nature chemical biology

Discovery of a natural thiamine adenine nucleotide

Lucien Bettendorff[1,4], Barbara Wirtzfeld[1,4], Alexander F Makarchikov[1], Gabriel Mazzucchelli[2], Michel Frédérich[3], Tiziana Gigliobianco[1], Marjorie Gangolf[1], Edwin De Pauw[2], Luc Angenot[3] & Pierre Wins[1]

Several important cofactors are adenine nucleotides with a vitamin as the catalytic moiety. Here, we report the discovery of the first adenine nucleotide containing vitamin B1: adenosine thiamine triphosphate (AThTP, 1), or thiaminylated ATP. We discovered AThTP in *Escherichia coli* and found that it accumulates specifically in response to carbon starvation, thereby acting as a signal rather than a cofactor. We detected smaller amounts in yeast and in plant and animal tissues.

Until now, only three natural thiamine phosphate derivatives (the mono-, di- and triphosphates) were known to be present in most cells[1], and of these only thiamine diphosphate (ThDP, 2) has a well-known cofactor role[2]. We previously showed that *E. coli* accumulate thiamine triphosphate (ThTP, 3) when grown in minimal medium supplemented with a carbon source such as glucose[3] (4) (**Fig. 1a**). However, in the absence of a carbon source no ThTP was detected, and instead we noticed the appearance of an additional peak eluted after ThTP (**Fig. 1b**).

We purified this compound using different chromatographic steps (**Supplementary Table 1** online). We checked the purity by HPLC (**Supplementary Fig. 1** online), and then we collected the peak, lyophilized it and used it for mass spectrometry and ^{1}H NMR.

High-resolution ESI-FT-ICR MS gave an *m/z* of 754.097486, which corresponds to the formula $C_{22}H_{31}N_9O_{13}P_3S^+$ (theoretical monoisotopic mass of 754.096939) at sub-p.p.m. mass accuracy (**Supplementary Fig. 2** online). ESI-MS/MS fragmentation (**Supplementary Fig. 2**) suggested the presence of an AMP moiety (*m/z* of 348.1). These data are in agreement with a structure containing a thiamine moiety and an adenosine moiety linked by three phosphates—that is, adenylated ThDP (AThTP) or thiaminylated ATP (**Fig. 1c**). We confirmed the presence of the thiamine moiety and the adenosine moiety by ^{1}H NMR. Comparison with the spectra of commercial 5′-AMP (5) and 3′-AMP (6) indicated that the 5′-hydroxyl group rather than the 3′-hydroxyl group is substituted in AThTP (**Supplementary Fig. 3** online).

We further confirmed the structure of AThTP through chemical synthesis by condensation of ThDP and 5′-AMP using dicyclohexylcarbodiimide as an activator[4] (**Supplementary Methods** online). We purified the synthesized compound as described for the natural product and confirmed the identity with AThTP isolated from bacteria by high-resolution ESI-FT-ICR MS (*m/z* = 754.096862), tandem ESI-MS/MS (**Supplementary Fig. 2**) and ^{1}H NMR (**Supplementary Fig. 3**). The ^{13}C NMR spectrum of the synthetic compound (**Supplementary Fig. 3**) confirmed the proposed structure of AThTP. The purified natural AThTP and the chemically synthesized AThTP were hydrolyzed at the same rate by an enzyme present in a bacterial membrane fraction (**Supplementary Fig. 4** online), thereby confirming their identity.

When *E. coli* were incubated in minimal medium devoid of any carbon source, AThTP gradually accumulated and reached a maximum concentration after 4 h (**Fig. 2a**), at which point it accounted for 10–15% of total thiamine in the cells. Subsequent addition of glucose (10 mM) induced a rapid disappearance of AThTP followed by a transient accumulation of ThTP (**Fig. 2b**). When malate (7) was added instead of glucose, the kinetics of AThTP disappearance were slower (half-time ~ 20 min), and, in agreement with previous results[3], no ThTP was formed. When the bacteria were directly

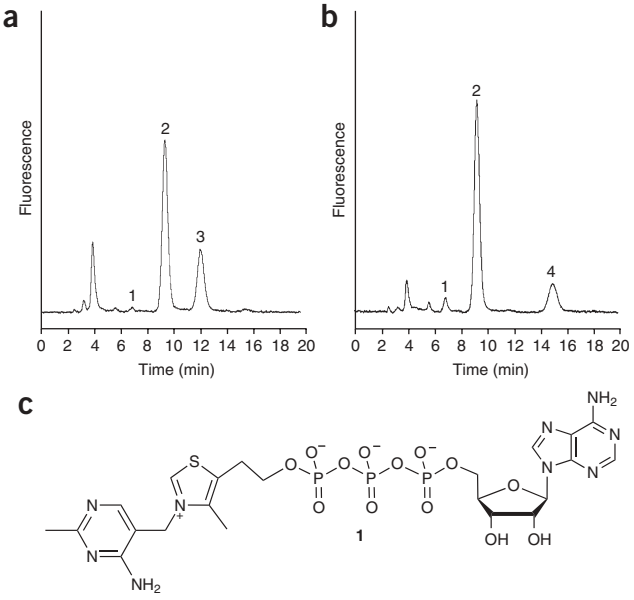

Figure 1 Chromatograms showing the presence of ThTP or the additional peak, according to culture conditions. The bacteria were grown overnight in LB medium and suspended in minimal M9 medium (**Supplementary Methods**). (**a**,**b**) They were then incubated (1 h, 37 °C) in M9 medium in the presence (**a**) or absence (**b**) of 10 mM glucose. Thiamine derivatives were determined by HPLC after oxidation to fluorescent thiochrome derivatives[8] (1, ThMP; 2, ThDP; 3, ThTP; 4, additional peak or AThTP). (**c**) Structure of AThTP.

[1]Center for Cellular and Molecular Neurobiology, University of Liège, Avenue de l'Hôpital 1, B-4000 Liège, Belgium. [2]Department of Physical Chemistry, University of Liège, Allée de la Chimie 3, B-4000 Liège, Belgium. [3]Laboratory of Pharmacognosy, Drug Research Center, University of Liège, Avenue de l'Hôpital 1, B-4000 Liège, Belgium. [4]These authors contributed equally to this work. Correspondence should be addressed to L.B. (l.bettendorff@ulg.ac.be).

Received 21 November 2006; accepted 12 February 2007; published online 4 March 2007; doi:10.1038/nchembio867

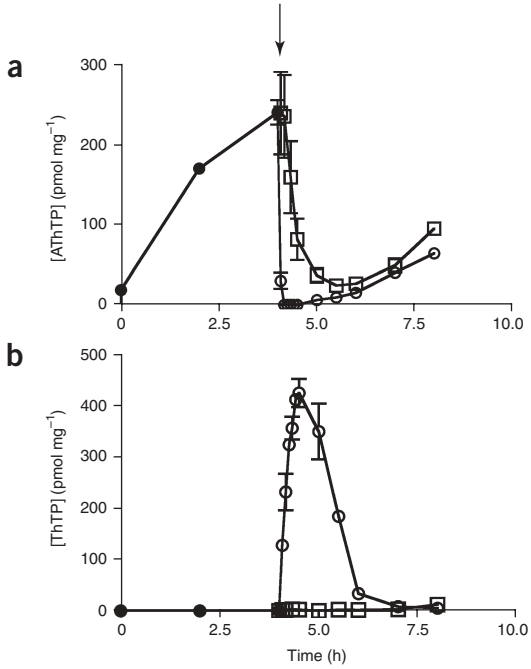

Figure 2 Effect of substrate addition on the content of AThTP and ThTP in *E. coli* BL-21 cells. (**a**,**b**) The bacteria were first incubated at 37 °C in M9 medium without a carbon source (●), and the concentrations of AThTP (**a**) and ThTP (**b**) were determined. After 4 h, glucose (○) or malate (□) was added at a final concentration of 10 mM (arrow). The results are expressed as mean ± s.d. for three experiments.

transferred from the LB medium to the minimal medium supplemented with glucose (10 mM), no significant amount of AThTP was formed. With other energy substrates such as pyruvate (8), lactate (9) and malate, AThTP concentrations also remained insignificant (**Supplementary Table 2** online).

These results suggest that AThTP is accumulated specifically in response to carbon starvation. This response was not appreciably modified by the presence or absence of a phosphate or nitrogen source (**Supplementary Table 2**). This specificity for carbon starvation differs from the synthesis of guanosine tetraphosphate (10) and guanosine pentaphosphate (11), which is known to trigger the so-called stringent response when the growth medium becomes deficient in either amino acids, carbon, phosphorus or nitrogen[5,6]. ThTP and AThTP seem to be more specific to particular types of stress: ThTP is produced in response to amino acid starvation, and a carbon source is required for its accumulation[3]. In contrast, AThTP specifically appears in response to a lack of any carbon source. Note that we never observed the simultaneous accumulation of ThTP and AThTP in large amounts, and both compounds were absent under most normal conditions of growth, such as in the presence of amino acids or in rich LB medium. Moreover, ThTP accumulation was always transient[3], whereas AThTP remained present as long as the cells were carbon starved (**Fig. 2a**).

AThTP is also present in eukaryotic cells. We found AThTP in yeast, in the roots of higher plants and in several animal tissues (**Supplementary Table 3** online). In the rat, it was present in most organs tested; the highest concentrations were found in the liver, heart, kidney and lung, and the lowest in skeletal muscle and brain. AThTP, much like ThTP, was present at much lower concentrations in eukaryotic organisms than in *E. coli*. It is possible that both compounds accumulate only in specific cells or organelles, under conditions that have not yet been defined in eukaryotes.

Among all the nucleotides found in living cells, adenine nucleotides are the most abundant and diverse. In coenzyme A (12), FAD (13), NAD^+ (14) and $NADP^+$ (15), the catalytic moiety is a vitamin: pantothenic acid, riboflavin or nicotinamide. Here, we report the discovery of the first nucleotide containing thiamine. Recently, an ADP-containing intermediate in the biosynthesis of the thiamine thiazole was described in eukaryotes[7]. The choice of the name adenosine thiamine triphosphate rather than thiamine ATP emphasizes its apparent close metabolic relationship to ThTP. The present data suggest that, at least in *E. coli*, AThTP acts as a signal rather than as a cofactor. Our findings highlight the diversity of thiamine biochemistry.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

ACKNOWLEDGMENTS

This work was supported by grant 2.4558.04 from the Fonds de la Recherche Fondamentale Collective and by grants from the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture to B.W. and M.G. M.F. and L.B. are respectively Research Associate and Senior Research Associate at the Fonds de la Recherche Scientifique–FNRS. The authors thank D. Baiwir for help with MS and I. Margineanu for helpful comments on the manuscript.

AUTHOR CONTRIBUTIONS

L.B. is the project leader and performed purification of the bacterial AThTP and chemical synthesis and purification of AThTP. B.W. discovered the AThTP peak on the chromatograms, performed part of the experiments with bacteria and started the purification. A.F.M. helped to identify AThTP and studied its enzymatic synthesis and hydrolysis. G.M. and E.P. performed MS experiments. M.F and L.A. performed NMR experiments. T.G. performed part of the experiments with bacteria. M.G. measured AThTP in animal tissues. P.W. helped to design the experiments and write the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturechemicalbiology
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Makarchikov, A.F. *et al. Cell. Mol. Life Sci.* **60**, 1477–1488 (2003).
2. Wille, G. *et al. Nat. Chem. Biol.* **2**, 324–328 (2006).
3. Lakaye, B., Wirtzfeld, B., Wins, P., Grisar, T. & Bettendorff, L. *J. Biol. Chem.* **279**, 17142–17147 (2004).
4. Bettendorff, L., Nghiêm, H.O., Wins, P. & Lakaye, B. *Anal. Biochem.* **322**, 190–197 (2003).
5. Cashel, M. & Gallant, J. *Nature* **221**, 838–841 (1969).
6. Artsimovitch, I. *et al. Cell* **117**, 299–310 (2004).
7. Chatterjee, A., Jurgenson, C.T., Schroeder, F., Ealick, S.E. & Begley, T.P. *J. Am. Chem. Soc.* **128**, 7158–7159 (2006).
8. Bettendorff, L., Peeters, M., Jouan, C., Wins, P. & Schoffeniels, E. *Anal. Biochem.* **198**, 52–59 (1991).