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Short Communication

Thiamin nutrition and catalysis-induced instability of thiamin diphosphate

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Thiamin (vitamin B₁) is required in animal diets because it is the precursor of the enzyme cofactor, thiamin diphosphate. Unlike other B vitamins, the dietary thiamin requirement is proportional to non-fat energy intake but there is no obvious biochemical reason for this relationship. In the present communication we show for two enzymes that the cofactor undergoes a slow destruction during catalysis, which may explain the interdependence of thiamin and energy intakes.

Cofactor instability: Dietary requirement: Energy metabolism: Enzyme cofactor: Thiamin diphosphate: Vitamin B₁

The B-group vitamins are required in the human diet for two reasons. The first is that man cannot make them *de novo*. And the second is that they, or derivatives of them, act as enzyme cofactors or carriers between enzymes. For example, biotin is itself a cofactor while riboflavin becomes a cofactor after conversion to one of the two nucleotides, FMN or FAD. Niacin is not a cofactor but, after conversion to NAD⁺ or NADP⁺, it acts as an electron carrier in virtually all metabolic pathways. All of the B vitamins must be continually replenished and the amount required for each to maintain health in various species has been measured.

Thiamin, or vitamin B₁, is another example of a B vitamin that is a precursor of a cofactor, thiamin diphosphate (ThDP; formerly known as thiamin pyrophosphate, TPP). In man, this cofactor participates in reactions catalysed by each of the 2-ketoacid dehydrogenase multienzyme complexes (pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and the branched-chain 2-ketoacid dehydrogenase), by transketolase and by a few other enzymes (e.g. 2-hydroxyphytanoyl-CoA lyase; Foulon *et al.* 1999). These enzymes are a subset of ThDP-dependent enzymes that are found in a wide range of organisms (Pohl *et al.* 2004).

The dietary thiamin requirement is known to be proportional to non-fat energy intake in birds and mammals, including man (Braddon & Cooper, 1914; Williams & Spies, 1938; Sauberlich *et al.* 1979). In contrast, the requirements for other B vitamins are never expressed in terms of substrate intake. The thiamin requirement has been calculated most reliably for human subjects, in whom it is 0.3 mg/1000 kcal (72 µg/MJ) non-fat energy intake (Williams & Spies, 1938; Sauberlich *et al.* 1979). This longstanding observation has always been attributed to the requirement for thiamin in

metabolism, and three of the ThDP-dependent enzymes (pyruvate dehydrogenase, 2-ketoglutarate dehydrogenase and transketolase) participate in the major routes of carbohydrate metabolism. However, there is no *a priori* reason why the requirements for a component of a catalyst should be dependent upon the extent of substrate utilization. In the present communication we show that ThDP is unstable when bound to ThDP-dependent enzymes and that this instability is exaggerated by catalysis. We suggest that thiamin destruction consequent upon ThDP-dependent catalysis would best explain the observed dependence of thiamin requirements on non-fat energy intake.

Experimental method

Recombinant *Escherichia coli* acetohydroxyacid synthase (AHAS) isoenzyme II was obtained as previously described (Hill *et al.* 1997). Recombinant pyruvate decarboxylase (PDC) from the bacterium *Zymomonas mobilis* (Huang *et al.* 2001) was prepared in a similar way, after modifying the expression plasmid by introducing a hexahistidine affinity-purification tag. For experiments with AHAS, control reaction mixtures contained 100 mM-KH₂PO₄/K₂HPO₄ (pH 7.9), 10 µM-FAD, 80 µM-ThDP and 1 mM-MgCl₂ in a total volume of 1250 µl. Where added, AHAS was present at 80 µM and pyruvate at 80 mM. During incubations for several hours at 37°C, duplicate 50 µl samples were taken at various intervals for assay of ThDP by the thiochrome method. This involved mixing the samples with 3 ml of 50% EtOH (v/v) before adding 500 µl of 0.04% (w/v) K₃Fe(CN)₆ in 15% (w/v) NaOH. The mixtures were allowed to stand for 2 min before adding 10 µl of 30% (v/v) H₂O₂. Fluorescence was

Abbreviations: AHAS, acetohydroxyacid synthase; PDC, pyruvate decarboxylase; ThDP, thiamin diphosphate.

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measured immediately with a Jasco (Easton, MD, USA) FP-770 spectrofluorimeter using excitation at 375 nm and emission at 430 nm, with both bandwidths set at 10 nm. ThDP concentrations were calculated by reference to standard solutions of 0, 20, 40, 60, 80 and 100 μM that were assayed in the same way. Experiments with PDC followed a similar protocol except that FAD was not added to reaction mixtures.

Results and discussion

The main focus of work in our laboratory over recent years has been the enzymes PDC and AHAS. While neither of these enzymes is found in man or other animals, we believe that our observations may be extrapolated to animal systems. Some support for this view will be offered later. We encourage others to perform similar experiments to those described here on enzymes that are of human origin.

During studies of the crystal structure of yeast AHAS (Pang *et al.* 2003) we observed some partial degradation of ThDP, and this has been confirmed subsequently (McCourt *et al.* 2005) and extended to the plant enzyme (McCourt *et al.* 2006). As shown in Fig. 1A, the mesh representing the electron density matches well with the structure of ThDP in the region of the diphosphate tail. However, in the region of the pyrimidine and thiazolium rings, there is a very poor match indicating that the cofactor has been fragmented. While these crystallization

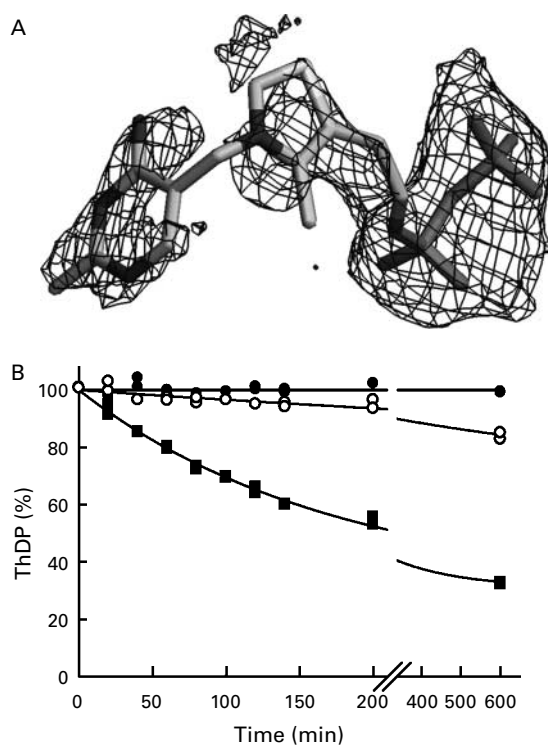


Fig. 1. Breakdown of thiamin diphosphate (ThDP) in reactions with aceto-hydroxyacid synthase (AHAS). (A) Electron density map, superimposed on the structure of intact ThDP. (B) Decay kinetics of the cofactor. ThDP (80 μM) was incubated in buffer alone (●), with 80 μM -AHAS (○), or with 80 μM -AHAS plus 80 mM-pyruvate (■). Residual ThDP was estimated in samples taken at intervals and is expressed as a percentage of that present initially.

experiments occupy days or weeks, we had also observed that *E. coli* AHAS loses activity during enzyme assays. Therefore, we investigated whether ThDP is stable in the presence of *E. coli* AHAS. Fig. 1B shows the results of one such experiment. ThDP in solution is perfectly stable but about 16% is lost over 10 h when bound to AHAS. Of greater interest is the result when pyruvate is added, when two-thirds of the ThDP is lost in 10 h. Clearly, flux of substrate through the cofactor–enzyme complex accelerates ThDP breakdown. We presume that there is not total loss of ThDP because pyruvate is used up after several hours and the complex ceases to turn over, thereby slowing ThDP destruction. A comparable experiment with *Z. mobilis* PDC (data not shown) yielded a less pronounced but still substantial 42% ThDP loss over 24 h.

From the data in Fig. 1B, it can be calculated that there is a probability of 0.0014 that ThDP will be destroyed in each catalytic cycle. While this may seem small, less than 3300 cycles would be sufficient to destroy 99% of the bound ThDP. It is relevant to ask whether this rate of destruction is sufficient to account for the human thiamin requirement of 72 $\mu\text{g}/\text{MJ}$. Metabolism by glycolysis and the citric acid cycle will require four turnover events of a ThDP-dependent enzyme (each molecule of glucose gives two molecules of pyruvate, which then pass through pyruvate dehydrogenase and 2-ketoglutarate dehydrogenase). Assuming an energy yield of 16.75 kJ/g glucose, we estimate that if ThDP is destroyed only once per 5.56×10^6 catalytic cycles, then the equivalent of 1 MJ glucose would result in the loss of 72 μg thiamin. Glucose metabolism via the pentose phosphate pathway would require twelve turnover events per molecule and a ThDP destruction rate of one molecule per 1.85×10^6 catalytic cycles. In either case, the observed ThDP destruction rate for AHAS is several thousand-fold greater than that required. Thus, our data easily explain the human thiamin requirement, unless human enzymes *in vivo* protect ThDP several orders of magnitude better than does this bacterial enzyme *in vitro*.

There have been previous observations of ThDP breakdown in ThDP-dependent enzymes. In the crystal structure of the bacterial PDC (Dobritzsch *et al.* 1998), a ThDP breakdown product is observed that is similar to that described in yeast AHAS (Pang *et al.* 2003). Recently, and perhaps of greater relevance to human metabolism, porcine pyruvate dehydrogenase was shown to lose activity with accompanying ThDP breakdown (Strumilo *et al.* 2004). However, as far as we are aware, our report is the first description of enhanced ThDP breakdown resulting from its participation in the normal catalytic reaction of an enzyme. Further work is required to verify our hypothesis of the connection between this ThDP breakdown during catalysis and the dependence of the human thiamin requirement upon metabolic activity.

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