Dissection of Contributions from Invariant Amino Acids to Complex Formation and Catalysis in the Heteromeric Pyridoxal 5-Phosphate Synthase Complex from *Bacillus subtilis*†

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ABSTRACT: Pyridoxal 5-phosphate (PLP), an active form of vitamin B_6, is one of the most versatile cofactors and is involved in numerous biochemical reactions. The main pathway for de novo PLP biosynthesis leads to direct formation of PLP from a pentose and triose. This reaction is catalyzed by the heteromeric PLP synthase, consisting of the synthase subunit Pdx1 and the glutaminase subunit Pdx2. L-Glutamine hydrolysis by Pdx2 supplies ammonia to Pdx1 for incorporation into PLP. Autonomous glutaminase Pdx2 is inactive; however, interaction with Pdx1 leads to enzymatic activity. Oxyanion hole formation in the active site of Pdx2 is required for substrate binding and was suggested as the prime event of enzyme activation. Here, we dissect interactions required for complex formation from interactions required for catalytic activation of the glutaminase. The three-dimensional structural analysis suggested a number of invariant residues that regulate complex formation and enzyme activation. We have replaced several of these invariant residues by site-directed mutagenesis in an effort to understand their function. In addition to the biochemical characterization of enzyme activity, the generated protein variants were studied by isothermal calorimetry to investigate their role in complex formation. The assembled data describe a multistep activation mechanism. Residues of helix αN of Pdx1 are essential for formation of the Pdx1−Pdx2 complex and also stabilize the oxyanion hole. Thus, these interactions describe the encounter complex. On the other hand, residues at the N-terminal face of the $(\beta\alpha)_8$ barrel of Pdx1 contribute to interface formation and are required for the organization of the catalytic center; thus, these interactions describe the Michaelis complex. However, the main players for formation of the Michaelis complex reside on Pdx2, as replacement of residues at the N-terminal face of the $(\beta\alpha)_8$ barrel of Pdx1 leads to reduction but not complete inactivation of the glutaminase.

Vitamin B_6 serves as one of the most versatile organic cofactors in biology. Besides its function in maintenance of the nervous and immune system in animals and humans (*1*), vitamin B_6 plays an important role as an antioxidant in various organisms (*2*−*4*). Pyridoxal 5-phosphate (PLP),[1] a biologically active form of vitamin B_6, is an essential cofactor in many enzyme reactions such as transaminations, decarboxylations, racemizations, and eliminations in amino acid metabolism, DNA biosynthesis, and biosynthesis of antibiotic compounds (*5, 6*). While bacteria, fungi, protozoa, and plants possess de novo biosynthetic pathways for vitamin B_6, these are missing in mammals, and hence, they require a constant supply of this vitamin in their diet.

Most PLP-synthesizing species, with the exception of the γ-subdivision of proteobacteria (*3, 7, 8*), utilize PLP synthase, a glutamine amidotransferase (GATase), consisting of a glutaminase subunit (Pdx2) with Rossmann topology and a synthase subunit (Pdx1) with a $(\beta/\alpha)_8$-TIM-barrel fold (*9*). In *Bacillus subtilis*, this PLP synthase is not a single heterodimer composed of a glutaminase and a synthase subunit, but a complex of approximately 650 kDa. Six Pdx1 molecules form a hexameric ring. Two such rings interdigitate to form the dodecameric core of the GATase. Each of the 12 subunits independently interacts with one glutaminase subunit, forming a larger complex of up to 24 subunits (*10*−*12*).

The two half-reactions—generation of ammonia from L-glutamine and generation of PLP from glyceraldehyde 3-phosphate, ribose 5-phosphate, and ammonia—are cata-

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[1] Abbreviations: APAD, 3-acetylpyridine adenine dinucleotide; *Bs*Pdx1, Pdx1 of *Bacillus subtilis*; CV, column volume; Δ*H*, enthalpy; DXP, deoxyxylulose 5′-phosphate; G3P, glyceraldehyde 3-phosphate; GATase, glutamine amidotransferase; Δ*G*, Gibbs free energy; IPTG, isopropyl thio-β-d-galactoside; ITC, isothermal titration calorimetry; K_a, association constant; K_d, dissociation constant; *N*, stoichiometry; PLP, pyridoxal 5-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; Δ*S*, entropy; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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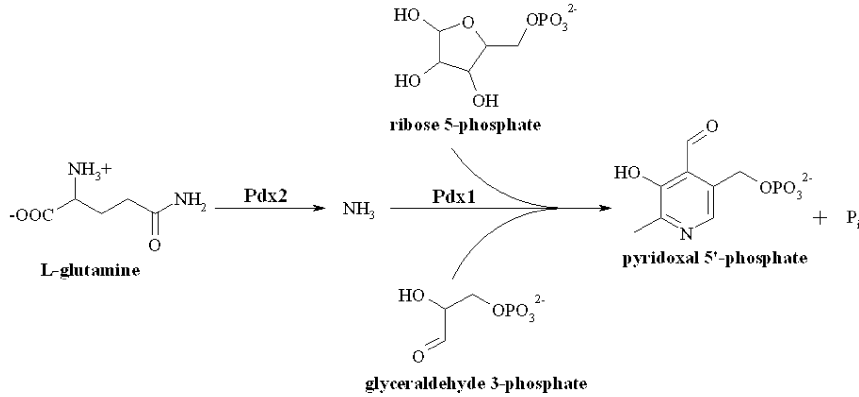
Scheme 1



lyzed by the respective subunits (Scheme 1). The reactive intermediate ammonia is transferred through an internal enzyme tunnel in the Pdx1 subunit from the glutaminase catalytic center to the synthase active site (compare Figure 1). The tunnel is transient in nature and lined by flexible methionine residues (*10*). It was reported earlier that the glutaminase Pdx2 is inactive in the absence of the synthase subunit Pdx1 (*13, 14*). Additionally, it was structurally predicted how the glutaminase is activated by the contact of the two enzyme subunits. A focal point of activation revolves around the formation of an oxyanion hole in the active site

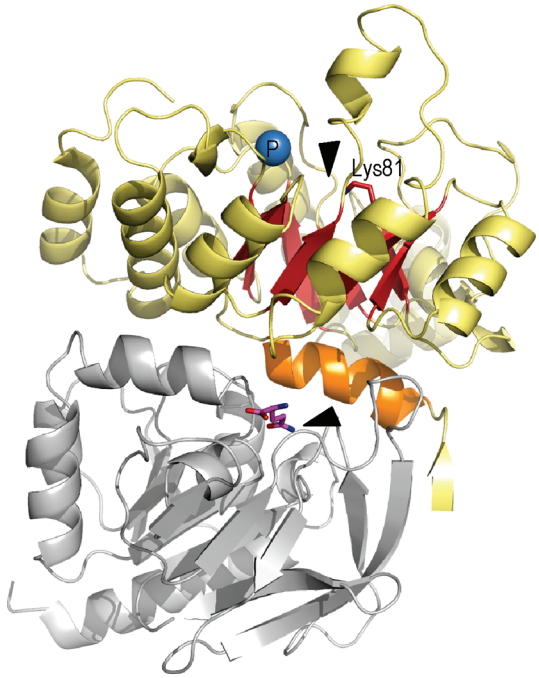


FIGURE 1: Structure of a Pdx1−Pdx2 heterodimer in presence of L-glutamine. PLP synthase subunit Pdx1 and glutaminase subunit Pdx2 are colored light orange and gray, respectively [Protein Data Bank entry 2NV2 (*10*)]. The substrate L-glutamine (magenta) is shown in stick representation in the active site of Pdx2. Ammonia is hydrolyzed from glutamine substrate by Pdx2. It then transfers through an internal enzyme tunnel formed by the central β barrel of the Pdx1 subunit [colored red (*10*)] to a second active site where the pentasaccharide is binding (*11*). The blue sphere represents bound phosphate near the putative Pdx1 active site. Starting and ending positions for ammonia passage are indicated by triangles. Helix αN of Pdx1 discussed in the text is highlighted in orange. The Pdx2 glutamine substrate binding region rotated by ∼180° around a vertical axis is shown in Figure 2. Figure 3 shows the glutaminase active site and the oxyanion region.

of the glutaminase. This event is triggered by a peptide flip induced by interaction with the synthase domain (*10*). Since the oxyanion hole is required to stabilize the negative charge of the reaction intermediate during glutamine hydrolysis, it is essential for catalysis. Whether oxyanion hole formation and glutamine binding occur at the same time is unclear. However, we were able to describe an encounter complex, the binary Pdx1−Pdx2 complex, that is tightened in the presence of the substrate L-glutamine and thus converts to a ternary Michaelis complex (Pdx1−L-Gln−Pdx2) (*15*). A precise match between enzymatic subunits is required, and a number of structural changes occur upon complex formation. Pdx1 residues Ser75 and Asp99 appear to be involved in the reorganization of the substrate binding region of Pdx2 crucial for the formation of the ternary Pdx1−L-Gln−Pdx2 complex (Scheme 2) (*10*). These two Pdx1 residues contact several residues of Pdx2 at the interface and L-glutamine binding site, most notably Glu48, Arg106, and Arg135. In addition, the Pdx1−Pdx2 complex structure has indicated that Pdx1 Lys18, Pdx2 Glu15, and Pdx2 Gln10 play an important role in formation of the oxyanion hole and the heteromeric complex. To analyze the contributions of these amino acids, we replaced these residues by site-directed mutagenesis and determined the effects on enzyme activity and protein complex formation for the created variant proteins.

MATERIALS AND METHODS

Reagents. Bovine glutamate dehydrogenase was from Fluka. L-Glutamine, APAD, ribose 5′-phosphate, and DL-glyceraldehyde 3-phosphate were from Sigma-Aldrich (Vienna, Austria). Tris, imidazole, and NaCl were from Roth (Karlsruhe, Germany). NaH_2PO_4 was from Merck (Darmstadt, Germany).

Recombinant Expression, Purification, and Quantification. In our study, we have used genes *pdx1* and *pdx2* from the Gram-positive bacterium *B. subtilis* for recombinant expression in *Escherichia coli* host cells [strain BL21(DE3), Stratagene]. *pdx1* and *pdx2* were cloned into pET21a(+) (Novagen) and pET24b(+) (Novagen), respectively, to afford generation of the C-terminally hexahistidine-tagged proteins. Heterologous protein expression was conducted at 37 °C and induced at an OD_{600} of 0.6 with IPTG (final concentration of 0.1 mM). After 3 h, bacteria were harvested by centrifugation, washed with 0.9% NaCl, and stored at −20 °C.

For purification of all proteins, cells were thawed and resuspended in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0)]. The cells were lysed by sonication, and the raw lysate was cleared by centrifugation at 40000*g* for 30 min at 4 °C. The supernatant was applied to a Ni-NTA agarose HP column (GE Healthcare), washed with 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8.0), and eluted with 50 mM NaH_2PO_4, 300 mM NaCl, and 150 mM imidazole (pH 8.0). Fractions containing the protein were combined and dialyzed against 20 mM Tris-HCl and 10 mM NaCl (pH 7.5). The protein was concentrated using Centriprep centrifugal filter devices (Amicon) with a molecular mass cutoff of 10 kDa. Protein concentrations were determined by measuring the absorbance at 280 nm using predicted extinction coefficients of 11460 M^{-1} cm^{-1} (Pdx1) and 2980 M^{-1} cm^{-1} (Pdx2) (*16, 17*). The purified proteins were flash-frozen and stored at −20 °C.

Site-Directed Mutagenesis. Site-directed mutagenesis of Pdx1 and Pdx2 for single and double mutants was carried out using the QuickChange XL Site Directed Mutagenesis Kit (Stratagene).

Isothermal Titration Calorimetry (ITC). Unless otherwise noted, microcalorimetric experiments were carried out in 20 mM Tris-HCl and 10 mM NaCl (pH 7.5). Both the purified enzymes and L-glutamine were dissolved in exactly the same buffer, and all solutions were degassed immediately before measuring. Binding of the titrant (Pdx2 wild type or variant proteins) to Pdx1 in the presence or absence of L-glutamine and the binding of L-glutamine to the complex were analyzed with a VP-ITC MicroCalorimeter (MicroCal) equilibrated to 25 °C. In a typical experiment, a total of one aliquot of 2 μL and 19 aliquots of 15 μL of the titrant (150 μM Pdx2 or Pdx2 variant) were injected into 1.421 mL of a 20 μM Pdx1 (wild type or mutein) or Pdx1 (wild type or mutein)/L-glutamine (20 μM and 1 mM, respectively) solution under constant stirring at 270 rpm. All titrations in the absence of L-glutamine were performed with Pdx2 and Pdx2-His170Asn, the glutaminase deficient form of Pdx2. For analysis of the binding of L-glutamine to the complex, aliquots of 15 μL of a 10 mM L-glutamine solution were injected into 1.421 mL of the preformed complex (20 μM) containing one variant protein. Every injection was carried out over a period of 30 s in intervals of 250 s. The heats of binding were determined by integration of the observed peaks. To correct for the heats of dilution of the titrant, the heats evolving in a reference measurement (titrant was injected into buffer or L-glutamine, or L-glutamine was injected into buffer) were subtracted from the heat of each injection. The corrected values were plotted against the ratio of titrant to protein concentration in the cell to generate the binding isotherm. Nonlinear least-squares fitting with Origin 7.0 (MicroCal) for ITC data analysis was used to obtain dissociation constants, heats of binding, and stoichiometries. The K_d and Gibbs free energy were calculated according to

$$K_d = \frac{1}{K_a} \quad (1)$$

$$\Delta G = -RT \ln K_a = RT \ln K_d \quad (2)$$

PLP Synthase Activity Assay. The PLP synthase activity of Pdx1 wild-type and variant proteins was determined as described in ref *14*; 40 μM protein, 1 mM ribose 5-phosphate, and 2 mM glyceraldehyde 3-phosphate were used. All measurements were carried out with ammonium sulfate (10 mM) or L-glutamine as the nitrogen source at 37 °C.

Glutaminase Activity Assay. The glutaminase activity of Pdx2 wild-type and variant proteins was monitored via coupled enzyme assays with glutamate dehydrogenase as described in ref *14*. All samples were assayed at 37 °C for 15 min.

RESULTS

Enzymatic Properties of Pdx1 Variant Proteins. Formation of PLP by Pdx1 can be monitored spectrophotometrically at 414 nm. Activities obtained from spectrophotometric measurements for Pdx1 wild-type and variant proteins, using ammonium sulfate as a nitrogen source, are summarized in Table 1A. In addition, we have assessed PLP formation by Pdx1 in the presence of Pdx2 and with L-glutamine as a nitrogen source (Table 1B).

Analysis of the structure of Pdx1 (Figure 2) had suggested that Ser75 plays an important role in the reorganization of the L-glutamine binding site upon formation of a complex with Pdx2, the glutaminase subunit (Scheme 2) (*10*). As one can see in Figure 2, Pdx1 Ser75 is involved in formation of a hydrogen bonding network with residues around the Pdx2 active site (*10*). To probe the role of Ser75, we generated a Pdx1-Ser75Ala variant protein and tested it for enzymatic activity. This exchange has no effect on enzymatic formation of PLP in the assay using ammonium as the nitrogen source (Table 1A) and reduces enzymatic activity 2-fold in the Pdx2/L-glutamine assay (Table 1B).

In the close vicinity of Ser75, Asp99 participates in a hydrogen bonding network with Pdx2 Arg135 and Arg111

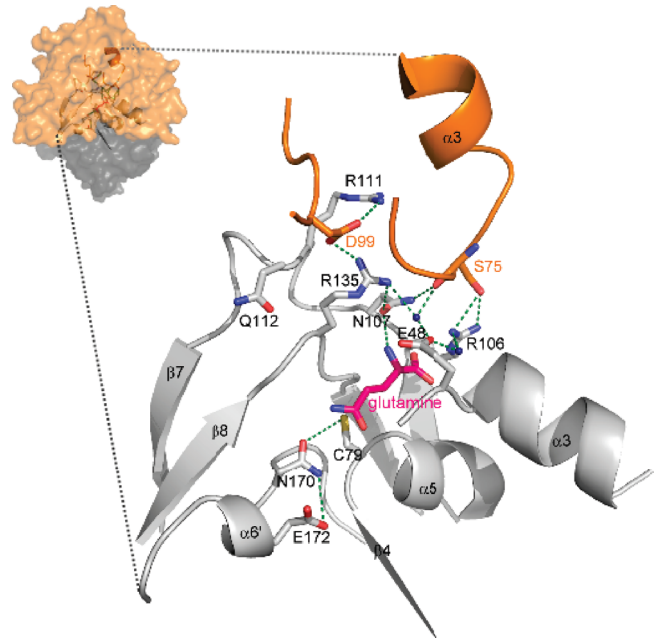


FIGURE 2: Surface representation of the Pdx1−Pdx2 heterodimer and a close-up of the "lid" interface with the substrate binding region of the ternary Pdx1−L-Gln−Pdx2 complex. PLP synthase subunit Pdx1 and glutaminase subunit Pdx2 are colored orange and gray, respectively. The substrate L-glutamine is colored magenta in the center of the catalytic site. Important amino acid residues are shown in stick representation, and the hydrogen bond network between crucial residues is represented as dashed green lines. The representation was generated from Protein Data Bank entry 2nv2 using Pymol Executable Build (DeLano Scientific LLC).

and was predicted to play a significant role in preparing a productive Pdx1−Pdx2 heteromeric complex (*10*). To analyze the contribution of Asp99, it was exchanged with an alanine (Pdx1-Asp99Ala). The resulting variant protein retained 49.2% of wild-type PLP synthase activity using ammonium as the nitrogen source (Table 1A). However, a 20-fold reduction in enzymatic activity was observed in the presence of Pdx2 and L-glutamine (Table 1B).

The third amino acid exchange introduced into Pdx1, located in the αN helix of Pdx1, was predicted to play a role in the binding of Pdx1 to Pdx2. As shown in Figure 3B, the amino group of the Lys18 side chain forms a salt bridge to the carboxylate group of the Pdx2 Glu15 side chain. An exchange of Lys18 with alanine was not expected to affect the PLP synthase activity of Pdx1, and indeed, this variant exhibits between 40.5 and 86.3% activity (depending on the type of activity assay used) compared to wild-type Pdx1 (Table 1A,B).

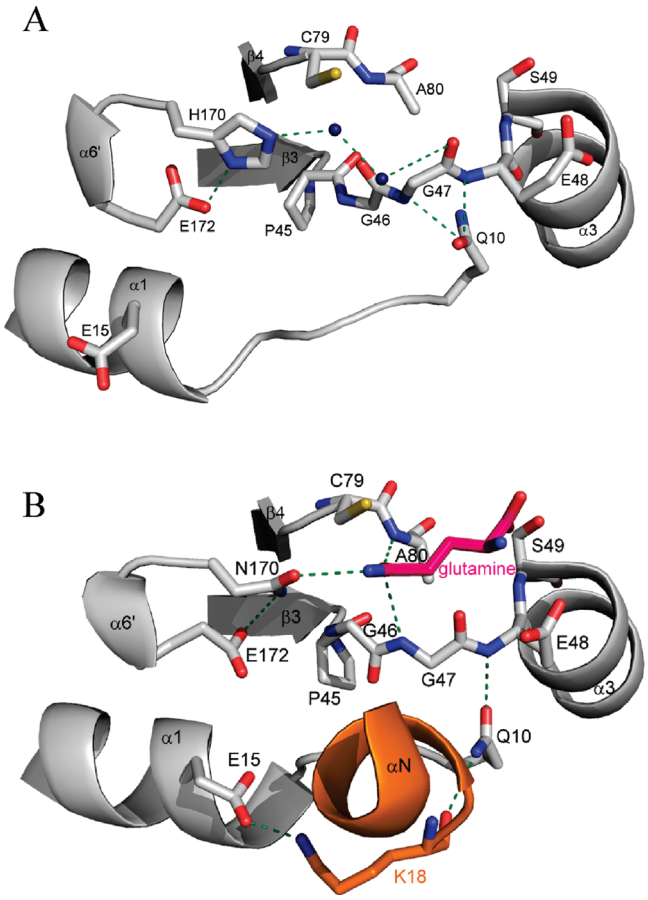


FIGURE 3: Structure of the glutaminase active site and the oxyanion region in the autonomous Pdx2 and in the ternary Pdx1−L-Gln−Pdx2 complex. (A) Representation of the glutaminase active site and the oxyanion region in the structure of the autonomous Pdx2. (B) Glutaminase active site and oxyanion hole in the structure of the ternary Pdx1−L-Gln−Pdx2 complex. The bound substrate L-glutamine is colored magenta. Pdx1 Lys18 and a part of the Pdx1 αN helix are colored orange. In panels A and B, important amino acid residues are shown in stick representation. Hydrogen bonds are plotted as green dashed lines. These structures were generated from Protein Data Bank entries 2nv0 and 2nv2, respectively, using Pymol Executable Build (DeLano Scientific LLC). The PLP synthase structure (Protein Data Bank entry 2nv2) shown in panel B was elucidated with a Pdx2 variant protein (H170N) lacking glutaminase activity (*10*).

Table 1: Specific PLP Synthase Activity of Wild-Type and Variant Pdx1[a] and with L-Glutamine as the N Donor[b]

	PLP synthase activity (nmol mg^{-1} min^{-1})	% activity
	(A) Without L-Glutamine	
Pdx1	0.197 ± 0.041	100
Pdx1-S75A	0.201 ± 0.091	100
Pdx1-D99A	0.097 ± 0.009	49.2
Pdx1-K18A	0.170 ± 0.016	86.3
	(B) With L-Glutamine	
Pdx1	0.945 ± 0.067	100
Pdx1-S75A	0.483 ± 0.046	51
Pdx1-D99A	0.047 ± 0.014	5
Pdx1-K18A	0.383 ± 0.013	40.5

[a] Values are means of at least three independent experiments. All measurements were carried out in 20 mM Tris-HCl (pH 8.0) at 37 °C. PLP synthase activity was observed with 40 μM Pdx1 wild-type or variant protein in the presence of 1 mM R5P, 2 mM G3P, and 10 mM ammonium sulfate. [b] Values are means of at least three independent experiments. All measurements were carried out in 20 mM Tris-HCl (pH 8.0) at 37 °C. PLP synthase activity was observed with 20 μM Pdx1 wild-type or mutant protein and 20 μM Pdx2 wild type in the presence of 1 mM R5P, 2 mM G3P, and 10 mM L-glutamine.

Table 2: Specific Glutaminase Activity of Wild-Type and Variant Proteins of Pdx1 and Pdx2[a]

	glutaminase activity (nmol min^{-1} mg^{-1})	% activity
Pdx1−Pdx2	328.2 ± 27.1	100
Pdx1-S75A−Pdx2	275.5 ± 4.2	84.0
Pdx1-D99A−Pdx2	56.5 ± 3.9	17.2
Pdx1-K18A−Pdx2	211.6 ± 28.2	64.5
Pdx1−Pdx2-R106A	no activity	0
Pdx1−Pdx2-R135A	no activity	0
Pdx1−Pdx2-E48A	no activity	0
Pdx1−Pdx2-Q10A	93.9 ± 19.7	28.6
Pdx1−Pdx2-Q10E	2.0 ± 0.3	0.6
Pdx1−Pdx2-Q10N	34.0 ± 7.7	10.4
Pdx1−Pdx2-E15A	280.0 ± 1.0	85.3

[a] Values are means of at least three independent experiments. All measurements were carried out in 20 mM Tris-HCl (pH 8.0) at 37 °C. Glutaminase activity was observed with each protein at 4 μM in the presence of 0.5 mM APAD, 23 units of bovine glutamate dehydrogenase, and 10 mM L-glutamine.

Influence of Pdx1 and Pdx2 Amino Acid Replacements on Pdx2 Glutaminase Activity. The glutaminase activity of Pdx2 wild-type and variant proteins was determined using an enzyme-coupled glutaminase assay (*14*). First, we analyzed the effect of the two Pdx1 variant proteins Pdx1-Ser75Ala and Pdx1-Asp99Ala on the glutaminase activity of Pdx2. While the Pdx1-Ser75Ala replacement showed a marginal effect on glutaminase activity (84% compared to that of the wild-type protein), the Pdx1-Asp99Ala replacement reduced glutaminase activity to 17.2% (Table 2).

Next, we determined glutaminase activity of Pdx2 in complex with variant protein Pdx1-Lys18Ala. In the ternary complex, Lys18 not only forms a salt bridge to Pdx2 Glu15 but also makes a main chain contact to the amide group of the side chain of Pdx2 Gln10 (Figure 3B). This residue was postulated to be involved in oxyanion hole formation in Pdx2, a prerequisite for catalysis (*10*). We observed only a small effect on the rate of glutamine hydrolysis (86.3% compared to wild-type Pdx2 activity) (Table 2). Likewise, replacement of Pdx2 Glu15 with alanine leads to a small decrease in enzymatic activity (85.3% of the wild-type value).

The glutaminase activity assay was then extended to Pdx2 wild type and variant proteins Pdx2-Arg106Ala, Pdx2-

Table 3: Thermodynamic Parameters for the Binding of Variant Proteins with the Respective Wild-Type Protein in the Absence and Presence of L-Glutamine[a]

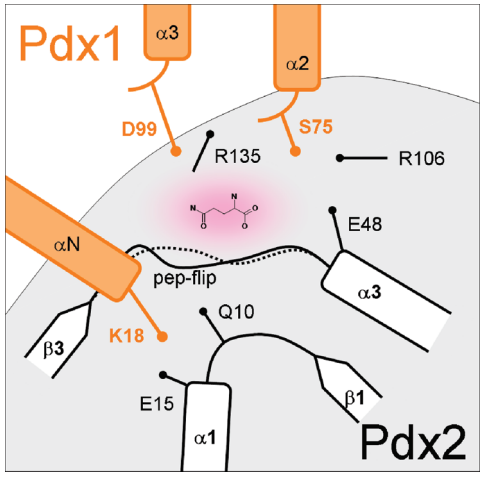
	without L-glutamine					with L-glutamine				
	N	K_d	ΔH	$-T\Delta S$	ΔG	*N*	K_d	ΔH	$-T\Delta S$	ΔG
Pdx1−Pdx2[b]	0.4 ± 0.10	6.9 ± 1.6	−34 ± 9	9 ± 1	−29 ± 2	1.0 ± 0.08	0.3 ± 0.1	−65 ± 3	29 ± 2	−37 ± 2
Pdx1-S75A−Pdx2[b]	0.5 ± 0.02	4.1 ± 1.3	−31 ± 8	5 ± 2	−30 ± 1	1.0 ± 0.20	3.4 ± 1.1	−25 ± 7	26 ± 5	−31 ± 2
Pdx1-D99A−Pdx2[b]	0.5 ± 0.04	8.2 ± 1.5	−31 ± 4	2 ± 3	−29 ± 3	0.5 ± 0.10	3.4 ± 1.7	−25 ± 5	7 ± 3	−30 ± 2
Pdx1-K18A−Pdx2[b]		<100[c]				0.6 ± 0.10	1.6 ± 0.3	−41 ± 1	8 ± 1	−33 ± 1
Pdx1−Pdx2-R106A		<100[c]					<100[c]			
Pdx1−Pdx2-R135A		<100[c]					<100[c]			
Pdx1−Pdx2-E48A[b]	0.5 ± 0.04	12.4 ± 0.1	−37 ± 5	10 ± 3	−29 ± 3	0.5 ± 0.01	16 ± 4.3	−36 ± 2	8 ± 2	−29 ± 2
Pdx1−Pdx2-Q10A[b]		100[c]				0.5 ± 0.07	5.7 ± 2.4	−6 ± 2	−24 ± 2	−30 ± 1
Pdx1−Pdx2-Q10E[b]		<100[c]				0.5 ± 0.11	7.4 ± 1	−6.3 ± 2	−23 ± 2	−29 ± 1
Pdx1−Pdx2-Q10N[b]		<100[c]				0.5 ± 0.07	6.4 ± 1.7	−5.8 ± 2	−24 ± 3	−27 ± 1
Pdx1−Pdx2-E15A[b]		<100[c]				1.0 ± 0.12	1.2 ± 0.3	−43 ± 2	9 ± 2	−34 ± 1

[a] Values are means of at least three independent experiments. Values of ΔH, $-T\Delta S$, and ΔG are in kilojoules per mole. K_d values are in micromolar. ΔG was calculated from the relation $\Delta G = -RT \ln K_a$, where K_a is the association constant determined by ITC. All measurements were performed in 20 mM Tris-HCl and 10 mM NaCl at pH 7.5 and 25 °C. [b] For measurements with glutamine, catalytic inactivation of Pdx2 by replacement of His170 of the catalytic triad with an Asn was required. [c] The lower limit for the dissociation constants was estimated to be in the range of a *c* value of ≈0.3, i.e., a concentration of Pdx2 at which a heat signal would still be discernible (*19*).

Arg135Ala, Pdx2-Glu15Ala, Pdx2-Gln10Ala, Pdx2-Gln10Glu, Pdx2-Gln10Asn, and Pdx2-Glu48Ala; observed glutaminase activities are listed in Table 2. Residues Pdx2 Arg106 and Pdx2 Arg135 are situated at the interface to Pdx1, forming intermolecular contacts with residues Ser75 and Asp99 of Pdx1 (Figure 2). For the Pdx2-Arg106Ala and Pdx2-Arg135Ala variant proteins, no glutaminase activity was detected (Table 2). Thus, both residues Pdx2 Arg106 and Pdx2 Arg135 are essential for the glutaminase activity of Pdx2. Similarly, replacement of Pdx2 Glu48 with alanine leads to a complete loss of glutaminase activity (Table 2), confirming its predicted role in binding of L-glutamine (*10*).

Mechanism of Oxyanion Hole Formation. A major consequence of formation of the Pdx1−Pdx2 complex is reorientation of amino acids Pro45-Gly46-Gly47 ("peptide flip"; see Scheme 2) to form an oxyanion hole that stabilizes the tetrahedral intermediate during L-glutamine hydrolysis (compare panels A and B of Figure 3). Structural considerations have led to the hypothesis that Pdx2 Gln10 plays a crucial role in this process (*10*). Pdx2 Gln10 was replaced by three different amino acid residues, alanine, glutamate, and asparagine, and glutaminase activity was determined for all three protein variants in the presence of wild-type Pdx1 (Table 2). The largest effect was observed with the Pdx2-Gln10Glu replacement (0.6% of wild-type activity), whereas the Pdx2-Gln10Ala and Pdx2-Gln10Asn variant proteins retained 28.6 and 10.6%, of the wild-type activity, respectively.

Scheme 2



Binding Properties of Pdx1 and Pdx2 Variant Proteins in Formation of a Complex. The influence of the various mutations on complex formation was analyzed by isothermal titration calorimetry (ITC). Binding of the individual Pdx1 or Pdx2 variant protein to the respective wild-type protein was investigated. Thermodynamic parameters such as the dissociation constant (K_d), enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG) as well as the stoichiometry (*N*) were determined at 25 °C. Thermodynamic data obtained from these measurements are listed in Table 3.

Heat changes due to glutaminase activity exceed heat changes arising from binding events. Thus, Pdx2 variant proteins (including wild-type Pdx2) were further catalytically inactivated via replacement of an essential histidine residue (His170) in the active site of Pdx2 with asparagine, generating a Pdx2-His170Asn variant protein (*10, 15, 18*). This exchange leads to complete catalytic inactivation of the glutaminase subunit but has no effect on the formation of the binary Pdx1−Pdx2 complex which was demonstrated previously (*15*). Pdx2 wild-type and variant proteins with either a histidine (catalytically active) or an asparagine (catalytically inactive) in position 170 were used to investigate complex formation in the absence of L-glutamine in the ITC experiments.

The first group of protein variants studied by ITC comprised proteins with replacements at the Pdx1−Pdx2 interface near the L-glutamine binding site of Pdx2 (Figure 2). Titrations were carried out in the absence and presence of L-glutamine (see Table 3). The presence of L-glutamine tightens the complex between Pdx1 and Pdx2 by a factor of 23 as reported previously (Table 3) and also changes the thermodynamic signature of the binding process (*15*). The Pdx1-Ser75Ala protein variant possesses interaction properties with Pdx2 similar to those of wild-type Pdx1 in the absence of L-glutamine. Addition of L-glutamine does not result in the same changes observed with wild-type Pdx1 (Table 3). In the case of wild-type Pdx1, the lowering of the dissociation constant is mainly driven by enthalpy

(decrease from −34 to −65 kJ/mol), while enthalpies do not change for Pdx1-Ser75Ala.

For Pdx1-Asp99Ala, enthalpies and additionally entropies do not change in absence or presence of L-glutamine. The exchange results further in a loss of the influence of the substrate L-glutamine on the stoichiometry of complex formation. Thus, the protein complex has lost its ability to respond to L-glutamine binding to the active site (Table 3).

The two interaction partners of Pdx1 Ser75 and Pdx1 Asp99, Pdx2 Arg106 and Pdx2 Arg135 (Figure 2), were also exchanged with alanine to generate two single-amino acid variant proteins, Pdx2-Arg106Ala and Pdx2-Arg135Ala. Thermodynamic analyses showed that the loss of any of these residues results in a complete abrogation of the protein−protein interaction, in the absence and presence of L-glutamine. This is in agreement with the importance of Arg106 and Arg135 for the catalytic activity of Pdx2 and suggests that these residues are involved in complex formation as well as catalysis.

Pdx2 Glu48 is required for glutaminase activity but is not essential for binding to Pdx1 (Table 3). For the Pdx2-Glu48Ala variant protein, binding enthalpies and entropies as well as stoichiometries are unchanged in the absence or presence of L-glutamine, similar to the Pdx1-Asp99Ala variant.

The next set of amino acids investigated are located at the interface of the Pdx1 αN helix with Pdx2. The crystal structure of the ternary complex suggested that Pdx1 Lys18 of helix αN may have a pivotal role for protein−protein interaction and trigger oxyanion hole formation, which is essential for the catalytic activity of Pdx2 (*10*). Pdx1 Lys18 makes a side chain contact with Pdx2 Glu15 and a main chain contact with Pdx2 Gln10 as shown in Figure 3B. Interestingly, ITC experiments failed to detect binding of Pdx1 Lys18 to wild-type Pdx2 in the absence of L-glutamine. However, in the presence of L-glutamine, interaction occurs, yielding a dissociation constant that is 5 times weaker as observed with the wild-type proteins (Table 3). Thus, L-glutamine binding to Pdx2 partially rescues the formation of the complex. This is despite the fact that the Pdx1-Lys18Ala replacement has no major effect on catalytic activity (Tables 1A, B and 2). The complementary protein variant Pdx2-Glu15Ala showed similar results: no interaction between Pdx2-Glu15Ala and Pdx1 was detected in the absence of L-glutamine; in its presence, the interaction was restored (Table 3). In contrast to Pdx1-Lys18Ala, the stoichiometry of complex formation with Pdx2-Glu15Ala fully recovered to unity as seen with wild-type protein (Table 3).

Helix αN was postulated to organize the oxyanion region in Pdx2 through main chain contacts of Pdx1 Lys18 with Pdx2 Gln10. As shown in Figure 3B, the amide side chain of Gln10 links Pdx1 Lys18 with Pdx2 Glu48, which is part of the peptide flip required for oxyanion hole formation (*10*). The three protein variants Pdx2-Gln10Ala, Pdx2-Gln10Asn, and Pdx2-Gln10Glu were tested in ITC measurements in the absence and presence of L-glutamine (Table 3). None of the three Pdx2 Gln10 protein variants was able to bind to Pdx1 in the absence of L-glutamine (Table 3). However, addition of L-glutamine restored interaction to some extent: in all cases, the dissociation constants are comparable to those for binding of Pdx1 to wild-type Pdx2 in the absence of L-glutamine. In contrast to the interaction of wild-type proteins, the thermodynamic signatures of all variant proteins deviate with regard to the contributions of the enthalpy and entropy terms; binding is favored by a large negative entropy term, while the contribution from the enthalpy term is much smaller than with any other protein variant investigated. Thus, formation of a complex between Pdx1 and Pdx2 protein variants is driven by entropy rather than enthalpy, which is in sharp contrast to the binding profile of wild-type proteins.

DISCUSSION

Previous studies have shown that complex formation is a basic prerequisite for inducing glutaminase activity of Pdx2 and thus is required to supply ammonia to Pdx1 for PLP biosynthesis (*14*). Pdx1 and Pdx2 interaction was demonstrated to occur in the absence of L-glutamine, albeit with a much higher dissociation constant and a different thermodynamic profile (*15*). This binary or encounter complex then binds L-glutamine, leading to tighter binding and priming of the glutaminase for catalysis (*15, 18*). The elucidation of the three-dimensional crystal structure of the *B. subtilis* ternary complex provided insights into complex assembly and the involvement of individual amino acid residues in the formation of the Pdx1−Pdx2 interface as well as the associated activation of the glutaminase (*10*). This initial work prompted us to analyze the contribution of single amino acid residues to complex formation by testing enzyme activities and binding properties of Pdx1 and Pdx2 variant proteins. In this study, we present biochemical and thermodynamic data obtained with several Pdx1 and Pdx2 variant proteins and we study their enzyme activity and their propensity to engage in formation of a protein−protein complex in the absence and presence of L-glutamine. The amino acid exchanges introduced into Pdx1 and Pdx2 focus on two interface areas between the proteins: the lid interface close to the L-glutamine binding site in Pdx2 and the interaction near the αN helix of Pdx1 (Figures 2 and 3, respectively). We will first discuss the impact of the amino acid replacements on enzyme activity and then the effects observed on the formation of the heteromeric protein complex as revealed by microcalorimetry.

The two replacements in Pdx1 near the lid interface concerned Asp99 and Ser75, both exchanged with alanine. The PLP synthase activity of both variant proteins (Pdx1-Asp99Ala and Pdx1-Ser75Ala) was not substantially diminished in the assay using ammonia as a nitrogen source compared to that of wild-type Pdx1 (Table 1A). This result was expected because neither one of these two residues is implicated in PLP synthesis as such (*10*). Since PLP synthase activity in the absence of the glutaminase depends on the uptake of ammonia from the medium, the 2-fold decrease in activity of the Pdx1-Asp99Ala variant protein may indicate an involvement of that residue in this process. This interpretation receives support from the finding that the activity of the Pdx1-Asp99Ala protein variant is even much lower when the Pdx2−L-glutamine complex is used as a nitrogen source. It is therefore conceivable that Pdx1 Asp99 plays a role in transferring ammonia from the active site of Pdx2 to a putative ammonia channel in Pdx1, a role that is not so critical when ammonia is taken up from the solvent, hence explaining the 10-fold lower activity in the Pdx2−L-

glutamine complex-based assay. Similarly, both mutations have no major effect on the glutaminase activity of Pdx2 (Table 2). While the glutaminase activitiy of Pdx2 in the presence of Pdx2 was not much affected by the serine to alanine replacement at position 75 (i.e., Pdx1-Ser75Ala variant protein), glutaminase activity is reduced 5-fold in the presence of the Pdx1-Asp99Ala variant protein, again suggesting a possible role of Pdx1 Asp99 in a necessary transfer of ammonia from Pdx2 to Pdx1.

These minor effects on the synthase and glutaminase activity are matched by the binding characteristics of the variant proteins in ITC experiments. In the absence of L-glutamine, the Pdx1-Ser75Ala variant protein behaves like the wild-type protein. In its presence, thermodynamic parameters do not shift in the same way as seen for wild-type protein with a much smaller contribution of ΔH (Table 3), and the protein interaction does not become tighter in the presence of L-glutamine as expressed in the K_d of the Pdx1−Pdx2 interaction. Therefore, it can be concluded that the side chain interaction of Pdx1 Ser75 with the guanidinium side chain of Pdx2 Arg106 contributes to the formation of a ternary complex. Pdx1 Ser75 also provides a main chain interaction to the side chain of Pdx2 Asn107, and this interaction is presumably in place in the Pdx1-Ser75Ala variant, thus explaining the mild effects on protein binding and enzymatic activity.

In contrast, the Pdx1-Asp99Ala variant deviates significantly from the wild-type protein with regard to its response to L-glutamine. The Pdx1-Asp99Ala variant shows a markedly lower affinity for Pdx2 in the presence of L-glutamine, and the switch in stoichiometry from 0.4−0.5 to unity as observed for wild-type Pdx1 is abolished (Table 3). Hence, replacement of Asp99 with alanine does not give rise to the same response to the presence of L-glutamine. This effect can be rationalized on the basis of the three-dimensional structure of the Pdx1−Pdx2 complex where two side chain interactions of Pdx1 Asp99 with the guanidinium side chains of Pdx2 Arg111 and Pdx2 Arg135 participate in the organization of the protein−protein interface as well as the glutaminase active site of Pdx2 (Figure 2). In summary, our results indicate that Pdx1 Asp99 is critically involved in setting up the proper interactions between Pdx1 and Pdx2 while it still retains a measurable enzyme activity (Tables 1A,B and 2).

Much more striking effects are observed when one of the interaction partners of Pdx1 Ser75 or Pdx1 Asp99 is replaced in Pdx2. Both, Pdx2-Arg106Ala and Pdx2-Arg135Ala have lost detectable glutaminase activity, emphasizing their role in organizing a network of interactions required for catalysis. Since neither of the arginine residues is directly involved in glutamine hydrolysis, it follows that their effect on glutaminase activity is indirect, i.e., results from the inability to form a binary protein complex. Moreover, neither of the arginine replacement variants was able to bind Pdx1 in the absence or presence of L-glutamine. Hence, Pdx2 Arg106 and Pdx2 Arg135 are essential for the formation of a binary encounter complex. This much more pronounced effect on binding as compared to the Pdx1 Ser75 and Pdx1 Asp99 protein variants is probably due to the ordering effect of the guanidinium group of both arginine side chains, which enable them to engage in multiple interactions with the neighboring amino acids, most notably Glu48 and Gln107 (Figure 2).

In addition to the two arginine residues, Glu48 is also implicated in substrate binding and activation of the glutaminase (*10*). Although Pdx2 Glu48 is not directly in the complex interface, its conformation changes upon formation of the heteromeric protein complex (Figure 3A,B). Interestingly, Pdx2 Glu48 is a direct neighbor of Pdx2 Gly46 and Pdx2 Gly47, which undergo a peptide flip in the ternary complex leading to the activation of the glutaminase by formation of the oxyanion hole. The Pdx2-Glu48Ala variant protein was devoid of detectable glutaminase activity (Table 2). In addition, this variant protein is unable to respond to the presence of L-glutamine as the thermodynamic signature remains similar to that of the binary encounter complex while formation of an encounter complex was observed with characteristics similar to those found for wild-type Pdx2 (Table 3). This suggests that Pdx2 Glu48 is critically involved in communicating the presence of L-glutamine to Pdx1.

The molecular mechanism underlying communication between Pdx1 and Pdx2 mainly involves the interaction between helix αN of Pdx1 and Pdx2 Gln10. The importance of helix αN for glutaminase activity and protein complex assembly was demonstrated recently for the PLP synthase from *Plasmodium falciparum* (*18*). As shown in Figure 3B, binding of L-glutamine to Pdx2 results in conformational changes in amino acid residues 45−48 (Gly46/Gly47 peptide flip), leading to the generation of the oxyanion hole required for glutaminase activity. The substrate-induced conformation is characterized by a hydrogen bond network among Pdx2 Glu48, Pdx2 Gln10, Pdx1 Lys18, and Pdx2 Glu15. Therefore, it appears that the catalytically active conformation forming upon binding of L-glutamine to the active site of Pdx2 is stabilized by the interaction of the αN helix of Pdx1. We replaced Pdx2 Gln10 (Figure 3B) with alanine, glutamate, or asparagine to investigate the importance of this residue for the interaction with Pdx1. Gln10Ala, Gln10Asn, and Gln10Glu cause 3-, 10-, and 200-fold reductions in glutaminase activity, respectively, in the presence of Pdx1 (Table 2). The introduction of a negatively charged amino acid side chain into the Pdx2-Gln10Glu variant protein obviously impedes binding of Pdx1 to Pdx2 and leads to a destabilization of the oxyanion hole conformation, while more conservative replacements have a less severe effect. On the other hand, complex formation is substantially affected in all three single-amino acid exchange protein variants. In the absence of L-glutamine, protein binding is not detectable (Table 3), indicating that Pdx2 Gln10 is important for the formation of a binary protein complex. In the presence of L-glutamine, binding is rescued but with a much lower affinity, exhibiting dissociation constants of ∼6 μM. Moreover, Pdx1−Pdx2 protein binding is mainly driven by entropy, differing from formation of the complex with wild-type Pdx2. Thus, it can be concluded that complex formation with any of the Pdx2 Gln10 variant proteins results in an entropic gain, probably by desolvation of the proteins and associated disruption of a highly ordered structure with favorable hydrogen bond interactions. Hence, it becomes apparent that Pdx2 Gln10 can regulate the interactions between Pdx1 and Pdx2.

The role of helix αN in formation of the complex is further underlined by the Pdx2 Glu15 and Pdx1 Lys18 variant proteins. Although neither of these single-amino acid exchange proteins has a strong effect on glutaminase activity (Table 2), no protein−protein interaction could be observed

in the absence of L-glutamine [K_d > 35 μM (Table 3)]. However, in the presence of L-glutamine, both variant proteins exhibit a dissociation constant that is 2−3 times higher than with the respective wild-type proteins, mainly resulting from a smaller enthalpic contribution (−41 and −43 kJ/mol for the Pdx1-Lys18Ala and Pdx2-Glu15Ala variant proteins, respectively, as compared to −65 kJ/mol for wild-type proteins) which is partially compensated by a smaller entropic term (Table 3). The Pdx1 Lys18−Pdx2 Glu15 side chain interaction provides ∼22−24 kJ/mol for protein complex formation, a typical value for this kind of side chain interaction. The gain of entropy of the Pdx1-Lys18Ala and Pdx2-Glu15Ala protein variants indicates that structuring and solvation in the ternary complex are not as pronounced as in the wild-type protein complex.

The ITC data for formation of a protein−protein complex between Pdx1 and Pdx2 show that interactions among Pdx2 Gln10, Pdx1 Lys18, and Pdx2 Glu15 are essential for formation of a binary encounter complex, giving further evidence that stabilization of helix αN is a prerequisite for formation of the encounter complex. L-Glutamine rescues protein−protein interaction between Pdx1 and Pdx2 by mediating additional interactions in the lid interface between Pdx1 Asp99 and Pdx1 Ser75 with Pdx2 Arg106 and Pdx2 Arg135, which compensate for the weaker interactions in protein variants with amino acid exchanges in the αN helix region. The lid interface of Pdx2 comprising residues Pdx2 Glu48, Pdx2 Arg106, and Pdx2 Arg135 is most critical for binding of Pdx1.

The three-dimensional structure of the ternary PLP synthase complex has revealed the structural changes in Pdx2 upon binding of L-glutamine which lead to the formation of a network of interactions in the glutaminase active site. Among these observed changes are conformational alterations in amino acid residues 45−48 (Figure 3A,B) contributing to the formation of the oxyanion hole that is assumed to stabilize the tetrahedral transition state during L-glutamine hydrolysis. The strong impact found for Pdx2-Glu48Ala on enzyme activity suggests that glutamate 48 operates as a transducer in maintaining the proper conformation for catalysis. Initially, L-glutamine binding causes an organization of the active site involving glutamate 48 and thereby affecting the conformation of glycine 47. This process then reorients Pdx2 Gln10 so that the amide side chain becomes available for an additional interaction, i.e., docking of Pdx1 via Lys18 to form helix αN with Pdx1 Glu15 providing further stabilization (Figure 3B). Therefore, we conclude that L-glutamine binding triggers a cascade of conformational changes that leads to a structure favorable for Pdx1 binding. Other processes in the lid interface are supporting Pdx1 binding but are less important for complex assembly as evidenced by our ITC data. Thus, Pdx1 selects the substrate-induced conformation of Pdx2, which primes the glutaminase for L-glutamine hydrolysis and subsequent channeling of the product into the PLP synthase active site.

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