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RECENT ADVANCES IN THE CHEMISTRY AND BIOCHEMISTRY OF THIAMINE

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Abstract

Recent advances in thiamine chemistry and biochemistry are reviewed, with emphasis on structure, biosynthesis, biological roles, and methods of assay.

This review covers advances in thiamine chemistry and biochemistry in the last 15 years. Literature covered by the 1962 "Thiamine" issue of the Annals of New York Academy of Sciences generally will not be repeated here. Owing to limitation of space and author's interest and knowledge, some omissions will inevitably be made without implying any value judgement on them.

The general structure for thiamine and its three biological phosphorylated derivatives is presented in Fig. 1

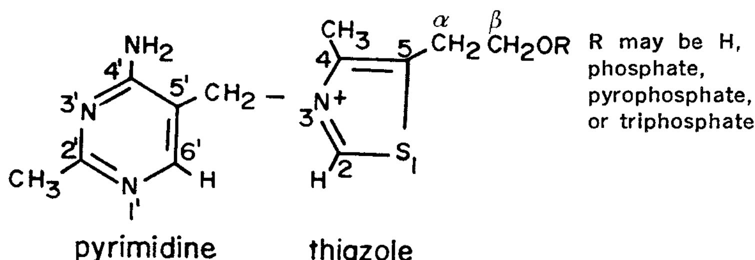

Fig. 1. General structure of thiamine and derivatives.

Thiazolium C-2

Breslow hypothesized in 1958 that thiamine diphosphate with the thiazole ring in the ylide form (ring N positively charged and C-2, having lost the proton, being negatively charged) is the active coenzyme form. The relative acidity of the C_2–H group has been supported by other experimental evidence[1] and the pK_a of this group in the free thiamine was found to be 12.6 in water by the stopped-flow method[2]. Scheffers-Sap and Buck[3] suggested that the ylide was stabilized predominantly by the greater polarizability of the sulfur atom rather than the polarization of the σ bond[4] or the $(d-p)\pi$ or the $(d-p)\sigma$ back bonding of the lone pair of the deprotonated C–2 into the vacant d orbitals of S[5].

Deuterium exchange in some 2–substituted thiazoles showed that the rate follows the order $5 > 2 \gg 4$ and this finding was attributed to the inductive effects and presence of S[6]. Model studies using variously substituted thiazoles and other five-membered N heterocycles have been carried out to test the influence of heteroatoms' inductive effects, steric hindrances of resonance on the kinetic acidity of the various side-chains and deductions have been made on their relevance to thiamine catalytic action[7,8]. ^{13}C–NMR studies suggest that the difference in the kinetic acidity of thiazolium C–2 of various analogues is not due to differences in the hybridization of the C–H but rather different electron withdrawing abilities of substituents bonded to thiazolium N–3[9]. The covalent intermediate between the keto substrate and thiamine diphosphate on the enzyme must have a chiral centre[10]. Attempts have been made to determine the absolute configuration of this centre[11]. Deuterium exchange experiments in acid conditions and cleavage of the exchanged thiamine, oxythiamine and imidazole thiamine have indicated the exchangeability of C–2' methyl group of the pyrimidine moiety[12].

Thiamine–Divalent Cation Complexes

^{1}H and ^{13}C nuclear magnetic resonance spectroscopy of thiamine and derivatives have been studied and assignments made of the signals. The pyrimidine N–1' and not the N of the amino substituent is first protonated. The H–D exchange rate at the C–2 of the thiazole moiety of thiamine is about 10 times that of oxythiamine over the pH range of 3 to 6. The pKa of thiamine ($\sim$ 5) decreases by $\sim$ 3.5 by complex formation with tryptophol in 50% methanol-water[13]. Ni^{2+} broadens thiamine diphosphate PMR signals indicating coordination between the metal ion and the pyrophosphate group and the N–1'. There is a strong temperature dependence of the broadening of C–6' hydrogen signal suggesting an equilibrium involving "folded" and "unfolded" forms with the latter constituting the major population[14]. Oxythiamine shows reduced basicity compared with thiamine. From line broadening and shifts of the C6'–H signals of thiamine monophosphate and diphosphate in the presence of Ni^{2+} and Mn^{2+} it was concluded that the amount of folded conformation in thiamine diphosphate (TDP)–metal complexes at neutral pH follows the order Ni^{2+} – TDP $\gg$ Mn^{2+} – TDP $>$ Mn^{2+} – oxy TDP[15].

It has also been found that the rate of H–D exchange at thiazolium C–2 increases with pH and that this increase is greater as the "phosphate" chain becomes longer[16,17]. T_1 relaxation measurements indicate that Mg^{2+} – TDP at a given pH value shows a more folded form than thiamine diphosphate (TDP) alone[18]. Cr (III) – TDP complex interaction with wheat germ pyruvate decarboxylase has indicated two metal binding sites on the enzyme[19]. ^{1}H and ^{31}P–NMR studies of Mn^{2+} – TDP complex indicate coupling between C–5–β–CH_2 and the adjacent P and support existence of proximity between C–2 and –NH_2 protons[20].

Crystallographic Studies

X-ray diffraction studies of thiamine, its diphosphate and their complexes with planar molecules have been carried out. The TDP·HCl has its negative pyrophosphate chain folded back over the positive ring portion of the molecule[21]. The N–1' of pyrimidyl moiety in thiamine chloride monohydrate is not hydrogen bonded in the crystals[22]. Picrolonate anion forms two different types of stacking interactions with the neutral pyrimidyl moiety of thiamine but not with its thiazole moiety (thiamine maintaining its characteristic F conformation in both[23]). Stacking between thiamine diphosphate and 1,10–phenanthroline in a ternary aquo-copper complex was not observed in the crystals. The metal ion bridges between the phenanthroline ligand and the TDP through the pyrophosphate group only[24]. Polymorphism of thiamine hydrochloride was also recently studied with respect to the water of crystallization[25].

Biosynthesis

It is well established that the pyrimidine and the thiazole moieties are biosynthesized independently and their phosphorylated derivatives are then joined together to give thiamine. However, the primary precursors of the two moieties and their detailed biosynthetic routes have not been elucidated.

It was shown that, in *E. coli* and *S. typhimurium*, the C–2 and N–3 of the thiazole ring was biosynthetically derived from C–2 and N of L-tyrosine[26] and the hetero-S was from S of cysteine[27]. Using a gas chromatography-mass spectrometry combination, the incorporation pattern of isotopes from L–(3–^{2}H, ^{18}O) glycerol into the trifluoroacetate of 4-methyl-5-hydroxymethylthiazole pointed to the transfer of the two hydrogens and the oxygen on the C–3 of glycerol[28]. This incorporation confirms the previous findings that the 5 contiguous carbon atoms in the 4-methyl–5-hydroxyethylthiazole moiety of thiamine are derived from pyruvate and a 3–C unit originating from glucose. In this the pyruvate provides the C–4 and C–6 of the thiazole and the C–4, –5 and –6 of glucose are thought to provide the C–5, 7 and 8 of the thiazole respectively[29].

A detailed analysis of mass fragmentation pattern of the pyrimidine derivative established that the N and C atoms of glycine are incorporated as a unit into the

pyrimidine moiety: the glycine N atom supplies the N-1 of the pyrimidine and the C-1 and C-2 supply the C-4 and C-6 of the pyrimidine respectively. This evidence is consistent with the substitution of a 2-C unit between the C-5 and C-4 of the 4-aminoimidazole ribonucleotide precursor during the biosynthesis of pyrimidine moiety of thiamine in *E. coli*[30]. A strain of *E. coli* derepressed for thiamine biosynthesis produces a growth factor for a thiazole-less mutant. This compound, 5-(β-hydroxyethyl)-4-methylthiazole-2-carboxylic acid, is likely not a precursor of thiazole biosynthesis[31]. In *Saccharomyces cerevisiae*, 2-^{14}C glycine radioactivity enters C-2 of the thiazole moiety and no other site. Neither L-[Me-^{14}C] methionine nor DL-[2-^{14}C] tyrosine could contribute their radioactivity to thiamine molecule[32]. A more general discussion of possible biogenetic scheme for thiazole has been proposed[33]. Other schemes based on different experimental evidence have also been advanced[34,35].

"Thiamine" Enzymes and Their Active Centres

A bacterial enzyme, thiamine dehydrogenase, catalyzes the oxidation of the β-OH group of thiamine to thiamine acetic acid. An interesting feature of this enzyme is its use of flavin adenine dinucleotide as the prosthetic group which is bound to the enzyme via the 8α-methylene group of the flavin ring to the N-1 position of the imidazole ring of a histidyl residue[36,37]. Thiaminases catalyze the cleavage of thiamine into the hydroxymethylpyrimidine and the thiazole moieties or, in the presence of amino containing bases, into the base conjugated pyrimidyl moiety and the thiazole moiety. The detailed mechanism of action at the active site is still not known[38]. Thiamine pyrophosphokinase from *Paracoccus denitrificans* has been purified and characterized. It exists as a dimer (MW 44,000) and is capable of further aggregation. Oxythiamine, chloroethylthiamine and thiamine diphosphate can inhibit the catalyzed reaction[39].

While the chemistry of thiamine itself has been the subject of intensive study, the way in which TDP interacts with proteins is still poorly understood. A TDP-requiring enzyme, transketolase (EC 2.2.1.1) from baker's yeast has two active centres[40]. By covalent modification with phenylglyoxal and 2,3-butanedione and by dual competitive inhibition with phenylphosphate and sulphate, it was shown that an arginine residue per active site is essential for catalytic activity probably by its binding action on sugar phosphate substrates and that a common binding region is used for both donor and acceptor substrates[14]. Thiamine has been found by PMR to form weak molecular complexes with the indole nucleus of tryptophan and it has been suggested that such complex formation may be of relevance to the coenzyme-enzyme binding[42,43].

Model reactions related to decarboxylation as catalyzed by pyruvate decarboxylase, a TDP-dependent enzyme, showed that thiazole-bound pyruvate decarboxylates about 10^4 times faster in ethanol than in water[44]. The solvent polarity dependence of the thiamine-tryptophan interactions, as studied by spectrophotometric methods, suggested that both electrostatic and dispersion forces contribute to the stability of the complex[45].

Studies of thiamine diphosphate binding site of *E. coli* pyruvate oxidase using thiamine thiazolonepyrophosphate, an analogue of thiamine diphosphate, show the essentiality of a tryptophan residue for binding the coenzyme at the active site and that the latter is hydrophobic[46].

A thiamine-binding protein has been isolated from chicken egg white[47]. It has a molecular weight of 38,000, interacts specifically with the riboflavin binding protein from the same source and binds thiamine with a molar ratio of 1 with a K_d of 0.3 μM. The protein is also present in blood plasma and is inducible by estrogen[48]. Thiamine-binding proteins from microbial systems have been identified and some isolated[49].

Transport and Metabolism

Using isolated liver cells, it has been found that thiamine transport is concentrative, Na^+ dependent and energy dependent[50]. There is evidence for extensive phosphorylation of internalized thiamine to the mono-, di- and tri-phosphate derivatives[51]. In the rat everted jejunal sacs, inorganic phosphate led to decrease in uptake and accumulation of free and phosphorylated thiamines. There was no effect of phosphate on phosphorylation of thiamine by thiamine pyrophosphokinase[52]. Because it had been difficult to dissociate the uptake from the phosphorylation step in the cellular thiamine incorporation, some investigators had proposed that these two processes in the mammalian systems (including the extensively investigated transport by the small intestine) were tightly coupled as in *E. coli*, which has both the thiamine transport carrier and the thiamine pyrophosphokinase in the membrane fraction[53,54]. However, recent data from utilization of various inhibitors suggested that thiamine transport and phosphorylation could be differentiated[55]. Also, using *E. coli* K-12 mutant lacking thiamine kinase, uptake of thiamine in its unphosphorylated form and its accumulation as such has been demonstrated[53]. This has been confirmed. Active uptake of thiamine diphosphate in *E. coli* has been found not to involve dephosphorylation and thiamine kinase[56]. Methylene blue competitively inhibits thiamine transport and overcomes inhibitory effect of pyrithiamine in *S. cerevisiae*[57]. Mg^{2+}-ATPase-deficient mutant of *E. coli* showed thiamine uptake to be dependent on glucose metabolism and H^+ conductors[58]. It is interesting to note that Mg^{2+}-, Ca^{2+}- and (Na^+ + K^+)-ATPase activities have been found to be different from alkaline phosphatase activity in the rat intestine in contradiction to the previous belief that the ATPase and the alkaline phosphatase were the same enzyme[59].

Ethanol was found to inhibit rat intestine basolateral membrane (Na^+ + K^+) -ATPase and this inhibition was associated with decreased thiamine transport[60]. However, Matsuda *et al*[57] found there was no change in the (Na^+ + K^+)-ATPase activity on ethanol administration. Chronic alcohol administration may impair the ability of rats to metabolize alcohol and such impairment is worsened by the deficiency state[61].

The metabolites of thiamine are numerous. In rats the compounds 3-(2'-methyl -4'-amino-5'-pyrimidylmethyl)-4-methylthiazole-5-acetic acid, 2-methyl-4-amino

-5-formylaminomethylpyrimidine and 5-(2-hydroxyethyl)-4-methylthiazole have been identified[62].

Role in Neuromuscular Transmission

The role of thiamine and derivatives in neuromuscular transmission has enjoyed more attention and elucidation. Thiamine interacts *in vitro* with acetylcholine, norepinephrine and serotonin yielding molecular complexes[63]. It also binds with cocaine, norcocaine, benzoylnorecgonine, benzoylecgonine, nicotine, caffeine, p-hydroxy-norephedrine[64]. Thiamine and derivatives inhibit acetylcholinesterase activity, and oxythiamine and thiamine release acetylcholine from *Torpedo* electric organ[65]. Pyrithiamine impairs neurotransmission in rat masseter muscle via a frequency-dependent mechanism. The effect of pyrithiamine and fern extract is reversed by thiamine. Oxythiamine has no effect on the rat masseter muscle's neurotransmission. Thiamine is released from the curarized cat phrenic nerve diaphragm when the phrenic nerve is stimulated. Thiamine binds to isolated nicotinic acetylcholine receptor from *Torpedo marmorata* and to nicotinic acetylcholine receptors of the frog end plate. Incorporation of thiamine into membrane proteins in squid giant axon has been studied[66].

Thiamine deficiency in cultured glial cells impairs their ability to synthesize fatty acids and cholesterol. The effect is related to reduced formation of key lipogenic enzymes. These changes could be the basis for degenerative changes seen in glial cells in early thiamine deficiency, e.g., 3-hydroxy-3-methylglutaryl CoA reductase—enzyme at the rate limiting step for cholesterol biosynthesis[67].

Ascorbic acid has been found to activate thiamine diphosphatase in rat brain microsomes and inhibit ATPase activity[68,69]. The possible role of thiamine triphosphate in nervous transmission is of great interest[70,71]. Also the inhibitory effect of noradrenaline and L-DOPA on thiamine pyrophosphatase in nervous tissues is of great significance[72].

Thiamine inhibits the rate of carbamoylcholine-induced acetylcholine receptor-mediated ^{22}Na efflux in postsynaptic membrane from *Torpedo californica*[73]. A structure-function relationship between thiamine compounds' inhibition of acetylcholinesterase from *Electrophorus electricus* has been performed. The positively charged quarternary nitrogen seems to be of major importance in the inhibition[74].

Thiamine Antagonists

Bisulphite/sulphite cleavage of thiamine has received renewed interest with a newly proposed reaction mechanism in which the anion forms a covalent intermediate with thiamine by first attacking at the C-6' position. In this scheme, the products, (6-amino-2-methylpyrimid-5-yl) methanesulphonic acid and 5-β-hydroxyethyl-4-methylthiazole, are then formed by the thiazole moiety acting as the leaving group and the final pyrimidyl-methanesulphonic acid product being formed by a second sulphite ion's attack on the "methylene bridge" carbon and eliminating the first bonded

sulphite in the process[75,76]. The finding that the rate of reaction is dependent on the square of [bisulphite] at low concentrations of bisulphite supports the above mechanism[77]. Regarding further elucidation of contributing factors in this cleavage reaction a number of thiamine derivatives has been synthesized and the kinetics have been studied[78]. A detailed kinetic study of thiamine cleavage by bisulphite was performed earlier[79,80]. The rate of cleavage of biological thiamine phosphates follows the order thiamine > TMP > TDP > TTP from pH 4.0 to 7.0 Also the Arrhenius activation energy was found to be the same from 30–60°C at pH 5.2[81]. A similar mode of thiamine cleavage by the thiaminase enzyme has been proposed[75,82].

Thiamine modification by hemin and hemoproteins[83,84], thiamine destruction by sodium nitrite[85] and hypochlorite in tap water[86] have been studied. Two good reviews of thiamine modification by various factors and compounds have recently appeared[38,87].

Nutritional studies have led to kinetic investigations of para-dihydroxy and ortho–di– and –trihydroxy phenolic compounds with respect to their thiamine modifying abilities[88-95]. The extremely rapid initial rate in the thiamine-polyphenol reaction found by several workers has been proved to arise from redox interference with the assay agent, $K_3Fe(CN)_6$, used for the conversion of thiamine to thiochrome, the fluorescence of which is used to reflect the concentration of intact thiamine[96,97]. A more explicit detailed mechanism of reaction has recently been proposed[98]. A form with an open thiazole ring exists in equilibrium with intact thiamine at higher pH[99]. Oxygen oxidizes and helps polymerize the ortho– and para-polyphenols. The semiquinones and quinones oxidize the "open" thiamine to thiamine disulphide. Oxidation and hydrolysis of the open form and/or thiamine may lead to more extensively degraded products of thiamine. Hemin, H_2O_2 and I_2 seem to affect thiamine in a similar way.

Evidence has accumulated in our laboratory to show that high concentrations of tannic acid can precipitate thiamine and its mono– and diphosphate derivatives. The presence of Ca^{2+} and Mg^{2+} helps increase the rate and amount of precipitation. Ascorbic, tartaric and citric acid can reduce this divalent-cations' promotion of tannic acid-thiamine precipitation (our unpublished results).

Assays of Thiamine and Derivatives

Chemical assays of thiamine have recently been based on its oxidation to thiochrome by $K_3Fe(CN)_6$, $HgCl_2$ and CNBr. The thiochrome may be extracted by an organic solvent and its fluorescence measured. Various variations on the basic assay technique have been proposed. Pre-extraction of samples to elminate endogenous fluorescence, internal compensation by measurement of non-oxidized and oxidized samples and preparation of appropriate standard curves have been attempted[100-105]. The decalso (zeolite) material may be used or omitted in various adaptations[105-112]. In a test for susceptibility to redox interference, it was found that CNBr is the least affected and $HgCl_2$ and $K_3Fe(CN)_6$ are more easily interfered with this way[113]. Various chromatographic and electrophoretic methods have been proposed for separation and quantitation of thiamine and its synthetic and phosphorylated biological derivatives[114-117]. The recent use of high performance liquid chromato-

graphy shows promise of sensitivity and freedom from interference[117-121]. The enzyme assays for thiamine pyrophosphate have been widely and effectively used despite the initial difficulties in mastering the techniques[122, 123].

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บทคัดย่อ

ได้พิจารณาความรู้ใหม่ ๆ ทางเคมี และชีวเคมีของไธอะมีน โดยเฉพาะอย่างยิ่ง ด้านโครงสร้าง, การสังเคราะห์ในธรรมชาติ, หน้าที่ทางชีววิทยา และวิธีการวิเคราะห์ปริมาณ