Vitamin B6 biosynthesis by the malaria parasite Plasmodium falciparum: biochemical and structural insights

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Abstract

Vitamin B6 is one of nature's most versatile cofactors. Most organisms synthesize vitamin B6 via a recently discovered pathway employing the proteins Pdx1 and Pdx2. Here we present an in-depth characterization of the respective orthologs from the malaria parasite, Plasmodium falciparum. Expression profiling of Pdx1 and -2 shows that blood-stage parasites indeed possess a functional vitamin B6 de novo biosynthesis. Recombinant Pdx1 and Pdx2 form a complex that functions as a glutamine amidotransferase with Pdx2 as the glutaminase and Pdx1 as pyridoxal-5'-phosphate synthase domain. Complex formation is required for catalytic activity of either domain. Pdx1 forms a chimeric bi-enzyme with the bacterial YaaE, a Pdx2 ortholog, both in vivo and in vitro, although this chimera does not attain full catalytic activity, emphasizing that species-specific structural features govern the interaction between the protein partners of the PLP synthase complexes in different organisms. To gain insight into the activation mechanism of the parasite bi-enzyme complex, the three-dimensional structure of Pdx2 was determined at 1.62 Å. The [...]
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Vitamin B6 is one of nature’s most versatile cofactors. Most organisms synthesize vitamin B6 via a recently discovered pathway employing the proteins Pdx1 and Pdx2. Here we present an in-depth characterization of the respective orthologs from the malaria parasite, Plasmodium falciparum. Expression profiling of Pdx1 and -2 shows that blood-stage parasites indeed possess a functional vitamin B6 de novo biosynthesis. Recombinant Pdx1 and Pdx2 form a complex that functions as a glutamine amidotransferase with Pdx2 as the glutaminase and Pdx1 as pyridoxal-5'-phosphate synthase domain. Complex formation is required for catalytic activity of either domain. Pdx1 forms a chimeric bi-enzyme with the bacterial YaaE, a Pdx2 ortholog, both in vivo and in vitro, although this chimera does not attain full catalytic activity, emphasizing that species-specific structural features govern the interaction between the protein partners of the PLP synthase complexes in different organisms. To gain insight into the activation mechanism of the parasite bi-enzyme complex, the three-dimensional structure of Pdx2 was determined at 1.62 Å. The obstruction of the oxyanion hole indicates that Pdx2 is in a resting state and that activation occurs upon Pdx1-Pdx2 complex formation.

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 vitamers 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, 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 bio-synthetic 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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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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6 The abbreviations used are: PLP, pyridoxal 5'-phosphate; APAD, 3-acytylpyridine adenosine dinucleotide; CPS, carbamoyl phosphate synthase; DHPA, dihydroxyacetone phosphate; GATase, glutamine amidotransferase; G3P, glyceraldehyde 3-phosphate; ImGP5, imidazole glycerol phosphate synthase; RBS, ribosomal binding site; MES, 2-(N-morpholino)ethanesulfonic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; r.m.s.d., root mean square deviation; TM, Spizizens minimal medium supplemented with 0.5 μM L-tryptophan and 10 mg ml⁻¹ FeSO₄·7H₂O.
Vitamin B6 Biosynthesis by Plasmodium

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.

Data Base 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 NdeI site allows the use of the APAD-DHAPase adenine dinucleotide (APAD), an analog of NAD+ (Sigma-Alrich), 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-aminopyrimidine 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).

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^-kt. The second order rate constant was calculated from a plot of k versus the initial acivicin concentration.

Comparison Assay—pSWEET-bgaB was kindly provided by Amit P. Bhavsar, McMaster University, Hamilton, Ontario, Canada (25). To create pSWEET-expressing Pdx1, a PacI 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 disruptant was grown at 37°C in TMM (Spizizen’s minimal medium supplemented with 0.5 mM L-trypophan 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-

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stream genes (27). For complementation assays, the disruptant plus RBS-Pdx1 strain and the control strain, B. subtilis yaaD disruptant plus Pdx1 were grown under the above-mentioned conditions except that pyridoxal was omitted from the minimal medium. Expression of Pdx1 was induced by supplementation with 2% xylose.

**Immunological Methods**—The recombinant Pdx1 and -2 proteins were used to raise polyclonal rabbit antisera by Sigma. For affinity purification, Pdx1 and -2 were purified as described above. The storage buffer was exchanged for coupling buffer (0.1 M NaCl, 0.05 M Na2CO3, and 0.05 M NaHCO3, pH 8.3). Coupling to Epoxy-activated Sepharose 4B (Amersham Biosciences) was carried out according to the manufacturer’s instructions. Antibodies were bound to the matrix at 4°C. The beads were washed with 1× phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5), and antibodies were eluted in 100 mM glycine, pH 2.8, and neutralized by the addition of 3 M Tris-HCl, pH 8.8.

Western blots were performed according to Kihse-Andersen (28). The membranes were incubated with either an anti-His antibody (mouse monoclonal, Novagen) at a 1:1,000 dilution, the affinity-purified AbPdx1 at a 1:30,000 dilution, the affinity-purified AbPdx2 at a 1:500 dilution, and the AbPPK5 at a 1:10,000. The blots were developed using the ECL system (Amersham Biosciences).

Confocal immunofluorescence analyses were performed as described previously (2, 29). Parasites were labeled with affinity-purified AbPdx1 at a dilution of 1:2000 and the affinity-purified AbPdx2 at a dilution of 1:100. The secondary antibody, a donkey anti-rabbit-Cy3 conjugate (Jackson ImmunoResearch Laboratories) was applied at a dilution of 1:400. Parasite DNA was stained with Hoechst dye (benzimide H33342, Calbiochem) at a concentration of 2 µg ml−1 during the secondary antibody incubation period. Microscope slides were examined using a confocal laser scanning microscope (LSM 510, Zeiss).

### TABLE 1

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<th>Crystallographic data</th>
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<td><strong>A)</strong> Data collection statistics</td>
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<th><strong>B)</strong> Refinement statistics</th>
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* Rmerge = \(\sum_{i} N_{i}(h)−\langle h\rangle/\sum_{i} N_{i}(h)\), where \(N_{i}(h)\) is the mean intensity after rejections.
* Rwork = \[\sum_{i} F_{i}(h)−\langle F_{i}(h)\rangle/\sum_{i} F_{i}(h)\], where \(F_{i}(h)\) and \(F_{i}(h)\) are observed and calculated structure factors, respectively.

* 5% of the data were excluded to calculate Rwork.
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](http://www.jbc.org/)

**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. 3B). 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 Yaa E 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,F - F,F 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 Gly185. 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).
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 glutaminase subunit of PLP synthase 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 from which the rate constants for inactivation (k) could be calculated as 0.08 ± 0.01 min⁻¹, 0.22 ± 0.04 min⁻¹, and 0.27 ± 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 ± 6 min⁻¹ M⁻¹. In addition, the IC₅₀ 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 β-sheet of the glutaminase component of the triad GATases (Fig. 4A) (37). A large β-sheet in the center of the structure contains β-strands β2, β1, β3, β4, β12, β11, and β10, with all except β11 in parallel orientation. This central β-sheet is flanked by two small β-sheets. The first one is formed by the strands β5 and β6 in parallel orientation and β9 in an anti-parallel orientation. The second one contains the anti-parallel strands β7 and β8 and is instrumental in contacting the synthase subunit (see below (38)). Helices α1, α2, α3, α4, and α6 pack against the central β-sheet. The catalytic cysteine, CysS⁶⁷, lies in a sharp γ-like turn at the end of the β-strand 4. The other two residues of the catalytic triad, HisH¹⁹⁶ and Glu¹⁹⁸, are in a small helical segment in the loop region between the last β-strand, β12 and helix α6.

To identify differences of potential functional significance, the primary sequence and structure of Pdx2 were 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 Cα positions from YaaE). Nonetheless, significant differences between the plasmodial and bacterial Pdx2s are observed. The most obvious one is an insertion between β5 and β6 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, α5–1. Helix α6 of PbPdx2 has an additional turn through an insertion at position 62–68. Both variable regions map to one side of the molecule.

**FIGURE 5. Cross-species interaction between Pdx1 and YaaE.** A, growth of the B. subtilis 168 (trpC2) YaaD disruptant complemented with control construct lacking the ribosomal binding site (Pdx1) (1) or the complementation construct (RBS-Pdx1) (2) on minimal plates (TMM) without and with additives (0.05 mM pyridoxal (TMM + pyridoxal) or 2% xylose (TMM + xylose)) in the presence or absence of IPTG. IPTG is required to induce the expression of the endogenous YaaE. B, growth curves of the B. subtilis 168 (trpC2) YaaD disruptant complemented with RBS-Pdx1 (filled symbols: ■, △, and □) or the RBS-lacking Pdx1 control construct (open symbols: ○, △, and □) in TMM plus IPTG without and with additives (0.05 mM pyridoxal or 2% xylose); ■, RBS-Pdx1 in TMM; ○, Pdx1 in TMM; △, RBS-Pdx1 in TMM plus 0.05 mM pyridoxal; □, Pdx1 in TMM plus 0.05 mM pyridoxal; ■, RBS-Pdx1 in TMM - Pdx1 expression was induced by the addition of 2% xylose; ○, Pdx1 in TMM plus 2% xylose. C, PLP formation by the Pdx1-YaaE complex in the presence of ribulose 5-phosphate, GTP, and 10 mM Gln: ■, YaaD-YaaE complex (1:1); ■, YaaD-YaaE complex (1:1); ■, Pdx1-YaaE (1:1); ○, Pdx1-YaaE (1:1); △, YaaD; □, Pdx1; ●, no enzyme.
and result in a surface that is structurally different from YaaE. Another insertion in Pdx2 175–180 elongates the two 3-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 225–30 and Pdx2 138–144.

Pdx2 is a typical triad glutaminase where the catalytic residues are Cys 79, His 170, and Glu 172. Other class I glutaminase active sites like HisH (IK9V) and carbamoyl phosphate synthase, CPS (1JD8 (40)) superimpose similarly well. The loop that connects β4 and α5 harbors Cys 79 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 Cys 79 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 (1J1N (38)) and of CPS in the free and glutamine-bound states (1JD8, Fig. 4D and 1A9X, Fig. 4E (42), respectively). The rotamer with lower occupancy pointing toward 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 GMP synthetase with a bound citrate (37), TnmlGPS with a phosphate (1KXJ (43)) and TnHisH with acetic acid in the substrate binding pocket (1K9V (39)).

A central feature of glutamine hydrolysis by triad GATases is the formation of an oxygen hole during catalysis (44). In the glutaminase subunit of CPS the backbone nitrogens of Leu 72 and Gly 241 were suggested to participate in this structure (Fig. 4, D and E) (40, 42). The corresponding residues in Pdx2 (Ala 48 and Gly 51) have a different mainchain conformation (Fig. 4C). The carbonyl oxygen of Gly 51 points toward the potential oxygen hole, suggesting that hydrolysis of glutamine cannot occur. Activation would then require the peptide bond of Gly 51/Gly 52 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 glyceral phosphate synthase of T. maritima (HisH/HisF) and Saccharomyces cerevisiae (His?) 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 α6 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) ∆yaaD (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 yaaD, 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 isofunctional subunit of another species is not able to fully re-constitute 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 reactive intermediate to the second active site where PLP is formed from ammonia, a pentose, and a triose.

Complementary 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 isofunctional subunit of another species is not able to fully re-constitute PLP synthase activity of the species-specific complex.
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). 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 (1GPW) the glutaminase subunit of *TmImGt*, 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 Gly⁶¹–Gly⁷². The carbonyl oxygen of the latter peptide bond points toward the putative oxynion 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 activation 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 β in the synthase subunit contributes a hydrogen bond from Gln⁶⁷ to carboxyamide nitrogen to the substrate (38, 39). In CPS (1KEE (36), Gln⁷₃ of Ne2 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/F 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

Supplementary Fig. 1. A C-terminal hexa-histidine tag was added to the protein sequence of Pdx2 to facilitate protein purification. This tag is involved in crystal contacts between two symmetry related chains. Side chains of all residues, His222 through His227, are visible in the electron density (not shown). Two tag sequences run antiparallel and a number of hydrogen bonding contacts between the heterocyclic nitrogen atoms of the histidine side chain and main chain carbonyl oxygen’s stabilise this arrangement. The pH of the crystallisation buffer was 6.0 at which the histidine side-chain is expected to be doubly protonated. The C-terminal carboxylate likewise is involved in two hydrogen bonds. This interaction is thought to have improved crystal quality as it provides a stable protein-protein contact in the crystal.
Supplementary Fig. 2. Structure based sequence alignment of Pdx2, PbPdx2, HisH and YaaE. Secondary structure elements are shown for Pdx2 and YaaE. The catalytic triad is marked with black triangles below these sequences. The green circles mark those residues that participate in the interface of the HisH/HisF bi-enzyme complex. The figure was prepared with ALSCRIPT (54).
Supplementary Fig. 3. *Cellular fractions of P. falciparum infected red blood cells* - Coomassie Brilliant Blue stained SDS-PAGE gel (12%); uRBC – uninfected red blood cell (control), iRBC – infected red blood cell, Para – parasite; M - membrane and C- cytosol are specifying the respective cellular fraction. Fifteen µg of each fraction was loaded.