Role of Arg-277 in the Binding of Pyridoxal 5′-Phosphate to *Trypanosoma brucei* Ornithine Decarboxylase[†]

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ABSTRACT: The pyridoxal 5′-phosphate (PLP) binding site in *Trypanosoma brucei* ornithine decarboxylase (ODC) has been studied by site-directed mutagenesis and spectroscopy. The β/α barrel model proposed for the eukaryotic ODC structure predicts that the phosphate group of PLP is stabilized by interactions with a Gly-rich loop (residues 235−237) and by a salt bridge to Arg-277 [Grishin, N. V., Phillips, M. A., & Goldsmith, E. J. (1995) *Protein Sci. 4*, 1291−1304]. Mutation of Arg-277 to Ala increases the K_m for PLP by 270-fold compared to that of wild-type ODC while reducing k_{cat} by only 2-fold at pH 8. PLP binding affinity was measured directly by ultrafiltration; the K_d for PLP is at least 20-fold higher in the mutant enzyme at pH 8. In addition, R277A ODC also has weaker binding affinities for a series of cofactor analogs than the wild-type enzyme. These results demonstrate that Arg-277 is necessary for high-affinity PLP binding by ODC. The ^{31}P NMR spectra of ODC suggest that the phosphate is bound in a strained conformation as a dianion to both wild-type and R277A ODC. However, the ^{31}P chemical shift for R277A ODC (6.7 ppm) is 0.5 ppm downfield from that observed for the wild-type enzyme, indicating that the environment of the enzyme-bound phosphate is altered in the mutant enzyme. The binding affinity of PLP for both wild-type and R277A ODC is weaker at high pH, corresponding to the titration of a protonated species with a pK_a of approximately 8.5. Concomitant with these changes are a decreased k_{cat} and an altered absorption spectra which arises from bound PLP. PLP bound to wild-type ODC has a ^{31}P chemical shift and a CD signal observable over the entire tested pH range (7−9). In contrast, for R277A ODC between pH 8 and 9, the ^{31}P chemical shift becomes solution-like and the CD signal is abolished. The data suggest that for R277A ODC the rigid PLP binding mode which characterizes the wild-type enzyme is lost at high pH. Thus, multiple interactions between the wild-type active site and PLP maintain the cofactor in a constrained conformation that is essential for efficient catalysis, tempering the consequence of the removal of any single interaction.

Ornithine decarboxylase (ODC)[1] catalyzes the conversion of ornithine to putrescine which is the first committed step in the biosynthesis of polyamines (Tabor & Tabor, 1984). Polyamines which are ubiquitous to both eukaryotic and prokaryotic organisms are required for cell growth and differentiation (Tabor & Tabor, 1984). In accord with these findings, inhibitors of ODC were found to arrest cell growth and to have a clinical role in the treatment of African sleeping sickness caused by *Trypanosoma brucei*, *Pneumocystis* pneumonia in AIDS patients, and in combination with other drugs for cancer chemotherapy (Wang, 1995; Pegg et al., 1995). We are attempting to elucidate the structural and mechanistic requirements for ODC activity as a prelude to the design of additional inhibitors of *T. brucei* ODC.

The eukaryotic ODCs are pyridoxal 5′-phosphate (PLP) dependent enzymes. They are unrelated to bacterial ODC (which is structurally related to aspartate aminotransferase; Momany et al., 1995) and instead share similarities with arginine and diaminopimelate decarboxylases and with alanine racemase (Grishin et al., 1995). The eukaryotic ODCs are obligate dimers with each monomer composed of two domains (Osterman et al., 1995b). The active sites are formed at the interface of the subunits between the N-terminal domain of one monomer and the C-terminal domain of the other (Tobias & Kahana, 1993; Coleman et al., 1994; Osterman et al., 1994). The N-terminal domain contains residues which have been identified to interact with PLP. Lys-69 forms a Schiff base with PLP (Poulin et al., 1992), and Glu-274 stabilizes the positive charge on the pyridine nitrogen of PLP (Osterman et al., 1995a). The C-terminal domain contains residues which are likely to be involved in interaction with substrate. Cys-360 is the covalent attachment site for the suicide inhibitor α-(difluoromethyl)ornithine (Poulin et al., 1992), and mutation of Asp-361 increases K_m for Orn by 2000-fold while having little impact on k_{cat} (Osterman et al., 1995a). Mutation of a number of other residues also reduces enzyme activity; however, the function

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[1] Abbreviations: ODC, ornithine decarboxylase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PDP, 4-deoxypyridoxine 5′-phosphate; PM, pyridoxamine; PD, 4-deoxypyridoxine; PPP, *N*-(5′-phosphopyridoxyl)putrescine; DTT, dithiothreitol. Mutant *Trypanosoma brucei* ODCs are referred to by their single-letter amino acid codes; e.g. the Arg-277 to Ala mutant enzyme is referred to as R277A.

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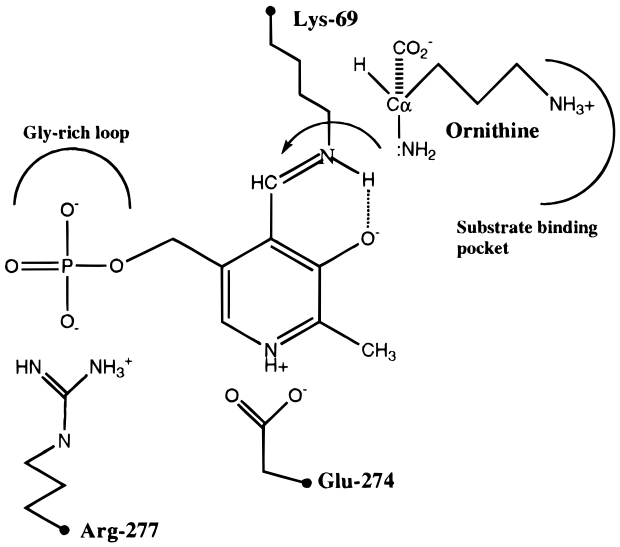


FIGURE 1: Schematic representation of the putative eukaryotic ODC active site. PLP forms a Schiff base with Lys-69 which is displaced upon interaction with Orn (Poulin et al., 1992). Glu-274 stabilizes the positive charge on the pyridine nitrogen (Osterman et al., 1995a,b), and the 5′-phosphate of PLP interacts with the Gly-rich loop (Grishin et al., 1995) and Arg-277 (this work).

of these residues in catalysis remains unknown (Lu et al., 1991; Tsirka et al., 1993; Osterman et al., 1995a). Modeling studies predict that the N-terminal domain of ODC folds into a β/α barrel which contains the PLP binding site (Grishin et al., 1995).

In many PLP-dependent enzymes, the phosphate moiety has been shown to be the major determinant of tight cofactor binding (Martines-Carrion, 1986). With the exception of glycogen phosphorylase, this structural element is not directly involved in the chemistry of the catalyzed reaction, but rather is dedicated to binding. Two structural motifs which account for strong interactions with the phosphate moiety, a Gly-rich loop and a basic amino acid, are found in many PLP-dependent enzymes despite great divergence in the overall protein fold; the described basic amino acids include Arg-266 in aspartate aminotransferase (Kirsch et al., 1984), Arg-50 in D-amino acid aminotransferase (Sugio et al., 1995), His-354 in bacterial ODC (Momany et al., 1995), and His-86 in tryptophan synthase (Hyde et al., 1988). Similarly, for eukaryotic ODC, the proposed β/α barrel model predicts that the phosphate group will be stabilized by interactions with a Gly-rich loop (residues 235−237) and by a salt bridge to Arg-277, which is conserved in all related decarboxylases (Grishin et al., 1995; Figure 1).

To further elucidate the role of Arg-277 in PLP binding, we have mutated Arg-277 to Ala in *T. brucei* ODC. We find that as expected Arg-277 promotes high-affinity PLP binding. Further, we demonstrate that the binding affinity of PLP for both wild-type and R277A ODC is weaker at high pH. Analysis of the absorption spectra provides insight into the structural basis for this finding. Characterization of the CD and ^{31}P NMR spectral properties of ODC-bound PLP, neither of which has been previously described, suggests that the PLP is held in a strained conformation which contributes to catalytic efficiency. In the R277A ODC mutant at high pH, the rigid PLP binding mode which characterizes the wild-type enzyme is lost.

EXPERIMENTAL PROCEDURES

Materials

The CO_2 detection kit, its components [NADH, phosphoenolpyruvate (PEP), and solution B], Orn, PLP, and its analogs, pyridoxamine 5′-phosphate (PMP), 4-deoxypyridoxine 5′-phosphate (PDP), pyridoxamine (PM), and 4-deoxypyridoxine (PD), and buffers (Hepes, Bicine, and Bis-Tris-Propane) were purchased from Sigma. Centricon-10 and Centriprep-10 are products of Amicon. Ni^{2+} resin was purchased from Qiagen. All nonlinear curve fitting was done with Sigma Plot (Jandel Scientific). Errors are the standard errors of the fit.

Methods

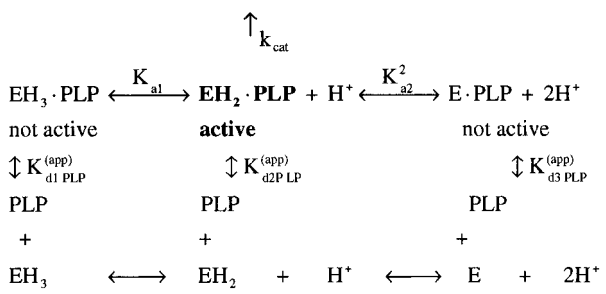
Site-Directed Mutagenesis. Mutagenesis of the *T. brucei* ODC gene, which was previously cloned (Phillips et al., 1987), was performed by the standard Kunkel technique (Kunkel, 1985) in the Bluescript vector (Stratagene) using the M13 helper phage R408 (Stratagene) and the Kunkel strain BO265. The primer used to convert Arg-277 to Ala was 5′-AGCCGGGAGCGTACTACGTTGC-3′; the replaced codon is underlined. DNA fragments containing the site of mutation were then subcloned into the expression vector. The structure of the subcloned fragment was verified by sequence analysis (Sanger & Coulson, 1975).

ODC Expression and Purification. *T. brucei* wild-type and R277A ODC were expressed in *Escherichia coli* using the His_6-TEV vector and purified as described previously by Ni^{2+}−agarose column chromatography and Superdex G-200 gel filtration (Grishin et al., 1996). ODC was concentrated to 0.4 mM in the presence of 2 mM PLP and 5 mM DTT. The excess of both reagents was removed using a Fast Desalting HR 10/10 column (Pharmacia) equilibrated with 20 mM Hepes/NaOH (pH 7.2) and 50 mM NaCl, and aliquots were stored at −80 °C. The protein concentration in all ODC samples was determined spectrophotometrically using a previously determined extinction coefficient $\epsilon^{280\ nm} = 0.85$ OD $(mg/mL)^{-1}$ cm^{-1} (Osterman et al., 1994). All calculations which require an enzyme concentration term are done using the concentration of monomeric ODC assuming a molecular mass of 49 kDa.

Synthesis and Characterization of N-(5′-Phosphopyridoxyl)putrescine (PPP). PPP was synthesized using a modification of a previously described procedure (Heller et al., 1975). Briefly, PLP (10 mg) and putrescine (50 mg) were incubated for 30 min at 37 °C in 2 mL of 20 mM sodium phosphate (pH 7.3). The sample was chilled, $NaBH_4$ (10 mg) added, and the pH adjusted to 2 with HCl after a 5 min incubation. PPP was purified on a AG 50W X8 (Biorad) 1 cm × 20 cm column equilibrated with 0.05 M ammonium formate at pH 3.2. An initial gradient from 0.05 M ammonium formate at pH 3.2 to 0.5 M ammonium formate (40%) at pH 7.5 was run to remove side products. PPP was eluted with a gradient to 0.5 M ammonium formate (100%) at pH 7.5. The structure of PPP was confirmed by NMR and mass spectroscopy.

ODC Activity Assay. Decarboxylation was followed using a modification of the previously described spectrophotometric assay where CO_2 production is coupled to NADH oxidation via phosphoenolpyruvate carboxylase and malate dehydrogenase (Osterman et al., 1994). The concentration of NADH

Scheme 1



was increased, and the reaction was followed at 380 nm to allow the reaction to be monitored for longer times. Unlike for wild-type ODC, Tris buffer (a component of the Sigma CO_2 detection kit) inhibits R277A ODC; thus, Tris was substituted with Hepes/NaOH (pH range of 7−8) or Bicine/NaOH (pH 8−9). The final reaction mixture contained solution A′ (300 μL of 100 mM Hepes/NaOH or Bicine/NaOH, 2 mM NADH, 4 mM phosphoenolpyruvate, and 12 mM $MgCl_2$), reagent B (40 μL of Sigma CO_2 detection kit), 2 mM DTT, Orn, and PLP in a final volume of 0.5 mL. The assay is initiated after 5 min of pre-equilibration at 37 °C, by the addition of ODC. NAD^+ formation was monitored in a Beckman DU 650 spectrophotometer at 380 nm, and the extinction coefficient was experimentally determined to be 1180 M^{-1} cm^{-1}.

Kinetic determinations were made over a range of pH (7−9). The Orn concentration was varied from 0.1 to 25 mM at fixed PLP concentrations (30−350 μM), and the PLP concentration was varied from 0.5 to 350 μM at fixed Orn concentrations (0.2−25 mM). The enzyme concentrations used to collect the wild-type (0.05−2 μM) and R277A (0.2−5 μM) data were all above the dimerization threshold. The overall PLP concentration in PLP and ODC stock solutions was determined by the phenylhydrazine method (see below). The ionic strength equivalent to the highest substrate concentration was maintained constant by addition of NaCl. Data were fit to the Michaelis−Menten equation to obtain the apparent $K_{m\ PLP}$, $K_{m\ Orn}$, and k_{cat}. The relationship between the apparent Michaelis−Menten constants and the microscopic constants has been described for the general model of an essential activator and a single substrate (Segel, 1975). In our case, this general model can be simplified by assuming that free E can bind PLP or the PLP−Orn conjugate but not free Orn. Additionally, we fix the assay conditions such that [Orn] ≫ [PLP] ≫ [ODC]. Data acquired for multiple PLP and Orn concentrations were either fit to the full model or collected under conditions in which either PLP or Orn was present at saturation. At saturating PLP or Orn, the apparent $K_{m\ PLP}$, $K_{m\ Orn}$, and k_{cat} closely approximate the theoretical values. Similar assumptions were made for the kinetic analysis of D-serine dehydratase (Dowhan & Snell, 1970).

pH Dependence of Kinetic Parameters. The minimal model (Scheme 1) which describes the pH dependence of k_{cat} requires a single proton titrating in the acidic limb (pK_{a1}) and two protons titrating with a similar pK_a in the basic limb (pK_{a2}). The relationship between the Michaelis−Menten parameters (k_{cat} and $K_{m\ PLP}$) and pH was derived from Scheme 1 using the rapid equilibrium assumption to obtain eqs 1A and 1B, assuming that EH_2·PLP is the only active species and that the active sites in the dimer function independently. Substrate (Orn) is assumed to be at saturation for all forms of bound and free PLP because the Orn concentration was maintained above $K_{m\ Orn}$ and above K_d for a typical Schiff base such as PLP−Orn in solution (K_d < 1 mM; Metzler et al., 1980). The k_{cat} data were fit to eq 1A to determine the values of pK_{a1} and pK_{a2}, where k_{cat} is the value determined at a given pH and $k_{cat}^{(EH_2\cdot PLP)}$ is the theoretical intrinsic value of the pure diprotonated active form EH_2·PLP. This model was extended for the global fit of all the kinetic data acquired at saturating Orn concentrations and various PLP concentrations. The data were fit to a form of the Michaelis−Menten equation where k_{cat} and $K_{m\ PLP}$ are defined by eqs 1A and 1B. K_{a1} and K_{a2} were fixed to the values obtained for the k_{cat} fit. Preliminary rounds of fitting established that $K_{d1\ PLP}^{(app)}$ is statistically identical to $K_{d2\ PLP}^{(app)}$, and for further analysis, they were assumed to be equal.

$$k_{cat} = \frac{k_{cat}^{(EH_2\cdot PLP)} K_{a1}[H^+]^2}{K_{a1}K_{a2}^{\ 2} + K_{a1}[H^+]^2 + [H^+]^3} \qquad (1A)$$

$$K_{m\ PLP} = \frac{K_{d3\ PLP}^{(app)} K_{a1}K_{a2}^{\ 2} + K_{d2\ PLP}^{(app)} K_{a1}[H^+]^2 + K_{d1\ PLP}^{(app)}[H^+]^3}{K_{a1}K_{a2}^{\ 2} + K_{a1}[H^+]^2 + [H^+]^3} \qquad (1B)$$

Inhibitory Analysis of R277A ODC with PLP Analogs. PLP analog concentrations were varied from 0.05 to 3.2 mM for PMP and PDP, from 8 to 30 mM for PM and PD, and from 0.5 to 30 μM for PPP. Inhibitor data were collected in Hepes buffer at pH 8 (as described above) at multiple concentrations of PLP. The data were fit to the standard Michaelis−Menten equation for competitive inhibitors to determine K_I. For PM and PD, the constants could only be estimated because solubility limited the concentration which could be used in the assay.

Estimation of PLP Dissociation Constants of R277A and Wild-Type ODC by Ultrafiltration. Free and enzyme-bound PLP were separated by ultrafiltration as described for D-serine dehydratase (Marceau et al., 1988), and PLP in the two fractions was quantitated by the colorimetric phenylhydrazine assay (Wada & Snell, 1961). ODC was preincubated (10 min) with PLP (up to a 4-fold excess in 2−10 mL) at 37 °C in the main reservoir of a Centricon-10 or Centriprep-10 (Amicon) unit. The filtrate did not exceed 10% of the total volume. Various concentrations of wild-type ODC (from 0.5 to 5 μM) and R277A ODC (from 0.5 to 15 μM) were analyzed over a pH range of 7−9 utilizing the buffer systems described for kinetic analysis. All concentration points were set up in duplicates or triplicates. Enzyme stock solutions contained a 1.4−1.7-fold molar excess of free PLP. Samples were brought to 0.2% phenylhydrazine and 0.5 N H_2SO_4, incubated for 30 min at 37 °C, and spun to remove precipitated protein. The concentration of PLP was determined by optical density at 410 nm using the standard calibration curve. Linear response and reliable detection (less than 10% error) were observed in a range of 0.2−5 μM PLP. The wild-type data and R277A ODC data collected at pH 8.0 and below gave a linear Scatchard plot and were fit to a one-site model, $R = [PLP]_b/[E]_T = [PLP]_f/(K_{d\ PLP} + [PLP]_f)$, to determine $K_{d\ PLP}$. The R277A data collected at pH 8.5 and 9.0 gave a curved Schatchard plot and were fit to a two-site model:

$$R = \frac{\frac{[PLP]_f}{K_{d1\ PLP}}\left(0.5 + \frac{[PLP]_f}{K_{d2\ PLP}}\right)}{1 + \frac{[PLP]_f}{K_{d1\ PLP}}\left(1 + \frac{[PLP]_f}{K_{d2\ PLP}}\right)}$$

^{31}P NMR Spectroscopy. Protein samples were equilibrated in 20 mM Hepes/NaOH, 50 mM NaCl, and 1 mM DTT at pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0 using a Fast Desalting HR 10/10 column (Pharmacia). The pH titration was started at pH 9.0, and ODC was recovered after each NMR experiment for reuse in subsequent experiments. Following these experiments, the protein remained fully active with the exception that the pH 6.0 and 6.5 samples precipitated significantly and were not reused. The pH of the samples was checked immediately prior to loading a 5 mm × 7 cm Wilmad NMR tube. The concentration of the protein was between 0.3 and 1.0 mM, on the basis of 280 nm absorbance. All ^{31}P NMR spectra were acquired on a Varian Unity 500 spectrometer operating at 202 MHz at 37 °C using a 60° pulse and a 3 s recycle time with no proton decoupling. The number of transients collected was between 1280 and 80 000 depending upon the sample concentration and pH. Phosphoric acid (85%) was used as an external chemical shift reference.

UV/Vis Spectroscopy. Spectra of both wild-type and R277A ODC were recorded on a Beckman DU-650 spectrophotometer over a pH range of 7−9 using 100 mM Hepes (pH 7−8) or Bicine (pH 8−9) buffers. DTT strongly affects the spectral properties of bound cofactor and was omitted from the spectral studies. ODC, depleted of the excess DTT and PLP by fast desalting (as described above), was diluted to a final concentration of 35 μM with the corresponding buffer, and the spectra were recorded immediately between 260 and 500 nm. To account for the contribution of free PLP, originating either from a molar excess of PLP still present in enzyme samples or from PLP dissociation, which is substantial at high pH, the sample was subjected to ultrafiltration using Centricon-10 units (Amicon). The flow-through for every sample was collected, and the spectra were recorded and subtracted from the corresponding initial spectra. The spectra were recalculated to millimolar absorptivities.

The pH dependence of millimolar absorptivities was analyzed at two wavelengths, 335 and 423 nm, representative of two titratable maxima in the spectra for both wild-type and R277A ODC. The simplest model that describes the pH titration requires two spectrally distinguishable forms (E and EH) with different extinction coefficients (ϵ_{EH}^{335nm}, ϵ_{E}^{335nm}, ϵ_{EH}^{423nm}, and ϵ_{E}^{423nm}) that interconvert at a single pK_a. It was necessary to correct for the PLP occupancy of ODC because PLP dissociation is significant at high pH, especially for R277A ODC. The molar fraction of ODC bound to PLP at a given pH ($R = [PLP]_b/[E]_T$) was calculated from the corresponding $K_{d\ PLP}$ values determined by ultrafiltration (above). The spectral data at both wavelengths were fit to eq 2, and the pK_a describing the dissociation of the spectrally sensitive proton was determined.

$$\epsilon_{(obs)}^{\lambda nm} = R\left(\epsilon_{EH}^{\lambda nm}\frac{[H^+]}{K_a + [H^+]} + \epsilon_{E}^{\lambda nm}\frac{K_a}{K_a + [H^+]}\right) \quad (2)$$

CD Spectroscopy. An AVIV 62DS circular dichroism spectrophotometer with a thermally regulated sample holder was used to acquire the CD spectra of the PLP cofactor of wild-type and R277A ODC (25 μM) from 300 to 500 nm at 25 °C using a 10 mm cell with a sample volume of 3 mL. Samples were collected both in the presence and in the absence of excess PLP (200 μM).

RESULTS

Mutagenesis and Expression of T. brucei ODC. The codon for R277 was replaced with that for Ala in *T. brucei* ODC by standard Kunkel mutagenesis (Kunkel, 1985). Both wild-type ODC and the mutant R277A ODC were expressed in *E. coli* as His_6 fusion proteins from the T7 promoter and purified for analysis as previously described (Osterman et al., 1994).

Kinetic Analysis of Wild-Type and R277A ODC. The apparent binding affinity of PLP ($K_{m\ PLP}$) to wild-type ODC and to R277A ODC was determined by kinetic analysis at multiple PLP and Orn concentrations over a pH range of 7−9. For both wild-type and R277A ODC, the data obeyed Michaelis−Menten kinetics with no evidence of cooperativity for either Orn or PLP. At pH 8, the $K_{m\ PLP}$ for R277A is increased by 270-fold compared to that for wild-type ODC, the k_{cat} for R277A ODC-catalyzed decarboxylation is decreased by 2-fold, and the $K_{m\ Orn}$ is increased by 7-fold (Table 1). At saturating PLP concentrations, the specific activity of wild-type ODC is constant over a wide range of enzyme concentrations ($[E]_T$ from 0.01 to 0.2 μM). In contrast, the specific activity of R277A increases as the enzyme concentration is increased until $[E]_T > 0.1$ μM, suggesting that the monomer/dimer equilibrium has been perturbed in the mutant enzyme. This effect is more pronounced at low concentrations of PLP (data not shown).

The plots of k_{cat} versus pH for both wild-type and R277A are bell-shaped (Figure 2A). The minimal model which fits the k_{cat} data for both wild-type and R277A ODC requires a single deprotonation to describe the acidic limb and the cooperative loss of two protons to explain the basic limb. The k_{cat} data sets were fit to eq 1A (Figure 2A) to obtain pK_{a1} and pK_{a2}. Analysis of the acidic limb is limited by the problem that data could not be collected below pH 7 because of limitations in the dye-linked assay and because ODC precipitates below pH 6.5. However, the data fit well to the model using a pK_{a1} of 6.6 ± 0.15 for wild-type ODC and 6.9 ± 0.10 for R277A ODC. The basic limb is well-defined and is described by $2H^+$ cooperative dissociation occurring with a pK_{a2} of 8.6 ± 0.05 for wild-type ODC and 8.5 ± 0.05 for R277A ODC (Figure 2A). The effect of pH on $K_{m\ PLP}$ for R277A ODC is also described by Scheme 1, in which the cooperative $2H^+$ loss (pK_{a2} = 8.5) causes an increase in $K_{m\ PLP}$ (Figure 2B). However, no acidic limb is observed. In contrast, $K_{m\ PLP}$ for wild-type ODC is constant over the entire pH range. In addition, the $K_{m\ Orn}$ for wild-type ODC is constant over the entire pH range, while the $K_{m\ Orn}$ for R277A ODC decreases toward the wild-type value above pH 8 (Table 1).

Analysis of PLP binding affinity by ultrafiltration. Because kinetic analysis cannot provide a measure of the true dissociation constants ($K_{d\ PLP}$), PLP binding affinity was assessed by ultrafiltration to separate ODC-bound PLP from free PLP. The PLP was detected spectrophotometrically after

Table 1: Kinetic and Equilibrium Constants of Wild-Type and R277A ODC[a]

	$K_{m\,Orn}$ (mM)		k_{cat} (s^{-1})		$K_{m\,PLP}$ (μM)		$K_{d\,PLP}$ (μM)	
pH	wild-type	R277A	wild-type	R277A	wild-type	R277A	wild-type	R277A
7.0	0.36 ± 0.01	3.3 ± 0.4	12.7 ± 0.2	4.9 ± 0.2	0.17 ± 0.03	8 ± 1	<0.05	0.5 ± 0.05
7.5	0.40 ± 0.01	3.3 ± 0.2	15.2 ± 0.3	7.1 ± 0.2	0.10 ± 0.02	11 ± 1	nd	nd
8.0	0.44 ± 0.03	3.2 ± 0.5	15.1 ± 0.3	7.1 ± 0.2	0.15 ± 0.03	41 ± 3	<0.05	1.0 ± 0.3
8.2	nd	nd	13.9 ± 0.6	6.3 ± 0.1	0.20 ± 0.09	58 ± 3	nd	nd
8.5	0.34 ± 0.03	2.0 ± 0.3	12.0 ± 0.3	4.3 ± 0.2	0.22 ± 0.06	115 ± 15	0.37 ± 0.07	0.9 ± 0.4 12 ± 5
8.8	nd	nd	4.3 ± 0.3	2.2 ± 0.2	0.24 ± 0.07	313 ± 40	nd	nd
9.0	0.26 ± 0.04	0.25 ± 0.15[b]	3.1 ± 0.1	0.5 ± 0.2	0.32 ± 0.14	900 ± 400	2.5 ± 0.1	1.5 ± 0.3 46 ± 14

[a] Kinetic data were collected at either a fixed Orn concentration (25 mM) while varying the PLP concentration (0.5−350 μM) or at a fixed PLP concentration (30 μM for wild-type ODC and 120 μM for R277A ODC) while varying the Orn concentration (0.1−25 mM) for each pH and fit to the Michaelis−Menton equation to obtain parameters. [b] For R277A ODC at pH 9, the $K_{m\,Orn}$ should be considered an apparent binding constant because assay conditions do not permit collection of data above 350 μM PLP. PLP dissociation constants were determined by ultrafiltration as described in Experimental Procedures. For wild-type ODC and R277A ODC at pH 7 and 8, a single-site (equivalent to noncooperative two-site) model was used to obtain $K_{d\,PLP}$. For R277A ODC at pH 8.5 and 9, values of $K_{d1\,PLP}$ and $K_{d2\,PLP}$ were obtained from a cooperative two-site model. nd represents data not collected.

coupling to phenylhydrazine. Unlike for other PLP-dependent enzymes, isolation of apo-ODC for these studies was not possible because the enzyme is irreversibly inactivated after incubation in the absence of PLP. This effect is more pronounced for low enzyme concentrations. Therefore, binding studies were performed with holoenzyme. Ultrafiltration was chosen for the binding studies over equilibrium dialysis because it could be performed rapidly, allowing measurements to be made prior to enzyme inactivation. The wild-type ODC data fit to a single-site model with the $K_{d\,PLP}$ ranging from <0.05 μM (the limit of detection of the method) at pH 7 to 2.5 μM at pH 9 (Table 1, Figure 3A). For R277A, the data also fit to a single-site model at the low end of the pH range (7−8). However, at pH 8.5 and 9, the Scatchard plot is clearly biphasic, indicating that the system contains two nonequivalent PLP binding sites (Figure 3B). The $K_{d\,PLP}$ for R277A increases with pH up to 50 μM at pH 9 (Table 1). Similar to the kinetic data, the binding data could also be fit to a global model in which a deprotonation event (pK_a of approximately 8.5) describes the pH dependence of the PLP binding affinity.

Inhibition of R277A ODC by Cofactor Analogs. The binding properties for several cofactor analogs were characterized to further analyze the effects of the R277A mutation. The PLP analogs, pyridoxamine 5′-phosphate (PMP), 4-deoxypyridoxine 5′-phosphate (PDP), pyridoxamine (PM), 4-deoxypyridoxine (PD), and *N*-(5′-phosphopyridoxyl)putrescine (PPP), were tested as competitive inhibitors of both wild-type and R277A ODC at pH 8. For wild-type ODC, PPP is the only analog which competes sufficiently well with PLP to allow steady-state kinetic analysis; the K_I for PPP is estimated to be 5 nM. For the R277A ODC, all five analogs are in rapid equilibrium with PLP and are competitive inhibitors of PLP. The K_I's for PMP and PDP were 210 and 94 μM, respectively, while the K_I's for the nonphosphorylated analogs, PM and PD, are at least 200-fold higher. The K_I for the bisubstrate analog PPP (0.9 μM) is similar to the $K_{d\,PLP}$.

^{31}P NMR Spectra of Wild-Type and R277A ODC. The ^{31}P NMR spectra of free PLP display a pH-dependent chemical shift which ranges from 1.8 to 4.5 downfield from 85% phosphoric acid with a pK_a of 6.5, similar to previously reported results (Martinez-Carrion, 1975; Schnackerz et al., 1979, 1989). Two resonances are observed in the ^{31}P NMR spectra of wild-type ODC (Figure 4A,B). One resonance is located at 6.25 ppm and accounts for 1 molar equiv of PLP per mole of enzyme. The line width of this signal (60 Hz) shows that the resonance arises from a phosphate which is bound tightly to ODC and thus can be assigned to the phosphoryl group bound in the enzyme active site. The chemical shift of the 6.25 ppm resonance is not affected by pH over a range of pH 6−9 (Figure 4A,B).

A second, sharper resonance is observed which has a chemical shift dependence on pH that is very similar to that of free PLP but that is systematically shifted ~0.2 ppm downfield of the solution value. This resonance originates from the molar excess of PLP in the ODC preparation, and its slight shift with respect to free PLP is likely to arise from loose association with surface lysine residues on ODC. Integration of this signal, and comparison with spectra obtained for different concentrations of free PLP, yielded a very good correlation between the excess of PLP estimated by ^{31}P NMR and that measured by the phenylhydrazine assay. To further confirm the identity of this resonance, we added free PLP to the holoenzyme and observed an increase in the resonance intensity as well as the appearance of a signal from free PLP. The observation of distinct ^{31}P NMR signals for free PLP and loosely bound PLP shows that the two species are in slow exchange, consistent with the slow exchange observed for the binding of PLP to free amines in solution (data not shown).

The ^{31}P NMR spectra of R277A ODC also display two resonance peaks, one resonance at 6.7 ppm arising from phosphate bound to the enzyme active site and a second which again arises from excess PLP in the preparation (Figure 4D). The resonance arising from the ODC-bound phosphate of PLP is shifted 0.5 ppm downfield from that observed in the wild-type enzyme. As for wild-type ODC, the chemical shift of this signal does not change over a pH range of 6−8. However, the signal intensity at this chemical shift reduces with increasing pH and becomes undetectable above pH 8.0 where a new resonance appears 0.2 ppm downfield of the loosely bound PLP peak (Figure 4C). The loss of the resonance at 6.7 ppm occurs in the same pH range as the decrease in PLP binding affinity (pK_a between 8 and 9).

UV/Vis and CD Spectra of Wild-Type and R277A ODC. PLP bound to either wild-type or R277A ODC displays

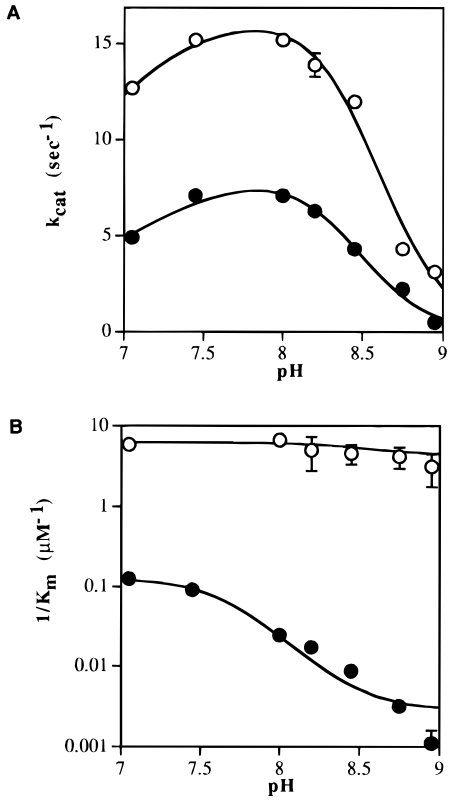


FIGURE 2: Comparative kinetic analysis of wild-type and R277A ODC. Decarboxylation rates were determined at various PLP and Orn concentrations over a pH rangeof 7−9. The kinetic parameters, k_{cat} and $K_{m\ PLP}$, were obtained using the Michaelis−Menten equation and plotted versus pH: (○) wild-type ODC and (●) R277A ODC. Standard errors are shown with error bars when they are larger than the size of the plotting symbols. (A) pH dependence of k_{cat}. Data were fit to eq 1A (Experimental Procdeures), and the fit is displayed by the solid lines. The values of the parameters $k_{cat}^{(EH_2\cdot PLP)}$, p$K_{a1}$, and p$K_{a2}$ from the fit are 17 ± 1.0 s^{-1}, 6.6 ± 0.15, and 8.6 ± 0.05 for wild-type ODC and 8.5 ± 0.5 s^{-1}, 6.9 ± 0.10, and 8.5 ± 0.05 for R277A ODC, respectively. (B) pH dependence of $K_{m\ PLP}$. Data were fit to eq 1B using fixed values of $k_{cat}^{(EH_2\cdot PLP)}$, p$K_{a1}$, and p$K_{a2}$ (shown in panel A). The values of the parameters $K_{d1\ PLP}^{(app)}$ ($K_{d2\ PLP}^{app}$) and $K_{d3\ PLP}^{(app)}$ from this fit are 0.16 ± 0.02 and 0.23 ± 0.09 μM for wild-type ODC and 7.7 ± 0.08 and 340 ± 20 μM for R277A ODC, respectively. The fit to the model-independent values of $K_{m\ PLP}$ simulated using these parameters and eq 1B is displayed as a solid line.

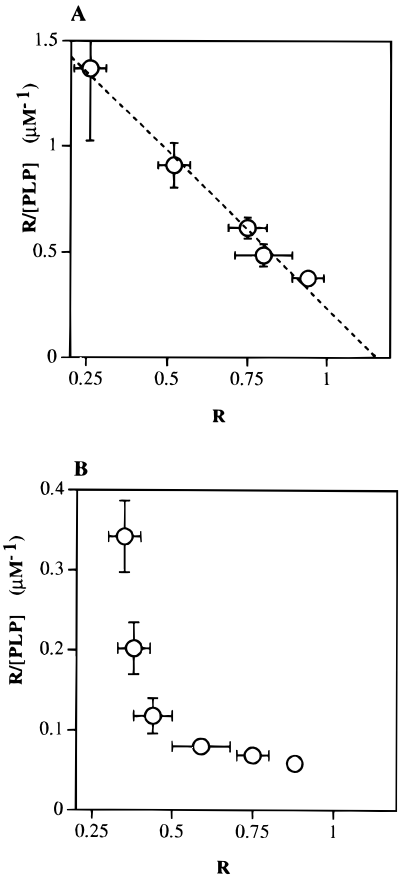


FIGURE 3: Analysis of PLP binding sites on R277A and wild-type ODC. Separation and quantitation of bound ($[PLP]_b$) and free ($[PLP]_f$) cofactor was performed using ultrafiltration and the phenylhydrazine assay as described in Experimental Procedures. Scatchard plots are shown for data collected at pH 8.5 for wild-type ODC (A) and R277A ODC (B) where $R = [PLP]_b/[E]_T$. Standard deviations are shown by error bars. The dotted line represents the fit for wild-type ODC to the single-site model (Experimental Procedures).

absorption spectra with band maxima at 335 and 423 nm (Figure 5A,B). The relative intensities of these bands depend on the pH; the 335 band increases at higher pH, while the 423 band decreases (Figure 5C,D). The simplest model that describes the pH titration requires two spectrally distinguishable forms, interconverting at a single pK_a (Figure 6); however, our data are also consistent with a cooperative 2H$^+$ loss similar to that observed in the kinetic data. The titration curve for wild-type ODC is described by a deprotonation event with a pK_a of 8.7 ± 0.13 (Figure 5C), while for R277A ODC, the titration occurs with a lower pK_a of 8.2 ± 0.15 (Figure 5D).

The interaction of ODC with PLP is characterized by a negative CD spectra in the visible region (300−500 nm) with the major peak occurring at 423 nm (Figure 7). PLP free in solution does not have CD spectra. The intensity of the signal is higher for wild-type ODC than for R277A ODC over the entire pH range. The signal intensity decreases at higher pH for both wild-type and R277A ODC; however, for R277A ODC, the signal is completely lost between pH 8 and 9 (Figure 7B). Similar trends were observed in the presence or absence of added exogenous PLP (200 μM).

DISCUSSION

As predicted by the model of the ODC structure (Grishin et al., 1995), Arg-277 contributes to high-affinity PLP binding. Mutation of R277 to Ala in *T. brucei* ODC decreases the $K_{m\ PLP}$ by 270-fold at pH 8 while having little effect on k_{cat}. Consistent with the increase in $K_{m\ PLP}$, the mutant enzyme has weaker affinity for PLP and the PLP analog, PPP; at least a 20-fold increase in $K_{d\ PLP}$ and a 200-fold increase in K_I for PPP are observed. In addition, the ^{31}P chemical shift of the 5′-phosphate of enzyme-bound PLP is 0.5 ppm downfield from the chemical shift observed for the wild-type enzyme, demonstrating that the environment of the phosphate has been altered in R277A ODC. A similar effect (0.5 ppm shift at neutral pH) was observed upon mutation of the positive charge (Lys-533) which interacts with the 5′-phosphate of PLP in phosphorylase (Schinzel et al., 1992). The changes both in the PLP binding affinity and in the ^{31}P NMR spectra are consistent with the prediction that Arg-277 forms a direct interaction with the 5′-phosphate of PLP.

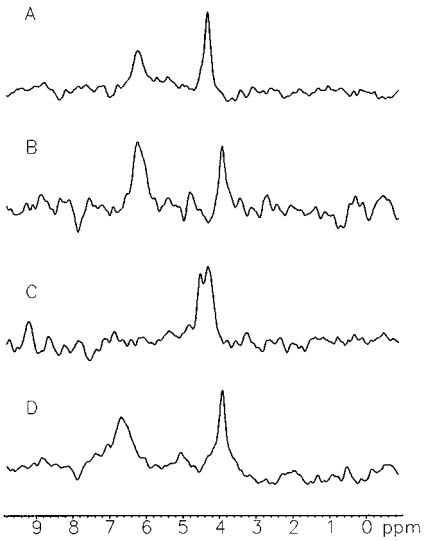


FIGURE 4: Representative ^{31}P NMR spectra of wild-type and R277A ODC. Spectra were acquired as described in Experimental Procedures using the following conditions: (A) wild-type ODC (600 μM) at pH 8.8 after 9024 transients, (B) wild-type ODC (600 μM) at pH 7.0 after 4992 transients, (C) R277A ODC (300 μM) at pH 8.8 after 12 352 transients, and (D) R277A ODC (600 μM) at pH 7.0 after 20 000 transients. All ^{31}P spectra are referenced to 85% phosphoric acid.

The model of the ODC structure predicts that in addition to Arg-277 the phosphate group will be stabilized by a Gly-rich loop (residues 235−237). The contribution of structural elements other than Arg-277 to phosphate binding can be estimated to provide at least an additional 3 kcal/mol to phosphate binding, by comparison of the K_I's for PMP and PDP to those of their unphosphorylated analogs at pH 8. The contribution of the Gly-rich loop to PLP binding affinity has been measured directly for *E. coli* D-serine dehydratase (Marceau et al., 1988) and for aminolevulinate synthase (Gong et al., 1996); in both cases, the $K_{d\ PLP}$ increased by 1−3 kcal/mol upon mutations in the Gly-rich loop. Beyond Arg-277 and the Gly-rich loop, other residues which are not revealed by the modeled ODC structure may also contribute to the interaction with the 5′-phosphate of PLP. For example, in aspartate aminotransferase (Toney & Kirsch, 1987) and tyrosine phenol-lyase (Chen et al., 1995), Tyr-70 was shown to contribute to PLP binding affinity through stabilization of the 5′-phosphate.

Deprotonation of a functional group (p$K_a \sim 8.5$) in the active site of both wild-type and R277A ODC at high pH causes concomitant changes in the kinetic parameters, the PLP binding affinity, and the spectral properties of the bound cofactor. These data suggest that these perturbations result from the same deprotonation event and that the deprotonation event is not a byproduct of the mutation. The PLP binding affinity (as measured by $K_{d\ PLP}$) is reduced at high pH for both wild-type and R277A ODC. However, for R277A ODC, two PLP binding sites of different affinity are evident at pH 8.5 and 9.0, suggesting that there may be negative cooperativity between the two active sites of the dimeric enzyme. There is no evidence for cooperativity in the binding data for the wild-type enzyme or the kinetic data collected for both wild-type and R277A ODC. Examples of apparent cooperativity triggered in nonallosteric enzymes by mutation have been described. Negative cooperativity is observed after mutation of Arg-106 in ornithine transcarbamoylase (Kuo et al., 1989), and biphasic kinetics were observed after mutation of Asp-222 to Ala in aspartate aminotransferase (Onuffer & Kirsch, 1994). It has been suggested that these effects arise because conformational changes are associated with the catalytic cycle (Goldsmith, 1996). However, it was found for D222A aspartate aminotransferase that the biphasic kinetics were not a result of negative cooperativity but were caused by the presence of two slowly interconverting enzyme forms, only one of which was active. The PLP binding data for R277A do not distinguish between negative cooperativity and the possibility that there is a mixed enzyme population.

While the kinetic data provide little insight into the structural basis for the effect of pH on ODC function, the absorption spectra have been demonstrated for other PLP-dependent enzymes to be a sensitive probe of the ionic and tautomeric state of the PLP ring. The absorption spectra of ODC are similar to that observed for tryptophanase (Metzler et al., 1991). Interestingly, the pK_a of the proton which affects the spectra in ODC is higher than that observed for tryptophanase (p$K_a = 8.5$ versus 7.5). For tryptophanase, the absorption maximum at 423 nm has been postulated to arise from the ketoenamine (**I**) and the maximum at 335 nm from the enolimine tautomer (**II**) where the proton on the imine nitrogen is transferred to the C3 hydroxyl (Figure 6; Metzler et al., 1991). Structures which could account for an increase in the 335 nm maxima at high pH arise from deprotonation of the pyridine nitrogen N1 (**III**) or from formation of an adduct upon the addition of a deprotonated nucleophilic group or a hydoxyl ion to the imine double bond (**IV**; Figure 6). Deprotonation of the imine nitrogen gives rise to a 360 nm maximum (Metzler et al., 1991), which is not evident in the spectra of ODC.

Glu-274 has been demonstrated to stabilize the positive charge on the pyridine nitrogen, enhancing the electron-withdrawing potential of the ring (Osterman et al., 1995a); therefore, the mutant provides a model for the effects of deprotonation at N1 on enzyme activity and PLP binding. Mutation of Glu-274 to Ala decreases k_{cat} and increases $K_{m\ PLP}$ as does the deprotonation which occurs at high pH for wild-type ODC. Thus, these data are consistent with the hypothesis that deprotonation of the pyridine nitrogen of PLP occurs with a pK_a of 8.5 and accounts, at least partially, for the changes, in k_{cat}, in PLP binding affinity, and in the spectral properties of the bound cofactor. Alternatively, if adduct formation accounts for the data, the pK_a of 8.5 is consistent with deprotonation of either a Lys or Cys residue; two candidate residues are Lys-169 and Cys-360, both of which have been demonstrated to be required for efficient catalysis (Lu et al., 1991; Osterman et al., 1994).

The effect of pH on k_{cat} and $K_{m\ PLP}$ is described by a model in which two protons with similar pK_a's (8.5) are cooperatively titrated, resulting in a decrease in k_{cat} for both wild-type and R277A ODC and a substantial increase in $K_{m\ PLP}$ for R277A ODC. The titration of the absorption spectra can be described by a single proton model; however, our data are also consistent with the possibility that a $2H^+$ cooperative deprotonation influences the spectral data. For tryptophanase, a $2H^+$ cooperative dissociation was proposed to explain the basic limb of the spectral pH titration curve (Metzler et al., 1991). The requirement for two protons in the pH titration can be explained if interaction between the subunits causes cooperative proton dissociation or if a nearby acidic group dissociates cooperatively with the group on PLP.

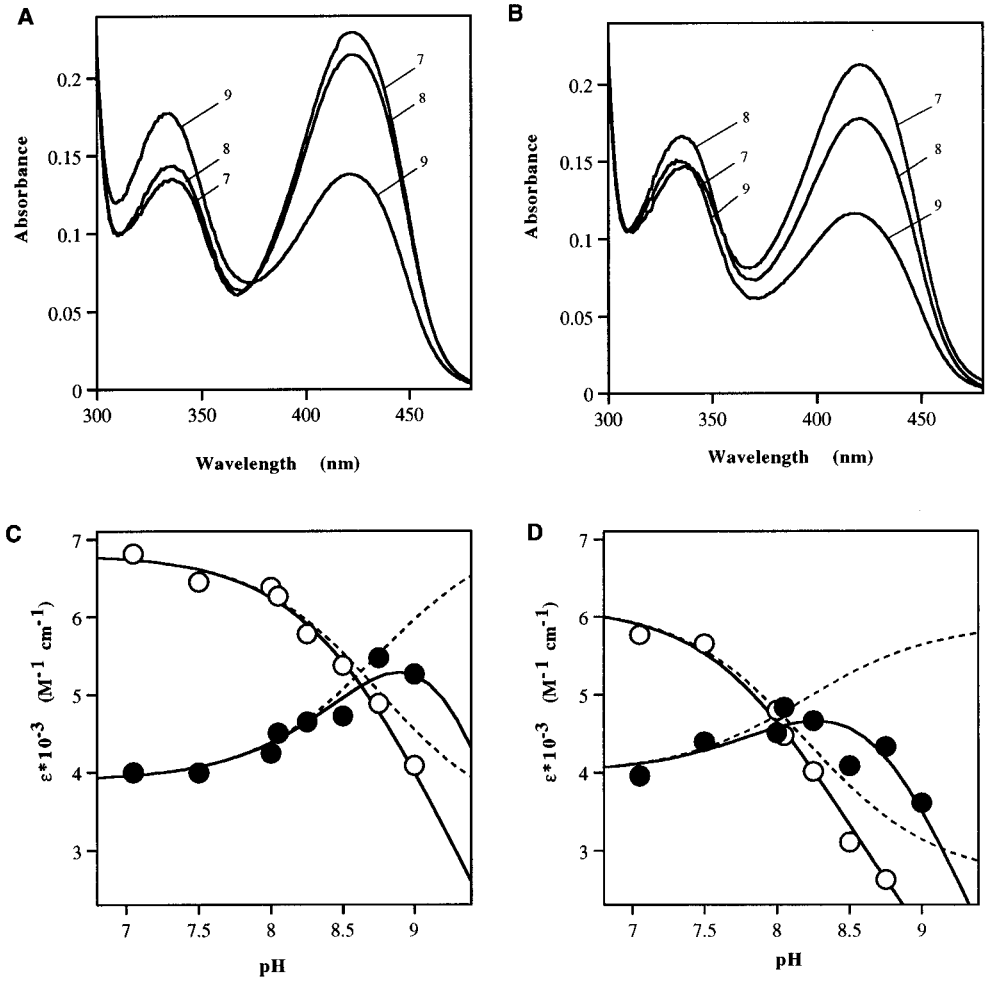


FIGURE 5: UV/vis spectra of wild-type and R277A ODC. Representative spectra at pH 7, 8, and 9 are shown for wild-type ODC (A) and R277A ODC (B). Millimolar absorptivities ($\epsilon^{\lambda nm}$, mM^{-1} cm^{-1}) at two wavelengths, 335 nm (●) and 423 nm (○), for both wild-type ODC (C) and R277A ODC (D) were fit to eq 2 (see Experimental Procedures); the solid lines represent the fit. The calculated pK_a for the ionizing group which influences the absorption spectra is 8.7 ± 0.13 for wild-type ODC and 8.2 ± 0.15 for R277A ODC. The values of the parameters, ϵ_{EH}^{335nm}, ϵ_{EH}^{423nm}, ϵ_{E}^{335nm}, and ϵ_{E}^{423nm}, are 3.9 ± 0.1, 6.8 ± 0.1, 7.1 ± 0.45, and 3.3 ± 0.45 mM^{-1} cm^{-1} for wild-type ODC and 4.0 ± 0.2, 6.15 ± 0.2, 5.9 ± 0.3, and 2.7 ± 0.04 mM^{-1} cm^{-1} for R277A ODC. The dashed lines show theoretical titration curves which would be obtained in the absence of PLP dissociation (simulated using eq 2, where $R = 1$ over the whole pH range).

The effect of this deprotonation on transition-state stabilization is more pronounced for R277A ODC than for wild-type ODC. The transition state is destabilized by 2.7 kcal/mol upon the loss of Arg-277 (measured by comparing $k_{cat}/K_{m\ PLP}$ for wild-type ODC to that for R277A ODC at pH 7.5), by 1.6 kcal/mol by the loss of the proton in wild-type ODC (measured by comparing $k_{cat}/K_{m\ PLP}$ for wild-type ODC at pH 7.5 and 9), and by 7.4 kcal/mol when both interactions are lost (measured by comparing $k_{cat}/K_{m\ PLP}$ for R277A ODC at pH 9 to $k_{cat}/K_{m\ PLP}$ for wild-type ODC at pH 7.5). Deviations from additivity for the energetic contribution of individual interactions have been observed when the sites function cooperatively (Wells, 1990). Thus, these data suggest that interactions between Arg-277 and the 5′-phosphate and interactions involving the protonated group may act cooperatively to bind PLP. The consequences of removing a single interaction are tempered because PLP is anchored to the wild-type ODC active site at multiple points (e.g. Arg-277 and the Gly-rich loop interact with the 5′-phosphate and Glu-274 interacts with the pyridine nitrogen); however, upon losing two interactions, a greater than additive loss in transition-state binding energy occurs.

The low-field resonances observed in the ^{31}P NMR spectra of wild-type and R277A ODC indicate that the phosphate of PLP is bound to the enzyme active site as a dianion. Free PLP displays a pH-dependent ^{31}P chemical shift sensitive to the mono to dianion transition which occurs with a pK_a of 6.3. In contrast, the ^{31}P chemical shift arising from PLP bound to either wild-type or R277A ODC is independent of pH over a range of 6−8, indicating that the pK_a of the mono to dianion transition of bound PLP is lowered by tight interactions with active site residues. Similar results have been reported for many other PLP-dependent enzymes [e.g. tryptophan synthetase (Schnackerz & Bartholmes, 1983), tryptophanase (Schnackerz & Snell, 1983), 4-aminobutyrate aminotransferase (Churchich et al., 1983), and *O*-acetylserine sulfhydrolase (Cook et al., 1992; Schnackerz & Cook, 1995)].

Interestingly, the magnitude of the downfield chemical shift for ODC is larger than that reported for these other PLP-dependent enzymes. The main factor which dictates the magnitude of the phosphate ^{31}P chemical shift is believed to be the smallest of the O−P−O bond angles (Gorenstein, 1975). For PLP free in solution, the measured ^{31}P chemical shift (4.25 ppm for the dianion) is expected to arise from the minimal energy conformations of the phosphate. For ODC-bound PLP, the magnitude of the downfield chemical shift (6.2 ppm for wild-type ODC) is significantly larger than that for free PLP in solution, suggesting that the phosphate

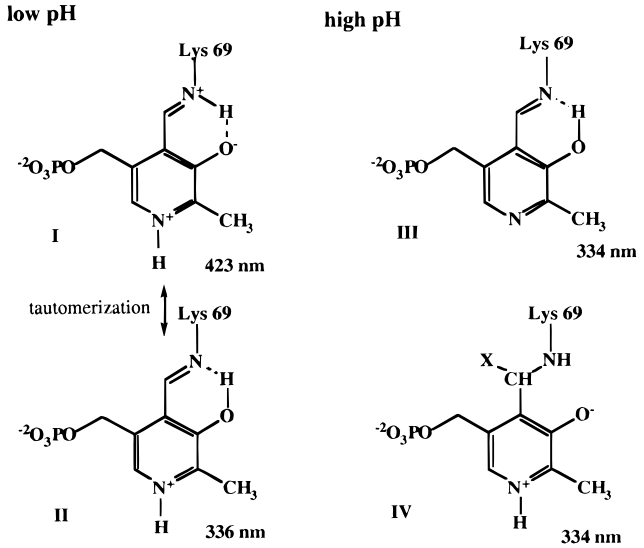


FIGURE 6: Proposed ionization states of PLP tautomers in the active site of ODC at low and high pH. The displayed structures were compiled from the similar structures proposed for tryptophanase (Metzler et al., 1991).

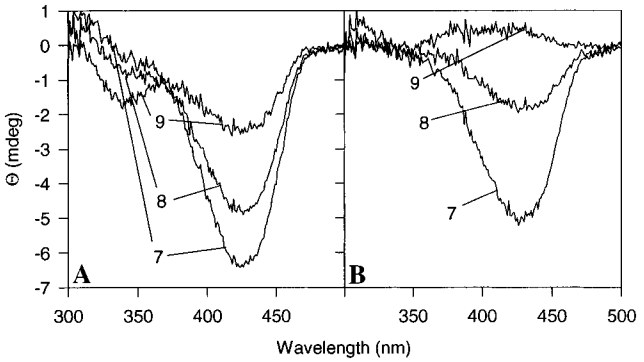


FIGURE 7: CD spectra of wild-type and R277A ODC. Representative spectra at pH 7, 8, and 9 are shown for wild-type ODC (A) and R277A ODC (B). The CD signal was averaged for 5 s at each wavelength and corrected for cuvette strain. Data were collected at 25 μM enzyme and 200 μM PLP in 0.1 M Bis-Tris-Propane-HCl buffer.

is bound to ODC in a strained conformation. For R277A ODC, the finding that the ^{31}P chemical shift (pH <8) is 0.5 ppm downfield of that observed for wild-type ODC suggests that the phosphate is still bound in a rigid conformation to the enzyme but that the O−P−O angles around the phosphorus atom have changed. Similar observations were made to account for ^{31}P chemical shift changes in phosphorylase upon mutating Lys-533 (Schinzel et al., 1992). In the absence of Arg-277, the phosphate may move closer to the remaining structural elements in the ODC active site, altering the position of the phosphate moiety (e.g. the Gly-rich loop). However, it is also possible that the change in chemical shift observed in the mutant enzyme could arize from other local environmental effects such as electrostatics.

While the ^{31}P NMR chemical shifts which arise from the R277A ODC-bound PLP are unaltered in the pH range of 6−8, the 6.7 ppm resonance disappears above pH 8.0 and a new resonance appears 0.2 ppm downfield of the loosely bound PLP resonance. Because the protein concentration in the NMR experiments is much higher than the $K_{d\ PLP}$, PLP is bound to R277A under these conditions. Thus, these observations suggest a model whereby the strained conformation of ODC-bound PLP arises from multiple interactions in the binding pocket. Either the deprotonation event occurring at pH ~8.5 for wild-type ODC or mutation of Arg-277 removes only one interaction and is not sufficient to release the strain. However, two of the interactions are disrupted for the R277A mutant at high pH, resulting in a much less strained conformation and a more solution-like structure for the ODC-bound PLP. This hypothesis is strongly supported by the observation that the CD signal that arises from the ODC-bound cofactor is entirely lost by pH 9 for R277A ODC but not for wild-type ODC. The induced CD signal from enzyme-bound PLP has been proposed to largely originate from out-of-plane twisting of the Schiff base double bond with respect to the pyridine ring of PLP (Judd & Ingraham, 1978). The loss of CD signal at high pH for R277A suggests that the angle between the Schiff base and the ring has relaxed at high pH. Alternate binding modes for PLP were also proposed for mutants of the Gly-rich sequence in aminolevulinate synthase on the basis of changes in the CD spectra (Gong et al., 1996). However, differences in dipole interactions between PLP and the active site of wild-type versus R277A ODC may also contribute to the effects on the CD signal upon loss of the proton.

Finally, analysis of structural features which contribute to the binding of PLP to ODC is complicated because the wild-type enzyme binds PLP tightly and most analogs are not in rapid equilibrium with PLP. Because R277A ODC binds PLP more weakly, it provides a good model for characterizing cofactor analogs. Comparison of the binding affinity of PLP to that of PMP and PDP suggests that the internal aldimine contributes 3 kcal/mol to PLP binding. The internal aldimine was estimated to contribute 4.7 kcal/mol in aspartate aminotransferase (Toney & Kirsch, 1991) and 2.8 kcal/mol in tyrosine aminotransferase (Voltattorni et al., 1975). The contribution of the external aldimine to binding of the substrate/cofactor complex is similarly estimated to be ~3 kcal/mol by comparing the K_I for binding of the bisubstrate analog PPP to the K_I for PMP or PDP. These data suggest that breakdown of the internal aldimine is paid for by formation of the external aldimine. For R277A ODC, the $K_{d\ PLP}$ and K_I for PPP are nearly identical, further supporting this conclusion. A similar result was reported for the relative binding affinity of *N*-(5′-phosphopyridoxal)-tyrosine and PLP to tyrosine aminotransferase (Borri-Voltattorni et al., 1975). These data suggest that amino acids which cannot bind to ODC [e.g. 2,4-diaminobutyruic acid (Osterman et al., 1995a)] are likely to be excluded from the active site because they cannot compensate for the loss of binding energy upon breaking the internal aldimine.

In conclusion, PLP is bound to the active site through a network of interactions which maintain a strained conformation that is conducive to catalysis. This conformation can be distinguished from the solution conformation by the ^{31}P chemical shift and by the induced CD spectra. The 5′-phosphate of PLP is bound as a dianion and interacts with Arg-277, but in addition, it also interacts with other structural elements in the active site that contribute at least equal binding energy to that of Arg-277. As previously reported, the pyridine nitrogen interacts with Glu-274 (Osterman et al., 1995a). The loss of activity and change in spectral properties which occur at high pH are consistent with a loss of this interaction upon deprotonation (pK_a = 8.5). The internal aldimine with Lys-69, or an energetically equivalent external aldimine to substrate, also contributes to the

energetics of PLP binding. Thus, the numerous contacts made between the enzyme and PLP provide plasticity to the system such that it is able to tolerate the loss of a single interaction without catastrophic consequences. However, the loss of two interactions, as occurs for the R277A mutant at high pH, greatly alters the conformation and the catalytic properties of the ODC-bound cofactor.

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