Vitamin B6 Biosynthesis by the Malaria Parasite *Plasmodium falciparum*

**BIOCHEMICAL AND STRUCTURAL INSIGHTS**

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Plasmodium falciparum is the causative agent of severe malaria. Each year up to two million human deaths and enormous economic losses are attributed to this parasite. Drug resistance in P. falciparum has been aggravating the problem in many parts of the world during the last two decades, which considering the lack of a protective vaccine, is the major obstacle to combat the disease. Hence, new antimalarials are urgently needed. Requirements for nutrients and vitamins have previously been discussed as possible novel targets (1). Indeed the P. falciparum genome contains genes that encode enzymes necessary for the syntheses of the vitamin precursor chorismate (2–4), vitamin B6 (5, 6), and the vitamin-like cofactor lipoic acid (7).

Vitamin B6 is renowned in the medical field as being involved in more bodily functions than any other single nutrient. It is required for the maintenance of physical as well as mental health. The term "vitamin B6" collectively refers to the vitamins pyridoxal, pyridoxine, and pyridoxamine, and their respective phosphate esters. The metabolically active form is pyridoxal 5′-phosphate (PLP), an essential co-enzyme in numerous pathways such as amino acid metabolism and the biosynthesis of antibiotic compounds. In contrast to mammals, which have to take up vitamin B6 from their diet, bacteria, fungi, and plants, and the protozoan P. falciparum have the ability to synthesize the vitamin de novo.

Analyses of a number of available genomes has demonstrated that most organisms, including all archaea, fungi, plants, and protozoa and most eubacteria use a class I glutamine amidotransferase (GATase) composed of two domains, a glutaminase and its associated acceptor/synthase domain to generate vitamin B6 (8–13). Structural knowledge on class I GATases in general demonstrates a common fold for the glutaminase subunit, whereas structure and function of the interacting synthase subunits vary (14). The synthase subunit appears to have a protein tunnel, which connects the glutaminase and synthase active sites and shields the labile ammonia produced by the glutaminase. The glutaminase is dependent on the interaction with the synthase, and both active sites are structurally and biochemically linked (14, 15).

The GATase involved in vitamin B6 biosynthesis is a bi-enzyme complex consisting of Pdx1, the acceptor/synthase, and Pdx2, the glutaminase domain (16, 17). Very recently, the substrates of Pdx1 and the biosynthetic pathway have been described for the Bacillus subtilis orthologs YaaD and YaaE. In contrast to Escherichia coli, which possesses an alternative enzymatic machinery for vitamin B6 biosynthesis (11), this pathway directly results in PLP formation (18, 19).

In the malaria parasite P. falciparum, single genes encoding Pdx1 and

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Pdx2 have been identified (5, 6). This study reports a detailed biochemical and cellular analysis of the Pdx1-Pdx2 bi-enzyme complex. Furthermore, we report the three-dimensional structure of Pdx2 and compare it with those of known class I GATases. In view of the absence of vitamin B6 biosynthesis in the mammalian host, this study is aimed to obtain further insights into the suitability of this metabolic pathway for the design of new antimalarials.

MATERIALS AND METHODS

Parasites—P. falciparum isolate K1 (Thailand), adapted to growth in horse serum, was a gift of Dr. Matile (F. Hoffmann-La Roche, Basel). P. falciparum HB3 and 3D7 were provided by Prof. Lanzer (University of Heidelberg, Germany). P. falciparum K1 was cultured as described (20). P. falciparum HB3 and 3D7 were cultivated according to Trager and Jensen in RPMI 1640 medium containing 5% human serum A− under reduced oxygen (21). The techniques of parasite synchronization, isolation, lysis, and protein preparation as well as the separation of the cellular fractions were performed as described previously (22). The concentration of acivicin required to inhibit parasite growth by 50% after 48 h (IC50) was determined by the method of [G-3H]hypoxanthine incorporation (23), using cultures of 1% hematocrit and 0.8% initial parasitemia.

Genome Database Searches and Sequence Analyses—Genes apparently encoding Pdx1 and Pdx2 were identified in the Plasmodium genome data base PlasmoDB (PlasmoDB.org (24)) by performing TBLASTN searches using the protein sequences from B. subtilis (accession numbers: P37527 for YaaD, B. subtilis Pdx1 ortholog; P37528 for YaaE, B. subtilis Pdx2 ortholog).

Molecular Biological Methods—The full-length Pdx1 and Pdx2 genes and/or cDNAs of P. falciparum were amplified by PCR using sequence-specific sense and antisense oligonucleotides and P. falciparum 3D7 genomic or cDNA as template. To express the predicted full-length proteins, the PCR fragments were cloned into the NdeI and XhoI sites of pET-21a (+) (Novagen). Cloning into the Ndel site allows the use of the native methionine for expression. Two extra amino acids plus a His6 tag are added to the C terminus using by the XhoI site. The Plasmodium berghei Pdx2 cDNA was amplified from cDNA of the P. berghei NK6 strain by PCR using sequence-specific sense and antisense oligonucleotides derived from the PbPdx2 gene of the P. berghei ANKA strain. The nucleotide sequences of all constructs were confirmed by automated sequencing.

Recombinant Expression, Purification, and Enzyme Assays—Pdx1 and -2 were expressed in the E. coli strain BL21-CodonPlus(R⃝) RIL (Stratagene) using standard protocols. After 3 h (Pdx1) or 5 h (Pdx2) bacteria were harvested by centrifugation, resuspended in lysis buffer (300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine), flash-frozen, and stored at −80 °C. Cells were disrupted by sonication, and the suspension was cleared by centrifugation. The crude supernatant was applied to nickel-nitritolactic acid (Qiagen), washed with 600 mM NaCl, 5 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 8.0, containing either 10 (Pdx2) or 40 mM imidazole (Pdx1) and eluted with 300 mM NaCl, 200 mM imidazole, 5 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 8.0. For storage, glycerol was added to a final concentration of 10%, and the protein was frozen at −80 °C.

Pdx2 was further purified on a Superdex™ 200 26/60 gel filtration column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 200 mM NaCl and 1 mM dithiothreitol. Pdx2 eluted at a volume of 240 ml corresponding to an apparent molecular mass of 25 kDa, consistent with Pdx2 being a monomer in solution. For crystallization, fractions containing monomeric Pdx2 were concentrated to >30 mg/ml.

To investigate complex formation of Pdx1 and -2, the respective genes were cloned into pETDuet(TM)-1 (Novagen). A stop codon and a Pac site were introduced at the 3′-end of Pdx1. In the case of Pdx2, a NcoI site was introduced at the 5′-end and a His6 tag, a stop codon, and a NotI site at the 3′-end. Pdx1 was cloned into the multiple cloning site 2 via Ndel/Pac and Pdx2 into multiple cloning site 1 via NcoI/NotI. The nucleotide sequence of the construct was confirmed by automated sequencing. Expression and purification of the complex were performed as described for Pdx2.

Different molar ratios of Pdx1 and -2 were analyzed for glutaminase activity using a coupled enzymatic assay with glutamate dehydrogenase as described (17). The reduction of NAD+ to NADH was monitored at 340 nm (εNAD = 6220 M−1 cm−1). Samples were prepared in a total volume of 300 μl containing 50 mM Tris-HCl, pH 8.0, 2 mM L-glutamine (Sigma-Aldrich), 9 μM Pdx2, and Pdx1 at varying concentrations covering a molar equivalent range of 0 to 2 (moles of Pdx1/moles of Pdx2). For kinetic analyses, glutaminase activity of Pdx2 was determined online using a modification of the above described method. For each measurement Pdx1 and -2 at equimolar concentrations were added to quartz cuvettes containing a freshly prepared mixture of 0.5 mM 3-acetylpyridine adenine dinucleotide (APAD), an analog of NAD+; 30 units of bovine glutamate dehydrogenase, and buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA). The reaction was started by adding 0–30 mM L-glutamine to a final volume of 600 μl. Reduction of APAD to APADH was measured at 363 nm (εAPADH = 8900 M−1 cm−1) at 30 °C.

The activity of Pdx1 was monitored according to a previous study (19). Reactions were carried out in 50 mM Tris-HCl, pH 8.0, at 37 °C containing 20 μM of the isolated Pdx1 and/or -2 and 1 mM of the substrates; either ribose 5-phosphate or ribulose 5-phosphate and either dihydroxyacetone phosphate or DL-glyceraldehyde 3-phosphate (2 mM). As a nitrogen source either 10 mM glutamine or 10 mM ammonium sulfate was added. In all cases samples were preincubated for 30 min in the presence of all components of the reaction apart from the triose sugar.

Acivicin inhibition was performed essentially as described before (19). The time course for the fraction of catalytic activity remaining displayed pseudo-first order kinetics from which the rate of inactivation (k) was determined by a fit of the data to the exponential function f = e−kx. The second order rate constant was calculated from a plot of k versus the initial acivicin concentration.

Complementation Assay—pSWEET- yaaB—which was kindly provided by Amit P. Bhavsar, McMaster University, Hamilton, Ontario, Canada (25). To create pSWEET-expressing Pdx1, a Pac site and nucleotides −24 to −1 of the B. subtilis tagD gene providing a ribosomal binding site (RBS) were introduced at the 5′-end and a stop codon and a BamHI at the 3′-end of Pdx1 and cloned into the PacI/BamHI sites of pSWEET. As a control, the same construct without the RBS was generated. Integration into the B. subtilis chromosome at the amyE locus via double recombination was selected with chloramphenicol (10 μg ml−1) and confirmed by integration PCR.

The B. subtilis 168 (trpC2) yaaD mutant was grown at 37 °C in TMM (Spizizen’s minimal medium supplemented with 0.5 mM L-tryp- tophan and 10 mg ml−1 FeSO4 × 7H2O) as described by Sakai and coworkers (26) in the presence of erythromycin (0.3 μg ml−1) and 0.05 mM pyridoxal (27). IPTG (0.05 mM) was added for expression of down-
Antibodies were eluted in 100 mM glycine, pH 2.8, and neutralized by the addition of 3M Tris-HCl, pH 8.8.

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**TABLE 1**

**Crystallographic data**

| **A) Data collection statistics** |  |
|---------------------------------|--|
| Spacegroup                      | C2 |
| Cell a/b/c (Å); α, β, γ (°)     | 88.0/67.9/70.3; 90/92.3/90 |
| Number of molecules per asymmetric unit | 2 |
| Resolution (Å)                 | 25.0–1.62 (1.63–1.62) |
| R_{sym} (%)                     | 6.4 (46.6) |
| l/σ (l)                         | 19.3 (2.3) |
| Completeness (%)                | 99.9 (100.0) |
| Redundancy (%)                  | 5.2 (4.8) |
| Unique reflections              | 52,656 |
| Average B (Å²)                  | 19.1 |

**B) Refinement statistics**

|                                |                  |
|--------------------------------|------------------|
| R_{work} (%)                   | 15.3             |
| R_{free} (%)                   | 18.9             |
| Amino acids in model (range)    | A2–A123, A128–A212, and B2–B227 |
| Double conformations modeled    | A2, A4, A9–A11, A27–A29, A55, A87, A100–A101, A111, A120, A134, A181, B39, B59, B87, B98, B104–B105, B109, B111, B121–B122, B130, B134, B136, B159–B160, B173, B179–180, B193, B219, and B221 |
| No. of protein atoms            | 3648             |
| No. of water atoms              | 469              |
| No. of ligand atoms             | 15 (polyethylene glycol 200) |
| r.m.s.d. bond (Å)               | 0.023            |
| r.m.s.d. angle (°)              | 1.962            |

**Notes:**

\[ R_{sym} = \sum_i \sum_j I_i(h) / \langle I_i(h) \rangle, \] where \( I(h) \) is the mean intensity after rejections.

\[ R_{work} = \frac{\sum_i h \sum_j|F_{o}(h)| - 2 \sum_i F_{c}(h) |h|}{\sum_i h \sum_j |F_{o}(h)| - 2 \sum_i F_{c}(h) |h|}, \] where \( F_{o}(h) \) and \( F_{c}(h) \) are observed and calculated structure factors, respectively.

\% of the data were excluded to calculate R_{work}.

**Figure 1.** A, stage-specific immunoblot analysis of *P. falciparum* lysates. The blot was probed with antibodies against Pdx1, Pdx2, and PIPKs. Red blood cell stages: R, ring stage parasites (12 ± 2 h); T, trophozoites (25 ± 2 h); S, schizonts (34 ± 2 h); Seg, segmenters (44 ± 2 h); G, gametocytes, presexual stages. B, cellular fractions of *P. falciparum*-infected red blood cells: uRBC, uninfected red blood cell (control); IRBC, infected red blood cell; Para, parasite; M, membrane/organellae; and C, cytosol specify the respective cellular fraction. Either 15 μg of either the respective stage-specific lysate or cellular fraction was loaded. C, localization of Pdx1 and -2 within the parasite. Confocal immunofluorescence analysis of a trophozoite (Pdx1) and a schizont (Pdx2). Each row represents images from a single parasite: first column, transmitted light; second column, Pdx1 and -2 immunofluorescence, respectively; third column, parasite DNA; fourth column, overlay of the first three columns. The scale bar represents 2 μm.

**Crystallization, Data Collection, Structure Determination, and Refinement**—Purified Pdx2 was used for crystallization experiments using hanging drop vapor diffusion at 19°C. Crystals grew overnight from 30% polyethylene glycol 200, 5% polyethylene glycol 3000 buffered with MES at pH 6.0. The dimensions of the crystals were ~100 × 40 × 40 μm. Crystals were flash-frozen and stored under liquid nitrogen. Data to 1.62 Å were collected at 100 K at the beamline ID29 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The crystals belong to the monoclinic space group C2 with unit cell dimensions of a = 88.0 Å, b = 67.9 Å, and c = 70.3 Å with a β-angle of 92.3°. For data collection statistics see Table 1A.

The structure was determined with molecular replacement using the program MOLREP (30) and the structure of YaaE as a search model (PDB entry 1R9G (31)). The asymmetric unit contains two protein chains. For initial model update and refinement, the auto-building options of ARP/wARP were used (molecular replacement mode (32)). ARP/wARP was able to trace and assign 421 out of 454 protein residues, among those the His6 tag of chain B, and placed 421 water molecules in the model. Untraced residues were residues A1 to A2, A103 to A107, A122 to A129, A175 to A179, and A221 to A227 in chain A and B1 to B2, B145 to B147, and B227 in chain B. Refmac5 (33) refinement cycles with ARP/waterpicking was iterated in three cycles with model building in O (34). The final model contains residues 2 to 123 and 129 to 221 in chain A and 2 to 227 in chain B. Sixteen double conformations have been modeled for chain A and 21 for chain B. One polyethylene glycol molecule containing four ethylene glycol monomers has been observed bound to the protein. The seven Ramachandran outliers (disallowed and generously allowed) include the catalytic cysteines Cys^{17A} and Cys^{87B}. Statistics of the final refined model can be seen from Table 1B.
RESULTS

Pdx1 and -2 Are Expressed in *P. falciparum* Blood Stages and Reside in the Parasite Cytosol—To assess that Pdx1 and Pdx2 are expressed during the erythrocytic life stages of *P. falciparum*, stage-specific parasite lysates were analyzed by Western blotting using antibodies generated against both recombinant proteins. Both proteins were detectable as single bands of the expected sizes (33 kDa for Pdx1 and 25 kDa for Pdx2) in all intraerythrocytic stages as well as in gametocytes (Fig. 1). It appears that the level of both proteins increases during the intraerythrocytic development with highest protein levels found in schizonts and segmenters. The blots were re-probed with an antibody of a protein expressed at equal levels throughout the erythrocytic cycle of the parasites (PfPK5, *P. falciparum* protein kinase 5) to control for equal loading (35).

A mixed stage parasite culture was fractionated into infected erythrocyte (iRBC) and parasite (*Para*), membrane/organelle (M), and cytosol (C) fraction to analyze the cellular distribution of Pdx1 and -2 in the parasites. Both proteins were exclusively detected in the parasite cytosol (Fig. 1B). An antibody against the control protein PfPK5 that is solely found in the parasite cytosol demonstrates the integrity of this fraction (35).

To visualize the cytosolic localization of Pdx1 and 2, intraerythrocytic parasites were analyzed by confocal immunofluorescence microscopy. Both Pdx1 and -2 show a disperse distribution within the parasite that is characterized by the absence of any discrete organelle specific pattern (Fig. 1C). Although many proteins show an even distribution within the cytosol, Pdx1 and -2 concentrate in part to diffuse foci. This has also been observed for example with the cytosolic *P. falciparum* chorismate synthase (2).

Pdx1 and -2 Are Required for Their Respective Enzyme Activities—Glutaminase activity of Pdx2 is only observed in the presence of Pdx1 as has also been observed by Wrenger *et al.* (7). A 1:1 molar ratio of the two proteins appeared to be optimal for glutaminase activity, indicative of a stoichiometric protein complex (Fig. 2A). Variation of the glutamine concentration produced typical Michaelis-Menten kinetics (data not shown) from which the kinetic constants were determined (*k*\(_{\text{cat}}\) as 0.11 s\(^{-1}\) and *K*\(_{\text{M}}\) as 0.56 mM).

Both ribose 5-phosphate and ribulose 5-phosphate, as well as glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) are substrates for the plasmodial PLP synthase (Fig. 2C). The activity of the complex in the presence of G3P or DHAP was comparable when either ribose 5-phosphate (Fig. 2C, dashed and solid lines, respectively) or ribulose 5-phosphate (Fig. 2C, dashed-dotted and dotted lines, respectively) were used as the C5 sugars. Under the given assay conditions, the specific activity of the plasmodial PLP synthase was calculated to be 92.1 pmol min\(^{-1}\) mg\(^{-1}\) using ribose 5'-phosphate and G3P as substrates.

FIGURE 2. Enzymatic characterization of Pdx1 and -2. A, characterization of the glutaminase activity of Pdx2. Effect of Pdx1 on the glutaminase activity of Pdx2. B, effect of acivicin concentration on Pdx2 activity. The plot shows the fraction of glutaminase activity remaining (A/A\(_{0}\)) versus time in the presence of 0 mM (●), 5 mM (○), 10 mM (△), and 15 mM (■) acivicin. The data were fitted to the exponential equation \(f = e^{-kt}\). C and D, PLP synthase activity. C, PLP formation by the Pdx1-Pdx2 complex in the presence of glutamine (10 mM) or D, by Pdx1 in the presence of ammonium sulfate (10 mM): dashed line, ribose-5-phosphate and G3P; solid line, ribose-5-phosphate and DAHP; dashed and dotted line, ribulose 5-phosphate and G3P; and dotted line, ribulose 5-phosphate and DHAP. Graphs show representatives of five continuously recorded measurements.
When glutamine is used as the nitrogen donor (Fig. 2C), the activity of Pdx1 was found to be dependent on the presence of Pdx2 under all conditions. In the absence of Pdx2, however, ammonium sulfate can substitute for glutamine in the presence of either pentose sugar (Fig. 2D). However, ammonia could only be used by Pdx1 when G3P was used as the triose sugar (Fig. 2D: G3P plus ribose 5-phosphate (dashed line); G3P plus ribulose 5-phosphate (dashed and dotted line)). When DHAP was used, no reaction took place (Fig. 2D, solid and dotted lines), i.e. the reaction depended on complex formation with Pdx2.

In addition, it was shown that the two plasmodial proteins form a stable complex when co-expressed in the prokaryotic expression system used. The presence of both proteins during the expression and purification procedure was verified by Western blotting using the affinity-purified Pdx1 antibody and a monoclonal His tag antibody for the detection of Pdx2 (Fig. 2B). Both proteins were expressed at comparable levels, and affinity purification using nickel-nitrilotriacetic acid-agarose resulted in the co-purification of His-tagged Pdx2 and untagged Pdx1 (Fig. 3, A and B, lane 4).

**FIGURE 3. Complex formation of Pdx1 and -2.** Co-expression of His-tagged Pdx2 and untagged Pdx1. A, Coomassie Brilliant Blue-stained SDS-PAGE gel (12%); B, Western blot analysis using the affinity-purified AbPdx1 and mouse monoclonal anti-His antibody for the detection of untagged Pdx1 and His-tagged Pdx2, respectively. Lane 1, uninduced cultures harboring the co-expression construct; lane 2, cultures induced with 0.4 mM IPTG; lane 3, lysate after sonication of cells and removal of cellular debris; lane 4, pooled fractions after affinity-purification by nickel-nitrilotriacetic acid chromatography.

**FIGURE 4. Structural analysis of Pdx2.** A, ribbon representation of the x-ray structure of Pdx2. Amino acids Cys87, His196, and Glu198 make up the catalytic triad. The respective residues are shown in yellow in all panels. Residues involved in the putative interface with the synthase subunit are labeled in green. Differences to the YaaE ortholog are shown in red. B, stick representation of the active site of Pdx2. The loop carrying the nucleophilic cysteine comprises residues Gly85, Thr86, Cys87, Ala88, and Gly89. This loop is shown together with the $2F_{o} - F_{c}$ electron density at a level of 1.2σ. The double conformation of Cys87 is visible. The proposed binding site of the synthase subunit is indicated. C, the proposed oxyanion hole, which forms during catalysis, is obstructed in Pdx2 by the carbonyl of Gly85. D, the oxyanion hole is formed in the apo-form of CPS (1JDB) by the peptide nitrogens of Gly241 and Leu270. E, in the glutamine-bound state of CPS (1A9X), this conformation is maintained. The figure was prepared with PyMOL (53).
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Effect of Acivicin on Pdx2 Activity and Parasite Growth—Acivicin irreversibly inhibits glutaminases by covalent modification of an essential cysteine residue (14, 36) and has been shown to inhibit the glutaminases: Pdx2 from B. subtilis (19). Acivicin also inhibited Pdx2 in a concentration- and time-dependent manner (Fig. 2B). The loss in glutaminase activity appears as a pseudo-first order decay versus acivicin concentration was used to calculate the second order rate constant, 19 \( \pm 0.01 \) min \(^{-1}\), 0.22 \( \pm 0.04 \) min \(^{-1}\), and 0.27 \( \pm 0.04 \) min \(^{-1}\), for 5 mM, 10 mM, and 15 mM acivicin, respectively. A plot of the pseudo-first order rate constants versus acivicin concentration was used to calculate the second order rate constant, 19 \( \pm 6 \) min \(^{-1} \) M \(^{-1}\). In addition, the IC \(_{50}\) of acivicin on parasite growth was determined to be in the lower micromolar range (data not shown).

Structure Determination of Pdx2 and Comparison to That of Other GATases—To obtain insights into the precise architecture of the active site of Pdx2, the structure of the protein was determined by x-ray crystallography at 1.62 Å resolution. The mean coordinate error of the structure is 0.175 Å as derived from the Luzzati plot (30). The high resolution obtained is attributed at least in part to an interaction of two symmetry related molecules through a fully ordered hexa-histidine tag (see supplemental data).

The structure of Pdx2 is that of the classic mixed \( \beta \)-sheet of the glutaminase component of the triad GATases (Fig. 4A) (37). A large \( \beta \)-sheet in the center of the structure contains \( \beta \)-strands \( \beta2\), \( \beta1\), \( \beta3\), \( \beta4\), \( \beta12\), \( \beta11\), and \( \beta10\), with all except \( \beta11\) in parallel orientation. This central \( \beta \)-sheet is flanked by two small \( \beta \)-sheets. The first one is formed by the strands \( \beta5\) and \( \beta6\) in parallel orientation and \( \beta9\) in an anti-parallel orientation. The second one contains the anti-parallel strands \( \beta7\) and \( \beta8\) and is instrumental in contacting the synthase subunit (see below (38)). Helices \( \alpha1\), \( \alpha2\), \( \alpha3\), \( \alpha4\), and \( \alpha6\) pack against the central \( \beta \)-sheet. The catalytic cysteine, Cys \(^{97}\), lies in a sharp \( \gamma \)-like turn at the end of \( \beta \)-strand 4. The other two residues of the catalytic triad, His \(^{196}\) and Glu \(^{199}\), are in a small helical segment in the loop region between the last \( \beta \)-strand, \( \beta12\) and helix \( \alpha6\).

To identify differences of potential functional significance, the primary structure of Pdx2 was compared with other glutaminases: Pdx2 from P. berghei (PbPdx2; 63.8% amino acid identity), the bacterial ortholog YaaE from B. subtilis (37.1% amino acid identity) (31), and Thermotoga maritima HisH (16.5% amino acid identity; see supplemental data for alignment (39)). YaaE can be superimposed with Pdx2 with an r.m.s.d. of 1.47 Å (175 of 196 C \(_{\alpha}\) positions from YaaE). Nonetheless, significant differences between the plasmodial and bacterial Pdx2s are observed. The most obvious one is an insertion between \( \beta5\) and \( \beta6\) in the malarial sequences not found in YaaE or in fact HisH (Fig. 4A and see alignment in supplemental data). In the Pdx2 structure these amino acids (95–111) form an additional, small helical segment, \( \alpha5\)–1. Helix \( \alpha6\) of Pdx2 has an additional turn through an insertion at position 62–68. Both variable regions map to one side of the molecule.
and result in a surface that is structurally different from YaaE. Another insertion in Pdx2 against β strands β10 and β11 and points toward the potential interface with Pdx1 (see below). All other variations are located on the surface of the molecule opposite to the synthase interaction site, e.g., in loops Pdx2 and Pdx2.

Pdx2 is a typical triad glutaminase where the catalytic residues are Cys87, His190, and Glu190 (yellow in Fig. 4, A and B). The catalytic triad can be superimposed with an r.m.s.d. of 0.45 Å over all atoms with the respective YaaE residues Cys349, His170, and Glu172. Other class I glutaminase active sites like HisH (IK9V) and carbamoyl phosphate synthase, CPS (1JDB) superimpose similarly well. The loop that connects β4 and β5 harbors Cys87 in Pdx2 and is strictly conserved between Pdx2 and YaaE and highly conserved in glutaminases in general. The sequence Gly-Thr-Cys-Ala-Gly allows the catalytic residue in Pdx2 to adopt an unusual conformation in the disallowed region of the Ramachandran plot with phi/psi values of 54°–110° (Fig. 4B, amino acids are in red lettering). This constraint is essential for the catalytic function of triad GATases and has been described for the similar active sites of the structurally unrelated α/β hydrolase family of enzymes (41). In Pdx2 the side chain of Cys87 is observed in two conformations (Fig. 4, B and C). The ratio of these two rotamers was fitted to 0.55 and 0.45 in chain A and to 0.8 and 0.2 in chain B experimentally by setting the occupancy to obtain comparable B-values for the two rotamers after refinement. The rotamer with higher occupancy superimposed with the position of the catalytic cysteine of the His7 in the acivcin-bound state (1VN) and of CPS in the free and glutamine-bound states (1IDB, Fig. 4D (40) and 1A9X, Fig. 4E (42), respectively). The rotamer with lower occupancy pointing to the catalytic histidine (Fig. 4B) is seen in a number of structures where the substrate binding pocket is occupied by ligands different from either substrate or product. Examples are CMP synthase with a bound citrate (37), TmHGP with a phosphate (1KXJ) and TmHisH with acetic acid in the substrate binding pocket (1K9V).

A central feature of glutamine hydrolysis by triad GATases is the formation of an oxanion hole during catalysis (44). In the glutaminase subunit of CPS the backbone nitrogen of Leu270 and Gly241 were suggested to participate in this structure (Fig. 4, D and E) (40, 42). The corresponding residues in Pdx2 (Ala148 and Gly51) have a different main-chain conformation (Fig. 4C). The carbonyl oxygen of Gly51 points toward the potential oxanion hole, suggesting that hydrolysis of glutamine cannot occur. Activation would then require the peptide bond of Gly51/Gly52 to undergo peptide isomerization.

Because there is no structure of a PLP synthase complex present in the PDB data base (45), the putative interaction site between glutaminase and synthase subunits was analyzed based on the available GATase structures from different organisms. The closest structural relative to Pdx2 is HisH from T. maritima. Comparison with the imidazole glyceraldehyde phosphate synthase of T. maritima (HisH/HisF) and Saccharomyces cerevisiae (HisF) allows to predict the contact sites for the interaction between the Pdx1 and -2 subunits of the plasmodial PLP synthase (38, 39). The glutaminase-synthase interaction surface involves many backbone contacts that are highly conserved in the three-dimensional structure (see regions labeled in green in Fig. 4A) but is not strictly conserved in primary structure. Pivotal in complex formation are β7 and β8 of the glutaminase. The interface of the complex also includes the loop region between β12 and β6 carrying the catalytic histidine and glutamate.

Pdx1 Can Substitute for YaaD Function in Vivo and in Vitro—The number of similarities between the PLP synthase of B. subtilis and P. falciparum and the high conservation of the predicted interaction surface of the glutaminase with the synthase subunit inspired us to perform functional complementation studies. A B. subtilis strain in which the yaaD gene, the Pdx1 ortholog, has been disrupted (B. subtilis 168 trpC2 ΔyaaD (27)) was used for these investigations. Because this strain was generated using the vector pmutin 3, genes downstream of the target gene acquire an isopropyl-1-thio-D-galactoside -(IPTG)-inducible promoter to overcome polar effects transmitted from the disruption of the target gene (27, 46). Because yaaD is the downstream gene of yaaE, IPTG has to be added to the bacterial culture in minimal medium to induce expression of YaaE.

Two different constructs of Pdx1 cloned into the expression vector pSWEET (25) were used for the complementation assays. One construct contains the coding sequence of Pdx1 with a ribosome binding site (RBS-Pdx1), whereas the other is lacking the ribosomal binding site (Pdx1) and thereby prohibiting translation of the parasite protein. Complementation was assessed both on minimal plates and in minimal liquid medium (Fig. 5, A and B). Neither of these two strains grew on minimal medium without pyridoxal (Fig. 5, A (TMM, I and 2) and B (○ and ○)), whereas addition of pyridoxal rescued the growth of both strains (Fig. 5, A (TMM+pyridoxal, I and 2) and B (● and ●)). When the expression of Pdx1 was induced by addition of 2% xylose, no growth was observed in the absence of added pyridoxal (Fig. 5A, − YaaE and TMM+xylose, 2). However, when IPTG was included in the medium, growth was observed, implying that under these conditions prototrophy is absolutely dependent on YaaE (Fig. 5, A (compare − YaaE(−IPTG) and + YaaE(IPTG) of TMM+xylose, 2) and B, ■). This indicated complex formation between Pdx1 and YaaE in B. subtilis. Moreover, the growth rate approaches the values observed when the medium is supplemented with pyridoxal. The control strain did not grow under these conditions (Fig. 5, A (+ YaaE(IPTG) and TMM+xylose, 1) and B, □).

In agreement with the in vivo complementation results, YaaE partially activated Pdx1 as monitored by its ability to synthesize PLP (Fig. 5C). However, even with a 1:5 molar ratio of Pdx1 to YaaE, PLP synthesis is only 50% of that observed with a 1:1 molar ratio of Pdx1 and -2 (Fig. 5C). These results clearly show that maximum PLP synthase activity depends on species-specific interactions of the two subunits, because an isostructural subunit of another species is not able to fully reconstitute PLP synthase activity of the species-specific complex.

**DISCUSSION**

P. falciparum possesses a functional vitamin B6 biosynthesis pathway (5, 6). The plasmodial PLP synthase is a typical class I GATase consisting of the glutaminase subunit, Pdx2, that produces ammonia from glutamine and the synthase subunit, Pdx1, which most likely tunnels the erythrocytic cycle of P. falciparum, and both were shown to be cytosolic proteins thus able to functionally interact and generate vitamin B6 in these developmental stages of the parasites. In addition to the absence of this metabolic pathway from the human host, these are prerequisites that possibly make vitamin B6 biosynthesis a novel antimalarial drug target. This hypothesis is supported by reports that the water-soluble vitamins present in blood plasma and culture medium only pantothenic acid or vitamin B5 appear to be absolutely required for parasite growth (47). However, it cannot be excluded that vitamin B6 is taken up by the parasite especially when considering reports about pyridoxine and PLP uptake by human erythrocytes and their interaction with hemoglobin (48, 49). Whether specific uptake mechanisms exist in the parasitized erythrocyte needs to be addressed in future studies, although genome analyses do not suggest the presence of a transporter.
specific for pyridoxal or its derivatives similar to those found in *S. cerevisiae* and in *Schizosaccharomyces pombe* (50, 51).

The functionality of Pdx1 and Pdx2 in PLP biosynthesis was analyzed using the recombinant proteins. Both ribose 5-phosphate and ribulose 5-phosphate, as well as G3P and DHAP, are substrates for the plasmodial PLP synthase (Fig. 2, C and D), implying that it possesses a ribose-5-phosphate isomerase and triose phosphate isomerase activity as does YaAD (18, 19). With 92.1 pmol min⁻¹ mg⁻¹, the specific activity of the plasmodial PLP synthase approaches that of the *B. subtilis* PLP complex (140 pmol min⁻¹ mg⁻¹). Similar to YaAD, Pdx1 alone has no detectable PLP synthase activity with any of its substrates when glutamine is used as the ammonia donor (19). This is overcome when glutamine is substituted by ammonium sulfate. In that case, activity of Pdx1 depends on G3P as C3 sugar, whereas DHAP cannot be used as triose substrate in the absence of Pdx2, implying a lack of triose isomerase activity unless the bi-enzyme complex is formed. This is remarkable, because isomerization is thought to occur in the active site of Pdx1, which in analogy to other class I GATases is supposed to be on the opposite side of the Pdx1-2 interface. Similar to Pdx1, YaAD exhibits PLP synthase activity in the absence of YaAe, if ammonium sulfate is used as an ammonia donor. However, in contrast to the plasmodial system, it is the isomerase activity for the pentose sugar that seems to be dependent on complex formation, because only ribose 5-phosphate can be used as the C5 sugar under these conditions (19). These results emphasize the differences occurring between the PLP synthase complexes of these two organisms.

The overall layout of class I GATases requires a stoichiometric bi-enzyme complex. For the plasmodial Pdx1-2 complex, this functional model is supported by kinetic analyses that identified a 1:1 molar ratio as optimal for glutaminase activity (Fig. 2A). These results confirm those of Wrenger et al. (6). The presence of a complex between Pdx1 and Pdx2 *in vivo* was further corroborated by our findings that co-expression of Pdx1 and -2 resulted in a near stoichiometric complex formation of the purified recombinant proteins (Fig. 3A, lane 4). The necessity for the formation of a protein-protein complex was also supported by the results that Pdx1 rescues the growth of a YaAD-deficient *B. subtilis* strain only when YaAe expression is also induced during the complementation assay (Fig. 5, A (+YaAe(+IPTG) of TMM+xylose, 2) and B, C). This is confirmed by the *in vitro* studies with the recombinant parasite and bacterial proteins albeit the cross-species interaction between Pdx1 and YaAe result in sub-optimal PLP biosynthesis (Fig. 5C). Thus, it can be concluded that the interaction between glutaminase and synthase subunit has species-specific features.

Three major findings in the three-dimensional structure indicate how activation of Pdx2 could take place: 1) Cys87 of the catalytic triad is observed in two side-chain conformations (Fig. 4, B and C). The orientation with lower occupancy superimposes that identified in the structure of HisH (1GFW) the glutaminase subunit of TmImGPS, whereas the second one with higher occupancy superimposes that identified in the structure of the HisH/F complex (1K9V (39)) and with structures where either substrate or inhibitor are covalently bound (38). Thus, Cys87 in Pdx2 must attain this conformation during catalysis. 2) Oxynion hole formation is a general principle among enzymes with catalytic triads stabilizing the transient negative charge during the hydrolytic reaction. Within the triad GATase family, the oxynion hole has been conclusively described for ImGPS (43, 52). In general, the oxynion hole is formed by two amide nitrogens, one from the residues following the catalytic nucleophile and the second from an adjacent β-strand, referred to as the “oxynion strand.” In Pdx2, these amide nitrogens are part of the peptide bonds between Cys87-Ala88 and Gly51–Gly52. The carbonyl oxygen of the latter peptide bond points toward the putative oxyanion hole and obstructs it (Fig. 4C). Activation of Pdx2 would thus require peptide bond isomerization, probably induced by the binding of Pdx1. If this mechanism is characteristic of this class of enzymes, the structure of Pdx2 shown here represents the resting state of the enzyme. 3) The structure of the glutaminase domain by the synthase could further be explained, if residues of the synthase subunit contribute to the glutaminase active site. Indeed, in the structure of His7, the bifunctional *S. cerevisiae* ImGPS, as well as in the crystallographic complex of HisH/HisF (1JVN) a glutamine in the loop connecting α4 and β5 in the synthase subunit contributes a hydrogen bond from Gln579 carbonyl oxygen to the substrate (38, 39). In CPS (1KEE (36)), Gln273Ne2 makes a similar interaction. Because Pdx2 alone has no detectable residual glutaminase activity, the interaction with the synthase subunit could be crucial to stabilize a conformation required for glutamine binding and/or hydrolysis. Amino acids in Pdx2 that potentially interact with Pdx1 in the bi-enzyme complex were deduced from the His7 structure and the HisH/βF complex. Besides β7 and β8, the interface also includes the loop region between β12 and α6 carrying the catalytic histidine and glutamate. The interaction in this region might stabilize a distinct conformation allowing the glutaminase to gain activity.

The structural similarity between Pdx2 and other class I glutaminases possibly excludes this protein as a suitable drug target. However, the specific interaction with the PLP synthase subunit Pdx1 and the substrate specificities of the latter strongly suggest that specific inhibition of the PLP biosynthesis pathway in the parasites is feasible. Structural analyses of the Pdx1-Pdx2 complex are underway to substantiate our present findings and to elucidate specific features that may be exploitable for the design of specific inhibitors. In addition the pathway is currently investigated by reverse genetics to test our hypothesis that it is essential for parasite survival.

**REFERENCES**

1. Kirk, K. (2001) *Physiol. Rev.* 81, 495–537
2. Fitzpatrick, T., Ricken, S., Lanzer, M., Amrhein, N., Machereux, P., and Kappes, B. (2001) *Mol. Microbiol.* 40, 65–75
3. McConkey, G. A., Finney, J. W., Westhead, D. R., Pluckthun, K., Fitzpatrick, T. B., Machereux, P., and Kappes, B. (2004) *Trends Parasitol.* 20, 60–65
4. Roberts, F., Roberts, C. W., Johnson, J. K., Kyle, D. E., Krell, T., Coggins, J. R., Coombs, G. H., Milhous, W. K., Tzipori, S., Ferguson, D. J., Chakrabarti, D., and McLeod, R. (1998) *Nature* 393, 801–805
5. Cassera, M. B., Gozzo, F. C., D’Alessandro, F. L., Merino, E. F., del Portillo, H. A., Peres, V. J., Almeida, I. C., Eberlin, M. N., Wunderlich, G., Wiener, J., Jomaa, H., Kimura, E. A., and Katzin, A. M. (2004) *J. Biol. Chem.* 279, 51749–51759
6. Wrenger, C., Eschbach, M. L., Muller, I. B., Warnecke, D., and Walter, R. D. (2005) *J. Biol. Chem.* 280, 5242–5248
7. Wrenger, C., and Muller, S. (2004) *Mol. Microbiol.* 53, 103–113
8. Bean, L. E., Drvavec, W. H., Jr., Braun, E. L., Errett, A., Saenz, G. S., Giles, M. D., Werner-Washburne, M., Nelson, A. M., and Natvig, D. O. (2001) *Genetics* 157, 1067–1075
9. Ehrensbein, M., Bilek, P. L., Miy, M. Y., Chignell, C. F., and Daub, M. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 9374–9378
10. Ehrensbein, M., and Daub, M. E. (2001) *J. Bacteriol.* 183, 3383–3390
11. Mittenhuber, G. (2001) *J. Mol. Microbiol. Biotechnol.* 3, 1–20
12. Usmani, A. H., May, G. S., and Usmani, S. A. (1999) *J. Biol. Chem.* 274, 23565–23569
13. Padilla, P. A., Fuge, E. K., Crawford, M. E., Errett, A., and Werner-Washburne, M. (1998) *J. Bacteriol.* 180, 5718–5726
14. Zalkin, H., and Smith, J. L. (1998) *Adv. Enzymol. Relat. Areas Mol. Biol.* 72, 87–144
15. Raushel, F. M., Thoden, J. B., Reinhardt, G. D., and Holden, H. M. (1998) *Curr. Opin. Chem. Biol.* 2, 624–632
16. Belitsky, B. R. (2004) *J. Bacteriol.* 186, 1191–1196
17. Dong, Y. X., Sueda, S., Nikawa, J., and Kondo, H. (2004) *Eur. J. Biochem.* 271, 745–752
18. Burns, K. E., Xiang, Y., Kinlaw, C. L., McLafferty, F. W., and Begley, T. P. (2005) *J. Am. Chem. Soc.* 127, 3682–3683
19. Raschle, T., Amrhein, N., and Fitzpatrick, T. B. (2005) *J. Biol. Chem.* 280, 32291–32300
20. Graeser, R., Wernli, B., Franklin, R. M., and Kappes, B. (1996) *Mol. Biochem. Parasitol.* 82, 37–49
21. Trager, W., and Jensen, J. B. (1976) *Science* 193, 673–675

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