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An alternative role of F_oF_1-ATP synthase in *Escherichia coli*: synthesis of thiamine triphosphate

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In *E. coli*, thiamine triphosphate (ThTP), a putative signaling molecule, transiently accumulates in response to amino acid starvation. This accumulation requires the presence of an energy substrate yielding pyruvate. Here we show that in intact bacteria ThTP is synthesized from free thiamine diphosphate (ThDP) and P_i, the reaction being energized by the proton-motive force (Δp) generated by the respiratory chain. ThTP production is suppressed in strains carrying mutations in F_1 or a deletion of the *atp* operon. Transformation with a plasmid encoding the whole *atp* operon fully restored ThTP production, highlighting the requirement for F_oF_1-ATP synthase in ThTP synthesis. Our results show that, under specific conditions of nutritional downshift, F_oF_1-ATP synthase catalyzes the synthesis of ThTP, rather than ATP, through a highly regulated process requiring pyruvate oxidation. Moreover, this chemiosmotic mechanism for ThTP production is conserved from *E. coli* to mammalian brain mitochondria.

Thiamine (vitamin B1) is an essential compound for all known life forms. In most organisms, the well-known cofactor thiamine diphosphate (ThDP) is the major thiamine compound. Free thiamine and thiamine monophosphate (ThMP), which have no known physiological function, account for only a few percent of the total thiamine content. In addition, many organisms also contain small amounts of triphosphorylated thiamine derivatives, such as thiamine triphosphate (ThTP)[1–3] and the recently discovered adenosine thiamine triphosphate (AThTP)[4].

So far, the biological role of ThTP remains elusive, but it was recently shown that in vertebrate tissues ThTP can activate a large conductance anion channel[5] and phosphorylate certain proteins[6]. ThTP is not likely to act as a coenzyme (replacing ThDP), but may rather be part of an as yet unidentified cellular signaling pathway[2].

In mammalian cells, cellular concentrations of ThTP are generally kept relatively constant and low (0.1 to 1 μM) because it is continuously hydrolyzed by a specific 25-kDa cytosolic thiamine triphosphatase[7–9]. However, we have shown that, in the enterobacterium *E. coli*, the cellular ThTP content is highly dependent on growth conditions and on the composition of the medium: while ThTP is barely detectable when the cells grow exponentially in rich LB medium, it rapidly and transiently accumulates when the bacteria are transferred to a minimum medium devoid of amino acids, but containing a carbon source such as glucose[2]. When the specific human 25-kDa ThTPase was overexpressed in *E. coli*, ThTP did not accumulate after transfer to a medium devoid of amino acids and the bacterial growth displayed an intermediate plateau, suggesting that ThTP is required for the rapid adaptation of bacteria to amino acid starvation[2]. ThTP might thus be produced either through a very specific enzyme reaction or through a more general mechanism under tight regulatory control.

The mechanism of ThTP synthesis has been a long-debated question. A first mechanism involving a cytosolic ThDP:ATP phosphotransferase (ThDP kinase) has been proposed by several authors[10–14], but the reaction product was not well characterized and might have been AThTP rather than ThTP. Indeed, AThTP can be synthesized from ThDP and ATP (or ADP) by a cytosolic enzyme complex[15]. In our laboratory, despite numerous efforts, we have been unable to demonstrate ThTP synthesis by a mechanism involving a ThDP kinase.

On the other hand, Kawasaki and coworkers have shown that, in vertebrate skeletal muscle, ThTP can be produced through the reaction ThDP + ADP ⇆ ThTP + AMP catalyzed by adenylate kinase 1 (myokinase)[16,17]. However, the reaction is very slow and we have shown that adenylate kinase 1 knock-out mice have normal ThTP levels[18]. In *E. coli*, we found that the bacterial adenylate kinase could be responsible for a significant accumulation

of ThTP, but this was observed only when the enzyme was over-expressed. Furthermore, this synthesis occurred in the presence of amino acids, was not activated by glucose and was long-lasting, rather than transient[19,20]. Furthermore, ThTP is synthesized in high amounts in the *E. coli* CV2 strain after heat-inactivation of adenylate kinase[19]. Thus, a low-rate constitutive synthesis of ThTP might be a general property of adenylate kinases, but another mechanism for ThTP synthesis must exist. As we were unable to detect any ThDP kinase activity in cell-free extracts from *E. coli*, the enzyme responsible for ThTP production in response to amino acid starvation remains unidentified.

In a recent study[9], we proposed an alternative mechanism for ThTP synthesis in eukaryotic cells. In rat brain mitochondria, a rapid synthesis of ThTP was observed in the presence of ThDP, P_i and a respiratory substrate. The strong inhibitory effects of respiratory chain blockers, protonophores and oligomycin supported the proposal that ThTP synthesis occurred through the reaction ThDP + $P_i \leftrightarrows$ ThTP + H_2O, energized by the proton-motive force generated by respiratory chain complexes. This was the first demonstration that a high-energy phosphate compound other than ATP can be formed through a chemiosmotic mechanism. However, it was not clear whether F_oF_1-ATP synthase itself was the catalyst. The marked inhibition by oligomycin and DCCD strongly suggested the implication of the F_o proton channel but, in view of the structural dissimilarity between ThDP and ADP, the implication of F_1 in the catalytic mechanism remained uncertain. It could be an isoform preferentially binding ThDP or even a completely different enzyme, possibly functionally associated with F_o.

Here, we present evidence that F_oF_1-ATP synthase is the actual catalyst for ThTP synthesis in *E. coli* cells under physiologically relevant conditions. The chemiosmotic mechanism involved appears to be similar in both *E. coli* and rat brain mitochondria, suggesting that it has been conserved from bacteria to mammals. However, as ThTP is produced only under very particular conditions in *E. coli*, the catalytic activity of F_oF_1 must be subject to a specific (and still largely unknown) regulatory process in order to switch from its normal activity, i. e. ATP synthesis, to the production of a putative signaling molecule.

Results

ThTP is formed from an intracellular pool of free ThDP. In *E. coli* (BL21 and other strains) grown in LB medium, the total thiamine content is high (≥ 1 nmol per mg of protein). It is largely in the form of the coenzyme ThDP, but only 9% of the latter is protein-bound after separation on a molecular sieve. Most of the ThDP in the supernatant was eluted in the inclusion volume of the column (Supplementary Figure S1). Thus *E. coli* cells have an unusually high intracellular pool of free ThDP (intracellular concentration of about 250 μM).

As previously shown, cells transferred to minimal medium (devoid of amino acids) start to accumulate ThTP on addition of 10 mM glucose, the maximum intracellular concentrations of ThTP being reached after about 1 hour[2,4]. This maximum content amounted to about 20% of total thiamine in the BL21 and up to 60% in the CV2 strain. Accordingly, it was observed in both strains that the amount of ThDP had decreased by a corresponding proportion, the total thiamine content (essentially ThDP and ThTP) remaining constant (Figure 1). We have also shown that AThTP is produced from free intracellular ThDP[20]. Thus this pool appears to be used as a reservoir for the synthesis of triphosphorylated thiamine derivatives.

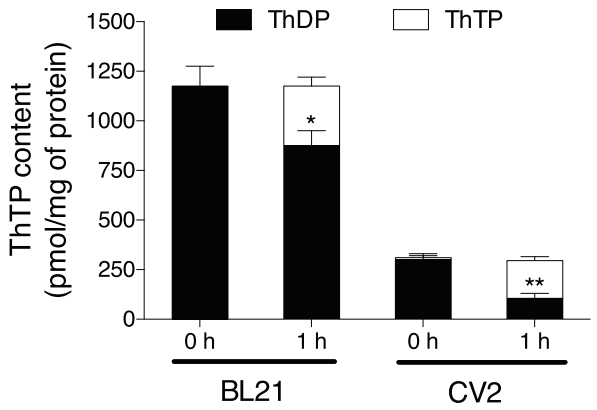

Figure 1 | ThTP is formed from ThDP. The bacteria (BL21 or CV2) were grown overnight in LB medium, transferred to minimal M9 medium and incubated at 37°C. Thiamine derivatives were determined at zero time and 1 h after addition of glucose (10 mM). In both strains, the ThDP content decreases after 1 h, but the total amount [ThDP] + [ThTP] remains constant. The results are expressed as mean ± SD for 3 experiments (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by the Dunnett post-test for comparison with ThDP levels at t = 0).

ThTP synthesis requires pyruvate oxidation through the Krebs cycle and the respiratory chain. In a previous work[2], we have shown that ThTP accumulation specifically requires a carbon source that can be converted to pyruvate, the best sources being glucose, mannitol, gluconate and pyruvate itself. Here we show that L-lactate (which can be readily converted to pyruvate) is also a good substrate for ThTP production.

We checked whether active aerobic metabolism is required for the synthesis of ThTP (Figure 2). In the presence of either glucose or L-lactate, significantly less ThTP appeared during O_2 deprivation (replaced by N_2), suggesting that O_2 is required for optimal ThTP synthesis (Figure 2).

KCN, an inhibitor of quinol oxidase bo3, was found to strongly inhibit ThTP production. Iodoacetate, which inhibits the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase[21], blocked ThTP synthesis only when glucose was the substrate. This was expected, as iodoacetate is supposed to block pyruvate formation

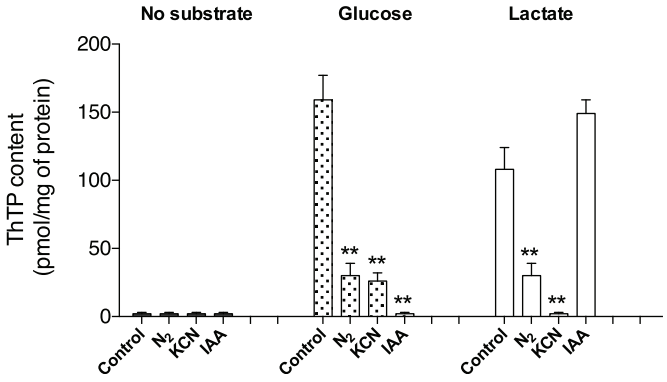

Figure 2 | Effect of metabolic inhibitors and anoxia on the ThTP content of BL21 cells. The bacteria were grown overnight in LB medium, transferred to minimal M9 medium and incubated 20 min at 37°C either in the absence of substrate or in the presence of D-glucose (10 mM) or L-lactate (10 mM). In each case, the control experiment was carried out in the presence of O_2. For growth in the absence of oxygen, the bacteria were incubated in sterile tubes with screw caps (Greiner Bio-One BVBA/SPRL) and the culture was sparged with N_2 for 1 min and the tubes were hermetically closed before incubation. N_2: oxygen replaced by nitrogen; KCN: 1 mM cyanide was added in the presence of O_2; IAA: 1 mM iodoacetete was added in the presence of O_2. (**, $p < 0.01$; *, $p < 0.05$: two-way ANOVA followed by the Dunnett test for comparisons with the respective control, Means ± SD, n =4).

from glucose. In contrast, iodoacetate was ineffective in the presence of lactate, which can still be converted to pyruvate.

These results suggest that ThTP synthesis requires the oxidation of pyruvate and electron flow through the respiratory chain. Under anoxia or in the presence of KCN, glycolytic activity is unable to support ThTP accumulation, even though ATP is produced in sufficient amounts. The requirement for pyruvate oxidation seems to indicate that a product such as acetyl-CoA or a downstream intermediate in the Krebs cycle is required for ThTP production. Presumably, this unidentified activator required for ThTP synthesis is not produced (or is produced only very slowly) when the oxidizable substrate is succinate or malate rather than pyruvate[2].

ThTP synthesis requires a proton-motive force. As the above results suggest that ThTP synthesis requires an electron flow through the respiratory chain, we wanted to test whether a proton-motive force was required, as it was found to be the case in rat brain mitochondria[9]. As shown in Figure 3a the protonophore CCCP indeed exerts a rapid and dramatic effect on ThTP accumulation in BL21 cells (this was also demonstrated with strains MG1655 and CV2, not shown). This strong effect was found using either glucose or lactate as substrate.

In order to confirm the requirement for a sufficiently high Δp, we tested the effects of ionophores such as valinomycin and nigericin. Valinomycin (an ionophore specific for K^+ ions) is known to collapse the membrane potential in the presence of external K^+. In contrast, nigericin is an electroneutral K^+/H^+ exchanger that collapses ΔpH in the presence of K^+. Both compounds (at 50 μM) had no or only a slight effect on ThTP and ATP levels in intact bacteria (not shown). When either of them was tested on O_2 consumption in the presence of glucose, their effect was less than 10%. This was likely due to the fact that those ionophores do not readily cross the outer bacterial membrane[22]. Therefore, we permeabilized the outer membrane with EDTA[23,24], and valinomycin or nigericin were used at 0, 22 and 64 mM external K^+ concentrations (Supplementary Table S2). The K^+ ionophore valinomycin (50 μM) inhibited over 90% of ThTP accumulation at 22 and 64 mM K^+, while it had no effect when all the K^+ was replaced by Na^+ in the medium. Note that in the complete absence of external K^+, ThTP production was lower than when K^+ was present. Nigericin (50 μM) had much less effect than valinomycin (50 μM). This may be because, in our experimental conditions, ΔpH is relatively small while Δψ is the essential component of Δp.

Although the above results suggest that Δp is required for ThTP synthesis, they do not rule out the possibility that ATP or ADP might also act as energy sources or phosphate donors. In order to study ThTP production in cells with very low ATP or ADP content, we used the CV2 strain, containing a heat-sensitive adenylate kinase. Inactivation of this enzyme at 37°C results in accumulation of AMP and very low levels of ADP and ATP[25]. In agreement with our previous results[19], we find that after 1 hour at 37°C, the ATP content of CV2 bacteria drops from 10–20 nmol per mg to 1 nmol per mg of protein. However, addition of CCCP induced a rapid stimulation of oxygen consumption, indicating that, despite the low energy charge (around 0.2), a significant proton-motive force can be maintained at 37°C. Furthermore, when CV2 cells are incubated in minimal medium containing glucose or lactate, they accumulate high amounts of ThTP (over 50% of total thiamine) at 37°C. As shown in Figure 4, addition of 50 μM CCCP after incubation for 1 hour in the presence of 10 mM L-lactate induced a rapid decrease in ThTP content. The drop was even faster at 37°C than at 25°C (at the latter temperature the adenylate kinase is stable and the energy charge is high). These

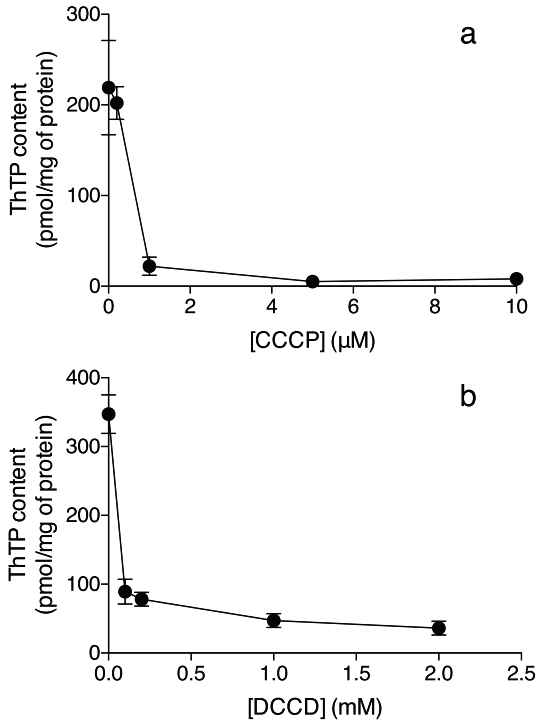

Figure 3 | Dose-dependent effects of CCCP and DCCD on intracellular ThTP content in the *E. coli* BL21 strain. The bacteria were grown overnight in LB medium, transferred to minimal M9 medium containing 10 mM D-glucose and incubated (37°C, 20 min) in the presence of CCCP (a) or DCCD (b) at the concentrations indicated. Stock solutions of CCCP and DCCD were made in dimethyl sulfoxide and used at a final solvent concentration of 1%. (Means ± SD, n = 3).

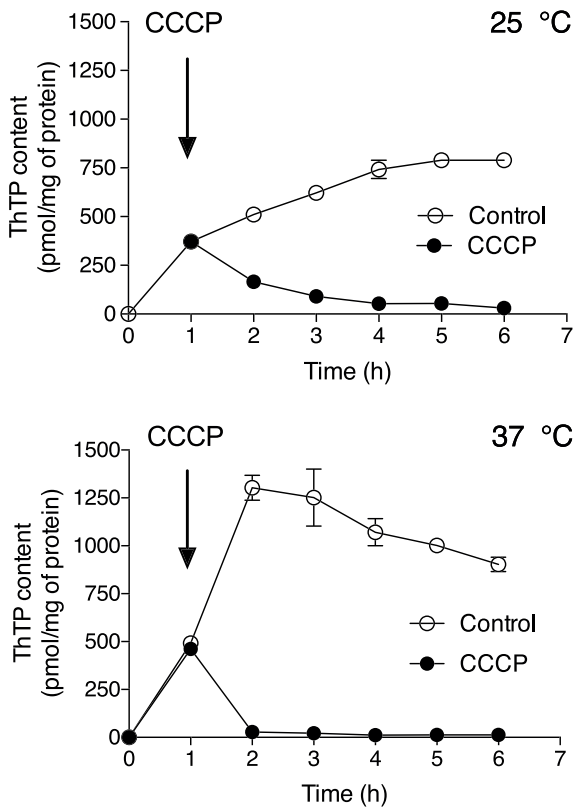

Figure 4 | Effect of CCCP on intracellular ThTP levels in the *E. coli* CV2 strain. The bacteria were grown overnight in LB medium and transferred to a minimal M9 medium containing 10 mM L-lactate either at 25 or at 37°C. CCCP (50 μM) was added after 1 h. (Means ± SD, n = 3).

results support the conclusion that the driving force for ThTP synthesis is Δp, without consumption of ATP (through a hypothetical ThDP kinase reaction) or ADP (through adenylate kinase activity). This conclusion is in agreement with our previous results showing no correlation between rate of ThTP synthesis and cellular ATP content[2].

ThTP synthesis requires F_oF_1-ATP synthase. The observation that ThTP accumulation is highly sensitive to uncouplers raises the possibility that it is synthesized by a chemiosmotic mechanism similar to ATP synthesis by oxidative phosphorylation. Previous data obtained on isolated mitochondria showed that ThTP synthesis is highly sensitive to inhibitors of F_oF_1-ATP synthase such as oligomycin and DCCD[9]. However, in *E. coli*, ATP synthesis by oxidative phosphorylation is relatively insensitive to oligomyin and our results show that high concentrations are also required for the inhibition of ThTP synthesis (Supplementary Table S3). However, as its eukaryotic counterpart, *E. coli* F_oF_1-ATP synthase is sensitive to DCCD. Figure 3b shows that ThTP synthesis is nearly completely inhibited at 0.1 mM DCCD. This is in agreement with the effect of DCCD on ATP hydrolysis by F_oF_1[26]. Therefore, we tested mutants carrying mutations on F_1, making them unable to carry out oxidative phosphorylation. In minimal medium containing glucose, no significant ThTP production could be shown in any of the strains tested (Figure 5). AN120 (atpA401 or uncA401) carries a point-mutation in the gene coding for the α subunit of F_1, leading to the replacement of serine 373 by a phenylalanine, resulting in defective steady-state catalysis[27]. Purified F_1 has less than 1% of the ATPase activity of the wild-type, but the structure seems to be intact. AN718 (atpA401 or unc1401) also carries a mutation in the α subunit of F_1 resulting in loss of ATPase activity, but to our knowledge, the exact mutation has not been characterized[28,29]. On the other hand, strain AN382 (atpB402) carries a mutation in subunit a of F_o. It has a normal F_1, but is defective in energy transducing capacity[30,31]. Small amounts of ThTP are already present in AN382 in the absence of glucose. However, ThTP levels are decreased in the presence of glucose and increased by CCCP, suggesting that it may be synthesized by a different mechanism (adenylate kinase for instance[19]).

In order to verify that F_oF_1-ATPase is required for ThTP synthesis, we tested the *E. coli* strain DK8, lacking the entire ATP operon (*ΔuncBEFHAGDC*)[32,33] (Figure 6). In this strain, no ThTP synthesis could be measured after transfer to minimal medium containing either glucose or lactate. However, when the plasmid encoding the entire *atp* operon was incorporated into the same strain, ThTP accumulated as in the wild-type strain. These results unambiguously demonstrate that F_oF_1-ATPase is required for ThTP synthesis in *E. coli*.

Labeled P_i is directly incorporated into ThTP. The above results suggest that ThTP is synthesized by a chemiosmotic mechanism according to the reaction ThDP + P_i ⇆ ThTP + H_2O, catalyzed by F_oF_1-ATP synthase. Thus, we may expect that when the bacteria are depleted in internal P_i, ThTP synthesis will depend on extracellular phosphate concentration. Therefore, we first incubated the cells for 4 h in a minimal medium devoid of phosphate. We used the wild-type MG1655 strain as well as the CF5802 strain, which is devoid of polyphosphate kinase. The latter, lacking polyphosphate, should have a much lower phosphate storage capacity than the wild-type strain. Then, 10 mM glucose and increasing concentrations of Na_2HPO_4 were added. In both strains, the ThTP production increased with increasing external phosphate concentration, the relationship being much steeper in the polyphosphate-deficient than in the wild-type strain (Figure 7).

If the above mechanism is correct, labeled P_i should be directly and rapidly incorporated into ThTP under the usual conditions of ThTP production. Indeed, after incubation of the bacteria in minimal

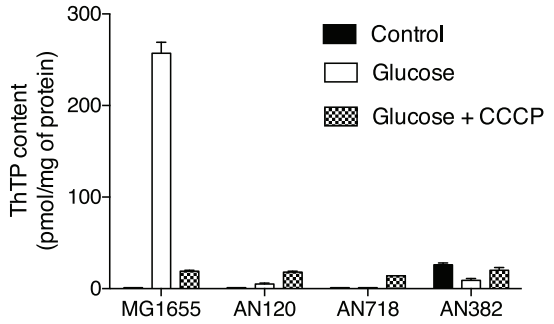

Figure 5 | ThTP synthesis in *E. coli* mutants carrying mutations in the α subunit of F_1 (AN120 and AN718) or in the a subunit of F_o (AN382). The wild-type (MG1655) and the mutants were grown overnight in LB medium (37°C, 250 rpm). For the mutant strains, streptomycin (200 μg/ml) was added to the medium. The bacteria were transferred to M9 medium and incubated (1 h at 37°C) in the absence of glucose (control) or in the presence of glucose (10 mM) with or without CCCP (50 μM). (Means ± SD, n = 3).

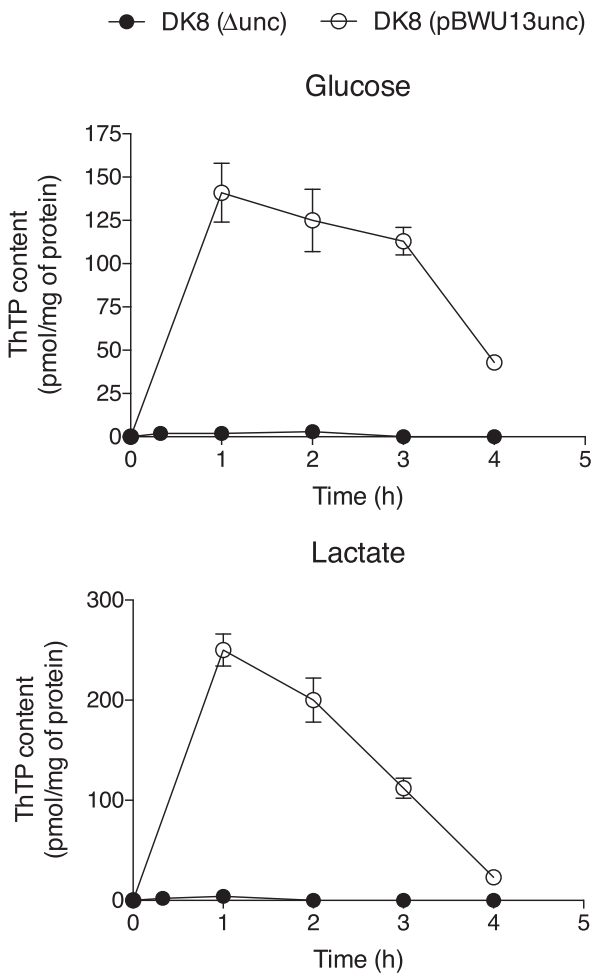

Figure 6 | ThTP synthesis in the DK8 strain and the DK8 strain containing the whole *atp* operon. The DK8(Δunc) strain was grown overnight in LB medium in the presence of 30 mg/l tetracycline (37°C, 250 rpm). For the DK8 (pBWU13unc) strain, the medium also contained in addition 100 mg/l ampicillin. Then, the bacteria were transferred to M9 medium containing 10 mM of either D-glucose or L-lactate and incubated at 37°C. (Means ± SD, n = 3).

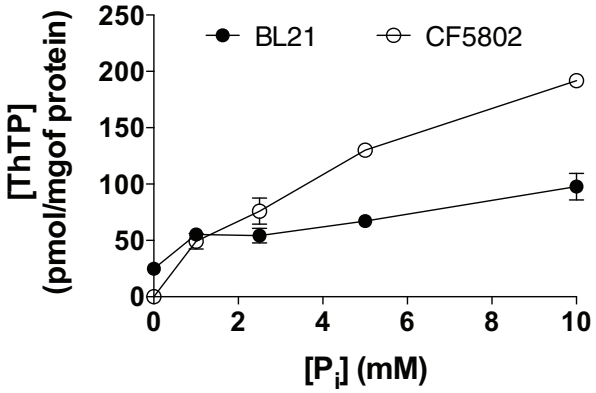

Figure 7 | Dependence of ThTP synthesis on external phosphate concentration in intact P_i-depleted bacteria. The bacteria (wild-type MG1655 or CF5802) were grown overnight in LB medium, transferred to minimal M9 medium devoid of P_i (replaced by chloride) and preincubated for 4 h at 37°C to deplete them of endogenous P_i. Then, glucose (10 mM) and Na_2HPO_4 (at the concentrations indicated) were added and ThTP was determined after 20 min. (Means ± SD, n = 3).

medium in the presence of $[^{32}P]PO_4^{3-}$ (10 GBq/mmol) for 1 h at 37°C in the presence of 10 mM glucose, the specific radioactivity of the ThTP synthesized was 8.9 ± 5.6 GBq/mmol (n = 6, mean ± SD), very close to the specific radioactivity of the $^{32}PO_4^{2-}$ used. This indicates that $[^{32}P]PO_4^{3-}$ is directly incorporated into ThTP rather than into a precursor (such as ATP for instance), which should result in a significant dilution of the specific radioactivity. Therefore, these data together with those shown in Fig. 1 indicate that ThTP is synthesized from free ThDP and P_i in vivo.

Hydrolysis of ThTP by purified *E. coli* F_1 subunit. Our initial aim was to demonstrate that ThTP can be synthesized in vitro, using either purified reconstituted F_oF_1 or inverted membrane vesicles[34]. These attempts were unsuccessful, although, in both preparations we were able to synthesize ATP from ADP and P_i (not shown). Failure to observe a net synthesis of ThTP in vitro in the presence of ThDP and P_i (even if a Δp is established) is not unexpected as data described above suggest that an unidentified activator (produced through pyruvate oxidation) is required for ThTP synthesis.

We could nonetheless demonstrate a significant hydrolysis of ThTP by soluble F_1 purified from *E. coli*. The apparent K_m was 40 µM, suggesting a reasonably high affinity of the catalytic sites for ThTP, but V_{max} was only 2 nmol $mg^{-1}.min^{-1}$, which is four orders of magnitude lower than for ATP hydrolysis under the same conditions (4.5 µmol. $mg^{-1}.min^{-1}$). Again, this very low k_{cat} (1.5 min^{-1}) may be explained by the absence of the putative activator. The activity was nearly completely inhibited by 0.1 mM ADP, suggesting that the same sites are responsible for the hydrolysis of ATP and ThTP. It is well known that Mg-ADP binds rather tightly to the active sites of F_1, thus inhibiting ATP hydrolysis[35].

Discussion

The present data show that, in *E. coli* cells, relatively high amounts of ThTP can be produced from free ThDP and P_i by a chemiosmotic mechanism requiring pyruvate oxidation. This process appears as alternative to ATP synthesis. Moreover, we demonstrate for the first time that, under particular conditions, F_oF_1-ATP synthase is able to catalyze the synthesis of ThTP instead of ATP.

In MG1655 as well as BL21 cells, ThTP may account for up to 20% of total thiamine while in CV2 cells this value may increase to 60%. At its peak, ThTP concentration may reach 400 pmol/mg of protein[4]. This represents an intracellular ThTP concentration of at least 0.1 mM. These results are in agreement with the view that, in *E. coli*, ThDP is not only a cofactor, but it can have a different fate, i. e. serve as a reservoir for the production of ThTP and AThTP, under specific conditions of stress[4,20].

Our work with brain mitochondria suggested that ThTP is synthesized by a chemiosmotic mechanism according to the reaction ThDP + P_i ⇆ ThTP + H_2O coupled to the respiratory chain[9]. Though this synthesis was inhibited by DCCD, oligomycin and aurovertin, it was not clear whether the reaction was catalyzed by the common F_oF_1-ATP synthase or by a ThTP-specific isoform, possibly linked to a specific cell population.

The present results demonstrate that in *E. coli* cells ThTP is synthesized by F_oF_1-ATP synthase in vivo. However, we have not been able to demonstrate a synthesis of ThTP in vitro by reconstituted F_oF_1. This underscores an important difference between ThTP and ATP synthesis: while the latter requires only a proton-motive force, ThTP synthesis requires at least one additional factor, as emphasized by the observation that ThTP synthesis occurs with substrates such as glucose, lactate or pyruvate, but not with malate, while ATP synthesis occurs with all permeant energy substrates. Therefore, at least three factors are simultaneously required for ThTP synthesis: amino acid starvation, a proton-motive force generated by the respiratory chain and a specific activator (Figure 8). The presence of the activator during amino acid starvation might induce a conformational change in F_oF_1-ATP synthase, so that the affinity for ThDP is increased. It is important to emphasize that the rate of ThTP synthesis is orders of magnitude slower than ATP synthesis. Initial accumulation of ThTP may proceed at a rate of approximately 170 pmol/mg of protein within 20 min[2], or 8.5 pmol mg^{-1} min^{-1}. This value is probably underestimated, as it does not account for ThTP hydrolysis during this time period. However, ThTP hydrolysis is slow in *E. coli* extracts (unpublished results). Oxidative phosphorylation-dependent ATP levels may increase from 0.1 to 1.8 mM within 1 min[36], i.e. at a rate > 5 nmol mg^{-1} min^{-1}, considering an intracellular volume of 3.2 µl/mg of protein[37]. For that reason, ATP synthesis is not significantly impaired when ThTP is synthesized in parallel. Thus, ATP synthesis may continue during ThTP synthesis, suggesting that only a relatively small population of F_oF_1-ATP synthase is recruited for ThTP synthesis.

It could be argued that ThTP synthesis is only a side-reaction of F_oF_1-ATP synthase. This would, however, hardly explain the tight regulation of ThTP synthesis by amino acids and the requirement of an activator, as well as the systematically transient character of the accumulation. Indeed, when a second addition of glucose is made four hours after the first addition (when all the glucose has been consumed), no further ThTP synthesis observed, though the first addition of glucose induced a transient accumulation of ThTP (results not shown). This suggests that once the cells have adapted to the amino acid downshift, metabolic conditions are such that no further ThTP synthesis can occur. This might suggest that ThTP is produced as the first step of a sequence of molecular events: if ThTP were simply a by-product of F_oF_1 activity, it would accumulate as long as the energy substrate (i. e. glucose) is available.

In conclusion, our results demonstrate that F_oF_1-ATP synthase is responsible for ThTP synthesis in *E. coli*, as has also been suggested in isolated brain mitochondria[9]. Interestingly, it was recently suggested that inorganic polyphosphate could be synthesized in mammalian cells by a chemiosmotic mechanism requiring F_oF_1-ATP synthase[38]. Hence, alternative roles must be considered for this important enzyme complex. The fact that ThTP is synthesized only under very specific conditions (amino acid starvation) and seems to require an activator suggests that this reaction is of physiological significance and under tight regulatory control. Furthermore, the fact that this mechanism is observed in *E. coli* and mammalian brain suggests that it is evolutionary conserved, possibly going back to the earliest living organisms.

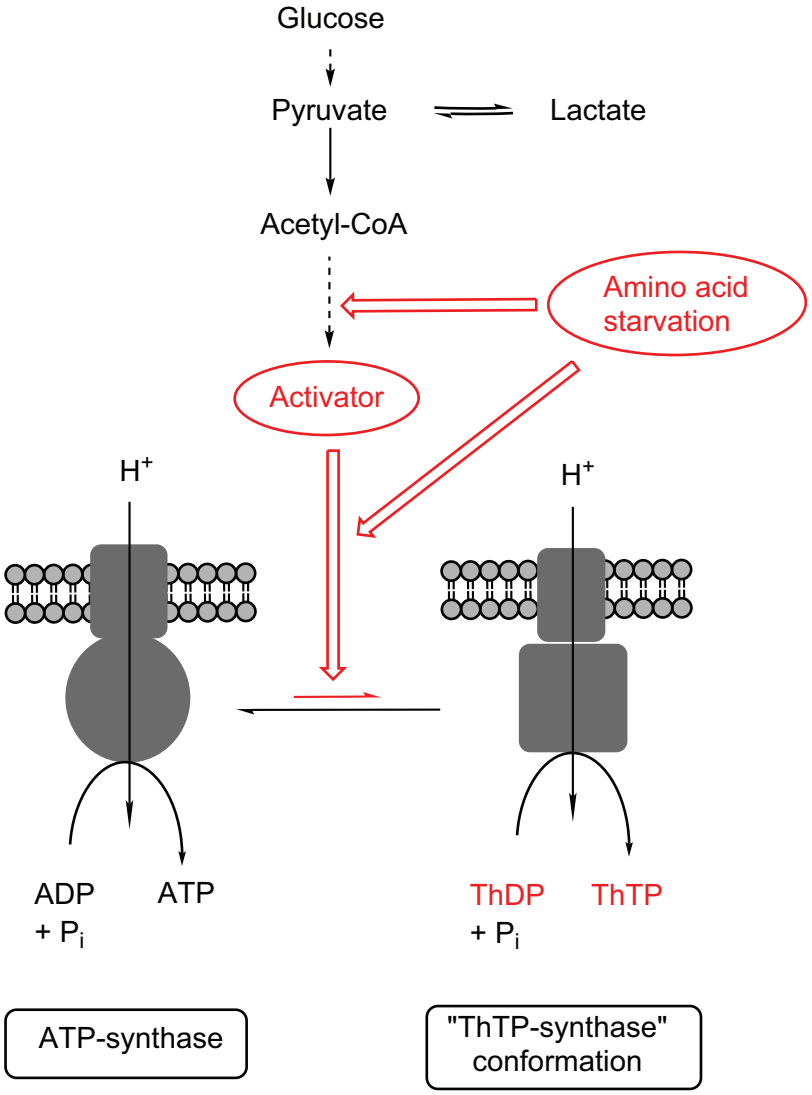

Figure 8 | Mechanism and regulation of ThTP synthesis in *E. coli*. Under conditions of amino acid starvation and in the presence of an energy substrate yielding pyruvate, a hypothetical activator is formed (presumably from acetyl-CoA). This would shift F_1 from the normal conformation (catalyzing ATP synthesis or hydrolysis) to a ThTP synthase conformation, binding ThDP or ThTP rather then ADP or ATP. Both ATP and ThTP synthesis are energized by the proton-motive force generated by the respiratory chain.

Methods

***E. coli* strains.** The BL21 strain, lacking PmpT and Lon proteases, was from Amersham Biosciences. The MG1655 (wild-type K-12) and the CF5802 (Δ*ppk-ppx::km*)[39] strains were a gift from Dr. M. Cashel (Laboratory of Molecular Genetics, NICHD, National Institutes of Health, Bethesda, MD). The CV2 (CGSC # 4682), AN120 (CGSC # 5100), AN382 (CGSC # 5101), AN718 CGSC # 6308), JW0110 CGSC # 8392, JW0111 (CGSC # 8393), JW0112 (CGSC # 8394) strains were obtained from the *E. coli* Genetic Resource Center (Yale University, New Haven, CT, U.S.A.). *E. coli* strain DK8, lacking the *unc* operon and plasmid pBWU13, coding for the *unc* operon of *E. coli* were obtained as described[33]. The genotype of each strain is given in Supplementary Table S1. Purified F_1 was a gift of J. E. Walker and Sidong Liu (Medical Research Council, Mitochondrial Biology Unit, Cambridge CB0 2XY, UK).

Growth and processing of the bacteria for determination of thiamine derivatives. The bacteria were grown overnight (37°C, 250 rpm) in 50–100 ml lysogeny broth (LB) medium (tryptone, 10 g/l; yeast extract, 5 g/l; NaCl, 10 g/l, pH 7.0). The bacteria were pelleted (5 min, 10,000 × g) and suspended in the initial volume of M9 minimal medium (Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; NH_4Cl, 1 g/l; $CaCl_2$, 3 mg/l; $MgSO_4$, 1 mM, pH 7.0) containing various metabolic substrates in sterile 14-ml PS-tubes (Greiner Bio-One BVBA/SPRL, Wemmel, Belgium). Thiamine derivatives were determined by HPLC as previously described[2,20,40].

Incorporation of [^{32}P]P_i into ThTP. The bacteria (MG1655 strain) were incubated in low-phosphate minimal medium (NaCl, 64 mM; KCl, 22 mM, NH_4Cl, 1 g/l; $CaCl_2$, 3 mg/l; $MgSO_4$, 1 mM; Na_2HPO_4, 1 mM, pH 7.0) for one hour in 1 ml aliquots in the presence of 10 mM glucose and 30 µl $H_3{}^{32}PO_4$ (314–337 TBq/mmol, 370 MBq/ml, PerkinElmer, Waltham, Massachusetts, USA). The bacteria were lysed by addition of 200 µl trichloroacetic acid (72%), centrifuged (15000 g, 15 min) and the specific radioactivity of ThTP was determined in the supernatant as previously described[9]. Briefly, after extraction of the trichloroacetic acid by diethyl ether, the supernatants were analyzed by HPLC column, the fractions containing ThTP were collected and the radioactivity was determined by liquid scintillation counting.

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Authors contributions

TG and MG made most of the experimental work. BP studied the phosphate dependence of ThTP synthesis. BL was responsible for the microbiology and molecular biology work done by the Liège group. CvB contributed plasmid pBWU13 and the DK8 strain. PW and LB wrote the manuscript. LB was the initiator of the project. All authors read the manuscript, contributed to the interpretation of the data and approved the final manuscript.

Additional information

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