Intersubunit Cross-talk in Pyridoxal 5′-Phosphate Synthase, Coordinated by the C Terminus of the Synthase Subunit

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Vitamin B₆ is essential in all organisms, due to its requirement as a cofactor in the form of pyridoxyl 5′-phosphate (PLP) for key metabolic enzymes. It can be synthesized de novo by either of two pathways known as deoxyxylulose 5-phosphate (DXP)-dependent and DXP-independent. The DXP-independent pathway is the predominant pathway and is found in most microorganisms and plants. A glutamine amidotransferase consisting of the synthase Pdx1 and its glutaminase partner, Pdx2, form a complex that directly synthesizes PLP from ribose 5-phosphate, glyceraldehyde 3-phosphate, and glutamine. The protein complex displays an ornate architecture consisting of 24 subunits, two hexameric rings of 12 Pdx1 subunits to which 12 Pdx2 subunits attach, with the glutaminase and synthase active sites remote from each other. The multiple catalytic ability of Pdx1, the remote glutaminase and synthase active sites, and the elaborate structure suggest regulation of activity on several levels. A missing piece in deciphering this intricate puzzle has been information on the Pdx1 C-terminal region that has thus far eluded structural characterization. Here we use fluorescence spectrophotometry and protein chemistry to demonstrate that the Pdx1 C terminus is indispensable for PLP synthase activity and mediates intersubunit cross-talk within the enzyme complex. We provide evidence that the C terminus can act as a flexible lid, bridging as well as shielding the active site of an adjacent protomer in Pdx1. We show that ribose 5-phosphate binding triggers strong cooperativity in Pdx1, and the affinity for this substrate is substantially enhanced upon interaction with the Michaelis complex of Pdx2 and glutamine.

It was long assumed that de novo biosynthesis of vitamin B₆ could only be achieved by the DXP-dependent pathway, characterized extensively for Escherichia coli (1, 2), which generates the active cofactor form via pyridoxine 5′-phosphate from erythrose 4-phosphate and glyceraldehyde 3-phosphate through a sequence of reactions requiring the participation of seven enzymes. Recently, an alternative route has come to light that directly results in the biosynthesis of the cofactor form of the vitamin, pyridoxal 5′-phosphate (PLP). Moreover, most organisms that can make this essential compound (i.e. microorganisms and plants) in fact utilize this alternative pathway. Remarkably, the newly discovered pathway involves the interplay of just two proteins, a synthase (named Pdx1) and a glutaminase (named Pdx2), jointly displaying glutamine amidotransferase activity (3, 4). Pdx1 and Pdx2 assemble into a 24-subunit complex (12 Pdx1 and 12 Pdx2 subunits) to form the functional vitamin B₆ biosynthesis machinery (5, 6). The intrinsic reactions of the synthase and glutaminase occur at sites distant from each other, separated by ∼26 Å. The Pdx2 active site, where glutamine is hydrolyzed to glutamate and ammonia, is located at the interface between Pdx2 and the N terminus of Pdx1, whereas the active site of Pdx1 is located at the C-terminal end of its (β/α)₈-barrel (5, 6). During the course of PLP synthesis, ammonia from the hydrolysis of glutamine in the Pdx2 active site is channeled to the Pdx1 active site via a putative tunnel, assumed to be inaccessible to solvent, that runs through the core of the Pdx1 (β/α)₈-barrel (5). At the Pdx1 active site, a pentose phosphate and triose phosphate sugar (i.e. ribose 5-phosphate and glyceraldehyde 3-phosphate) are combined along with ammonia to form PLP. Since the active sites of Pdx1 and Pdx2 are remote from each other, tight regulation of enzyme activities coordinated by interdomain signaling is expected. So far, studies have primarily focused on the interaction of Pdx1 with Pdx2 and the direct effect of Pdx1 on the glutaminase activity of Pdx2. Signaling from Pdx2 to the Pdx1 active site and transmission of an interdomain signal between the individual Pdx1 monomers to explain the ornate dodecameric architecture have not yet been explored.

X-ray crystallography and biochemical studies have facilitated characterization of the heteromeric PLP synthase as well

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4 The abbreviations used are: DXP, deoxyxylulose 5-phosphate; pBpa, p-benzoyl-phenylalanine; PLP, pyridoxal 5′-phosphate; ESI, electrospray ionization; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight.
as of the individual subunits at the molecular level. The structure of the individual glutaminase subunit Pdx2 was the first to be reported and provided insight into the active site architecture (7, 8). This was followed by the description of the Pdx1 oligomer (9), and shortly thereafter the Pdx1-Pdx2 complex was solved independently by two groups (5, 6). A comparison of the structures of the individual subunits with that of the Pdx1-Pdx2 complex revealed areas that become ordered upon assembly of PLP synthase. In Pdx1, these include the N-terminal tail (residues 2–6), an N-terminal α helix (residues 7–17), and residues 47–56 that comprise α helix 2’. (Note that annotation and numbering is that of the Bacillus subtilis protein (5).) The N-terminal ordered regions are mainly involved in the interaction with Pdx2, whereas the function of helix 2’ is not yet clear. However, in no case could structural information be provided on residues 270–294 of the Pdx1 C terminus, presumably due to inherent flexibility. Interestingly, in the presence of the pentose phosphate substrate, part of the C-terminal region is observed in the Thermotoga maritima protein (residues 270–280) (6). However, the remainder of the C terminus of Pdx1 (15 residues) has eluded structural analysis and remains to be characterized.

Here we report on conformational changes in the Pdx1 protein upon assembly of the heteromeric PLP synthase complex. These occur at two levels, comprising interdomain cooperation between the individual Pdx1 monomers and signaling from the Pdx2 subunit to relay tighter binding of the pentose phosphate substrate. Employing fluorescence and UV-visible spectrometry combined with protein mutagenesis, we demonstrate that the C terminus of Pdx1 is essential in mediating these functionalities in PLP synthase, and we provide insight into the dynamics of this highly flexible region, which has a major role to play in the mechanism of this polymorphic enzyme. Furthermore, through the use of the recently developed technique of specific incorporation of a photoactivatable unnatural amino acid, we demonstrate that the vital role of the Pdx1 C terminus is accomplished by acting as a lever to a neighboring protomer, where it fulfills its catalytic role.

**EXPERIMENTAL PROCEDURES**

Materials—p-Benzoyle-phenylalanine (pBpa) was purchased from Bachem AG (Bubendorf, Switzerland). Recombinant trypsin (proteomics grade) was from Roche Applied Science.

Equilibrium Binding Monitored by Intrinsic Protein Fluorescence—All steady-state fluorescence measurements were conducted on a QuantaMaster 4 spectrofluorometer (Photon Technology International, Seefeld, Germany). All spectra were acquired in 20 mM Tris-Cl, pH 7.5, containing 10 mM NaCl and at 23 °C. The fluorescence emission spectra of the individual proteins were acquired from 305 to 550 nm after excitation at 295 nm using 2 μM either Pdx1 or Pdx1 ΔW294 and either a 4 or 20 μM concentration of Pdx2 H170N in the presence or absence of 1 mM glutamine, as indicated. Data were recorded in increments of 1 nm with an integration time of 1 s. Each spectrum was corrected for dilution effects. Bandwidths of the excitation and emission monochromator were set to 4 and 8 nm, respectively. For the fluorimetric titration, Pdx1 (either 0.3 or 2 μM) was incubated with varying concentrations of Pdx2 H170N (0–6 μM) in the presence of 1 mM glutamine, and the fluorescence emission at a λmax of 350 nm was measured for 10 s, averaged, and corrected for dilution effects and for any fluorescence signal caused by Pdx2 H170N (photobleaching was negligible with these experimental settings). Samples were preincubated for 5 min at 23 °C prior to the measurement. Equilibrium binding data were analyzed employing nonlinear fitting routines to Equation 1,

\[
F([\text{Pdx2H170N}]) = F_0 - \frac{\Delta F}{2 \cdot [\text{Pdx1}]} \cdot ([\text{Pdx1}] + [\text{Pdx2H170N}] + K_d) - \sqrt{[\text{Pdx1}] + [\text{Pdx2H170N}] + K_d \cdot 4([\text{Pdx1}] \cdot [\text{Pdx2H170N}])} \tag{1}
\]

where \(F([\text{Pdx2H170N}])\) represents the fluorescence signal at a given Pdx2 H170N concentration, \(F_0\) is the maximal fluorescence signal, \(\Delta F\) is the maximal change in fluorescence intensity, \([\text{Pdx1}]\) is the Pdx1 concentration, \([\text{Pdx2H170N}]\) is the Pdx2 H170N concentration, and \(K_d\) is the dissociation constant. The steady-state kinetics for ribose 5-phosphate binding displayed sigmoidal behavior and were thus determined by nonlinear curve fitting to the Hill equation,

\[
F([\text{R5P}]) = F_0 + \frac{\Delta F \cdot [\text{R5P}]}{K_{0.5} + [\text{R5P}]} \tag{2}
\]

where \(F([\text{R5P}])\) represents the fluorescence signal at a given ribose 5-phosphate concentration, \(F_0\) is the maximal fluorescence signal, \(\Delta F\) is the maximal change in fluorescence intensity, \([\text{R5P}]\) is the ribose 5-phosphate concentration, \(K_{0.5}\) is the ribose 5-phosphate concentration at half-maximal fluorescence signal, and \(h\) is the Hill coefficient.

Pre-steady-state Kinetics Followed by Intrinsic Protein Fluorescence—Rapid kinetic measurements were performed using an Applied Photophysics SX.18MV stopped-flow spectrometer. The excitation wavelength was set at 295 nm, and the slit width was 15 nm. Light emitted from the sample was monitored after passing through a 320-nm cut-off filter. The concentration of Pdx1 was held constant at 0.3 μM, whereas that of Pdx2 H170N was varied in the range of 1.2–4.2 μM. Experiments were performed in the presence of 1 mM glutamine in 20 mM Tris-Cl and 10 mM NaCl, pH 7.5, at 23 °C. In each experiment, 8000 data points were recorded during 200 s, and data from 5–10 traces at identical conditions were averaged. Photo-bleaching of Pdx1 over the time course of the reaction was subtracted from the fluorescence signal, and the end signal was normalized according to the fluorescence titration of Pdx2 H170N binding to Pdx1. The normalized data were fitted to a double exponential according to Equation 3,

\[
F(t) = F_o + \alpha_1 \cdot \exp(-t/\tau_1) + \alpha_2 \cdot \exp(-t/\tau_2) \tag{3}
\]

where \(F(t)\) represents the fluorescence at time \(t\), \(\alpha_1\) and \(\tau_1\) are the amplitude and the observed relaxation time of the fast relaxation (\(\alpha_2\) and \(\tau_2\) for the slow relaxation, respectively), and \(F_o\) is the fluorescence intensity after infinite time due to incomplete Pdx1-Pdx2 assembly.

Cross-linking Studies—Site-directed incorporation of pBpa into Pdx1 was accomplished using the technique established by

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Schultz and colleagues (10, 11). This method employs an engineered plasmid coding for an orthologous aminoacyl tRNA synthetase pair allowing the incorporation of unnatural amino acids at the position of the amber stop codon. The amber stop codon (TAG) was introduced at either Arg-8, Tyr-261, Arg-288, or Trp-294 in Pdx1 using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. As forward and reverse primers, the oligonucleotides (mutation underlined) 5′-CAACAAGGATTAAATAGGTAAACGGCGAATG-3′ and 5′-GATCTCCGCCTTTTACCATTTTCAGTACCTTGG-3′ were used for Arg-8, 5′-CATTCTTACTGATTAAATTAATCGCT-3′ and 5′-CAGCGATTAAATTTCTAATCGTAAAGTTG-3′ for Tyr-261, and 5′-CTTCGATGACCTCTTGTTCTCTG-GAA-3′ for Arg-288, whereas 5′-CAAGAAGCGCTATTGACGACACCAACAC-3′ and 5′-GGTGTGTTGTCCAGACAGCTGCGCTCTG-3′ were used for Trp-294, respectively. As a template, pETBsPdx1-His<sub>6</sub> (5) was employed. The mutation was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland). Two plasmids, one (pSup-BpaRS-6TRN: p-benzoyl-phenylalanine) endowing the host cell with the ability to incorporate pBpa through an amber codon and another directing the expression of the amber mutant form of Pdx1, were introduced into <i>E. coli</i> BL21 cells using sequential heat-shock transformation. The expression of the amber mutant form of Pdx1, were introduced into <i>E. coli</i> BL21 cells using sequential heat-shock transformation. The transformed cells were grown at 37 °C in LB medium to an <i>A<sub>600</sub></i> of 0.6. Prior to the induction of protein expression by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside, the medium was supplemented with 1 mM pBpa. Cells were incubated for another 6 h at 37 °C and then harvested by centrifugation and subsequently stored at −80 °C. The respective hexahistidine-tagged proteins, Pdx1 R8:pBpa, Pdx1 Y261:pBpa, Pdx1 R288:pBpa, and Pdx1 W294:pBpa were purified by Ni<sup>2+</sup>-nitrilotriacetic acid chromatography according to the protocol supplied by the manufacturer (Qiagen). Incorporation of the unnatural amino acid was verified by electrospray ionization-mass spectrometry (ESI-MS) (Protein Service at the Functional Genomics Center, Zurich, Switzerland). Cross-linking reactions were performed in a 96-well microtiter plate with a total reaction volume of 100 μl in 50 mM Tris buffer, pH 7.5, on ice. Samples were irradiated at 365 nm using the UV Stratalinker 1800 (EIKO lamps F8T5/BL; Stratagene) for 1–120 min (depending on the experiment). Samples were analyzed by SDS-PAGE on 12.5% polyacrylamide gels. Where shown, immunodecoration with antibodies against Pdx1 and Pdx2 was carried out as described in Ref. 3.

**Nano-liquid Chromatography Separation and MALDI Analysis of Tryptic Peptides**—One hundred μm Pdx1 protein sample (wild type or W294:pBpa in a total volume of 100 μl that had been subjected to 60 min of irradiation at 365 nm using the UV Stratalinker 1800) was digested with 2 μg of trypsin for 3 h at 37 °C in 100 mM NH<sub>4</sub>HCO<sub>3</sub>. After digestion, trifluoroacetic acid was added to a final concentration of 0.1% (v/v). Peptide separation was performed on an Ultimate chromatography system (Dionex-LC Packings, Sunnyvale, CA) equipped with a Probot MALDI spotting device. Five μl of the samples was injected by using a Famos autosampler (Dionex-LC Packings) and loaded directly onto a 75 μm × 150 mm reversed-phase column (PepMap 100; 3 μm; Dionex-LC Packings). Peptides were eluted at a flow rate of 300 nl/min by using a water/acetonitrile gradient. For MALDI analysis, the column effluent was directly mixed with MALDI matrix (3 mg/ml α-cyano-4-hydroxycinnamic acid in 70% acetonitrile, 0.1% trifluoroacetic acid) at a flow rate of 1.1 μl/min via a μ-Tee fitting. Fractions were automatically deposited every 10 s onto a MALDI target plate (Applied Biosystems, Toronto, Canada) using a Probot microfraction collector. A total of 416 spots were collected from each HPLC run. MALDI plates were analyzed on a 4800 MALDI TOF/TOF system (Applied Biosystems) equipped with a Nd:YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode and generated by accumulating data from 500 laser pulses in the mass range from <i>m/z</i> 750 to 6000. In the MSMS mode, collision-induced dissociation spectra of selected precursor ions were recorded at a collision energy of 1 kV and a collision gas pressure of ~2 × 10<sup>−6</sup> torr. Typically, spectra from 10,000 laser pulses were accumulated.

**Enzyme Assays**—Distinct enzyme activities of PLP synthase can be separately measured by i.e. 1) the rate of glutamine hydrolysis by Pdx2, 2) the formation of the end product PLP, and 3) the formation of the chromophoric reaction intermediate (12). All of these enzyme activities are detectable by UV-visible spectrophotometry. Glutaminase activity of Pdx2 was monitored by a coupled enzyme assay as described in Ref. 3. PLP formation was monitored by the change in absorbance at 414 nm in the presence of ribose 5-phosphate, glyceraldehyde 3-phosphate, Pdx2, and glutamine, as described in Refs. 3 and 12. Formation of the chromophoric reaction intermediate was determined by the increase in absorbance at 315 nm in the presence of Pdx1, Pdx2, ribose 5-phosphate, and glutamine (12).

**Protein Expression and Purification**—The constructs pETBsPdx1 and pETBsPdx2-His<sub>6</sub> described in Ref. 3, in addition to pETBsPdx1-His<sub>6</sub> and pETBsPdx2-His<sub>6</sub> H170N described in Ref. 5, were used in this study. The mutant Pdx1 ΔW294 was generated using the QuickChange site-directed mutagenesis kit (Stratagene, Basel, Switzerland) employing the oligonucleotides 5′-GCAAGACGCGCTCGAGGATTCGTCGTCGTTTGC-3′ and 5′-GGTGGTGGTGCTCGAGGATTCGTCGTCGTTTGC-3′ as forward and reverse primer, respectively, and pETBsPdx1-His<sub>6</sub> as the template. The Pdx1 ΔW294–294 mutant was generated using the forward and reverse oligonucleotides, 5′-CTAGCTAGCATTGGCTCAACAGGTACGATG-3′ and 5′-CCGCTCGAGAACGTTCCTTGCGACACACAC-3′, respectively, to amplify the DNA, with pETBsPdx1-His<sub>6</sub> as the template. The fragment was cloned into the Nhel/XhoI restriction sites of pET21a (EMD Biosciences) such that when expressed, a hexahistidine tag would be incorporated at the C terminus. Protein expression and purification, for both the native and hexahistidine-tagged proteins, were carried out as described in Ref. 3 and monitored by SDS-PAGE on either 12.5% or 15% polyacrylamide gels and staining with Coomassie Blue. Protein concentration was determined by the method of Bradford (13) using bovine serum albumin as a standard.

**Partial Proteolytic Digestion**—Thirty-two μg of Pdx1 was incubated with either 0.0005, 0.0028, or 0.005 μg of trypsin and, when indicated, Pdx2 H170N (30 μg) in 20 mM Tris-Cl, pH 7.5, in a total volume of 100 μl for 10 min at 37 °C. When present,
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ribose 5-phosphate, glyceraldehyde 3-phosphate, and glutamine were each at a final concentration of 1 mM. The reaction was stopped by the addition of formic acid to a final concentration of 1% (v/v). One-fifth of the reaction volume was utilized to monitor the progress of proteolytic cleavage by SDS-PAGE on 12.5% polyacrylamide gels. The remainder of the reaction volume was dialyzed against 0.4% (v/v) formic acid overnight, and the size of the protein fragments was deduced from mass spectrometry (Waters quadrupole time-of-flight Ultima API, Milford, MA).

Light Scattering—PLP synthase complex formation was monitored by changes in 90° scattered light intensity as described in Ref. 14. The increase in scattered light intensity was measured at 23 °C on an Applied Photophysics SX.18MV stopped-flow spectrometer with excitation and emission monochromators set at 400 nm. The excitation and emission bandwidths were set to 2 nm. The increase in scattered light intensity was measured at an angle of 90° upon mixing equimolar concentrations of Pdx1 and Pdx2 H170N (1 μM) in 20 mM Tris-Cl and 10 mM NaCl, pH 7.5, in the presence of 1 mM glutamine. In order to relate these measurements with the observed changes in intrinsic tryptophan fluorescence, identical measurements were carried out following tryptophan fluorescence essentially as described above with the exception that 1 μM Pdx1 and Pdx2 H170N were used.

RESULTS

Equilibrium Binding of B. subtilis Pdx1 and Pdx2—To date, the C terminus of Pdx1, the synthase component of the heteromeric PLP synthase, has escaped both structural and functional characterization. We recognized that PLP synthase from B. subtilis is a particularly amenable system to monitor perturbations in this region by fluorescence spectroscopy. This is because Pdx1 from this organism contains only a single tryptophan residue that is at the very C terminus, whereas its partner protein, Pdx2, does not have any tryptophan residues. Consequently, the tryptophan residue in Pdx1 is an ideal probe to monitor changes in the C-terminal region of the protein during complex formation and substrate binding. The fluorescence emission spectrum of Pdx1 shows a maximum at 350 nm upon excitation at 295 nm (Fig. 1A). Site-directed mutagenesis confirmed that the fluorescence is due to the single tryptophan residue (Fig. 1A). The Pdx1 fluorescence decreases 2.7% upon incubation with a 2-fold excess of Pdx2 H170N, whereas a 10-fold excess of Pdx2 H170N results in a 6% decrease in Pdx1 fluorescence (Fig. 1B). If in addition to a 2-fold excess of Pdx2 H170N, glutamine was added, a larger quenching of the Pdx1 fluorescence was observed (23%) with no readily apparent shift in the wavelength maximum (Fig. 1A). We therefore propose that the fluorescence signal may comprise two components; one is due to binding of Pdx2 H170N to Pdx1, and the other is due to a conformational change of the Pdx1-Pdx2 complex that can only be reached when glutamine is bound to Pdx2 H170N. However, we noted that, depending on the preparation of Pdx2 H170N, the magnitude of the Pdx1 fluorescence decrease, without the addition of glutamine, varies slightly. We suspect that this is a result of Pdx2 H170N co-purifying with a small amount of glutamine, which does not dissociate from the protein under the conditions used. Based on these observations and the previously reported dissociation constant of 6.9 μM for the Pdx1-Pdx2 complex in the absence of glutamine (15), we conclude that the association of Pdx1 with Pdx2 H170N causes only a fraction of

FIGURE 1. Analysis of PLP synthase complex formation by fluorescence spectroscopy. A, fluorescence emission spectra of 2 μM Pdx1 (black solid line), 2 μM Pdx1 with 4 μM Pdx2 H170N (black dashed line), 2 μM Pdx1 ΔW294 (gray solid line), and 2 μM Pdx1 ΔW294 with 4 μM Pdx2 H170N (black dotted line) in the presence of 1 mM added glutamine, after excitation at 295 nm. B, fluorescence emission spectra of 2 μM Pdx1 (black solid line), 2 μM Pdx1 with 4 μM Pdx2 H170N (black dotted line), and 2 μM Pdx2 with 20 μM Pdx2 H170N (black dashed line) in the absence of added glutamine. C, relative fluorescence change of Pdx1 (2 μM) at 350 nm (excitation λ = 295 nm) in the presence of increasing concentrations of Pdx2 H170N (0.1–6 μM) and added glutamine (1 mM). D, quenching of Pdx1 fluorescence under nonsaturating conditions (i.e. 0.3 μM Pdx1 with increasing concentrations of Pdx2 H170N (0.05–3.0 μM)). Shown is a plot of the relative fluorescence intensity at 350 nm (excitation λ = 295 nm) versus the concentration of Pdx2 H170N. Binding data were fitted using nonlinear regression according to Equation 1, yielding a value of 0.42 ± 0.04 μM for the $K_d$. All measurements were carried out at 23 °C.
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The total fluorescence signal change, whereas the remaining larger contribution results from the allosteric effect of glutamine bound to Pdx2.

Pdx2 H170N is unable to catalyze glutamine hydrolysis but binds glutamine in a dead-end Michaelis complex, resulting in an at least 23-fold increase in the affinity of Pdx2 for Pdx1. Titration of Pdx1 with Pdx2 H170N in the presence of added glutamine is characterized by a decrease in fluorescence intensity as a function of Pdx2 H170N concentration (Fig. 1C). Saturation occurs at a Pdx1 to Pdx2 H170N molar ratio of 0.82. The binding constant $K_d$ could be estimated by titration of Pdx1 with Pdx2 H170N in the presence of added glutamine under nonsaturating conditions (Fig. 1D). A plot of the relative fluorescence change against the ligand concentration results in hyperbolic binding behavior, yielding a $K_d$ of 0.42 ± 0.04 μM. This value is similar to the $K_d$ of 0.3 μM determined by isothermal titration calorimetry under equivalent conditions (15).

Real Time Analysis of PLP Synthase Complex Assembly—The observed change in the intrinsic fluorescence of Pdx1 upon interaction with Pdx2 H170N in the presence of added glutamine can be used to determine the kinetics of PLP synthase complex assembly. In a stopped-flow setup, equimolar amounts of Pdx1 and Pdx2 H170N were rapidly mixed in the presence of glutamine, and either the intrinsic fluorescence change or the change in light scattering intensity at 400 nm was followed (Fig. 2A). The recorded fluorescence decrease coincides with an increase in light scattering intensity, indicating that the fluorescence time course indeed reports on complex formation.

To obtain information on the involved individual rate constants, stopped-flow fluorescence measurements were performed under pseudo-first order conditions with an excess of Pdx2 H170N in the presence of added glutamine. The concentration dependence of the association reaction was analyzed by varying the Pdx2 H170N concentration (1.2–4.8 μM) while keeping the concentration of Pdx1 constant (0.3 μM). The time courses obtained under these conditions can be described by sums of exponentials. The association time courses display biphasic behavior and were analyzed by a double exponential fit (Fig. 2B). The fast and slow relaxation occurs in time windows that differ by about 1 order of magnitude. Their relaxation times are dependent on the concentration of Pdx2 H170N, as would be expected for a bimolecular reaction. However, in the presence of added glutamine (1 mM) were rapidly mixed in a stopped-flow apparatus, resulting in an increase in the scattered light intensity (gray trace) with a concomitant decrease in fluorescence intensity (black trace). B, time-dependent change in the intrinsic protein fluorescence of Pdx1 (0.3 μM) upon rapid mixing with Pdx2 H170N (1, 2, 2.4, and 4.2 μM) in the presence of added glutamine (1 mM). The black curves through the experimental data (gray traces) correspond to a double exponential fit. The fits are described by Equation 3. The inset displays a global numerical fit to the fluorescence time courses using the two-step model A + B ⇌ C ⇌ D, where the first equilibrium is described by $k_1$ and $k_{-1}$, and the second is described by $k_2$ and $k_{-2}$, respectively. C, values of the observed relaxation rate constants (1/τ) for the fast phase of PLP synthase complex formation as a function of Pdx2 equilibrium concentration. The concentration of Pdx1 was held constant at 0.3 μM, whereas Pdx2 H170N was varied in the range of 1.2–4.2 μM. The black line through the experimental data corresponds to a linear fit yielding the rate constants $k_1$ and $k_{-1}$ of the Pdx1-Pdx2 association reaction. The inset shows the relaxation rate constants (1/τ) of the slow relaxation as a function of Pdx2 concentration. All measurements were carried out in 20 mM Tris-Cl, 10 mM NaCl, pH 7.5, at 23 °C.
although the accessible concentration range showed a linear increase for the first relaxation rate constant ($1/\tau_1$), the second relaxation rate constant ($1/\tau_2$) appeared to be close to saturation. Since both steps are associated with a change in the intrinsic fluorescence of the terminal tryptophan residue, yet the C terminus is not involved in the formation of the binding interface, a fast conformational change must already occur concomitant with formation of the Pdx1-Pdx2 H170N complex and is then followed by a slower conformational change, leading to a further decrease in the signal. The data were therefore evaluated according to a two-step mechanism with a fast equilibrating association step followed by a slower unimolecular step. Fitting the concentration dependence of the first relaxation rate constant to a linear function allows extraction of the forward and reverse rate constants of the second, unimolecular step associated with formation of the Pdx1-Pdx2 H170N complex and is then followed by a slower conformational change, leading to a further decrease in the signal. The data were therefore evaluated according to a two-step mechanism with a fast equilibrating association step followed by a slower unimolecular step. Fitting the concentration dependence of the first relaxation rate constant to a linear function allows extraction of the forward and reverse rate constants of the second, unimolecular step.

To confirm that saturation was reached for the second relaxation rate constant, numerical analysis employing a global fit to all fluorescence time courses using the proposed two-step model was carried out and corroborated the relaxation kinetic analysis while in addition providing values for $k_2$ and $k_{-2}$ of 0.05 ± 0.01 and 0.015 ± 0.005 s⁻¹, respectively (Fig. 2B, inset). The best numerical fit was obtained when the Pdx1-Pdx2 H170N complex held 30% of the total signal. In the ternary complex (Pdx1-Pdx2 H170N-glutamine) the tryptophan fluorescence is further quenched (70% of the total signal), demonstrating that glutamine affects the active site of Pdx1 in an allosteric manner.

Substrate Modulation of the Proteolytic Cleavage of the Flexible Pdx1 C Terminus—We frequently noted that if Pdx1 (either without or with a C-terminal hexahistidine tag) is purified in the absence of protease inhibitors, a protein band with slightly higher mobility, in addition to that expected, is observed after SDS-PAGE analysis. In the presence of a protease inhibitor mixture (Roche Applied Science), this band is substantially reduced. N-terminal sequencing and Western blot analysis with a specific antibody confirmed that the truncated mutant has the correct three-dimensional structure.

Site-specific Incorporation of an Unnatural Amino Acid and Cross-linking Further Define the Role of the C Terminus of Pdx1—To further characterize the molecular role of the C terminus of Pdx1, we employed the recently established powerful approach of site-specific unnatural amino acid incorporation developed by the group of Schultz (11). The molecule pBpa was chosen as the probe, which is a photoreactive phenylalanine derivative that upon activation at 350–360 nm reacts preferentially with C-H bonds within a radius of 3.1 Å of the carbonyl oxygen. Advantages of pBpa over similar probes include that it can be
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FIGURE 3. Mapping flexible sites of Pdx1 by limited proteolysis and mass spectrometry. A, SDS-PAGE analysis of Pdx1 (32 µg) incubated with trypsin (0.0005–0.005 µg) in the presence and absence of ligand(s). Lane 1, Pdx1 as isolated; lanes 2–4, Pdx1 in the presence of 0.0005, 0.0028, and 0.005 µg of trypsin, respectively; lanes 5–7, as for lanes 2–4 but in the presence of 1 mM ribose 5-phosphate; lanes 8–10, as for lanes 2–4, but in the presence of 1 mM D-glyceraldehyde 3-phosphate; lanes 11–13, as for lanes 2–4 but in the presence of 1 mM ribose 5-phosphate and 1 mM D-glyceraldehyde 3-phosphate; lanes 14–25, as for lanes 2–13 but in the presence of Pdx2 H170N (30 µg) and glutamine (1 mM). In all cases, the samples were incubated for 10 min at 37 °C in 20 mM Tris-Cl, pH 7.5, before application to the gel. For Pdx1 and Pdx2 H170N, 0.8 and 0.75 µg of each protein, respectively, was loaded per lane. Protein was visualized by Coomassie Blue staining, and the mobilities of protein mass standards are as indicated. The black blocks indicate a progressive increase in the concentration of trypsin. B, ESI mass spectra of selected samples from A. Samples were digested with 0.005 µg of trypsin, in the presence or absence of ligands, as indicated. The left top panel is the spectrum of untreated Pdx1, whereas the diagram beside it depicts the observed protease cleavage site. The numbers in the panels of the spectra represent the ratio of full-length to truncated Pdx1 as judged by the intensity of the respective peaks.

manipulated in ambient light, is chemically more stable than diazo esters, aryl azides, or diazirines, and can be activated at wavelengths that do not cause protein damage (16, 17). The experiment was designed in such a way that pBpa would be co-translationally integrated into hexahistidine-tagged Pdx1 at the position of either Arg-288 (Pdx1 R288pBpa) or the C-terminal tryptophan residue, Trp-294 (Pdx1 W294pBpa). Successful expression of either Pdx1 R288pBpa or Pdx1 W294pBpa was achieved, and both were purified by Ni²⁺-nitrilotriacetic acid chromatography. Incorporation of the unnatural amino acid was confirmed by ESI mass spectrometry (observed masses 32,928.0 Da for Pdx1 R288pBpa (calculated 32,929.9 Da) and 32,898.0 Da for Pdx1 W294pBpa (calculated 32,899.9 Da), respectively). These proteins showed a reduced ability to catalyze chromophore formation and an undetectable level of PLP formation but could activate glutaminase activity in Pdx2 to the same extent as the wild type protein, indicating correct folding (Table 2; example shown in supplemental Fig. 1). The attenuation of PLP synthase activity may be due to steric hindrance of the active site from the incorporated pBpa molecule. Irradiation of the purified proteins resulted in the time-dependent formation of oligomers of Pdx1 as judged by SDS-PAGE analysis (Fig. 4A). In each case, the predominant oligomer formed corresponded to a dimer, indicating cross-linking to a neighboring Pdx1 subunit (Fig. 4A). As a proof of principle and to control the study, a second set of experiments were performed where we inserted pBpa at position Tyr-261 in Pdx1. This site was chosen based on the available three-dimensional structure as it resides at the interface of the individual Pdx1 subunits in a single hexameric ring (5) and would there-
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Indeed, substitution of Tyr-261 with pBpa, followed by cross-linking, results in the time-dependent appearance of higher order oligomers, almost sequentially from monomer to hexamer (Fig. 4B). Furthermore, we also inserted pBpa at position Arg-8 in Pdx1. This N-terminal region is known to be the site of interaction with Pdx2 in the PLP synthase complex (5, 6). Irradiation of Pdx1 R8:pBpa in the presence of Pdx2 H170N and glutamine resulted in the formation of a higher order complex (Fig. 4C, left). Subsequent immunodecoration of the samples with antibodies specific to Pdx1 and Pdx2 confirmed exclusive heterodimer formation, whereas in the case of Pdx1 W294:pBpa and Pdx2 H170N, exclusive Pdx1 homomeric formation was observed (Fig. 4C, right).

In an attempt to map the site of cross-linking in Pdx1 W294:pBpa, the cross-linked protein (Fig. 4A) was subjected to tryptic digestion followed by nano-liquid chromatography and MALDI-TOF/TOF mass spectrometry. The identification of peptide masses corresponding to the sum of any expected peptide mass in the natural protein (supplemental Table 1) plus that of the modified peptide (i.e. the peptide in which Trp-294 has been substituted by pBpa) (GpBpaLEHHHHHHH: the underlined residues are additionally expressed due to incorporation of a hexahistidine tag, calculated mass 1391.6 Da) permits the identification of the site of cross-linking. Using this approach, a mass corresponding to the size of the modified peptide plus that of the peptide containing residues 270–276 of Pdx1 (i.e. ELGTAMK) could be clearly identified (calculated mass 2139.99 Da, observed mass of 2139.93 Da). The assignment of the cross-linked peptide was confirmed by a MALDI-MS/MS spectrum (supplemental Fig. 2). This implies cross-linking of the very C terminus of Pdx1 of one subunit to the region following helix a8 in the C-terminal region of a neighboring subunit. This suggests that the C terminus of one subunit is in close proximity to that of a neighboring subunit under these conditions. To bring this into the context of the known three-dimensional structure of Pdx1, the spatial arrangement in this region of the protein is highlighted (Fig. 5).

Ribose 5-Phosphate Triggers Cooperativity within the Pdx1 Oligomeric Complex—It has been demonstrated that binding of the pentose phosphate substrate to an active site lysine residue in Pdx1 is one of the earliest steps in the mechanism of this multifaceted enzyme (4, 12). The propensity of ribose 5-phosphate to perturb the protease-sensitive sites of Pdx1, in particular the C terminus (Fig. 3 and Table 1), indicates a conformational change upon binding of this substrate and prompted us to examine the effect of the pentose phosphate on the fluorescence of Pdx1. A comparison of the fluorescence intensity of Pdx1 with ribose 5-phosphate bound with that of the substrate-free enzyme revealed an approximately 24% increase in the emission maximum at 350 nm in the presence of the pentose phosphate substrate (Fig. 6A). Indeed, titration of Pdx1 with ribose 5-phosphate resulted in changes in the intrinsic protein fluorescence. A plot of the relative fluorescence change at 350 nm as a function of the ribose 5-phosphate concentration exhibits sigmoidal binding behavior (Fig. 6B). Nonlinear regression analysis employing Equation 2 yields a Hill coefficient of 3.1 ± 0.2 and a $K_{0.5}$ of 324.8 ± 5.5 μM, indicative of cooperativity within the Pdx1 protein complex. Intrigued by this observation, we went on to investigate the effect of ribose 5-phosphate on preassembled PLP synthase. To this end, Pdx1 was preincubated with Pdx2 H170N in the presence of added glutamine and titrated with ribose 5-phosphate. A plot of the Pdx1 fluorescence emission maximum as a function of ribose 5-phosphate again resulted in sigmoidal binding behavior (Fig. 6B). However, although the Hill coefficient of the preassembled PLP synthase (2.8 ± 0.2) was similar to that of Pdx1 alone, there was a strong reduction in $K_{0.5}$ (88.7 ± 2.1 μM). This indicates substantially enhanced binding of the substrate to Pdx1 in the presence of the Michaelis complex of Pdx2 H170N and glutamine.

### TABLE 1
Susceptibility of Pdx1 to partial proteolytic cleavage

<table>
<thead>
<tr>
<th>Pdx2</th>
<th>Pdx1 mass observed</th>
<th>Pdx1 cleavage site</th>
</tr>
</thead>
<tbody>
<tr>
<td>H170N-glutamine</td>
<td>Da</td>
<td></td>
</tr>
<tr>
<td>No substrate</td>
<td>–</td>
<td>31,484.4 Arg-288</td>
</tr>
<tr>
<td>+</td>
<td>31,692.5</td>
<td></td>
</tr>
<tr>
<td>R5P</td>
<td>–</td>
<td>31,484.0 Arg-288</td>
</tr>
<tr>
<td>+</td>
<td>31,693.0 Arg-288</td>
<td></td>
</tr>
<tr>
<td>G3P</td>
<td>–</td>
<td>31,693.5 Arg-288</td>
</tr>
<tr>
<td>+</td>
<td>31,690.6 Arg-288</td>
<td></td>
</tr>
<tr>
<td>R5P/G3P</td>
<td>–</td>
<td>31,691.5 Arg-288</td>
</tr>
<tr>
<td>+</td>
<td>30,905.0 Arg-288</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>30,905.5 Arg-288</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 2
Specific enzyme activity of Pdx1 variants

<table>
<thead>
<tr>
<th></th>
<th>Chromophore-specific activity</th>
<th>PLP-specific activity</th>
<th>Glutaminase-specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol min$^{-1}$ mg$^{-1}$</td>
<td>μmol min$^{-1}$ mg$^{-1}$</td>
<td>μmol min$^{-1}$ mg$^{-1}$</td>
</tr>
<tr>
<td>Pdx1 wild type</td>
<td>5.2 ± 0.3</td>
<td>480.7 ± 13.93</td>
<td>142 ± 0.2</td>
</tr>
<tr>
<td>Pdx1 R288pBpa</td>
<td>1.1 ± 0.2</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>Pdx1 W294pBpa</td>
<td>1.3 ± 0.1</td>
<td>138 ± 0.2</td>
<td>133 ± 0.2</td>
</tr>
<tr>
<td>Pdx1 ΔW294</td>
<td>4.7 ± 0.3</td>
<td>413.1 ± 19.2</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>Pdx1 Δ273–294</td>
<td>27.7 ± 1.6</td>
<td>147 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ ND, not detectable.
$^b$ Not measured.
Here we report on the use of fluorescence spectrophotometry, limited proteolysis, and unnatural amino acid incorporation to study conformational changes induced in Pdx1 upon substrate binding and upon assembly with Pdx2 to form the PLP synthase complex. In particular, the study focuses on the structurally and functionally undefined C-terminal region of Pdx1. Despite the availability of several three-dimensional structures of both the autonomous Pdx1 protein (9) and its complex with Pdx2 (5, 6), this region of the protein had eluded characterization, presumably due to its highly flexible nature. Our study was facilitated by the fortuitous presence of a single tryptophan residue at the very C terminus of \textit{B. subtilis} Pdx1 and the absence of such a residue in Pdx2. With the data reported in hand, several conformational states can be distinguished for Pdx1. These states are characterized by different chemical environments on account of observed differences in tryptophan fluorescence.

Binding of Pdx2 shifts the “resting” conformational equilibrium of the Pdx1 C terminus and is manifested as a decrease in fluorescence intensity (Fig. 1). In the presence of glutamine, this decrease is substantially enhanced such that the majority of the conformational change can be attributed to the Michaelis complex of Pdx2 and glutamine (Pdx2-Gln). In spatial terms, this can be interpreted as movement of the C terminus to a position that brings it into the proximity of fluorescence-quenching residues. It must be noted that the C terminus is spatially separated from the site of Pdx1 interaction with Pdx2. Thus, the change in Pdx1 fluorescence cannot be the result of direct quenching by Pdx2. Instead, it must be assumed that binding of Pdx2-Gln to Pdx1 relays conformational changes to the C terminus of Pdx1. Moreover, the observed decrease in Pdx1 fluorescence in the presence of Pdx2-Gln can be used to estimate the stoichiometry and kinetics of PLP synthase assembly. Interestingly, two distinct phases could be resolved, a fast concentration-dependent phase followed by a slower phase. A combination of stopped flow fluorescence and light scattering established that the fast phase corresponds to bimolecular protein association and represents formation of a 1:1 PLP synthase complex (Fig. 2). The slower phase must correspond to an isomerization step after protein association. That Pdx2-Gln modulates Pdx1, albeit remotely, upon assembly of the PLP synthase complex is supported by other evidence. X-ray crystallography of the indi-
individual Pdx1 protein compared with that in complex with Pdx2 has demonstrated that in Pdx1, helix H9251–H11032, a region remote from the site of interaction with Pdx2, becomes ordered upon assembly of the PLP synthase complex (5, 6). Furthermore, the use of fluorescence spectroscopy allowed us to demonstrate that Pdx2-Gln dramatically enhances the affinity of Pdx1 for the substrate ribose 5-phosphate (Fig. 6).

Although this indicates that binding of the pentose phosphate is thermodynamically favored in the fully assembled complex, it is noteworthy that Pdx1 alone can bind ribose 5-phosphate, as shown here (see below) and as reported earlier (12). However, the increased affinity for the substrate in the preassembled PLP synthase complex provides evidence that Pdx2-Gln must alter the distantly located substrate-binding pocket of Pdx1. In summary, Pdx2-Gln is an allosteric effector of Pdx1.

Another distinct conformational state of the Pdx1 C terminus is observed upon binding of the pentose phosphate substrate alone and can be quantitatively described by fluorescence spectrophotometry (Fig. 6). In contrast to the association of Pdx1 and Pdx2-Gln, binding of the substrate to Pdx1 leads to an increase in tryptophan fluorescence, which indicates movement of the C terminus to an environment that enhances its fluorescence relative to other states of the enzyme. This latter conformation in the presence of the pentose phosphate synthase protein complex (Protein Data Bank code 2ISS). Pdx1 monomers are shown in orange and white, whereas Pdx2 is shown in green. The area between adjacent Pdx1 monomers is expanded. On monomer one (white), the terminal residue of the resolved structure is highlighted (yellow) (note that residues 280–293 were unresolved and are therefore missing in the structure), whereas on monomer two (orange), the area following helix α8”, which is implicated to be in close proximity to the C-terminal residue based on cross-linking, is highlighted (red) (i.e. residues 271–277 (note T. maritima numbering)). The co-crystallized substrate ribulose 5-phosphate is shown in a ball and stick representation. This figure was prepared using PyMOL (25).

**FIGURE 5.** The architecture of the PLP synthase protein complex (Protein Data Bank code 2ISS). Pdx1 monomers are shown in orange and white, whereas Pdx2 is shown in green. The area between adjacent Pdx1 monomers is expanded. On monomer one (white), the terminal residue of the resolved structure is highlighted (yellow) (note that residues 280–293 were unresolved and are therefore missing in the structure), whereas on monomer two (orange), the area following helix α8”, which is implicated to be in close proximity to the C-terminal residue based on cross-linking, is highlighted (red) (i.e. residues 271–277 (note T. maritima numbering)). The co-crystallized substrate ribulose 5-phosphate is shown in a ball and stick representation. This figure was prepared using PyMOL (25).

**FIGURE 6.** Probing ribose 5-phosphate binding to Pdx1 by fluorescence spectroscopy. A, normalized fluorescence intensity of 2 μM apoPdx1 (solid line) and with R5P bound (dashed line) after excitation at 295 nm. B, titration of either apoPdx1 (2 μM) or preassembled PLP synthase (apoPdx1 (1 μM) and Pdx2 H170N (2 μM) in the presence of glutamine (1 mM) incubated for 5 min at 37 °C) with increasing concentrations of ribose 5-phosphate (25–910 μM). A plot of the relative fluorescence change at 350 nm (excitation λ = 295 nm) versus the concentration of ribose 5-phosphate demonstrates sigmoidal binding behavior. For apoPdx1 alone, fitting to Equation 2 using nonlinear regression yields values of 3.1 ± 0.2 and 324.8 ± 5.5 μM for the Hill coefficient and K<sub>0.5</sub>, respectively. Preassembled PLP synthase exhibits sigmoidal binding behavior similar to that of apoPdx1 but with a Hill coefficient and K<sub>0.5</sub> of 2.8 ± 0.2 and 88.7 ± 2.1 μM, respectively.
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tose phosphate sugar shows decreased susceptibility of the C
terminus of Pdx1 to protease digestion. Thus, it appears that
ribose 5-phosphate protects the Pdx1 C-terminal residues.

Of major significance is the sigmoidal dependence of the Pdx1 fluorescence signal on the concentration of ribose 5-phosphate. This phenomenon with a Hill coefficient of 3.2 ± 0.2 provides unequivocal evidence for positive coop-
erativity in Pdx1. Generally, positive cooperativity is a mech-
anism to make enzymes more sensitive to changes in the concen-
tration of their substrate(s) (18). Given that many enzymes utilize ribose 5-phosphate as a substrate, the fea-
tures described make PLP synthase appealing by “luring” the pentose phosphate substrate into the Pdx1 active site. In
genereal, the alterations in protein structure that produce cooperativity normally involve only minor changes in the protein subunit. In almost all cases where protein structures have provided a detailed picture of the conformational change, it is most commonly observed that one domain in the structure moves a small amount relative to another domain. Such a motion can either be a hinge-like closure or opening or a rotational movement. The question then arises as to what is triggering this signaling cascade in Pdx1. Based on the results reported herein and the available three-di-
dimensional structures of Pdx1 (5, 6), a hypothesis explaining the cooperative behavior and suggested allostery induced by Pdx2-Gln can be attempted at the structural level. Of partic-
ular interest is the identity of the signal(s), which either reports ribose 5-phosphate binding to a neighboring sub-
strate binding site in Pdx1, thus conferring cooperativity, or indeed the nature of the signal instigating the allosteric rearrangement by Pdx2-Gln. To this end, a brief recapitulation of the quaternary structure of Pdx1 is appropriate. Pdx1 is a
(β/α)8-barrel, six subunits of which assemble to form a hex-
amer ring. Two hexameric rings join in a head-to-head fashion to form a dodecameric architecture (5, 6, 9). Inter-
subunit contacts between adjacent (β/α)8-barrels within one hexamer are mainly established by a bulge created by helix
α8′ at the C terminus running parallel to helix α8. The active site of Pdx1 is located at the C-terminal side of the (β/α)8-
barrel and faces the center of the ring-like structure. Insight into the organization of the active site was obtained from the
solving of the three-dimensional structure of pent(ul)ose-
complexed Pdx1 and various mutagenesis studies (5, 6). The pent(ul)ose substrate is covalently linked to an active site
lysine residue of Pdx1 as an imine adduct at C2 and anchored through its phosphate group at C5 (6, 12). Structural ele-
ments participating in phosphate anchoring involve residues from loop 6, α-helices α2′ and α8′, and the macrodipole of
α8′ (6). A comparison of apoenzyme with the pentose phos-
phate substrate-bound enzyme reveals that alterations occurring in the protein structure are almost exclusively re-
stricted to helices α2′ and α8′ (19). In particular, both helices move closer to the pentose phosphate substrate, presum-
ably tightening the active site. It is therefore suggested that these helices are key players in inducing the conforma-
tional change(s) that alter the pentose phosphate-binding pocket of Pdx1. Since ordering of helix α2′ in Pdx1 is only
triggered upon assembly with Pdx2, it must be assumed that
this part of the protein is mainly responsible for the observed increase in substrate affinity in the presence of the glutamin-
ase subunit. However, neither α2′ nor α8′ directly contact bound substrate at a neighboring subunit, thereby necessi-
tating the existence of structural elements that function as a relay, to explain cooperativity. Since the C terminus of Pdx1
is downstream of α8′, it would appear to fulfill the require-
ments. Indeed, this implication is not without precedent,
since an extra 10 residues (positions 270–280; T. maritima
numbering) of the C terminus are observed in the Pdx1 crys-
tal structure when the pent(ul)ose phosphate substrate is bound (6). Moreover, these residues are observed to fold
over toward the active site of a neighboring subunit. In par-
ticular, contacts to loop β6-α6, helix α8′, and residues 57–59
of the active site loop of an adjacent monomer were observed. However, the remaining 14 residues of the C ter-
minus have eluded structural characterization, and their
location in three-dimensional space has continued to be a
matter of intrigue. In this study, cross-linking of the C-ter-

minal residue through incorporation of a photoactivatable
probe combined with mass spectrometry allowed us to dem-
strate that this region of Pdx1 resides in the region of
residues 270–276 of a neighboring subunit (Fig. 4). This
implies that under the conditions used in the experiment, the C terminus of one monomer stretches over an adjacent
monomer and is within 3.1 Å of the C-terminal residues
270–276, just beyond helix α8′. Thus, the inherent flexibility
in the C terminus of Pdx1 as seen in the crystal structures is
confirmed by this study and shown to be functionally rele-
vant. The conformational states supposed are depicted dia-
grammatically in Scheme 1. Conformational flexibility is a
common feature of enzymes, often used to control chemical
reactivity or to deliver a signal from one macromolecule to
another. It is, therefore, proposed that the C terminus of Pdx1 acts as a “lever” bridging adjacent Pdx1 monomers.
Indeed, the triggering of a change in the flexibility of the C
terminus by the pentose phosphate substrate could be
explained by helices α2′ and α8′ acting as “ropes,” pulling
the lever into a less exposed environment. One cannot help
but speculate that it is the binding of the triose substrate
that bestows rigidity to the C terminus and closes the active site
to allow catalysis to occur. Indeed, this conclusion is corro-
borated by the limited protease digestion experiments, where
in the first instance a decrease in susceptibility is observed in
the presence of glyceraldehyde 3-phosphate (albeit not as
pronounced as that observed with ribose 5-phosphate) (Fig.
3). Moreover, the presence of both substrates almost com-
pletely protects the C terminus from digestion under these
conditions. It is noteworthy that two phosphate-binding
sites (P1 and P2) have been identified in Pdx1 (5, 6). Only one
phosphorylated substrate can be accommodated at any one
time in either P1 or P2 because otherwise the phosphate
groups would clash. The obvious additive protection from
protease digestion afforded by the presence of both sub-
strates compared with either one alone (Fig. 3) suggests the
interesting possibility that the phosphorylated substrates
individually occupy the separate phosphate binding sites.
Future studies directed at the assignment of the triose-binding site should clarify this matter.

In light of the above data, a functional role for the C terminus of Pdx1, either as a mediator of cooperativity or as a protective agent shielding the substrate binding pocket and perhaps recruiting an essential catalytic group to the active site, is justified. In this context, it is interesting to note that although the amino acid residues of the Pdx1 C terminus are not highly conserved (supplemental Fig. 3), a number of conserved residues could be conceived to play a catalytic role (e.g. Arg-288 or Arg-292). Logically, the elucidation of the structure of the entire C terminus of Pdx1 should assist in defining the exact role of these residues in catalysis.

In summary, this study provides evidence for a highly regulated PLP synthase. On the one hand, cooperative binding of the pentose phosphate substrate is observed between the Pdx1 active sites, a phenomenon that is further regulated by Pdx2-Gln in that it enhances substrate affinity. The fact that Pdx2-Gln enhances the affinity for ribose 5-phosphate in Pdx1 is most likely to signal that it is "ready" to proceed with glutamine hydrolysis, a step that is necessary for the transformation of the pentose phosphate substrate into the chromophoric intermediate (12). In this way, the efficacy of the reaction is ensured. The intersubunit cross-talk features appear to be manifested either directly or indirectly through the C terminus of Pdx1. Conformational coupling between monomers provides a mechanism for tight regulation, thereby increasing the dynamic range of sensing events by positive cooperativity. This not only provides an explanation for the functional essentiality of the C terminus but also rationalizes the ornate Pdx1 architecture that manifests cooperativity. Many enzymes use segmental motions to regulate catalysis. A common element in these mechanisms is an active site loop, wherein an open form can facilitate ligand binding and release, and a closed form prepares, controls, and protects a reaction intermediate (20, 21). Indeed, this feature has already been demonstrated for E. coli pyridoxine 5'-phosphate synthase, the key enzyme of the DXP-dependent pathway of vitamin B6 biosynthesis. Extensive structural studies have highlighted the importance of an active site loop (loop 4) in catalysis by this enzyme, where the loop acts as a lid, opening or closing the active site depending on whether substrate is present (22–24). The C terminus of the unexpectedly closely related Pdx1 with respect to tertiary structure and function (19) would appear to provide the same function in PLP synthase.

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Cooperativity and Allosterism in Pyridoxal 5'-Phosphate Synthase