The Glycine-rich Region of \textit{Escherichia coli} d-Serine Dehydratase

ALTED INTERACTIONS WITH PYRIDOXAL 5'-PHOSPHATE PRODUCED BY SUBSTITUTION OF ASPARTIC ACID FOR GLYCINE*

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Replacement of glycine by aspartic acid at either of two sites in a conserved, glycine-rich region inactivates the pyridoxal 5'-phosphate-dependent enzyme d-serine dehydratase (DSD) from \textit{Escherichia coli}. To investigate why aspartic acid at position 279 or 281 causes a loss of activity, we measured the affinity of the G→D variants for pyridoxal 5'-phosphate and a cofactor:substrate analog complex and compared the UV, CD, and fluorescence properties of wild-type D-serine dehydratase and its inactive variants.

The two G→D variants DSD(G279D) and DSD(G281D) displayed marked differences from wild-type D-serine dehydratase and from each other with respect to their affinity for pyridoxal 5'-phosphate and for a pyridoxal 5'-phosphate:glycine Schiff base. Compared to the wild-type enzyme, the cofactor affinity of DSD(G279D) and DSD(G281D) was increased 225- and 50-fold, respectively, and the ability to retain a cofactor:glycine complex was decreased 765- and 1970-fold. The spectral properties of the inactive variants suggest that they form a Schiff base linkage with pyridoxal 5'-phosphate but do not hold the cofactor in a catalytically competent orientation. Moreover, the amount of cofactor aldaminium in equilibrium with cofactor Schiff base is increased in DSD(G279D) and DSD(G281D) relative to that in wild-type DSD. Collectively, our findings indicate that introduction of a carboxyl methyl side chain at G-279 or G-281 directly or indirectly disrupts catalytically essential protein-cofactor and protein-substrate interactions and thereby prevents processing of the enzyme-bound cofactor:substrate complex. The conserved glycine-rich region is thus either an integral part of the d-serine dehydratase active site or conformationally linked to it.

The pyridoxal 5'-phosphate (PLP) dependent enzyme d-serine dehydratase from \textit{Escherichia coli} loses the ability to convert d-serine (a growth inhibitor) to pyruvate and ammonia upon substitution of aspartic acid for glycine at either position 279 or 281. G-279 and G-281 lie in an unusually glycine-rich region that is highly conserved in several PLP enzymes that catalyze α,β-eliminations and well-removed from the active site lysyl residue (K-118) in the primary structure of DSD (1). These observations, together with X-ray crystallographic studies that indicate the presence of glycine-rich sequences at the PLP-binding site of glycogen phosphorylase (2-4) and the phosphoryl-binding sites of many nucleotide-binding proteins (5-13), prompted us to suggest that the glycine-rich region of DSD may constitute part of the PLP-binding site (1). The earliest chemical transformation in the catalytic pathway for DSD is a transamination reaction whereby d-serine displaces the ε-amino group of K-118 from the cofactor Schiff base linkage. Glycine and certain other amino acids behave as inhibitory substrate analogs by transaminating the Schiff base linkage between PLP and K-118 to form a PLP:amino acid Schiff base that is not further processed by DSD. Studies of the interactions between substrate analogs and DSD have thus provided useful information about the initial steps in DSD catalysis (14). Transamination requires proper orientation of the Schiff base linkage between PLP and K-118 for nucleophilic attack by an amino acid substrate or substrate analog and reorientation of the DSD-PLP-amino acid gem diamine intermediate to ensure elimination of K-118 rather than the substrate (14, 15). Moreover, the product of transamination (the PLP-substrate Schiff base) must remain bound to the active site to permit further processing of the substrate. To gain further information regarding the manner in which the G→D replacements prevent catalysis, we compared wild-type DSD, DSD(G279D) and DSD(G281D) with respect to their interactions with cofactor and their ability to form an enzyme-bound Schiff base between PLP and glycine. Our findings indicate that the presence of an asparatyl residue at position 279 or 281 alters covalent and noncovalent interactions between cofactor and protein in a manner that impairs PLP binding and prevents productive interaction with amino acid substrates.

\textbf{MATERIALS AND METHODS}

\textbf{Chemicals}

DTT was ordered from Behring Diagnostics. 2-Hydroxyethylamine and guanidine hydrochloride were purchased from Aldrich. d-Serine and glycine were ordered from Sigma. All other reagents were analytical grade. Buffers for UV, CD, and fluorescence measurements were filtered through 0.22-μm Millex-GS filter units (Millipore Corporation, Bedford, MA) prior to use.
**General Considerations**

Purification of DSD and DSD variants, assays of enzyme activity, separation of free and enzyme-bound PLP by the spun column method of Freyssex (16), and determination of the total protein content of enzyme samples in 6 M guanidine hydrochloride, 0.5 M EA, 0.1 M KH$_2$PO$_4$, pH 7.8 (quenching buffer), have been described (1). Sphadex G-50 (Pharmacia LKB Biotechnology Inc.) used in the preparation of 1-ml centrifuged columns was pre-equilibrated in Buffer A (0.1 M KH$_2$PO$_4$, 19 mM EDTA, 5 mM DTT, pH 7.8). Absorption spectra (520–270 nm) were recorded on a Cary 219 spectrophotometer at 25 °C.

**Determination of $K_p$ and $K_{PS}$, the Equilibrium Constants for Dissociation of PLP and PLP:GLY from DSD**

**Preparation of Incubation Mixtures**—Since DSD binds PLP very tightly, direct evaluation of the equilibrium constant ($K_p$) for dissociation of PLP from DSD is difficult. $K_p$ was thus determined by perturbing the DSD:PLP equilibrium with EA, an amine that competes with DSD for cofactor by forming a Schiff base with PLP in the enzyme. Four ways of dissociation of PLP from DSD is difficult. $K_p$ was thus determined by perturbing the DSD:PLP equilibrium with EA, an amine that competes with DSD for cofactor by forming a Schiff base with PLP in the enzyme (empirically determined as 1 M EA in Buffer A for wild-type DSD and 0.01–0.05 M EA for DSD variants).

**Separation of Free and Enzyme-bound PLP in Equilibrium Mixtures**—Following incubation at room temperature for 2–3 h (empirically determined as the time required to reach a constant value of [PLP]/[E], as described under Calculations of $K_p$ and $K_{PS}$), free and enzyme-bound PLP were determined by the spun column method (16) or by ultrafiltration as follows. An aliquot of each incubation mixture was loaded on top of a dry Centricon-10 microconcentrator (Amicon) and centrifuged at 5,000 g at room temperature for 18 h (t$_{th}$ for equilibration across the dialysis membrane was approximately 1.5 h) using a plexiglass chamber equipped with 0.3-ml microdialysis cells (Technilabs, Fiscaletawny, N.J.). $K_p$ or $K_{PS}$ was determined as described in the next section.

**Calculations of $K_p$ and $K_{PS}$**

The equilibrium constants for dissociation of PLP ($K_p$) and PLP:GLY ($K_{PS}$) from DSD, DSD(G279D) and DSD(G281D) were determined from the equilibrium constant of PLP bound to enzyme ([PLP]$_b$), unbound PLP ([PLP]$_u$), and the total enzyme concentration ([E]). For each enzyme, [PLP]$_b$, and [PLP]$_u$ were obtained by one or more of the three methods just described to determine free and enzyme-bound PLP. (i) In the spun column method (16), the PLP and enzyme concentrations in the eluent yielded an estimate of $r$, the ratio of [PLP]$_b$ to [E]. The product of $r$ and [E], yielded [PLP]$_u$, and the difference between the total concentration of PLP in the equilibrium mixture ([PLP]$_b$) and [PLP]$_u$ was obtained by one or more of the three methods just described to determine free and enzyme-bound PLP. (ii) In the spun column method, the concentration of free PLP in the incubation mixture ([PLP]$_u$) was obtained at less than 10% of the volume of the equilibrium mixture was taken as [PLP]$_u$. The difference between [PLP]$_b$, [PLP]$_u$, and [PLP]$_u$ gave [PLP]$_b$. [E]$_b$, and [PLP]$_u$, were the average of values determined for the retentate before and after ultrafiltration. (iii) For determination of [PLP]$_u$, and [PLP]$_b$, following equilibrium dialysis, [PLP]$_u$ was taken as the equilibrium concentration of PLP in the chamber lacking enzyme, and [PLP]$_b$ was determined from the difference in PLP concentration in the chambers with and without enzyme.

Experimental values of [PLP]$_b$ and [PLP]$_u$ were corrected for nonspecific binding of excess PLP to ϵ-amino groups on the surface of DSD and for the binding of excess PLP to other components of the incubation mixture (i.e., DTT, EA, or GLY (17)). These PLP solution complexes may all theoretically bind to the active site, PLP that is not bound to DSD at equilibrium (either specifically or nonspecifically) is the sum of free, uncomplexed PLP ([PLP]$_u$) and the various PLP solution complexes:

$$[\text{PLP}]_{u} = [\text{PLP}] + [\text{PLP:DTT}] + [\text{PLP:EA}] + [\text{PLP:GLY}]$$ (1)

It follows that

$$[\text{PLP}]_b = [\text{PLP}](1 + \Sigma[X]/K_p)$$ (2)

where $X$ represents the concentration of the PLP-binding species in the incubation mixture and $K_p$ represents the equilibrium constant for dissociation of the PLP complex in solution (PLP:$X$ → PLP + $X$). In our experiments:

$$\Sigma[X]/K_p = [G]/K_G + [D]/K_D + [E]/K_E$$ (3)

where $[G]$, $[D]$, and $[E]$ represent the concentrations of glycine, DTT and EA, respectively. The values of $K_p$, were determined spectrophotometrically by previously described methods (17). For analysis of the resolution of G → D variants, $K_D$ (7.0 mM) and $K_E$ (2.4 mM) were determined in 0.1 M KH$_2$PO$_4$, pH 7.8, at room temperature. For analysis of the resolution of wild-type DSD, $K_D$ (6.0 mM) and $K_E$ (3.0 mM) were determined in 0.1 M KH$_2$PO$_4$, 1.0 M KCl, pH 7.8. One M KCl was added to the buffer in the case of the wild type to approximate the higher ionic strength (i.e. higher concentration of 2-hydroxyethylammonium ion) of solutions used to resolve wild-type DSD. A value of 12.7 mM for $K_D$ (determined in 0.1 M KH$_2$PO$_4$, pH 7.8) was used in all analyses. The observed value of $r$ ([PLP]/[E],) was corrected for nonspecific binding of PLP to ϵ-lysyl groups other than K-118 by assuming all 19 nonactive site lysyl residues of DSD were available for Schiff base formation (although this may not be the actual case, the assumption allows an upper limit determination of $K_p$). PLP specifically bound to the active site ($r' \times [E]$) is equivalent to the experimentally determined value of total enzyme-bound PLP ($r' \times [E]$) minus the concentration of PLP bound to ϵ-lysyl groups other than K-118:

$$r' = r - 19/[1 + K_{PS}][\text{PLP}]_u$$ (4)

where $K_{PS}$ is the average equilibrium constant for dissociation of a PLP Schiff base of a nonactive site DSD lysyl residue. A value of 4.4 mM was determined for $K_{NS}$ of the model Schiff base formed between PLP and ϵ-aminocaproic acid (0.1 mM KH$_2$PO$_4$, pH 7.8) and used in equation 4 (17).

If the complexes that PLP forms with DTT, EA, and glycine can bind to the active site of DSD (in addition to uncomplexed PLP), then the total concentration of PLP bound to the active site represents the sum of these species:

$$r' \times [E] = [\text{DSD:PLP}] + [\text{DSD:PLP:EA}] + [\text{DSD:PLP:DTT}] + [\text{DSD:PLP:GLY}]$$ (5)

If the equilibrium constant for dissociation of a PLP complex from the active site (DSD:PLP:$X$ → DSD + PLP:$X$) is denoted by $K_{PS}$, where $X$ is DTT, EA, or glycine and $K_p$ is again the equilibrium constant for dissociation of the PLP complex in solution, then equation (5) can be rewritten as

$$r' = [\text{PLP}]/[K_p + \Sigma[X]/K_p]/[1 + [\text{PLP}]/[K_p] + \Sigma[X]/K_p]$$ (6)

2 DTT serves to prevent the oxidation of DSD thiol groups and does not resolve wild-type DSD at the 5 mM concentration of DTT used in the incubation buffer, since $K_D >> K_p$. After determining that the $K_p$ values for the DSD variants were much greater than $K_p$ for the wild type (such that $K_D$ was no longer insignificant with respect to $K_p$), it became necessary to correct the $K_p$ values for the small amount of resolution of DSD(G279D) and DSD(G281D) that occurs due to the presence of 5 mM DTT in the buffer.

3 The effect of variation in the values of $K_D$ and $K_{NS}$ with ionic strength was negligible.

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Equation 8 leads to the relationship

\[ r'/(1 - r')([PLP]_f - 2([X]/K_{Na})) = 1/K_p \]  
(7)

where \((1 - r')\) is the fraction of \([E]\) present as apoenzyme.

In experiments where glycine was absent, we observed that the first term in Equation 7 was independent of both EA and DTT concentration (Table I). This finding strongly suggests that under our experimental conditions, the solution complexes PLP:DTT and PLP:EA do not bind to DSD (i.e., the second term of Equation 7 is negligible). Equation 7 was thus simplified for determinations of \(K_p\) (in the absence of glycine):

\[ K_p = ([PLP]_f - [PLP]_t)/[E] \]  
(8)

The values of \(K_p\) listed in Table I were obtained using Equation 8. In the presence of glycine, however, the experimentally measured first term in Equation 7 was dependent on the glycine concentration, indicating that PLP:GLY (unlike PLP:EA) bound to DSD. The average \(K_p\) values (Table I) together with measurements of [PLP]_f in the presence of glycine were used in Equation 7 to calculate the equilibrium constant \((K_p')\) for dissociation of PLP:GLY from DSD for each enzyme (Table II).

**UV Spectra**

Enzymes (100–250 mM) were dialyzed against 0.1 M KH_2PO_4, 0.1 mM DTT, 1 mM EDTA, 0.3 mM PLP, pH 7.8, and then centrifuged through successively spun column (equilibrated in buffer without PLP) to remove excess cofactor. An aliquot was removed for determination of [E]_t and r ([PLP]_f/[E]) prior to record the spectrum from 270–500 nm (25°C). For the data plotted in Fig. 1, the values of [E]_t and r were: WT-DSD, 0.100 mM r = 1.18; DSD(G279D), 0.211 mM, r = 0.97; DSD(G281D), 0.250 mM, r = 0.39. Spectra were normalized for PLP concentration by dividing the observed absorbance (A) by the millimolar concentration of PLP in the cuvet ([PLP]_f). Spectra were also corrected for the contribution to the absorbance of free PLP (resulting from dissociation in the assay cuvet) and PLP nonspecifically bound to lysyl residues other than K-118. The millimolar absorbivity of active site bound PLP (\(\epsilon_{PLP}\)) was calculated from the observed absorbance (A) using the relationship

\[ \epsilon_{PLP} = \epsilon/([PLP]_f - [PLP]_t)/[E] \]  
(9)

where the millimolar absorbivities of free PLP (\(\epsilon_{PLP}\)) and nonspecifically bound PLP (\(\epsilon_{PLP}\)) were obtained from spectra PLP and PLP complexed to e-aminocaproic acid. The fraction of PLP present as free PLP \(\epsilon_{PLP}\), nonspecifically bound PLP \(\epsilon_{PLP}\), or active site bound PLP \(\epsilon_{PLP}\), was calculated by iterative solution of Equation 10 for [PLP]_f and substitution of the solution in Equation 11 and 12 along with the average values of \(K_p\) listed in Tables I and the value of 4.4 mM previously reported for \(K_{Na}\) (17).

**CD Spectra**

Wild-type DSD, DSD(G279D), and DSD(G281D) were dialyzed for 18 h at 4°C against two changes (1 liter each) of 20 mM KH_2PO_4, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 250 mM PLP, pH 7.8, and then for 3.5 h against buffer lacking EDTA and DTT. After removing an aliquot of the sample for determination of ([E]_t)/([PLP]_f), the CD concentration was adjusted to 75 mM for 300–305 nm spectra or to 5.0 mM for 305–305 nm spectra. CD spectra were recorded on a Jasco model 40C spectropolarimeter at 17°C using a cuvette of path length, 1 (1 cm for 300–305 nm or 0.2 cm for 305–305 nm spectra). The observed rotation in degrees \(\theta_{222}\) was converted either to molecular circular dichroism \((\Delta E = \theta_{222}/3300\text{c}cm^{-1}\text{dmol}^{-1}\text{mol}^{-1})\) or to millimolar ellipticity \((305–305 \text{nm spectra})\). Molecular circular dichroism \((\Delta E = \theta_{222}/3300\text{c}cm^{-1}\text{dmol}^{-1}\text{mol}^{-1})\) was calculated from the expression \(\Delta E = \theta_{222}/3300\text{c}cm^{-1}\text{dmol}^{-1}\text{mol}^{-1}\) where \(c\) is the molar concentration of protein \((75 \times 10^{-3} \text{M})\). Millimolar ellipticity in degree-cm²/dnmol \((\Delta E)\) was calculated from the expression \(\theta_{222}/3300\text{c}cm^{-1}\text{dmol}^{-1}\text{mol}^{-1}\).

**RESULTS**

**Kp and Kp’ Determinations—Equilibrium constants for dissociation of PLP (Kp) and PLP:GLY (Kp’).**

The values of \(K_p\) and \(K_p’\) determined by the centrifuge column procedure, ultrafiltration, and equilibrium dialysis corresponded within experimental error. The spun column method proved to be the most convenient and was used to determine most of the values listed in Tables I and II. The close agreement of values obtained by the three different methods suggests that centrifugation of incubation mixtures through the spun columns did not perturb the equilibrium established during incubation.

The value of the equilibrium constant for dissociation of PLP from wild-type DSD in Table I (120 nM) is higher than the values of 7–35 nm reported in earlier studies (18, 19). The increased ionic strength and medium effects from the buffer used to resolve wild-type DSD (due to the presence of 0.5–1.0 M 2-hydroxyethylammonium chloride) may be responsible in part for the increased dissociation constants. Examination of the data in Table I reveals that the inactive variants buffer to minimize cofactor loss from DSD variants. DSD samples for fluorescence spectra in the presence of 0.5 mM glycine consisted of 90 µl of enzyme solution (final enzyme concentration 2.4 mM), 450 µl of buffer, and 500 µl of 1.0 mM glycine (preincubated in buffer and adjusted to pH 7.8 with potassium phosphate). The contribution of the sample buffer to fluorescence was determined from the spectra of 80 µl PLP and 0.5 M glycine in 0.1 M KH_2PO_4, pH 7.8.

The equilibrium constant \((K_{p'})\) for dissociation of glycine from DSD was determined from the enhancement of DSD fluorescence (400 nm excitation, 470 nm emission) by glycine. The dependence of DSD fluorescence on glycine concentration was analyzed in terms of Equation 13.

\[ 1/(F - F_0) = 1/(F_M - F_0) + K_{p'}/([G] - (F_0)/G) \]  
(13)

where \(F_0\) and \(F_M\) are the measured values of fluorescence in the absence of glycine, a concentration of glycine \((\text{[G]}\)), and at saturating glycine, respectively. The value of \(K_{p'}\) was estimated from the ratio of the slope to the intercept of a linear plot (r = 1.00) of 1/(F – F_0) versus 1/[G]. The use of Equation 13 to determine \(K_{p'}\) assumes that dissociation of PLP and PLP:GLY from DSD is negligible under the conditions used for the titrations.

**Fluorescence Spectra**

Fluorescence emission and excitation spectra were recorded at 25°C on a spectrophotometer built by Dr. David Ballou and Gordon Ford of the Department of Biological Chemistry. Emission spectra (400–650 nm) were obtained using an excitation wavelength of 400 nm. Excitation spectra (250–500 nm) were obtained using 490 nm as the emission wavelength. DSD samples consisted of 100 µl of a 48 mM solution of wild-type DSD, 0.5 mM PLP, and 0.1 M KH_2PO_4, 80 µM PLP, pH 7.8, diluted into 900 µl of the same buffer (final enzyme concentration 4.8 µM). Excess PLP was included in the
DSD(G279D) and DSD(G281D) bind PLP 225- and 50-fold less tightly than does wild-type DSD. It is important to note that the binding of PLP to the weaker binding variant is still ~160-fold tighter than the binding of PLP to free lysine or \( \epsilon \)-aminoacrylamic acid in solution (17), suggesting that the variants retain some of the features of a functional PLP-binding site. Comparison of the values of \( K_d \) and \( K_{D} \) (Table II) indicate that the affinity of wild-type DSD for PLP is reduced only 2.8-fold when the active site bound cofactor exists in a Schiff base linkage with glycine despite the fact that the covalent interaction between K-118 and PLP has been broken. In contrast, DSD(G279D) and DSD(G281D) disfavor the binding of PLP/GLY relative to free cofactor by 9.6- and 112-fold, respectively, indicating that the \( G \rightarrow D \) replacements diminish the ability of DSD to retain a transamidation complex even more than they lower the affinity of the enzyme for uncomplexed PLP. Relative to wild-type enzyme, the affinity of both \( G \rightarrow D \) variants for PLP/GLY was decreased markedly, with the ratio \( K_{D} \) (variant)/\( K_{D} \) (wild type) 765 for DSD(G279D) and 1970 for DSD(G281D).

**UV Spectra**—Figs. 1 and 2 compare the UV absorption spectra of wild-type DSD, DSD(G279D), and DSD(G281D) with those of free PLP, PLP-\( \epsilon \)-aminoacrylamic acid and PLP:EA. The 388 nm absorption maximum of free PLP (Fig. 1, curve C) reflects the predominant species in solution at neutral pH, a zwitterion (\( \epsilon \)/OH unprotonated, N1 protonated) with C4’ in the aldehyde form (20, 21). About 20% of the cofactor exists in solution with the formyl group hydrated, an equilibrium reflected in the 330 nm shoulder of the spectrum (20). The absorption maximum of PLP shifts to ~415 nm upon Schiff base formation with \( \epsilon \)-aminoacrylamic acid (Fig. 1, curve A), EA (Fig. 1, curve B), or wild-type DSD (Fig. 1, curve D, and Refs. 22 and 23). The Schiff base formed between \( \epsilon \)-aminoacrylamic acid and PLP was included as a model for the Schiff base linkage between PLP and K-118 of DSD, since \( \epsilon \)-aminoacrylamic acid resembles lysine in carbon chain length and \( pK_a \) of the free amino group (17). The small shoulder in the 350 nm region of the PLP:EA spectrum (Fig. 1, curve B) may represent a small amount of cyclic alamine resulting from addition of the 2-hydroxyl group of EA to the imine bond.

The pronounced 415 nm absorbance maximum resulting from the alamine linkage between PLP and DSD (Fig. 1, curve D and Fig. 2, curve A) is typical of PLP-dependent holoenzymes (22, 24–29). The small shoulder around 325 nm probably represents one or more species of enzyme bound
PLP that contain $sp^3$ hybridization at C4' rather than an $sp^2$ aldimine linkage (see “Discussion”). The absorption spectra of DSD(G279D) and DSD(G281D) (Fig. 2, curves B and C, respectively), display considerably less Schiff base absorbance ($A_{415}$) than the wild-type enzyme as well as a pronounced increase in 325 nm absorbance characteristic of $sp^2$ hybridized PLP complexes. As noted earlier, the observed spectra (inset, Fig. 2) have been normalized for PLP concentration by plotting millimolar absorptivity (the ratio of observed absorbance to total PLP concentration) and corrected for the small amount of PLP dissociation that may have occurred in the assay cuvette, thus the decrease in 415 nm absorbance (main panel, Fig. 2) cannot reflect a simple loss of cofactor. Moreover, the spectra of DSD(G279D) and DSD(G281D) differ markedly from that of free PLP (compare Fig. 2, curves B and C with Fig. 1, curve C).

Fluorescence Spectra of DSD and DSD Variants—The fluorescence spectra of the three enzymes in the absence and presence of 0.5 mM glycine are given in Fig. 3. The wild-type enzyme is weakly fluorescent with an excitation maximum at 425 nm corresponding to the absorbance maximum of the internal Schiff base and an emission maximum around 500 nm (14, 30). This intrinsic fluorescence increases markedly and shifts to slightly lower wavelengths upon transfer of the aldimine linkage to an incoming amino acid, a phenomenon attributed to a significant conformational change at the active site upon transaldimination (14). In contrast, DSD(G279D) displayed $\sim 70\%$ less intrinsic fluorescence than wild-type DSD, and DSD(G281D) displayed virtually no intrinsic fluorescence. The addition of 0.5 mM glycine induced no measurable fluorescence enhancement in the emission or excitation spectrum of either variant. The lack of fluorescence enhancement of DSD(G281D) may be due in part to its inability to retain PLP:GLY. The $K_F$ and $K_{FG}$ values of this variant predict that only $\sim 9\%$ of DSD(G281D) should have been present as DSD-PLP:GLY, despite the 33-fold molar excess of PLP used in the fluorescence assays. A larger proportion of DSD(G279D) should have existed as a DSD-PLP:GLY transaldimination complex under the assay conditions ($\sim 22\%$). The observation that this variant did not exhibit a fluorescence enhancement comparable to $\sim 22\%$ of wild-type DSD (where $>86\%$ of the enzyme exists as DSD-PLP:GLY) strongly suggests that the orientation of PLP:GLY in DSD(G279D) is different from that in wild-type DSD.

The discrepancy between the two values may reflect differences in the ionic strength used for the two determinations.
The experimentally determined values of \( K_{G'} / K_G \) and \( K_{P'} / K_P \) were 2 (i.e., 12 mM/6 mM) and 2.8 (i.e., 0.34 \( \mu \)M/0.12 \( \mu \)M), respectively. The discrepancy between these ratios may be due to experimental error and/or medium effects from the high concentration of 2-hydroxyethylammonium ion used in the determination of \( K_{P'} / K_P \). The lack of glycine induced fluorescence enhancement prevented direct measurement of \( K_{P'} / K_P \), the ratio of slope/intercept for at least squares fit of the plotted data to Equation 13.

\[ \frac{1}{[\text{Glycine}]} = \frac{1}{[\text{Glycine}]}_{\text{equilibrium}} + \frac{1}{K_{P'}} \times \frac{1}{[\text{Glycine}]} \]

Equation 13.

\[ \frac{1}{[\text{Glycine}]} = \frac{1}{[\text{Glycine}]}_{\text{equilibrium}} + \frac{1}{K_{P'}} \times \frac{1}{[\text{Glycine}]} \]

The values of \( K_{G'} / K_G \) and \( K_{P'} / K_P \) were determined indirectly from the measured values of \( K_G \), \( K_P \), and \( K_{G'} \). The values of \( K_G = 60 \text{ mM} \) and \( K_P = 600 \text{ mM} \) for DSD(G279D) and DSD(G281D) relative to a \( K_{G'} \) value of \( 12 \text{ mM} \) observed for wild-type DSD indicate that G \( \rightarrow \) D replacement at position 279 or 281 inhibits transimination.

**CD Spectra**—The CD spectra of wild-type DSD, DSD(G279D), and DSD(G281D) did not differ significantly in the far UV region (Fig. 5), consistent with our earlier suggestion that the inactivity of G \( \rightarrow \) D variants is not due to gross changes in secondary structure (1). We note that amide ellipticity may be insensitive to subtle changes in secondary structure or small regional conformational changes, however (31, 32). In the visible region (Fig. 6), wild-type DSD shows the strong positive CD maximum characteristic of many PLP dependent enzymes (24, 25, 27–29), with the maximum rotation at 415 nm corresponding to the absorption maximum of aldimine-bound PLP. In contrast, DSD(G279D) and DSD(G281D) show almost no measurable CD at 415 nm.

CD spectra were recorded under conditions of excess PLP (3.3 to 50 \( \times \) molar excess for the visible and far UV spectra, respectively) to minimize cofactor loss from G \( \rightarrow \) D variants. Excess PLP in the buffer should not have affected the CD measurements, however, since free PLP is symmetrical and thus optically inactive. Additionally, in control experiments where the concentration of excess PLP in a sample of 100 \( \mu \)M wild-type DSD was increased from 1 to 250 \( \mu \)M so as to increase the concentration of nonspecifically bound PLP, the CD spectrum was not affected. Balk et al. (24) have similarly found that nonspecific binding of PLP to \( \varepsilon \)-lysyl groups outside the active center did not contribute to the visible CD spectrum of tryptophan synthetase.

**DISCUSSION**

Our findings suggest that the presence of a carboxymethyl side chain at position 279 or 281 alters both covalent and noncovalent interactions required for proper function of the...
DSD active site. The nature of the covalent linkage between PLP and DSD may be inferred from the UV spectra (Figs. 1 and 2) and an extensive body of literature regarding the spectral properties of PLP and PLP derivatives (17, 18, 20, 21, 28, 34-39). Absorbance maxima around 330 nm have frequently been correlated with sp² hybridization at C4' of PLP. Buell and Hansen (36) and Tobias and Kallen (34) have shown, for example, that seminal diamines and aldimines formed upon reaction of PLP with aminothiols in aqueous medium at neutral pH have absorbance maxima around 335 nm. Derivatives of enzyme-bound PLP with sp² hybridization at C4' display similar absorption characteristics. For example, treatment of DSD or other PLP enzymes with sodium borohydride reduces the sp²-hybridized Schiff base of a secondary amine, with a simultaneous shift in the visible spectrum from ~420 to ~330 nm and loss of catalytic competence (22, 29, 40, 41). An inactive derivative of cytosolic aspartate aminotransferase absorbing at 340 nm has been attributed to an aldamine derivative of PLP linked to group "X" of the protein (26).

And PLP bound at the active site of tryptophanase normally displays both 330 and 410 nm absorbance maxima, the former being attributed to an internal aldime (28).

The 325 nm absorbance observed in DSD(G279D) and DSD(G281D), and to a lesser extent in the wild-type enzyme (Fig. 2), may represent a carbinolamine formed by the addition of water to the internal Schiff base, an aldime wherein C4' of the cofactor is bonded to a nucleophilic group X on the protein, or a mixture of these species (Scheme I). The small 325 nm shoulder in the UV spectrum of wild-type DSD may or may not represent the same sp² species or combination of species as the one(s) present in DSD(G279D) and DSD(G281D). The possibilities for group X on the protein include the ε-amino group of lysine, a ring carbon of histidine, the —OH of serine or threonine, the —SH of cysteine, or the carboxyl group of glutamate or aspartate.

Although the existence of PLP hydrates in aqueous solution is well established (20, 21), there is a lack of consensus regarding the amount of carbinolamine in equilibrium with PLP Schiff bases. Both NMR and structure-stability correlations have argued for an and against the presence of substantial equilibrium levels of carbinolamines (34, 39, 42-44). The observation that the PLP:N-acetylaspartic acid complex shows no shoulder in the 330 nm region (Fig. 1) suggests that little carbinolamine is present in aqueous solutions of the model Schiff base. A carbinolamine might be selectively stabilized at the active site of the enzyme, however. Thus, we cannot exclude the possibility that all or part of the increased 330 nm absorbance in the UV spectra of the G → D variants reflects a more favorable equilibrium constant for the addition of water to the azomethine bond. Since the enzyme may similarly perturb the equilibrium constant for addition of any nucleophilic amino acid side chain to the DSD:PLP imine bond, identification of the enzyme-linked nucleophile X from the propensity of X to add to model Schiff bases is difficult.

It is tempting to speculate that X is a thiolate anion, however. The rapid, reversible reaction of thiol groups with model or enzyme-bound Schiff bases has been well demonstrated (17, 18, 35, 36, 38), and Buell and Hansen (36) have shown that a wide variety of PLP complexes with aminothiols display absorbance maxima around 330 nm at neutral pH. Moreover, Dowhan and Snell (22) have shown that the presence of PLP shields two of the three titratable thiol groups of DSD from reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) and substantially reduces the reactivity of the third. Incubation of apoDSD with 5,5'-dithio-bis(2-nitrobenzoic acid) leads to modification of all three thiol groups and inactivation due to an inability to recombine with cofactor (22). These observations suggest that one or more thiol groups may lie close to the DSD active site. We should note one further possibility for the 330 nm absorbance maximum, namely that insertion of the intact imine linkage into an exceptionally hydrophobic microenvironment might cause the Schiff base itself to absorb around 330 nm (45). Further studies will be required to distinguish between these possibilities.

Comparison of the UV spectra of DSD, DSD(G279D), and DSD(G281D) (Fig. 2) suggests that 20-60% of the PLP bound to the active site of the G → D variants exists in a Schiff base linkage. The observation that the CD of both variants at 415 nm was markedly less than 20-60% that of wild-type DSD suggests that the orientation of the DSD:PLP Schiff base is different in the G → D variants. The altered orientation of bound cofactor together with the reduced affinity of DSD(G279D) and DSD(G281D) for PLP suggests that non-covalent interactions between cofactor and protein have been disrupted in the inactive variants.

The sensitivity of the PLP-binding site of DSD to subtle changes in structure is well demonstrated by studies with a wide variety of PLP analogs (19, 46, 47). Even minor alteration of the pyridine ring substituents severely compromises the ability of the cofactor to bind or else to bind in a catalytically competent manner (19). For example, N-methyl-PLP (where 1-H → 1-CH3) and 3-O-methyl-PLP (where 3-OH → 3-0CH3) do not bind to DSD. Replacement of the 6-H by a methyl group reduces DSD affinity 370-fold and kcat 5-fold.

DSD has 5 cysteiny1 residues and no disulfide bridges (22).

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**Scheme 1.** Formation of a carbinolamine or internal aldmine at the DSD active site.
(19). Substitution of the 2-methyl group by an ethyl group decreases DSD affinity 3-fold and kcat 4-fold (19). The PLP analog containing an isopropyl group at C2 (i.e., 2', 2'-dimethyl PLP) exhibited only a 7-fold decrease in affinity for DSD but was inactive (19). The inability of enzyme bound 2', 2'-dimethyl PLP to support catalysis strongly suggests that the activity of DSD is extremely sensitive to the orientation of the bound cofactor.

The limited tolerance of the active site to structural perturbations of the cofactor strongly implies the reverse is also true, i.e. that small alterations in active site structure might severely compromise the ability of normal cofactor to bind or to bind in a catalytically competent fashion. D-279 or D-281 might perturb the active site directly through unfavorable electrostatic or steric interactions with PLP or indirectly by inducing conformational changes in the vicinity of the active site. Either type of perturbation could weaken noncovalent interactions between cofactor and protein necessary for constraining PLP in a catalytically competent orientation at the active site. The virtual inability of DSD(G281D) to retain methyl PLP to support catalysis strongly suggests that the presence of D at 281 of the conserved glycine-rich region substantially de-

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