the PyMOL program using the PDB file 1IG0.

Two thiamin molecules bind at both ends of a cleft which occurs in the intersubunit interface within the protein dimer. The substrate-binding site is built of two β-strands (12 and 13) from the β-sandwich domain of one subunit and a β5-αF loop from the αβ domain of the other subunit. The arrangement of active-site amino acid residues surrounding the bound substrate is shown in Fig. 4.

The bound thiamin adopts the "F" conformation, which is characteristic for free thiamin in solution and essentially different from the stressed "V" conformation of TDP utilized by TDP-dependent enzymes (Fig. 1). The Glu7a and Tyr123b residues, where a and b mean different subunits, gate the bound ligand from the solvent. The pyrimidine moiety enables van der Waals interactions with Tyr123b and Trp275a, a stacking (π-π) interaction with Trp270a, and hydrogen bonds to the side-chain oxygen atom of Ser285a (via the N1'-ring atom), to the main-chain oxygen of Glu122b (via the 4'-amino group nitrogen) and via fixed water molecules, to Trp275a (via the N3' ring nitrogen). The thiazolium nitrogen interacts with the main chain oxygen atoms of Gln122b and Ser286a. The hydroxyethyl chain is fixed by hydrogen bonds via water molecules to the nitrogen and carboxyl oxygen atoms of Asp126b.

A putative ATP binding site may be located in the vicinity of the hydroxyethylthiazole end of the thiamin molecule, where several well-conserved amino acid residues occur. For example, three aspartic acid residues, Asp69a, Asp97b and Asp126b, may be involved in the coordination of magnesium ions required for the enzyme's catalytic action.

Thiazole synthase (THI4)

The *THI4* gene of *S. cerevisiae* codes for a 35-kDa protein which was recently obtained in a recombinant form. Also, its three-dimensional structure was determined to 1.8 Å resolution [17]. The protein contained a tightly bound ligand, adenosine diphospho-5-(2-ethyl)-4-methylthiazole-2-carboxylic acid, which was assumed to be a product of a THI4-catalyzed reaction, so the protein was named thiazole synthase. This enzymatic activity was not directly demonstrated in the wild-type THI4 protein, probably because the bound ligand could only dissociate under denaturing conditions. However, some mutants of THI4 protein presented a partial activity and were used to identify some reaction intermediates and to suggest a convincing mechanism of adenosine diphospho-5-(2-ethyl)-4-methylthiazole-2-carboxylic acid biosynthesis [11].

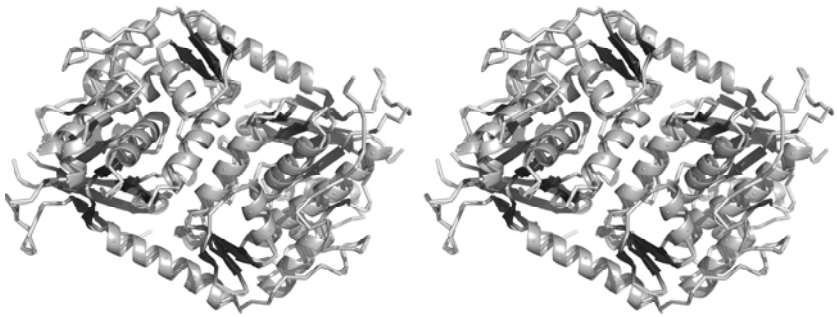

Fig. 5. A stereo ribbon diagram of the THI4 dimer. After [17]. The figure was generated with the PyMOL program using the PDB file 2GJC.

THI4 monomers asymmetrically dimerize to form a final ring-shaped homooctamer. The overall structure of the THI4 dimer is shown in Fig. 5.
Each monomer contains nine α-helices, ten β-strands and several disordered regions, the largest being the 15- to 16-amino acid N-terminal fragment. The subunit has a central core of a five-stranded parallel β-sheet flanked by three α-helices on one side and an antiparallel three-stranded β-sheet on the opposite side. The first (N-terminal) and the last (C-terminal) α-helices are 17 to 18 amino acids long and amphipathic, and the latter interacts with the disordered N-terminal fragment of the second monomer. The N-terminal parts of the subunits are largely hydrophilic as they are exposed on the octamer surface.
The ligand-binding sites are located near the inner ring of the octamer. The arrangement of amino acid residues surrounding the bound ligand is shown in Fig. 6.

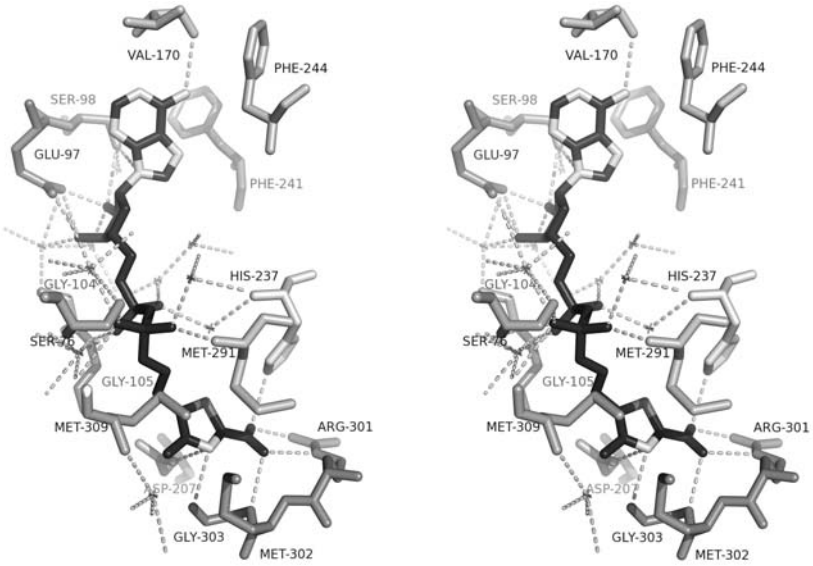

Fig. 6. A stereo stick representation of the arrangement of amino acid residues around adenosine diphospho-5-(2-ethyl)-4-methylthiazole-2-carboxylic acid bound to the yeast THI4 protein. All the residues indicated are from one subunit, except Asp207, which is provided by the other (fifth) subunit within the THI4 octamer. Further comments in the text. After [17]. The figure was generated with the PyMOL program using the PDB file 2GJC.

The adenine moiety is fixed through a stacking interaction with Phe241 and Phe244 and the hydrogen bonds of the N1, N6 and N3 atoms to the amide nitrogen and the carbonyl oxygen of Val170 and the amide nitrogen of Ser98. The ribose hydroxyl groups form hydrogen bonds to Glu97, Ser98, and active site-bound water molecules. The phosphate groups are immobilized through

hydrogen bonds between their oxygen atoms and Gly104, Gly105, Ser76, Met309 and some bound water molecules. Three methionine residues, Met291, Met302 and Met309, surround the thiazole moiety, which is additionally fixed through a hydrogen bond between the thiazolium nitrogen and the carbonyl group of Gly303, a direct contact of the thiazolium ring with Asp207' (from another monomer), and several hydrogen bonds between the thiazole-2-carboxylate and Arg301, Gly303 and His237.

Thiamin phosphate diphosphorylase/hydroxyethylthiazole kinase (THI6)
A natural bifunctional product of the *THI6* gene was purified and characterized in the early nineties [28]. The two activities of the purified protein (EC 2.5.1.3 and EC 2.7.1.50, respectively) were inseparable, though their pH optima differed. A classic steady-state kinetic description is available, primarily for its TMP diphosphorylase activity. The purified enzyme is a homooctamer of 60 kDa subunits.

CONCLUDING REMARKS

Although thiamin was discovered nearly a hundred years ago, the biosynthesis of this essential biomolecule in eukaryotic organisms is still poorly recognized. Only recently, studies of the genetic regulation of thiamin biosynthesis and the structure and catalytic mechanism of the enzymes involved have been intensified in some model eukaryotic organisms such as the baker's yeast *Saccharomyces cerevisiae*.

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