Assembly of the Eukaryotic PLP-Synthase Complex from *Plasmodium* and Activation of the Pdx1 Enzyme

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DOI 10.1016/j.str.2011.11.015

SUMMARY

Biosynthesis of vitamins is fundamental to malaria parasites. Plasmodia synthesize the active form of vitamin B₆ (pyridoxal 5’-phosphate, PLP) using a PLP synthase complex. The EM analysis shown here reveals a random association pattern of up to 12 Pdx2 glutaminase subunits to the dodecameric Pdx1 core complex. Interestingly, *Plasmodium falciparum* PLP synthase organizes in fibers. The crystal structure shows differences in complex formation to bacterial orthologs as interface variations. Alternative positioning of an α helix distinguishes an open conformation from a closed state when the enzyme binds substrate. The pentose substrate is covalently attached through its C1 and forms a Schiff base with Lys84. Ammonia transfer between Pdx2 glutaminase and Pdx1 active sites is regulated by a transient tunnel. The mutagenesis analysis allows defining the requirement for conservation of critical methionines, whereas there is also plasticity in ammonia tunnel construction as seen from comparison across different species.

INTRODUCTION

*Plasmodium falciparum* (Pf), *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi* are human pathogens of the malaria disease. Malaria is a serious threat to human health with nearly 250 million cases worldwide, and about 1 million deaths annually (World Health Organization, 2010). Despite significant advances in the understanding of the disease as well as the parasite’s biology and metabolism (Lakshmanan et al., 2011), malaria is still one of the leading causes of morbidity and mortality in developing countries, especially in sub-Saharan Africa, but also in Asia and Latin America (World Health Organization, 2010). A major factor hindering malaria control is the high degree of resistance developed by *Plasmodium* species against currently available drugs (Bustamante et al., 2009; Petersen et al., 2011; Travassos and Lauffer, 2009). Hence, there is still an urgent need for the identification of novel drug targets as well as antimalarial chemotherapeutics (Burrows et al., 2011; Kappes et al., 2011).

Although plasmodia rely on host-derived nutrients (Divo et al., 1985), certain essential cofactors need to be produced by the parasite. Vitamin biosynthetic pathways in general (Müller et al., 2010; Müller and Kappes, 2007; Wrenger et al., 2008) and vitamin B₆ biosynthesis in particular (Gengenbacher et al., 2006; Wrenger et al., 2005) have attracted interest as potential drug targets. Although humans depend on vitamin uptake, plasmodia possess a de novo pathway for vitamin B₆ biosynthesis, constituted by the pdx1 and pdx2 genes. *Pf* expresses functional orthologs of the pdx1 and pdx2 genes in blood stages (Gengenbacher et al., 2006; Wrenger et al., 2005), a prerequisite for an antimalarial drug target. Furthermore, the expression in intra-erythrocytic stages suggests that de novo vitamin B₆ biosynthesis is vital for parasite development. Other pathogenic organisms that express the pdx1 and pdx2 genes are *Toxoplasma* (Knöckel et al., 2007) and *Mycobacterium* (Dick et al., 2010).

Pyridoxal 5’-phosphate, PLP, an active form of vitamin B₆, is a widely used enzymatic cofactor in enzymes involved in amino acid metabolism (Eliot and Kirsch, 2004; Percudani and Peracchi, 2003); PLP has also an important role as an antioxidant (Ehrenshaft et al., 1999; Mooney and Hellmann, 2010). This function may indeed be required for intracellular survival within red blood cells, a developmental phase concurrent with marked oxidative stress (Müller, 2004; Wrenger et al., 2005).

De novo PLP biosynthesis by Pdx1 requires the substrates ribose 5-phosphate (R5P) and glyceraldehyde 3-phosphate (G3P) (Burns et al., 2005; Fitzpatrick et al., 2007; Raschle et al., 2005). The heterocyclic nitrogen is derived through hydrolysis of L-glutamine by Pdx2 (Tanaka et al., 2000; Tazuya et al., 1995). The two enzymes form the PLP synthase complex, functionally classified as a glutamine amidotransferase (Zalkin and Smith, 1998). PLP synthase assembles a total of 12 Pdx1 PLP synthase subunits (Neuwirth et al., 2009; Strohmeier et al., 2006; Zhu et al., 2005) and up to 12 Pdx2 glutaminase subunits (Strohmeier et al., 2006; Zein et al., 2006; Figure 1). The architecture of the complex is known from 3D structures...
of bacterial enzymes, but to our knowledge, no structure of a eukaryotic Pdx1/Pdx2 PLP synthase complex is available to date.

Prokaryotic Pdx1 proteins had previously shown to be mainly dodecameric (Strohmeier et al., 2006; Zhu et al., 2005). In contrast, Pdx1 from the yeast Saccharomyces cerevisiae has been characterized structurally, and it differs from prokaryotic and eukaryotic orthologs by a small insertion responsible for a shift in hexamer-dodecamer equilibrium toward the hexameric form (Neuwirth et al., 2009). Hence, it was speculated that prokaryotic and eukaryotic enzymes have a different mode of regulation.

Biochemical and biophysical characterizations of Pf PLP synthases revealed further differences (Flicker et al., 2007). It was noted that the complexes of eukaryotic and prokaryotic systems have different thermodynamic signatures in isothermal titration calorimetry (Flicker et al., 2007; Neuwirth et al., 2007). This is surprising because activation of Pdx2 by interaction with Pdx1 was thought to be similar in all systems. It was shown that these differences are more pronounced in the absence of the substrate L-glutamine and vanished in the presence of substrate (Flicker et al., 2007; Neuwirth et al., 2007; Strohmeier et al., 2006). Despite all efforts, no suitable crystals for X-ray diffraction analysis were obtained.

To understand what interfered with crystal formation, the Pf proteins were further analyzed by electron microscopy (EM) and single-particle image processing. The analysis showed that the autonomous PPdx1 subunit formed cylinders (Figure 2A), which matched the dodecameric complex in shape and size observed in crystal structures of bacterial complexes (Strohmeier et al., 2006; Zein et al., 2006; see model of the Pf PLP synthase assembly in Figure 1). Furthermore, dynamic light-scattering experiments and size-exclusion chromatography independently confirmed this dodecameric species (see Figure S1A available online).

Surprisingly, PLP synthase complexes reconstituted from PPdx1 and PPdx2 formed fibers (Figure 2B). Comparing the spacing between the layers in the fibers (Figure 2B, inset) with the B. subtilis (Bs) crystal structure of BsPdx1/BsPdx2 (Strohmeier et al., 2006) suggested that the fibers consisted of stacked 24-mer with the longer distance between layers in the fibers being internal to the 24-mer. To address whether fiber formation is a specific property of the Pf PLP synthase, we studied PLP synthase complexes from the bacterium Bs and from the mouse malaria parasite P. berghei (Pb). In a similar EM analysis, we used the inactivated BsPdx2H170N (Neuwirth et al., 2007; Strohmeier et al., 2006) and PbPdx2H189N (constructed in analogy to the PPdx2H196N variant). Fiber formation was only observed when PPdx1 was present in the sample, and this fact may explain our failure to obtain suitable crystals for Pf PLP synthase, whereas the Bs PLP synthase (Strohmeier et al., 2006) and the Pb PLP synthase (see below) were successfully crystallized. The finding illustrates the potential of EM when working with samples that are difficult to crystallize, in this case revealing high-order assemblies of macromolecular complexes. Although we clearly observed aggregation of protein over time (Figure S1), we had not suspected that this was an ordered process, and fiber formation would have gone unnoticed had we not wanted to study complex assembly by EM.

RESULTS

Fiber Formation of Recombinant PLP Synthase from Plasmodium falciparum

In order to structurally characterize the malarial PLP synthase, Pdx1 and Pdx2 from Pf were recombinantly expressed and purified to homogeneity. Our dynamic light scattering confirmed that both proteins were monodisperse and suited for crystallization (data not shown). PPdx1 and PPdx2 proteins were reconstituted into the PLP synthase complexes. Crystallization trials were set up in the absence or presence of substrate L-glutamine. Instead of using the native PPdx2 enzyme, we employed a PPdx2H196N catalytically inactive variant to avoid hydrolysis of the substrate. Exchange of the catalytic triad histidine with asparagine allows formation of a stable PLP synthase complex in the presence of substrate (Flicker et al., 2007; Neuwirth et al., 2007; Strohmeier et al., 2006). To our knowledge, whether Pdx2 forms complexes with Pdx1 hexamers is unknown.
Pb in the presence of L-glutamine because the Pf proteins were inaccessible to this analysis due to their tendency to form fibers. When a 1:1 stoichiometric mixture of PbPdx1 and PbPdx2 was used, multiple oligomeric species were observed (Figure 2C, compare Figure 1). Generally, PbPdx1 was seen as dodecameric core complex, but the occupancy with PbPdx2 subunits varied without discernable pattern in the distribution of PbPdx2 subunits on the PbPdx1 core, suggesting that attachment of Pdx2 is at random and, thus, most likely not cooperative. This is an important finding considering that the reaction catalyzed by Pdx1 is cooperative (Knöckel et al., 2009; Raschle et al., 2007).

Pdx1 dodecamers that were fully occupied with 12 PbPdx2 subunits were rarely observed, consistent with the behavior of the bacterial complexes in analytical ultracentrifugation (Strohmeier et al., 2006; Zhu et al., 2005). In an attempt to generate a uniform population of fully occupied complexes, a 1:1 stoichiometric mixture of PbPdx1 and PbPdx2H196N variant proteins was analyzed in the presence of the substrate L-glutamine. Class averages showed highly occupied as well as fully occupied complexes (data not shown). Thus, the inactivation of the Pdx2 subunit and the addition of the substrate L-glutamine increased the stability of the plasmodial PLP synthase complexes, as noted previously with bacterial samples (Strohmeier et al., 2006).

We were able to crystallize the Pb PLP-synthase complex, using the PbPdx1 and PbPdx2H196N proteins in the presence of substrate L-glutamine. Needle-shaped crystals were obtained that diffracted anisotropically to 4 and 5.5 Å at the ESRF microfocus beamline ID23-2. Data analysis suggested a low-symmetry space group with pseudo-merohedral twinning, which complicated data collection and structure determination. Because no significant improvement in crystal growth was made, structure determination was not further pursued.

However, we discovered that a complex from Pb Pdx1 and Pf Pdx2 subunits formed readily in the presence of glutamine. PLP synthase complexes with PbPdx1 dodecamers highly occupied with PbPdx2H196N subunits were formed in vitro (Figure 2D). Complex formation is probably helped by the high sequence conservation: Pf and Pb Pdx1 and Pdx2 proteins are 84.5% and 63.8% identical, respectively (compare alignments in Figure S2). To test whether the chimeric complexes represented a viable model for an active PLP synthase complex, it was essential to determine enzymatic activities. Both chimeric combinations of Pf and Pb proteins were tested and compared with single-species complexes (Table 1). Complexes that contained PbPdx1 showed slightly higher PLP synthase activities than complexes that contained PfPdx1. The PLP synthase activity was not affected by the choice of the Pdx2 subunit. Similarly, when the Pdx2 glutaminase activity was assayed, we found slightly higher activities when PfPdx2 was present, regardless of the source organism for the Pdx1 subunit in the complex. Both, PfPdx2 or PbPdx2 were inactive in the absence of the respective Pdx1 subunit (Gengenbacher et al., 2006).

The summarized data in Table 1 show that plasmodial chimeric complexes form catalytically competent PLP synthase species and that the Pdx1 and Pdx2 proteins within these complexes behave as in native complexes. We, therefore, carried out crystallization experiments with chimeric complexes, a strategy often employed when combining proteins from different species in structure determination of macromolecular complexes, e.g., as in the structure of Pf Falcipain-2 with Pb cystein protease inhibitor (ICP-C (Hansen et al., 2011) or the structure of the Gsa-adenyl cyclase complex, which is a rat/dog/cow chimera (Tesmer et al., 1997).

Critical Differences between Plasmodium and Bacterial PLP Synthase Complexes

Although Pf PLP synthase complexes formed fibers, and crystals of the Pb PLP synthase were of insufficient quality, we were able to grow crystals of a chimeric PLP synthase complex made up from PbPdx1 and PfPdx2H196N in the presence of L-glutamine. Crystals grew in a hexagonal space group, and complete data...
to a resolution of 3.6 Å were collected (Table 2). Structure determination by molecular replacement was facilitated by the availability of the high-resolution models of PbPdx1, determined earlier at 1.62 Å (Gengenbacher et al., 2006) (PDB code 2ABW).

As in the EM analysis, the PLP synthase complex contains a dodecameric Pdx1 core occupied with 12 Pdx2 subunits (Figure 2D). Superposition of the Plasmodium PLP synthase complex with the Bs complex (Strohmeier et al., 2006) showed that the Pdx2 subunit is rotated by 8.5° and displaced by 1.5 Å (Figure 3A). This analysis is based on superposition of 261 Cz positions of a single Pdx1 subunit from the 2 complexes resulting in a root-mean-square deviation (rmsd) of 0.93 Å, using lsqkap (Collaborative Computational Project, Number 4, 1994).

A good quality electron density map resulted after rigid body refinement (Figures 3B and 3C), and a number of protein segments were seen in different conformations from the input models. We carried out model building in sharpened electron density maps, followed by Translation Library Screw-motion (TLS) refinement using Non-Crystallographic Symmetry (NCS) averaging (see Experimental Procedures). Comparison with the Bacillus complex (Strohmeier et al., 2006) (PDB code 2NV2) aided in the building of the critical stabilizing catalytic center loop and oxyanion hole loop regions in Pdx2, residues 10–14 and 52–54 (Figure 3C), as well as in the building of Pdx1 helix α2* (Figure 3B), residues 52–58. In addition to these regions, few segments at the interface or at the surface of the proteins were rebuilt, and these were in Pdx2, the N-terminal residues 3–9 (Figure 3B); in Pdx2, loops 95–111, 124–127, and 140–146 (Figure 3C); as well as the side chains of Arg121 and Arg154, two residues shown previously to be important for the organization of the Pdx1-Pdx2 interface (Wallner et al., 2009).

In Bs (Strohmeier et al., 2006) and Thermotoga maritima (Zein et al., 2006) PLP synthase complexes (PDB codes 2NV2 and 2ISS), helix αN of the Pdx1 subunit is essential for complex formation with and activation of the Pdx2 glutaminase subunit (Strohmeier et al., 2006; Wallner et al., 2009; Zein et al., 2006).

In the two bacterial structures, the β strand βN preceding helix αN interacts with the central β sheet of Pdx2 by a mechanism called β completion (see Figure 3B). Our earlier deletion analysis indicated that in Pf Pdx1 the region that forms βN in bacterial complexes is not involved in interaction between Pdx1 and Pdx2 (Flicker et al., 2007). Indeed, β completion does not occur in plasmoidal PLP synthase. The electron density map in this region suggests that the very N terminus is oriented toward Pdx1 and does not contact Pdx2 (Figure 3B).

The electron density map for the plasmoidal PLP synthase complex resolves the loop region 124–127 that was disordered in the 1.62 Å structure of autonomous PfPdx2 (Gengenbacher et al., 2006) (PDB code 2ABW). This loop is at the interface to the Pdx1 protein, and superposes well with the equivalent regions in bacterial complexes (Figure 3C). PfPdx2 has a seven amino acid insertion between β5 and β6 (ten amino acids in the PfPdx1 sequence).
Figure 3. The Structure of the Plasmodium PLP Synthase Complex

(A) The PLP synthase/L-glutamine complex structure in two views, 90° rotated around an axis horizontal in the paper plane. The *Pb* Pdx1 dodecamer is shown in blue; the *Pf* Pdx2 subunits are shown in green. Superposition of plasmodial and bacterial (Strohmeier et al., 2006) PLP synthases, based on Pdx1, reveals different relative positioning of plasmodial and bacterial Pdx2 subunits (shown in yellow), as visualized in the schematic representation: the rotation by 8.5° is associated with a shift of 1.5 Å.

(B) 

(C)
Plasmodium PLP Synthase

PbPdx2), compared with BsPdx2 (Gengenbacher et al., 2006). This region, labeled loop 95-111, is shown red in Figure 3C, and forms helix α5-1 at the interface to the α2-α3 loop in PIPdx1 (compare alignment in Figure S2A). The segment and, thus, the interaction are absent in bacterial complexes (Strohmeier et al., 2006; Zein et al., 2006). We conclude that the insertion sequences in plasmodial proteins, the differences in the N-terminal region of PbPdx1, and the different structure of the loop regions together explain the divergent thermodynamic signatures for complex formation between Pdx1 and Pdx2 in plasmodial and bacterial proteins as detected by calorimetry (Flicker et al., 2007; Neuwirth et al., 2007).

The Mode of Reciprocal Enzyme Activation, Derived from a Substrate Complex

The interaction between Pdx1 and Pdx2 fine-tunes the activity of the Pdx2 glutaminase (Wallner et al., 2009), and the mechanism of glutaminase activation has been well known for some time (Burns et al., 2005; Gengenbacher et al., 2006; Raschle et al., 2005; Strohmeier et al., 2006). Reorganization of the Pdx2 catalytic center is a major event in enzyme activation (Wallner et al., 2009). The Pdx2 subunit in the plasmodial PLP synthase complex reveals changes in the oxynian region. The experimental electron density fit much better with the PbPdx2 structure, as seen in the BsPdx1/BsPdx2 complex (Strohmeier et al., 2006), than with the input model (Gengenbacher et al., 2006) (PDB code 2ABW) and, hence, was rebuilt. These similarities observed in the BsPdx2 catalytic center imply that the mechanism of Pdx2 activation is conserved, despite the aforementioned documented differences in bacterial and plasmodial Pdx1/Pdx2 interfaces (Flicker et al., 2007; Neuwirth et al., 2007).

An open question is how Pdx1 is activated by complex formation and how ammonia production and incorporation into the forming PLP molecule are coordinated. In the Bs structures, helix α2’ was observed in the vicinity of the active site in the hetereric BsPdx1/BsPdx2 PLP synthase complex but was disordered in autonomous Pdx1, which led us to postulate that this helix may prime the enzyme for catalysis (Strohmeier et al., 2006). However, helix α2’ was ordered in the yeast Pdx1 structure in the absence of Pdx2 (Neuwirth et al., 2009; Zhang et al., 2010). In plasmodial PLP synthase we now observe a “ground state” in this important region, giving additional insight into Pdx1 activation. Helix α2’ does not cover the proposed active site and is leaving ample access to the catalytic center (Figure 4A). This differs from the previously observed closed conformation in PLP synthase complexes from Bs or T. maritima (Strohmeier et al., 2006; Zein et al., 2006).

We wanted to investigate the significance of this finding and determine structures of Pdx1 in the absence or presence of substrate R5P (Table 2). Co-crystallization with the carbohydrate yielded crystals of the PbPdx1-R5P substrate complex (PbPdx1-R5P) that diffracted to 2.4 Å. Interestingly, helix α2’ is in a different position from the chimeric PLP synthase complex (Figure 4), closer to what was observed for Bs or T. maritima PLP synthase complexes and consistent with the active state of the PbPdx1 enzyme.

The 3D structure of the adduct complex showed the carbohydrate R5P in covalent linkage to Lys84, forming a Schiff base (Figure 4B). The structure is of considerable interest, given the ongoing controversies regarding the catalytic mechanism of PLP formation (Burns et al., 2005; Hanes et al., 2008a, 2008b, 2008c; Moccand et al., 2011; Raschle et al., 2007; Zhang et al., 2010). In the previous 2.9 Å resolution structure of T. maritima Pdx1, substrate attachment of the ribose at C2 was suggested (Zein et al., 2006), which was later corrected by chemical analysis (Hanes et al., 2008b). Accordingly, the complex reported here at higher resolution now confirms attachment at C1 (Figure 4B). The residues Asp105 and Ser107 of the totally conserved DESE motif were in close proximity to the substrate but did not make polar contacts. These residues may be involved in Schiff base formation and removal of water from C1. The covalent intermediate was shielded from the environment by helix α2’ and made very few polar contacts with the enzyme. One such polar contact occurred between the 2-OH group of the substrate and the carboxylate of the Asp27 side chain, which is totally conserved among Pdx1 enzymes. Asp27 could, therefore, have a catalytic role in water elimination from the carbon backbone. Finally, there were polar interactions between the oxygen atoms of the substrate phosphate group with three glycines (Gly156, Gly217, and G238) and the Ser239 hydroxyl group from helix α8’ (Figure 4B). Helix α8’ points toward the phosphate group with its N-terminal dipole.

Methionine Residues Involved in Ammonia Transfer Link Ammonia Production, Transfer, and PLP Biosynthesis

On the reaction path toward the forming PLP molecules, ammonia is incorporated into the R5P adduct to form a stable chromophoric intermediate (Hanes et al., 2008b; Raschle et al., 2007). The site of ammonia production by Pdx2 is about 22 Å away from the site of ammonia incorporation in Pdx1. A transient hydrophobic tunnel operated by shifting methionine residues was proposed for the passage of ammonia (Strohmeier et al., 2006). With the exception of Met46 and Met148, these methionine residues are not conserved between eukaryotic and bacterial orthologs (Figures 5A, 5B, and S2A).

Three amino acid exchanges give the plasmodial Pdx1 ammonia tunnel a character different from the Bs protein. First, Trp16 in PbPdx1 replaces BsMet13 in helix αN. Variation at this position was analyzed in the Arabidopsis protein, where
Leu30 is found in the equivalent position (Figure S2A). Our earlier mutagenesis study showed that this residue influences the coupling between Pdx2 glutaminase and Pdx1 synthase activities and possibly alters entrance into the tunnel (Tambasco-Studart et al., 2007). Interestingly, the exchange of PbTrp16 for BsMet13 is matched by a reciprocal exchange of PbMet103 for BsTyr100. Finally, PbLeu82 replaces BsMet79. These three exchanges lead to differences in the construction of the putative ammonia tunnel, as seen from the position of cavities (Figure 5C). The reciprocal exchanges suggest a functional swap in methionines and flexibility in the construction of the tunnel.

We sought to confirm the role of methionine residues by site-directed mutagenesis and determination of associated enzymatic activities and determine Pdx2 glutaminase activity as well as PLP synthase activity both with ammonium salts or Pdx2 as source for the required ammonia. We also calculated the efficiency of enzymatic coupling as the ratio between Pdx2-dependent and ammonium salt-dependent PLP synthesis rates. Using this calculation, wild-type proteins show a ratio of 1.6. This indicates that the presence of Pdx2 has a stimulating effect on Pdx1 activity (Figure 6).

Replacement of PbMet19 located in helix αN with valine halved the glutaminase activity but did not significantly affect PLP synthesis rates in the Pdx2-dependent assay, suggesting that this residue plays a role in glutaminase activation. In the ammonia-dependent assay we observed a doubling of PLP

Figure 4. The PbPdx1-R5P Substrate Complex
(A) Comparison of Pdx1 monomers from autonomous PbPdx1 (left) and the PbPdx1-R5P substrate complex (right). The side chain of Lys84 and the covalent intermediate complex are shown in stick representation. Helix α2' is observed in the closed conformation in the PbPdx1-R5P substrate complex, whereas it is in the open conformation in the PbPdx1/R5P-glutamine PLP synthase complex (blue).
(B) Two views showing selected structural elements that define substrate binding, rotated by about 60° around a horizontal axis. The following protein-carbohydrate interactions are shown: the covalent R5P attachment between Lys84 and the C1 atom of R5P through a Schiff base; the contact between Asp27 and the C2-OH of R5P; backbone interactions with the R5P phosphate group (loop β6-α6, green; loop β7-α7, pink; loop β8-α8, light blue); and the contact between the hydroxyl group of Ser239 on α8' with the R5P phosphate group. Residues Asp105 and Ser107 of the totally conserved DESE motif are seen in close proximity to the substrate and Lys84. Also shown are helices α2' (blue) and α8' (light blue) that change conformation upon substrate binding.
synthase activities, and thus, exchange of this methionine with the smaller valine may have released a block at the tunnel entrance.

We grouped residues lining the tunnel through the central core of the β barrel in three layers. Layer 1 contains PbLeu82, PbMet103, and PbMet148. Although variation of PbLeu82 adversely affected PLP synthase activity in the ammonium salt-dependent assay, PbMet103 lowered PLP synthase activities in both the ammonium salt and Pdx2-dependent assays. Complete loss of activity in the PbMet103Phe variant suggested that the tunnel was impassable for ammonia. Even replacement of PbMet103 with alanine showed lowered PLP synthase activity, suggesting that Met103 is required for regulation of ammonia transfer. The most significant changes are observed in variation of Leu82 and Met148. Exchange of these residues resulted in approximately 4- to 5-fold higher ratios between Pdx2-dependent and ammonium salt-dependent PLP synthase rates. These residues, therefore, directly link glutamine hydrolysis, ammonia transfer, and PLP production. This is in contrast to variation of the conserved PbMet46 in layer 2, which leads to significant increase in PLP synthase activity in both types of assays, suggesting that this residue influences the function of the ammonia tunnel but does not function in enzyme communication.

**Figure 5. Ammonia Tunnel in Pb Pdx1**

(A) View of the PbPdx1 β barrel (i2 and i3 removed for clarity) and part of the αN helix. Selected side chains are shown in stick representation and labeled (gray), and residues from a superposed BsPdx1 are shown for comparison (light blue, labels in brackets, PDB code 2NV2) (Strohmeier et al., 2006). (B) Schematic organization of the NH3 tunnel with a comparison of PbPdx1 (top) and BsPdx1 (bottom). Residues pointing into the β barrel are highlighted in gray. Differences discussed are marked in blue (PbPdx1) and green lettering (BsPdx1). (C) Cavities in the NH3 tunnel for PbPdx1 (left) and BsPdx1 (right), produced with a 1.2 Å (blue) and 1.4 Å (green) probe radius in PyMOL (DeLano, 2002).

Structure 20, 172–184, January 11, 2012 © 2012 Elsevier Ltd All rights reserved 179
DISCUSSION

PLP biosynthesis has received renewed interest since the discovery of the new route catalyzed by the Pdx1 and Pdx2 enzymes (Ehrenshaft et al., 1999; Mittenhuber, 2001). Several mechanistic studies on PLP biosynthesis by the enzymes Pdx1 and Pdx2 have recently appeared (reviewed in Fitzpatrick et al., 2007, 2010; Mukherjee et al., 2011). Structural descriptions were so far focused on prokaryotic systems (Strohmeier et al., 2008; Zein et al., 2006), and insight into orthologous eukaryotic PLP synthases is sparse (Neuwirth et al., 2009; Zhang et al., 2010). Further structural knowledge is required to understand documented differences between prokaryotic and eukaryotic systems in their biochemistry and in the interaction between Pdx1 and Pdx2 proteins (Flicker et al., 2007; Neuwirth et al., 2007). The potential impact as a drug target (Müller et al., 2008; Müller and Kappes, 2007) further provides a strong motivation to characterize the malarial enzymes.

*Pf* PLP synthase proved a difficult target for crystallographic 3D structure determination due to the observed aggregation into fibers in vitro, as detected by EM analysis. In contrast, fiber formation was not seen with the *Pb* proteins, and it will require further data to establish whether this finding can be generalized to other Pdx1 orthologs. We have compared the surfaces of the proteins to study the cause of fiber formation, but a mechanism was difficult to assign. An insertion in the *PfPdx2* sequence (residues 95–111), varying between *Pf* and *Pb* proteins and not present in prokaryotic sequences, could mediate contacts in fibers. Because the chimeric complex contains *PfPdx2* elements in the highly conserved Pdx1 protein must contribute to fiber formation. Indeed, the EM analysis not only detected fiber formation preferentially of the *PfPdx1/PfPdx2* complex but also of the *PfPdx1* proteins (data not shown). Circumstantial evidence comes from gel filtration analysis that shows that *PfPdx1* proteins form higher-order oligomers after prolonged storage at 4°C, whereas this is also true for the *PfPdx1/PfPdx2* PLP synthase complex, albeit at a higher proportion (Figure S1).

Our previous study on *Plasmodium* Pdx1 demonstrated that hexamers are required for formation of the covalent enzyme-substrate adduct (shown in Figure 4) (Derrer et al., 2010). The C terminus of the protein responsible for hexamer formation probably has a function in trans, influencing the catalytic activity in a neighboring subunit in the hexameric form of Pdx1. To our knowledge, no data are currently available to give a molecular rationale for dodecamer formation. Indeed, the yeast Pdx1.1 protein is hexamerically equal or higher catalytic activity than dodecameric Pdx1 homologs (Neuwirth et al., 2009). Although an even higher organization into fibers shown here may not be catalytically relevant, fibers may provide a storage pool, perhaps protecting the viable C terminus that is prone to degradation (Raschle et al., 2009). A similar behavior of higher-order oligomerization has been seen in the AAA-ATPase Vps4p (Hartmann et al., 2008). It is an open question whether fibers could be identified at physiological conditions; however, fiber formation may be relevant to explain our previous data that revealed an uneven distribution of the two proteins within the cytosol in blood stages of the parasite (Gengenbacher et al., 2006).

<table>
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<th>Protein</th>
<th>Glutaminase activity (% of wild-type)</th>
<th>PLP synthesis using NH4Cl as NH donor mol/min mg</th>
<th>PLP synthesis using Pdx2/Gln as NH donor mol/min mg</th>
<th>Pdx2-dependent PLP synthesis rate</th>
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<td><em>PbPdx1M103A</em></td>
<td>178 ± 18 (97)</td>
<td>0.31 ± 0.0 (34)</td>
<td>0.42 ± 0.1 (30)</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td><em>PbPdx1M103F</em></td>
<td>126 ± 37 (69)</td>
<td>no activity</td>
<td>no activity</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>PbPdx1M148L</em></td>
<td>229 ± 7 (125)</td>
<td>0.8 ± 0.4 (89)</td>
<td>3.7 ± 1.2 (274)</td>
<td>4.6</td>
<td></td>
</tr>
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</table>

Figure 6. Mutagenesis and Kinetic Analysis of Pdx1 Variants

The columns in the table show the *PbPdx1* glutaminase activity in the presence of *PbPdx1* proteins and variants, as well as the PLP synthase activities of *PbPdx1* proteins and variants in the presence of *PbPdx2* or in the absence of *PbPdx2* with ammonium salt as ammonium donor. The effect of Pdx2 was calculated as ratio of glutamine-dependent and -independent assay-specific activities and is plotted in the bar diagram. Values are the mean of two independent measurements.
Autonomous PbPdx1 was dodecameric, as is characteristic for prokaryotic Pdx1 proteins (Strohmeier et al., 2006; Zein et al., 2006; Zhu et al., 2005). This observation emphasizes the difference with the Pdx1 protein from S. cerevisiae that was hexameric in the crystal structure and almost exclusively hexameric in solution (Neuwirth et al., 2009). In the yeast protein a unique sequence variation not present in PbPdx1 was responsible for the different oligomerization behavior (Neuwirth et al., 2009). The Plasmodium structure confirms that eukaryotic Pdx1 proteins, in general, can assemble into dodecameric structures.

From the EM studies we gained new insights into PLP protein complex assembly. The PbPdx1 dodecamers were randomly occupied by PbPdx2 subunits, and none of the electron microscopic data showed any preference for the PbPdx2 association, and in particular there seemed to be no requirement for neighboring or alternating binding sites to be occupied. We tested against the presence of Pdx1 substrate R5P but did not detect differences in complex formation. Fully occupied complexes require the presence of glutamine and inactivation of the Pdx2 subunit by exchange of catalytic residues (Strohmeier et al., 2006). Under these conditions the encounter complex between Pdx1 and Pdx2 is converted to the more stable Michaelis complex (Flicker et al., 2007; Neuwirth et al., 2007). The electron microscopic data presented here now suggest that after glutamine hydrolysis, Pdx2 not only reverts to the encounter complex but also leaves the complex. Alternatively, encounter complexes must have high off rates.

Aggregation of Pf PLP synthase samples into fibers, whereas in itself an interesting result, prohibited crystallization. We resorted to crystallize a chimeric complex between PbPdx1 and PfPdx2. The reconstituted PLP synthase was fully functional with respect to glutaminase and PLP synthase activities, suggesting that the PbPdx1 and PfPdx2 proteins interact in a way that allows for enzyme activation. Availability of this enzymatically fully active complex enabled not only crystallization of a Plasmodium PLP synthase but also the 3D structure determination at resolution higher than that possible with the Pb PLP synthase complex. The approach to address relevant biological questions by combining proteins from different species in multiprotein complexes has proven to be a valuable approach in crystallography in general and may, with the Plasmodium proteins in particular, help to circumvent some of the pertinent problems in protein production (Mehlin, 2005; Mehlin et al., 2006).

Structure determination of PLP synthase by molecular replacement and subsequent NCS refinement at 3.6 Å benefited from availability of high-resolution models (2.4 Å for PbPdx1, this study, and 1.6 Å for PfPdx2, PDB entry 2ABW). The resulting electron density map visualized structural differences from the input models, in well-ordered regions even for side chains. Structural differences mainly occurred at protein interfaces and explain the differences in the interaction of prokaryotic and eukaryotic PLP synthases, as described earlier (Flicker et al., 2007; Neuwirth et al., 2007). Eukaryotic structures differ from their prokaryotic counterparts by sequence insertion in Pdx2, which in PfPdx2 is found in the loop comprising amino acids 95–111 (Gengenbacher et al., 2006), and a smaller insertion can also be identified in the Arabidopsis thaliana Pdx2 sequence (Figure S2B). This loop is observed in different positions in the structures of autonomous Pf Pdx2 and the PbPdx1/PfPdx2 PLP synthase complex. Furthermore, the loop connecting β6 and β7 is disordered in autonomous Pdx2 but ordered in the PLP synthase complex where it is part of the Pdx1-Pdx2 interface. The segment N terminal to helix αN in PbPdx1 does not interact with PfPdx2, in contrast to previous bacterial structures. This explains why we could not detect differences in the interaction of PfPdx1 and PfPdx2 when deleting this segment (Flicker et al., 2007). The latter abates the general importance of β augmentation for plasmodial PLP synthase.

Pdx1 is cooperative (Raschle et al., 2009) and relies on the C-terminal region for catalysis. The entire C terminus is required for full PLP synthase activity (Derrer et al., 2010), and a large part of the C terminus is resolved here, missing only the 14 most C-terminal residues. The resolved protein region is sufficient for full PLP synthase activity (Derrer et al., 2010), and a large part of the C terminus is resolved here, missing only the 14 most C-terminal residues. The resolved protein region is sufficient for PLP synthase, as demonstrated in our previous C-terminal deletion studies in Pf Pdx1 (Derrer et al., 2010). The resolved C terminus contacts the loop between β6 and α6 of the same subunit and is in close proximity to helix α2’ of an adjacent subunit. Importantly, we show here two different structures for the α2’ region, depending on either formation of the PLP synthase complex or substrate binding (Figure 4A). The cooperative mechanism of PLP biosynthesis might thus rely on the active role of the C terminus in moving the helix α2’ that subsequently covers the active site and sequesters the reaction intermediates from the aqueous environment. Conversion from the open to the closed state of the helix α2’ is not dependent on Pdx2 because Pdx1 alone forms covalent R5P intermediates. However, Pdx2 promotes an ordering of helix α2’, explaining why contact between Pdx1 and Pdx2 enhances R5P binding (Raschle et al., 2009). These findings give structural evidence how Pdx2 actively influences the catalytic state of the Pdx1 synthase.

Finally, the structures of the autonomous PbPdx1 and covalent enzyme substrate complex allow three important advances in the understanding of PLP synthase: (1) the alternative positioning of helix α2’, suggesting a regulatory layer in PLP synthase activation; (2) the assignment of the pentose substrate in Schiff base with the catalytic Lys84 attached to the ribose C1 atom; and finally, (3) the role of the β barrel in NH4 transfer. Remarkably, the plasmodial proteins reveal plasticity in the construction of the ammonia tunnel. The features of ammonia transfer and catalytic mechanism allow us to understand—arguably—one of the most complex enzymatic systems known to date, and several of these unique features are exploitable in drug design.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

3-acetylpyrimidine adenine dinucleotide (APAD), L-glutamic dehydrogenase from bovine liver, L-glutamine, DL-G3P, and R5P were from Sigma-Aldrich, Vienna. Imidazole was from Merck Darmstadt, Germany. KCl, NaH2PO4, and EDTA were from AppliChem GmbH, Darmstadt, Germany. NaCl was from AnalAr Normapur, Belgium. All other chemicals and reagents were from Roth, Karlsruhe, Germany.

**Molecular Biology, Protein Production, and Purification**

The pdx1 gene was amplified from cDNA of the Pb NK65 strain using custom primers (Invitrogen Karlsruhe, Germany) and subsequently cloned into pET21a expression vector (Novagen, USA), using NdeI and XhoI restriction sites (enzymes from New England Biolabs, Frankfurt). The pdx1 and pdx2 genes

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**Notes:**

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from Pf3D7 strain and Pdx2 from P. berghei were cloned as described (Gengenbacher et al., 2006). Pbpdx2 was subcloned into PET24a.

His-tagged Pbpdx1 or Pbpdx1 and Pbpdx2 or Pbpdx2 proteins were recombinantly expressed at 37°C in Escherichia coli BL21-CodonPlus (DE3)-RIL cells (Strategene, USA). Cells were grown to an OD600 of 0.6, induced by addition of 0.1 mM isopropyl thiogalactoside, and then further grown for 4 hr. The cells were harvested by centrifugation and stored at −20°C. Proteins were purified by immobilized metal ion affinity chromatography using Ni-ntriutriacetic-acid agarose beads (GE Healthcare) equilibrated with 20 mM Tris/HCl (pH 8.0) buffer containing 10 mM NaCl, 1 mM EDTA, 0.5 mM mono-thioglycerol (MTG) was used. For Pbpdx2 or Pbpdx2 a Superdex 75 26/60 column (GE Healthcare) equilibrated with 20 mM HEPES (pH 7.0) buffer containing 300 mM KCl, 0.5 mM MTG was used. Fractions containing the desired protein were pooled and concentrated using Amicon Ultra filters (Millipore, Ireland).

**Determination of Enzymatic Activities**

The enzymatic activity for PLP synthesis was monitored spectrophotometrically at λ = 414 nm using a Jasco V550 Spectrophotometer (Jasco, Gross-Umstadt, Germany) according to the protocol described earlier (Raschle et al., 2005). Here, the purified proteins were exchanged into reaction buffer 50 mM Tris/HCl (pH 8.0), 20 mM NaCl using Amicon Ultra filters (Millipore). The reaction mixture contained 40 μM Pdx1, 1 mM R5P, 1 mM G3P, and 10 mM NaCl. For Pdx1/Pdx2 complexes, additionally 40 μM Pdx2 was present, and NaCl was replaced with 10 mM L-glutamine. Measurements were carried out at 37°C.

The enzymatic activity for glutamine hydrolysis was analyzed using a coupled enzyme assay with glutamate dehydrogenase as described earlier (Gengenbacher et al., 2006). The reaction mixture contained 5 μM Pdx1, 5 μM Pdx2, 10 mM L-glutamine, 10 μM glutamate dehydrogenase, and 0.5 mM APAD+ in 50 mM Tris/HCl (pH 8.0), 20 mM NaCl. The reduction of APAD+ to APADH was monitored for 20 min at 363 nm using a Jasco V550 Spectrophotometer.

**Crystallography**

Pbpdx1 at a concentration of 20 mg/ml was crystallized by sitting or hanging drop vapor diffusion in 0.1 M bicine (pH 8.5) buffer containing 1.6 M LiCl buffer at 19°C. For the Pbpdx1-RSP, crystallization was carried out in the presence of 10 mM RSP. The Pbpdx1/Pbpdx2H196N/glutamine chimeric complex was prepared by mixing the two purified proteins at 1:1 stoichiometric ratio in a buffer containing 10 mM L-glutamine. The complex was crystallized at a concentration of 15 mg/ml in 0.1 M Na/K phosphate-phosphate buffer (pH 6.2) containing 0.2 M NaCl, 6% PEG 8000 by hanging drop vapor diffusion at 19°C. All crystals were flash frozen in liquid nitrogen, using additional 15% 2% uranyl acetate using the sandwich technique (Diepholz et al., 2008). Micrographs were recorded either with a CM120 Biotwin electron microscope at 100 kV and a nominal magnification of 52,000× on a 4kx4k Tietz-CCD-camera, or with a FEI F20 electron microscope at 200 kV with a nominal magnification of 29,000× on a Tietz 8x8k CMS camera. The pixel size was 2.36 Å/pixel for the CM120 and 2.68Å/pixel for the F20. For image analysis, particle images were semiautomatically selected with the autobox option of Beter (Ludtke et al., 1999). Subsequent alignment and classification were done with IMAGINE 5 (van Heel et al., 1996).

**ACCESSION NUMBERS**

The PDB accession numbers for the structures reported in this paper are 4ads, 4ad4, and 4adu.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes two figures can be found with this article online at doi:10.1016/j.str.2011.11.015.

**ACKNOWLEDGMENTS**

Staff at the European Synchrotron Radiation Facility is gratefully acknowledged for support during data collection. We thank Jürgen Kopp and Claudia Stauffer from the cryocrystallization platform of the Cluster of Excellence CellNetworks (BZH, Heidelberg) for protein crystallization. Electron microscopy investigations were carried out at EMBL-Heidelberg and in the high-resolution electron cryo microscopy facility Edinburgh (funded by Wellcome Trust: WT087658MA and the Scottish Universities Life Science Alliance). We would like to thank Kai Matuschewski for the possibility to grow P. berghei. K.H. was funded by the Scottish Universities Life Science Alliance. B.B. was funded by the Darwin-Trust of Edinburgh. This work was supported in part by grants from the European Commission (VITBIOMAL-012158) and by the DFG (TE368).

Received: July 28, 2011
Revised: November 4, 2011
Accepted: November 5, 2011
Published: January 10, 2012

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