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Vitamin B1 and B6 in the malaria parasite: requisite or dispensable?

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Vitamins are essential compounds mainly involved in acting as enzyme co-factors or in response to oxidative stress. In the last two years it became apparent that apicomplexan parasites are able to generate B vitamers such as vitamin B1 and B6 *de novo*. The biosynthesis pathways responsible for vitamin generation are considered as drug targets, since both provide a high degree of selectivity due to their absence in the human host. This report updates the current knowledge about vitamin B1 and B6 biosynthesis in malaria and other apicomplexan parasites. Owing to the urgent need for novel antimalarials, the significance of the biosynthesis and salvage of these vitamins is critically discussed in terms of parasite survival and their exploitation for drug development.

Key words: Malaria; Apicomplexa; Pyridoxal phosphate synthase; Vitamin B6; Vitamin B1

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Introduction

Apicomplexan parasites have a significant impact on human and livestock health, and chemotherapy remains a problem. The most important apicomplexan parasite is *Plasmodium*, the pathogenic agent of malaria. Malaria is a devastating and quite often a deadly parasitic disease which causes important public health problems in the tropics. However, coccidiosis caused by *Cryptosporidia* and *Toxoplasma* infections also pose a serious threat especially to immunocompromised people such as HIV patients all over the world and first infection with *Toxoplasma* during pregnancy can lead to developmental damage to the fetus.

Due to the high mutational rate of *Plasmodium falciparum* and its resulting rapid adaptation to environmental changes, drug resistance to the standard medication with chloroquine and antifolates is increasing. Therefore, continuous discovery of novel drug targets and development of new chemotherapeutic agents are inevitable. It has been claimed that antiparasitic compounds should preferably be created to target only the parasite without harming the human host. In this respect, the biosynthesis of parasite-specific vitamin B1 and B6 represents an ideal drug target.

Alternative pathways of vitamin B6 biosynthesis

Two different pathways for the synthesis of vitamin B6 are currently known. While 4-phosphohydroxy-L-threonine and 1-deoxyxylulose 5'-phosphate (DOXP) are substrates in the *Escherichia coli* (DOXP-dependent) pathway (1,2), vitamin B6 is synthesized from ribose 5'-phosphate, glyceraldehyde 3'-phosphate and glutamine in a DOXP-independent or fungus-like pathway (3-8). The DOXP-independent pathway has been identified in fungi, plants and some bacteria and was originally considered to be in-

volved in the detoxification of singlet oxygen ($^1O_2$) (3,9). However, the analysis of fungal mutants deficient in singlet oxygen resistance (SOR1), and therefore sensitive to singlet oxygen, demonstrated that the product of this gene also participates in pyridoxine biosynthesis (3,4,9). The SOR1 enzyme (also named Pdx1) belongs to the highly conserved enzyme family SNZ in *Saccharomyces cerevisiae*. The SNZ1 protein has been shown to interact with the SNO1 protein (5), also named Pdx2, which is a member of another preserved family in yeast consisting of three SNO enzymes (10). The involvement of Pdx2 in *de novo* vitamin B6 synthesis was confirmed by complementation assays of mutants deficient in pyridoxine biosynthesis (11). This pathway for vitamin B6 biosynthesis has been demonstrated in various other organisms such as *Bacillus subtilis* and *Arabidopsis thaliana* (6,9,12-14). In contrast to the *E. coli*-like formation of vitamin B6 leading to pyridoxine 5'-phosphate, the synthesis of the latter pathway results directly in pyridoxal phosphate (PLP), the active form of vitamin B6.

A few years ago Cassera et al. (15), using labeling experiments, suggested the presence of a vitamin B6 metabolite in *P. falciparum* which we later reported to be produced via the DOXP-independent pathway (16) (Figure 1). Moreover, we demonstrated a homologous pathway in the other apicomplexan parasite *Toxoplasma gondii*, where PLP synthesis also is a product of the single copy genes Pdx1 and Pdx2 (17). The two proteins need to act together for enzyme activity, similar to the homologous yeast proteins SNZ1 and SNO1 (6). The functionality of the two proteins of the apicomplexan parasites was verified by complementation of vitamin B6-deficient yeast strains (16). As is the case for yeast, in apicomplexa vitamin B6 is directly synthesized in its active form PLP (7,8,17,18). The Pdx1 protein is responsible for the creation of PLP by utilizing pentose, triose as well as ammonia. The ammonia is provided by the Pdx2 protein, which possesses glutaminase activity. Interestingly, the activity of Pdx1 is not dependent on the presence of Pdx2 if glutamine is replaced by ammonia, whereas the activity of Pdx2 relies on its interaction with Pdx1 (16,17).

The crystal structures of the *B. subtilis* and *Geobacillus stearothermophilus* Pdx1 and Pdx2 homologues have already been solved (19,20). The structures of the entire Pdx1/Pdx2 complex from *B. subtilis* and *Thermotoga maritima* have been analyzed only recently, with the identification of PLP synthase as a multimeric protein complex consisting of 12 Pdx1 proteins decorated by 12 Pdx2 proteins (21,22). These data suggest a channel between the individual Pdx1 and Pdx2 proteins for the transfer of the ammonia produced from Pdx2 towards the active site of Pdx1. Amino acid residues involved in the enzyme activity of Pdx1 proteins (21,23) are also present in the Pdx1 enzymes of the apicomplexan parasites *P. falciparum* and *T. gondii*. Interestingly, analyses of the genome data available for *Eimeria* or *Cryptosporidia* and for other protozoan parasites such as *Trypanosoma* and *Leishmania* did not reveal any indices for genes involved in *de novo* vitamin B6 synthesis. This clearly indicates that PLP biosynthesis is not a general event in protozoan parasites. Parasites which are dependent on external sources of vitamin B6 need to take up this nutrient like the human host. After import, B6 vitamers are immediately phosphorylated and thereby trapped within the cell. This catalysis is carried out by pyridoxine kinase (PdxK) (24-27). In addition to performing *de novo* synthesis, the malaria parasite also holds

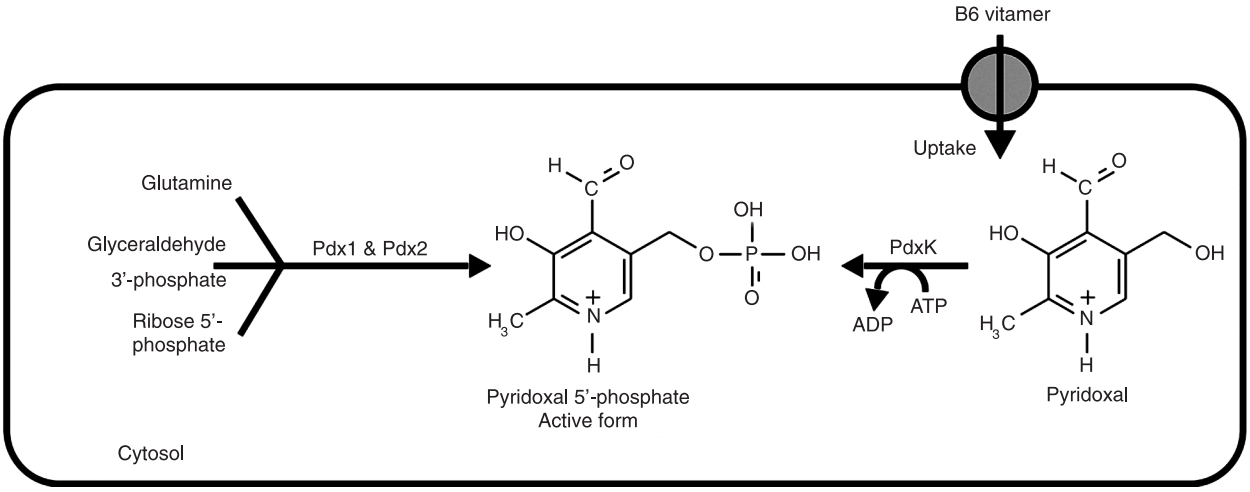

Figure 1. Vitamin B6 biosynthesis in *Plasmodium falciparum*. For explanation of the abbreviations see text.

a PdxK which enables *P. falciparum* to salvage B6 vitamers (16) (Figure 1). However, the value of the dual PLP provision in *Plasmodium* is not known and needs to be further analyzed.

Suitability of vitamin B6 biosynthesis for chemotherapy

The question arises whether vitamin B6 metabolism can be exploited to develop novel antimalarials. In general, there are two options to interfere with the biosynthesis of plasmodial vitamin B6. i) Since the crystal structure of PLP synthase was solved for *T. maritima* and *B. subtilis*, the design of inhibitors targeting this enzyme complex in order to block biosynthesis was facilitated (21,22). Nevertheless, rational drug design will benefit from structural analyses of the plasmodial PLP synthase crystal to detect parasite-specific targets. ii) On the other hand, emphasis should also be placed on unphosphorylated B6 vitamers, which after uptake are phosphorylated by PdxK and trapped within the parasite (16). Derivatives of these vitamers offer the strategy of channeling inactive co-factors into the depot of PLP, thereby poisoning PLP-dependent enzymes. Both strategies have a high potential for the design of novel drugs in order to interfere with the parasite's vitamin B6 metabolism.

Vitamin B1 and its biosynthesis

Until recently only prokaryotes, fungi and plants were reported to synthesize vitamin B1. Mammals entirely depend on the salvage of vitamin B1 from their diet. The active form of vitamin B1 is thiamine pyrophosphate (TPP) which is a co-factor for various enzymes mainly involved in carbohydrate metabolism such as 2-oxoglutarate dehydrogenase, pyruvate dehydrogenase or transketolase (28,29). The biosynthesis of thiamine occurs by the combination of two different branches. The pyrimidine branch provides 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP), which has to be phosphorylated to HMP diphosphate (HMP-PP) in two consecutive steps by HMP/HMP-P kinase (ThiD; Figure 2) (30-33). In bacteria HMP itself is formed from precursors of the purine biosynthesis by the HMP synthesis enzyme ThiC (34), whereas in yeast the Thi5 protein is suggested to link pyridoxine 5'-phosphate and histidine to form HMP-P (35-37). The product of the other branch is 5-(2-hydroxyethyl)-4-methylthiazole phosphate (THZ-P), which can also be provided by the salvage of THZ upon phosphorylation by THZ kinase (ThiM) (38,39). Subsequently, the respective phosphorylated pyrimidine and thiazole moieties, HMP-PP and THZ-P, are amalgamated by thiamine phosphate synthase (ThiE) to form thiamine phosphate (TMP) (40). An additional phosphorylation step by TMP kinase (ThiL) would be necessary for this to become the active diphosphorylated form (32). However, in yeast as well as in some bacteria ThiL is absent. Therefore, it has been suggested that TMP is first dephosphorylated by a phosphatase before it is pyrophosphorylated by thiamine pyrophosphokinase (TPK) (41,42). The malaria parasite contains the genes encoding for the vitamin B1 synthesis enzymes ThiM, ThiD, and ThiE and their expression and functionality was confirmed (33,43) (Figure 2). Consistent with the situation in other organisms, an open-reading frame encoding for ThiL is not found in the plasmodial genome database.

Creation of a "thiamine pool" in *plasmodium falciparum*

Instead of ThiL, a gene with homology to TPK has been identified (43,44) and analyzed for its kinetic parameters (45) (Figure 2). The plasmodial TPK does not accept the *de novo* synthesized TMP as substrate, which means that TMP must first be dephosphorylated to thiamine before it is pyrophosphorylated to TPP by *Pf*TPK (45). The dephosphorylation of TMP is thought to be carried out by a non-specific phosphatase (46). Recently, such a phosphatase was characterized and its substrate specificity for PLP and TMP shown (Knöckel J, Müller IB, Walter RD, Wrenger C, unpublished data).

Pyrophosphorylation of thiamine to TPP takes place solely in the cytosol of the parasite, as demonstrated by immunofluorescence assays of *Pf*TPK (45). However, TPP-dependent enzymes are located not only in the cytosol but also in different compartments, a fact that raises the question of TPP transport in these plasmodial organelles (Figure 2). For instance, the TPP-dependent pyruvate dehydrogenase complex is targeted to the apicoplast, whereas the 2-oxoglutarate dehydrogenase complex has been reported to be mitochondrial (47,48). Assuming that thiamine is pyrophosphorylated exclusively in the cytosol, *P. falciparum* must hold specific transporters to fulfill the need for TPP of both organelle-specific dehydrogenase complexes. The relocation of TPP into the yeast mitochondrion by a specific transporter has been reported (49).

Does plasmodial vitamin B1 biosynthesis serve as a target for chemoterapy?

Promising attempts to exploit vitamin B1 biosynthesis for chemotherapy have been made in bacteria by using the naturally occurring HMP analogue bacimethrin (MeO-HMP)

or the synthetic CF_3-HMP compound (50,51). Although the former compound was successfully tested as a substrate for the plasmodial ThiD protein, bacimethrin did not affect the proliferation rate of the parasite, a fact that could be explained by an inefficient salvage of this analogue (33). Nevertheless, HMP derivatives as such should be considered as promising drug candidates against the malaria parasite, as already shown for bacteria (33,51).

Culturing *P. falciparum* in thiamine-free medium for a few days showed no adverse effect on parasite growth behavior (52); however, extending the period of culture in vitamin B1-deficient medium to ten days revealed a significant need for the externally supplied thiamine precursor HMP or thiamine itself for growth (33). The necessity of HMP in the medium clearly indicates that the parasite is able to create thiamine but depends on added HMP. Consistent with these results a homologue of the yeast-like Thi5 enzyme was not found in the plasmodial genome database, indicating the absence of a linkage of the vitamin B6 and B1 biosynthesis pathways (36,53-55). Furthermore, there is no plasmodial homologue coding for the bacterial ThiC protein, the enzyme that catalyzes the formation of HMP from aminoimidazole ribonucleotide, an intermediate of purine synthesis (32). The latter is not surprising since purine biosynthesis is absent in the malaria parasite (56). Considering that *Plasmodium* can either salvage HMP - as mentioned above - or thiamine for the formation of TPP offers novel chemotherapeutic strategies to import pro-drugs in the form of HMP or thiamine derivatives. Indeed, pyrithiamine and thiochrome, both

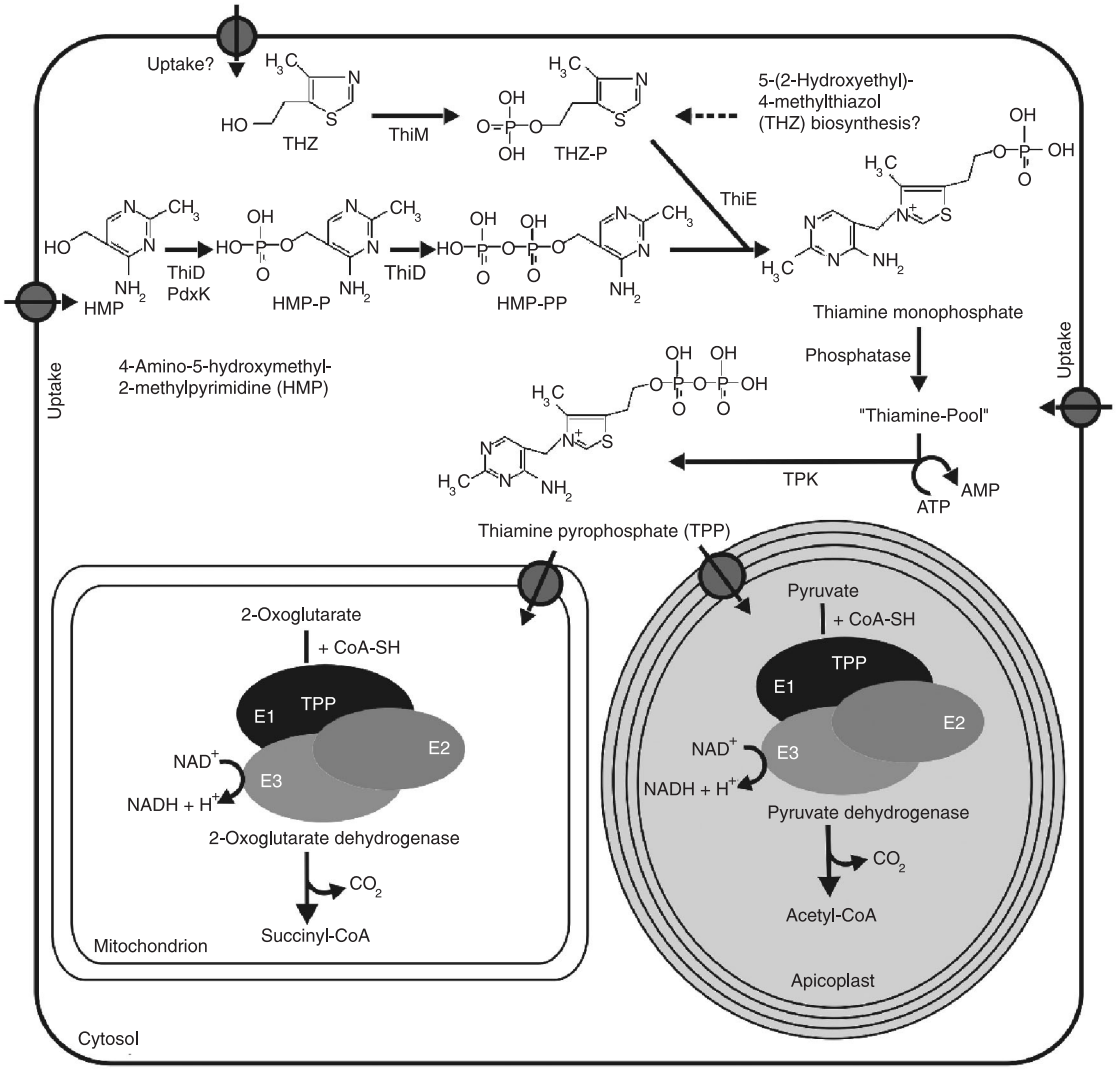

Figure 2. Generation of thiamine pyrophosphate and its trafficking within *Plasmodium falciparum*. Transport mechanisms are indicated by ⇳. For explanation of the abbreviations see text.

thiamine derivatives, were tested as substrates for plasmodial TPK. While pyrithiamine is accepted equivalently to thiamine as substrate, thiochrome is not phosphorylated by *Pf*TPK (45). However, testing pyrithiamine on cultured parasites did not reveal any anti-proliferative effect even at a concentration of 0.5 mM, implying an insufficient uptake of this analogue by the parasite or poor targeting of vitamin B1-dependent enzymes by its pyrophosphorylated form (Müller IB, Walter RD, Wrenger C, unpublished data).

Interestingly, cultivation of the parasite in THZ-deficient medium led to normal growth, suggesting that *P. falciparum* might be able to generate THZ *de novo*. Despite the use of bioinformatic tools, genes coding for THZ biosynthesis enzymes could not be identified in the plasmodial genome database (57). The synthesis of either the pyrimidine or the thiazole moiety was discussed for various other organisms, requiring the other part to be salvaged (28,58,59). This might be also the case for the malaria parasite; however, we cannot exclude a THZ biosynthesis pathway different from the prokaryotic pathway in *P. falciparum*.

Does vitamin B1 biosynthesis occur in other apicomplexan parasites?

Enzymes involved in vitamin B1 synthesis as shown for *P. falciparum* are apparently absent in the genome of the apicomplexan relatives *E. tenella* (http://www.sanger.ac.uk/Projects/E_tenella/), and *T. gondii* (http://www.toxodb.org/toxo/home.jsp) (17). This major difference poses questions about evolutionary adaptation of these parasites to their hosts. Assuming that the ancestor of apicomplexa had the potential to synthesize vitamin B1 *de novo* or at least to salvage the THZ- and HMP-moieties, apicomplexan parasites like *T. gondii* have lost their ability to synthesize vitamin B1 *de novo* during evolution. Therefore, these parasites entirely depend on thiamine uptake to satisfy their needs, a fact that might be explained by their perfect accommodation to their host cells. The malaria parasite, however, still expresses a rudimentary set of vitamin B1 synthesis enzymes pointing out an insufficient vitamin B1 supply from its host. This distinct adaptation of apicomplexan parasites may result from life cycle and host cell specificity. Further analyses are required to solve the riddle concerning *de novo* co-factor synthesis, uptake and salvage.

In summary, vitamins B1 and B6 are essential co-factors and their biosyntheses offer a powerful potential as drug targets, since these pathways are absent in the human host. Ongoing studies on the structures of the enzymes involved in vitamin B1 and B6 synthesis will allow the design of specific inhibitors blocking these crucial pathways and thereby impairing parasite growth.

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