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Adenylate kinase 1 knockout mice have normal thiamine triphosphate levels

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Abstract

Thiamine triphosphate (ThTP) is found at low concentrations in most animal tissues and it may act as a phosphate donor for the phosphorylation of proteins, suggesting a potential role in cell signaling. Two mechanisms have been proposed for the enzymatic synthesis of ThTP. A thiamine diphosphate (ThDP) kinase (ThDP + ATP ⇔ ThTP + ADP) has been purified from brewer's yeast and shown to exist in rat liver. However, other data suggest that, at least in skeletal muscle, adenylate kinase 1 (AK1) is responsible for ThTP synthesis. In this study, we show that AK1 knockout mice have normal ThTP levels in skeletal muscle, heart, brain, liver and kidney, demonstrating that AK1 is not responsible for ThTP synthesis in those tissues. We predict that the high ThTP content of particular tissues like the *Electrophorus electricus* electric organ, or pig and chicken skeletal muscle is more tightly correlated with high ThDP kinase activity or low soluble ThTPase activity than with non-stringent substrate specificity and high activity of adenylate kinase.

Keywords: Thiamine triphosphate; Adenylate kinase; Thiamine triphosphatase; Skeletal muscle

1. Introduction

Thiamine triphosphate (ThTP) occurs in most animal tissues [7] and recent data suggest that it may act as a phosphate donor for the phosphorylation of proteins. For instance, in *Torpedo* electric organ, ThTP is specifically used for the phosphorylation of histidyl residues of rapsyn [20], a protein required for the clustering of nicotinic receptors at the neuromuscular junction [12]. ThTP is also the donor for phosphorylation of as yet unidentified proteins in rodent brain [20]. In isolated patches of neuroblastoma cells, ThTP activates a high conductance chloride channel possibly by phosphorylation [2]. Thus, protein phosphorylation by ThTP could be part of a new phosphorylation signal conduction pathway.

Although ThTP generally accounts for less than 1% of total thiamine, a few tissues such as pig skeletal muscle [11], electric organ of *Electrophorus electricus* [4] and chicken white muscle [18] contain even more ThTP than the cofactor thiamine diphosphate (ThDP). Shikata et al. [27] have suggested that this high ThTP content is correlated with the high content of adenylate kinase (AK; EC 2.7.4.3) in skeletal muscle and electric organ. Indeed, these authors have shown that, in vitro, the adenylate kinase isoform 1 (AK1) may synthesize ThTP according to the reaction

$$\mathrm{ThDP} + \mathrm{ADP} \Leftrightarrow \mathrm{ThTP} + \mathrm{AMP} \quad (1)$$

The same group suggested that this enzyme was also responsible for ThTP synthesis in chicken white muscle in vivo [18,28]. However, the significance of this reaction in

Abbreviations: GPS, gastrocnemius–plantaris–soleus muscle complex; TCA, trichloroacetic acid; ThDP, thiamine diphosphate; ThMP, thiamine monophosphate; ThTP, thiamine triphosphate; ThTPase, thiamine triphosphatase

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vivo can be questioned as the conversion of ThDP to ThTP catalyzed by AK1 in vitro has a sharp pH profile with an optimum at 10 [26]. Moreover, physiological concentrations of free ThDP in the cytosol are less than 10 μM [8], much lower than the K_m for ThDP reported for AK1 (830 μM).

In nerve cells and hepatocytes, the main mechanism for ThTP synthesis, as originally proposed by Eckert and Möbus in 1964 [10] is thought to be:

$$\mathrm{ThDP} + \mathrm{ATP} \Leftrightarrow \mathrm{ThTP} + \mathrm{ADP} \quad (2)$$

This reaction is presumably catalyzed by a ThDP kinase (ThDP:ATP phosphoryltransferase; EC 2.7.4.15) but, until now, the enzyme remains poorly characterized. A purification procedure from baker's yeast has been described [9], but the enzyme's specific activity was very low. A similar ThDP kinase was also partially purified from rat liver [29].

Nishino et al. [21] reported the purification of a ThDP kinase from beef bovine brain but, in this case, the substrate was protein-bound ThDP rather than free ThDP. In any event, the specific enzymatic activity of this enzyme preparation was so low that its biological relevance is doubtful. Concerning the in vivo synthesis of ThTP, previous results suggest that it is fairly rapid in the mammalian brain. Using crude rat brain synaptoneurosomes [6], we observed a synthesis of ThTP occurring in a few minutes in the presence of ThDP and ATP. Yet, ThTP did not accumulate, presumably because of rapid hydrolysis by a specific thiamine triphosphatase (ThTPase, EC 3.6.1.28) present in the brain [16]. Using cultured neuroblastoma cells, we also observed that incubation in the presence of ^{14}C-thiamine resulted in rapid incorporation of radioactivity into cellular ThTP [1]. Likewise, when labeled thiamine was injected to rats intraperitonelly, a relatively rapid incorporation into brain ThTP was observed [8]. These results suggest that intact brain cells can rapidly synthesize ThTP but, for unknown reasons, it has not been possible so far to obtain a soluble enzyme that catalyzes ThTP synthesis at an appreciable rate. On the other hand, the presence of ThTPase appears to prevent any significant net synthesis of ThTP in intact cells or crude extracts [1,6].

Taken together, these studies suggest that the intracellular concentration of ThTP is highly regulated, especially in neurons, and that a specific ThTPase plays a critical role in this regulation. We have recently characterized a specific ThTPase [15,16] which is widely distributed in mammalian tissues. For this protein, no sequence homology with any other known protein or hypothetical protein sequence deduced from expressed sequence tags could be established [15].

Before starting a detailed investigation of the enzymatic mechanism(s) of ThTP synthesis, it was important to rule out the possibility that ThTP might simply be a by-product of adenylate kinase activity. Here, we show that AK1 knockout and control mice have the same ThTP levels in all tissues examined, even in those rich in AK1, such as heart and skeletal muscle. Our results thus indicate that AK1 is not responsible for ThTP synthesis in mice, supporting a dedicated role for ThTP-specific metabolizing enzymes.

2. Materials and methods

2.1. AK1-knockout and control mice

The homozygous AK1 deficient ($AK^{-/-}$) mice were generated as previously described [14]. Age and sex-matched wild-type animals with a 50–50% C57BL/6 × 129/Ola mixed inbred background (similar to that of the knockout mice) were used as controls. Tissues, one liver lobe, left kidney, heart, whole brain or gastrocnemius–plantaris–soleus (GPS) muscle complex, were removed from freshly killed animals (3 months old) and snap-frozen in liquid nitrogen.

2.2. Determination of thiamine and its phosphate esters

Thiamine and thiamine phosphate esters were determined as previously described [3,5]. Samples of mouse tissues were homogenized in 5 volumes of 10% trichloroacetic acid (TCA) in a glass–glass homogenizer and centrifuged (5000 × g; 15 min). The supernatant was extracted with diethyl ether and thiamine derivatives were determined by HPLC [5]. The method of Peterson [23] was used for the determination of protein concentration.

The authenticity of endogenous ThTP was checked by enzymatic hydrolysis. To 70 μl of tissular TCA extract, we added 10 μl Bis–Tris–propane buffer (500 mM, pH 8.9), 10 μl $MgSO_4$ (50 mM) and 10 μl purified bovine ThTPase ([15], specific activity 1.7 μmol s^{-1} mg^{-1}). The mixture was incubated at 37 °C for 10 min and the reaction was stopped by addition of 20 μl TCA (72%). After extraction of the TCA by 3 × 1.5 ml diethyl ether, the extract was analyzed by HPLC [5].

2.3. Determination of adenylate activity

Tissue samples were homogenized with a teflon-glass Potter–Elvehjem homogenizer in 10 volumes of medium containing Hepes–Tris (20 mM, pH 7.5), 0.5 mM EDTA, 0.2 mM dithiothreitol and 0.2% NP-40. The total AK activity of the homogenate was measured by the method of Oliver [22] with slight modifications. The assay medium contained 40 mM K–Hepes, pH 7.6, 80 mM KCl, 5 mM MgO_2, 1 mM Na_2EDTA, 0.2 mM dithiothreitol, 16 mM D-glucose, 2.5 mM NaADP, 0.6 mM $NADP^+$, 5 U/ml hexokinase and 2 U/ml glucose-6-phosphate dehydrogenase. The reaction was initiated by

the addition of homogenate (40–200 μg of protein per ml).

2.4. Determination of ThTPase activity

Tissue samples were homogenized in a teflon-glass Potter–Elvehjem homogenizer in 10 volumes of Tris–Cl buffer (50 mM, pH 7.5) containing 150 mM KCl and 0.2 mM EDTA. After centrifugation (15,000 × *g*; 30 min), ThTPase activity was measured as previously described [15]. The reaction medium contained 70 μl Bis–Tris–propane buffer (50 mM, pH 8.7), 10 μl $MgCl_2$ (50 mM), 10 μl ThTP (1 mM) and 10 μl of the enzyme preparation at the appropriate dilution. After incubation (20 min, 37 °C), the reaction was stopped by addition of 500 μl of TCA 12%. After extraction with 3 × 1.5 ml diethyl ether, the amount of ThDP formed was estimated by HPLC [5].

2.5. Gel filtration

The chicken muscle extract was run on a Sephadex G-75 column (2.5 × 36 cm), equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.1 M NaCl and calibrated with molecular weight markers (Amersham Biosciences). The eluate (0.4 ml/min) was analyzed for the presence of ThTPase activity in the 20–40 kDa range.

2.6. Statistical analyses

Statistical analyses were made by ANOVA followed by the Fisher's protected LSD test for post hoc comparisons. If necessary, a multivariate analysis of variance (MANOVA) is carried out beforehand.

3. Results and discussion

It was previously shown that AK1 knockout had surprisingly mild effects on skeletal muscle and the heart, tissues where the enzyme is expressed at relatively high levels. AK1 deficiency had no overt effect on physiological performance, but was associated with a clear decrease in energetic efficiency [14]. In skeletal muscle (GPS complex), genetic ablation of the AK1 gene resulted in an almost complete loss of adenylate kinase activity (99.7% decrease measured by incorporation of ~ phosphoryl groups at the β-position of ADP), and no compensatory up-regulation of other AK genes was observed. Response to genetic stress did, however, result in a rewiring of phosphoryl transfer reactions, with increased flux through the glycolytic and creatine kinase pathways. In the heart of AK1 knockout mice, AK1 activity was reduced by 94%, leading to compromised energetic metabolism under hypoxia [25]. Remaining activity could be attributed to the presence of mitochondrial isoforms AK2 and AK3 in both types of muscle.

Here we compared total AK activity in homogenates of skeletal muscle, heart, brain, liver and kidney between mutant and control animals. As anticipated, total AK activity was always lower in $AK1^{-/-}$ mice than in age-matched wild-type controls (Fig. 1). The most dramatic difference was observed in skeletal muscle, where the AK1 form is known to be predominant. A very strong reduction in total AK activity was also observed in heart and brain, but not in liver and kidney of $AK1^{-/-}$ mice. The large difference observed in brain was unexpected, as it was previously reported that another member of the adenylate kinase family, AK2, is predominant in this tissue [24]. In liver, the drop in activity did not exceed 30%, suggesting that the fractional contribution to total activity by mitochondrial AK2 and/or AK3, or any other AK-isoform, is high in this organ.

The content of thiamine compounds found in different tissues of mice is given in Table 1. Comparison between $AK1^{-/-}$ and wild-type mice showed no significant differences in the levels of thiamine derivatives (MANOVA), despite the dramatic decrease in AK1 activity in muscle and brain of knockout mice compared with the controls. The lack of difference in ThTP content is most conspicuous for skeletal muscle, where AK levels differ about 100-fold between controls and mutants. The authenticity of skeletal muscle ThTP was tested by hydrolysis with purified bovine brain ThTPase and found to be completely normal (not shown).

Remarkably, significant large variations were observed for the tissue concentrations of thiamine, ThMP, ThDP and ThTP between different tissues. Analysis by two-factor ANOVA confirmed these differences for all four thiamine compounds. For ThTP, the highest content was found in liver and the lowest in heart. ThDP was most abundant in the heart and occurred at 4-fold lower concentration in skeletal muscle. Thiamine monophosphate (ThMP) was the major thiamine compound in kidney and liver, but the significance of this finding is unclear. Free thiamine always represented

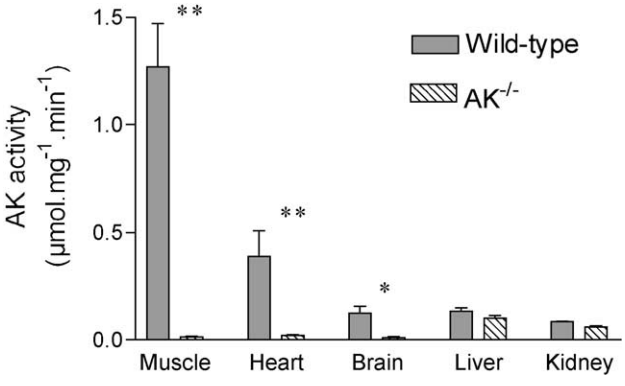

Fig. 1. Comparison of adenylate kinase activity in tissues of AK1 proficient (wild-type; $AK1^{+/+}$) and deficient ($AK1^{-/-}$) mice. The results are expressed as mean ± SD for three animals. A two-factor ANOVA revealed significant effects of genotype [$F(4, 20) = 62.12$, $P < 0.0001$], tissue [$F(1, 20) = 170.56$, $P < 0.0001$], and genotype × tissue interaction [$F(4, 20) = 71.94$, $P < 0.0001$]. Bonferroni post-test: **, $P < 0.001$; *, $P < 0.01$.

Table 1
Content of thiamine derivatives in normal (wild-type, wt) and AK1 knockout mice

Tissue[a]	Genotype	Mean (nmol/g wet weight) ± SD ($n=5$)				
		Thiamine[b]	ThMP[c]	ThDP[d]	ThTP[e]	Total
Brain	wt	0.44 ± 0.16	2.1 ± 0.3	7.8 ± 1.7	0.009 ± 0.003	10.4 ± 1.4
	$AK^{-/-}$	0.37 ± 0.10	1.9 ± 0.1	7.6 ± 1.5	0.014 ± 0.010	9.9 ± 1.5
Liver	wt	2.2 ± 0.5	17.2 ± 1.2	13.1 ± 1.4	0.14 ± 0.07	32.6 ± 1.9
	$AK^{-/-}$	1.7 ± 0.8	17.1 ± 2.2	11.4 ± 2.3	0.14 ± 0.03	30.3 ± 3.8
GPS muscle complex	wt	0.16 ± 0.02	0.49 ± 0.05	6.7 ± 0.7	0.026 ± 0.006	7.4 ± 0.7
	$AK^{-/-}$	0.21 ± 0.07	0.41 ± 0.05	6.7 ± 1.2	0.026 ± 0.008	7.3 ± 1.2
Kidney	wt	2.8 ± 0.5	17.8 ± 3.3	14.5 ± 2.1	0.018 ± 0.010	35.1 ± 4.7
	$AK^{-/-}$	2.1 ± 0.4	16.3 ± 2.1	11.1 ± 2.2	0.009 ± 0.004	29.5 ± 4.2
Heart	wt	0.35 ± 0.03	8.7 ± 1.0	26.6 ± 1.9	0.004 ± 0.002	35.7 ± 1.3
	$AK^{-/-}$	0.29 ± 0.05	6.0 ± 1.0	28.1 ± 6.0	0.008 ± 0.004	34.4 ± 6.3

[a] Overall significant effect of tissue by MANOVA (Wilks' $\lambda=0.001$, $P=0.0001$).
[b] Two-factor ANOVA $F(4, 40)=88.644$, $P<0.001$.
[c] Two-factor ANOVA $F(4, 40)=279$, $P<0.001$.
[d] Two-factor ANOVA $F(4, 40)=111$, $P<0.001$.
[e] Two-factor ANOVA $F(4, 40)=48{,}21$, $P<0.001$.

less than 10% of the total thiamine content in all tissues examined.

Our results demonstrate that AK1 proficiency or deficiency has no effect on the relative concentrations of Th, ThMP, ThDP and ThTP in any given tissue and does also not affect the tissue distribution profile of thiamine derivatives in vivo. This provides a strong argument against a role for AK in thiamine phosphorylation reactions. Though it has been demonstrated that AK1 will catalyze ThTP synthesis in vitro (reaction (1)), the high K_m for ThDP as well as the alkaline pH optimum for this reaction argue against a role under physiological conditions in vivo. In rat brain, most of the cytosolic ThDP is bound to transketolase and its free cytosolic concentration is only about 2–3 μM [1,8], much too low for a K_m of 830 μM.

Shikata et al. [26] reported that the rate of ThTP synthesis by purified skeletal muscle AK1 was 16 nmol/mg of protein in 1 h at physiological pH and 100 μM ADP and ThDP. Taking into account that the intracellular free ThDP concentration is about 2–3 μM and that the AK1 concentration is approximately 3 μM, we could calculate that it would take 28 days to reach an intracellular ThTP concentration close to 20 μM in pig skeletal muscle (Table 2), and this supposing that during this time, there is no hydrolysis.

These results are not in favour of the hypothesis that AK1 is responsible for ThTP synthesis. Based on our findings, it could still be argued that not the cytosolic AK1 but one of the mitochondrial isoforms would be responsible for ThTP synthesis. This is however not very likely, as in those tissues where ThTP is the most abundant, such as pig [11] and chicken skeletal muscle [18] or *Electrophorus* electric organ [4], AK1 is largely predominant and ThTP is essentially cytosolic. In brain, the amount of mitochondrial AK seems far too low to explain the rapid rate of ThTP synthesis. In liver, where mitochondrial AK is more abundant, we cannot exclude that the latter might be responsible for part of the ThTP synthetic capacity. However, most of the ThTP in hepatocytes was found to be cytosolic [17].

Our observations therefore suggest that ThTP is synthesized through a specific mechanism, presumably involving a ThDP kinase according to reaction (2). The characterization of this enzyme will be the subject of future investigations. We previously suggested the existence of two ThDP pools [1,8], the first essentially mitochondrial, associated with ThDP-dependent enzymes, and the second, cytosolic, precursor of ThTP. It is therefore highly probable that quick signaling events such as histidine phosphorylation [20] may require brief momentaneous flux alterations through the latter reaction, in response to as yet unknown signals.

Electrophorus electric organ, pig skeletal muscle and chicken muscle have a ThTP content two orders of magnitude higher than mouse muscle (Table 2). It is clear that the slight differences in AK content in pig, chicken and wild-type mouse skeletal muscle (Table 2) cannot account for

Table 2
ThTP content, ThTPase and adenylate activities in various animals ($n=3$)

	ThTP content (nmol/g of wet weight)	ThTPase activity (nmol g^{-1} of tissue min^{-1})	Adenylate kinase activity (μmol mg^{-1} min^{-1})
E. electricus electric organ	3.9 ± 0.5[a]	nd[b]	–
Pig skeletal muscle	20 ± 2, 18.24 ± 5.83[c]	12.5 ± 0.5	1.03 ± 0.15
Chicken white muscle (pectoralis)	3.2 ± 0.4, 1.2 ± 0.15[d]	nd[e]	1.6 ± 0.1
Mouse GPS skeletal muscle	0.026 ± 0.006	302 ± 54	1.3 ± 0.2

[a] According to Ref. [4].
[b] Below the detection limit.
[c] According to Ref. [11].
[d] According to Ref. [18], assuming that 1 g of muscle contains 171 mg of protein.
[e] After chromatography on a Sephadex G-75 column.

such large differences in ThTP content. As pointed out above (see Introduction), the intracellular ThTP concentrations seems to be tightly regulated in most tissues and the presence of a specific ThTPase is probably a key factor of this regulation. We thus suspected that the very high ThTP content of the abovementioned tissues might be linked to an abnormally low expression of this enzyme. We therefore determined the activity of the 25 kDa specific soluble ThTPase in *E. electric* organ as well as in chicken and mouse skeletal muscle. As shown in Table 2, the activity was more than 20-fold lower in pig than in mouse skeletal muscle. In non-mammalian tissues (*E. electricus* or chicken), the enzyme appeared to be totally absent.

In the chicken muscle homogenate, ThTP was rapidly hydrolyzed, but this hydrolysis was most probably due to myosin, which has previously been shown to be able to hydrolyze ThTP [13,19]. When the extract was chromatographed on a Sephadex G-75 column, no ThTPase activity was detected in the 20–40 kDa range, suggesting that the 25 kDa specific ThTPase is absent or has a very low activity in chicken muscle. It is to be noted here that we did not observe any difference in ThTPase activity between wild-type and $AK^{-/-}$ mice (not shown).

Although it is not excluded that the rate of ThTP synthesis may also differ from tissue to tissue, our data suggests an inverse correlation between the ThTP content of a tissue and soluble ThTPase activity. Together with the fact that this enzyme has a virtually absolute substrate specificity for ThTP and a high catalytic efficiency ($k_{cat}/K_m = 6 \times 10^6$ s^{-1} M^{-1}) [15], these observations strongly support a determining role for ThTPase in the regulation of intracellular ThTP levels. In mouse tissues (Table 1), and in many other mammalian tissues [7], mean ThTP levels are very low, suggesting that protein phosphorylation by ThTP is triggered by an increase in ThTP concentration, only under certain conditions and perhaps in particular cell populations. As ThTP is continuously newly synthesized [1,8], there may be an important role for (transient) down-regulation of ThTPase activity in this specific process.

In conclusion, our results indicate that AK1 is not involved in ThTP synthesis in vivo. The cytosolic ThTP concentration in various tissues may therefore be predominantly regulated by the soluble 25 kDa ThTPase.

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