Substitution of Pyridoxal 5′-Phosphate in D-Serine Dehydratase from Escherichia coli by Cofactor Analogues Provides Information on Cofactor Binding and Catalysis

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D-Serine dehydratase (DSD) is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the conversion of D-serine to pyruvate and ammonia. Spectral studies of enzyme species where the natural cofactor was substituted by pyridoxal 5′-sulfate (PLS), pyridoxal 5-deoxymethylene phosphonate (PDMP), and pyridoxal 5′-phosphate monomethyl ester (PLPMe) were used to gain insight into the structural basis for binding of cofactor and substrate analogues. PDMP-DSD exhibits 35% of the activity of the native enzyme, whereas PLS-DSD and PLPMe-DSD are catalytically inactive. The emission spectrum of native DSD when excited at 280 nm shows maxima at 335 and 530 nm. The energy transfer band at 530 nm is very likely generated as a result of the proximity of Trp-197 to the protonated internal Schiff base. The cofactor analogue-reconstituted DSD species exhibit emission intensities decreasing from PLS-DSD, to PLPMe-DSD, and PDMP-DSD, when excited at 415 nm. Large increases in fluorescence intensity at 530 (540) nm can be observed for cofactor analogue-reconstituted DSD in the presence of substrate analogues when excited at 415 nm. In the absence and presence of substrate analogues, virtually identical far UV CD spectra were obtained for all DSD species. The visible CD spectra of native DSD, PDMP-DSD, and PLS-DSD exhibit a band centered on the visible absorption maximum with nearly identical intensity. Addition of substrate analogues to native and cofactor analogue-reconstituted DSD species results in most cases in a decrease or elimination of ellipticity. The results are interpreted in terms of local conformational changes and/or changes in the orientation of the bound cofactor (analogue).

EXPERIMENTAL PROCEDURES

Chemicals—PDMP was kindly provided by Dr. O. Saiko (Merck). PLP monomethyl ester, prepared by the method of Pfeuffer et al. (17), was a gift of Dr. J. Ehrlich. Pyridoxal 5′-sulfate was prepared by the method of Kuroda (18). All other chemicals were of highest quality commercially available.

Enzymes—D-Serine dehydratase was purified from Escherichia coli K12 mutant C6 as described by Schlitz and Schnacke (19) or from a wild-type DSD expression plasmid (11). D-Serine apodehydratase was prepared by using the resolution procedure described by Dowhan and Snell (1). The apoenzyme had a residual activity of 1.9 units/mg of protein. The specific activity of PLP-reconstituted dehydratase was 100 units/mg of protein when measured at 25 °C. Reconstitution of apodehydratase with PLP analogues was achieved by incubating apoenzyme with 5-fold excess of the respective cofactor analogue for 1 h at 25 °C in the dark. Excess cofactor analogue was removed by passing the incubation mixture over a Sephadex G-25 (3.4 × 40 cm) or PD10 column equilibrated with 100 mM potassium phosphate buffer, pH 7.8. The specific activity of the PDMP-reconstituted dehydratase was 38 units/mg of protein.

Enzyme Assay—The enzymatic activity of DSD was determined at 25 °C as described by Dowhan and Snell (1). The assay mixture contains...
**Fluorescence and CD Studies on d-Serine Dehydratase**

**Table I**

| Substrate or inhibitors | PLP | PDMP | PLS, KS value | PLPMe, KS value |
|-------------------------|-----|------|---------------|-----------------|
|                         | $K_m$ value | $K_i$ value | $K_S$ value | $K_i$ value | $K_S$ value |
| d-Serine                | 0.35 | 0.40 | 0.85<sup>a</sup> | ND<sup>b</sup> | 0.053 |
| DL-DAP                  | 0.035 | 0.044 | ND |
| l-DAP                   | 0.09 | 0.047 | 0.03 | 0.03 |
| l-Serine                | 3.0 | 5.5 | 2.0 |
| O-Methyl serine         | 70 | 71 | ND |
| Isoserine               | 0.33 | 1.4 | 7.5 |
| Glycine                 | 4.9 | 5.1 | ND |
| l-Alanine               | 17 | 20.1 | 15.6 |

<sup>a</sup> Values are for $K_m$.
<sup>b</sup> ND, not determined.
<sup>c</sup> See Ref. 4.

The absorption spectrum for PDMP-DSD also exhibits a visible maximum at 415 nm with a slightly lower extinction coefficient compared with native DSD. Addition of glycine or DAP results in a decrease of absorbance at the maximum and in the case of DAP a red shift in the $\lambda_{max}$. l-Serine produces a slight decrease in the absorbance and a red shift in the $\lambda_{max}$ at the visible maximum and an increase in the absorbance at 330 nm. The absorption spectrum of PLPMe-DSD has a maximum at 413 nm with an extinction coefficient slightly higher compared with native enzyme. Addition of amino acids results in a decrease in the absorption and/or a red shift in the $\lambda_{max}$ at the maximum. In the case of the PLS-DSD, two maxima are observed at 415 and 330 nm, both with extinction coefficients higher than those for native enzyme. Addition of amino acids results in an increase in the extinction coefficients of both bands. Data are summarized in Table II.

**Fluorescence Spectra of d-Serine Dehydratase in the Presence and Absence of Amino Acids**—Fluorescence emission spectra have been very informative of the environment and orientation of the cofactor in PLP-dependent enzymes (12, 16, 20). The emission spectrum of native DSD when excited at 280 nm exhibits two maxima at 335 and 530 nm, respectively. In addition to the intrinsic tryptophan fluorescence ($\lambda_{max}$ 335 nm) an energy transfer band ($\lambda_{max}$ 530 nm) is generated as a result of the proximity of an enzyme tryptophan residue to the proteo- nized internal Schiff base of the active site PLP (8). The ratio of $F_{335}/F_{530}$ is about 3. Excitation of DSD at 415 nm results in an emission band at 530 nm (8, 10), typical of the emission of Schiff bases of PLP in an aqueous environment at neutral pH (21–23). The excitation spectrum for longer wavelength emission of DSD measured at 510 nm exhibits two bands. The shorter wavelength excitation band is located at 275 nm, whereas the longer wavelength excitation band is located at 400 nm (data not shown). Excitation of apoDSD at 280 nm yields an emission spectrum with a single maximum at 335 nm. Upon addition of PLP to apoDSD at a 1:1 ratio the emission band at 335 nm decreases with the concomitant appearance of the 510 nm emission band. The rates of these processes are identical when compared on a percent basis (data not shown). Furthermore, the rate at which enzymatic activity occurs is very similar if not identical to the rate at which the 510 nm band appears (8).

Excitation of DSD at 280 nm in the presence of l-diaminopropionate, DL-diaminopropionate, glycine, l-serine, and isoserine (10 × $K_i$) shows considerable enhancement of the fluorescence emission at 530 nm, indicating energy transfer from a tryptophanyl residue to the PLP-amino acid Schiff base, con-
emission band ranges from 1.7-fold for isoserine, 7.5-fold for glycine, and DL- and L-diaminopropionate, respectively (Fig. 2). In all cases, the tryptophan emission at 335 nm had the same intensity. Similar results were obtained for D-serine dehydratase in the absence and presence of amino acids. The enhancement of the long wavelength emission band centered on the visible absorption maximum with nearly identical intensity. Although the PLPMe-DSD also gives a fluorescence enhancement was 23-, 17-, and 14-fold for D-serine, DL-diaminopropionate, and glycine, respectively (data not shown). Apparent dissociation constants of cofactor analogue-reconstituted apoDSD were obtained from fluorescence measurements. The data are summarized in Table I.

Circular Dichroism Studies—Both far UV CD and visible cofactor-induced CD spectra have been useful in interpreting overall structural parameters of proteins and changes in the orientation of the cofactor. The far UV CD spectrum of native DSD exhibits a broad negative Cotton effect at 208–225 nm with positive Cotton effect below 200 nm (data not shown). Virtually identical CD spectra are obtained for apoDSD and other DSD species that carry PLP analogues. Data indicate that no gross conformational changes are introduced upon cofactor binding. Addition of reactant and analogues, known to form an external Schiff base based on UV-visible absorption spectra of native DSDs, also gave no change in the far UV CD spectrum (data not shown).

Circular dichroism spectra were also recorded in the visible region, 300–500 nm, for native and cofactor analogue-reconstituted DSD in the absence and presence of amino acids, Fig. 4A–D. In all cases, spectra are normalized to the same enzyme concentration. In the absence of amino acids, the visible CD spectra of native enzyme, PDMP-DSD, and PLS-DSD exhibit a band centered on the visible absorption maximum with nearly identical intensity. Although the PLPMe-DSD also gives a fluorescence band centered on the visible absorption maximum with nearly identical intensity.
maximum around 415 nm, it is about half the intensity of that observed for native DSD. Addition of amino acids to the native enzyme results in a decrease in ellipticity with isoserine giving a spectrum closest to native enzyme alone. Addition of amino acids to PDMP-DSD results in elimination of most of the ellipticity. In the presence of D-serine no change is observed in the CD spectrum of the PLS-DSD, whereas addition of DAP eliminates all ellipticity. Finally, a slight decrease is observed in the ellipticity of the PLPMe-DSD upon addition of DAP, l-serine, or isoserine, whereas glycine eliminates most of the ellipticity.

**DISCUSSION**

D-Serine dehydratase is a member of the b-family of PLP-dependent enzymes (24) and belongs to the fold type II family (25). Other members of this family include tryptophan synthase b subunits and O-acetylserine sulfhydrylase which have been extensively studied with respect to fluorescence and CD spectral properties (12, 16, 26–29) and structure (30–32). Threonine deaminase is also a member of the b-family that has been studied to a more limited extent (33–35). DSD is likely to have the same fold as the other enzymes in the b-family (see below).
and mechanistic similarities given a common α-aminoacylate intermediate. Data for d-serine dehydratase can thus be interpreted using the results of these other enzymes as a frame of reference.

Spectral Probes of PLP Enzymes—In order to interpret data obtained for D-serine dehydratase, it is important to understand generally what each of the spectral probes reflects with respect to the structure of a cofactor-bound PLP-dependent enzyme. Each of the spectral measurements used in these studies probes a different aspect of the bound PLP-cofactor and/or protein structure, and each will be considered below, as a frame of reference.

Ultraviolet-visible absorption spectra are useful in determining the tautomers of the bound PLP that exist in equilibrium at any step that can be accessed along the reaction pathway. Each of the potential intermediates along the reaction pathway has its own spectral signature, as shown in Scheme 1 (36). Note that each of the intermediates may exhibit multiple spectral bands dependent on the tautomeric equilibrium that exists. The tautomeric equilibrium will, of course, be influenced by the structure of the bound cofactor. OASS (37) and TRPS (38) exhibit maxima at 412 nm reflecting the ketoenamine tautomer and finite absorbance at 330 nm, likely reflecting the enolimine tautomer, although other species cannot be ruled out. The OASS external Schiff base with O-acetyl-L-serine absorbs maximally at 418 nm (39), whereas the red extreme (350 nm) indicates a shift in the enolimine/ketoenamine equilibrium (27).

Fluorescence spectra will provide information on the environment around the tryptophans when excited at 280 nm. Upon excitation in the 280–296 nm range tryptophan residues of a protein will emit in the 324–350 nm range (41). The blue extreme (324 nm) indicates protection of the tryptophanyl side chain from aqueous solvent in a hydrophobic environment, whereas the red extreme (350 nm) reveals full exposure of the residue to aqueous solvent. For TRPS (16), OASS (12), and TDA (34), the tryptophan emission occurs at 337, 335, and 342 nm, respectively, suggesting partial exposure of at least one of the residues contained in the individual subunits. All three enzymes also yield a long wavelength emission around 500 nm (which is cofactor-derived) upon excitation at 280 nm. The long wavelength band was suggested to result from delayed fluorescence of the ketoenamine tautomer of the internal aldimine, enhanced by triplet-singlet Förster resonance energy transfer from an enzyme tryptophan to the PLP cofactor (12), and this

FIG. 4. Circular dichroism spectra of native and cofactor analogue-reconstituted d-serine dehydratase in the absence and presence of amino acids. Circular dichroism spectra were obtained for native d-serine dehydratase (A), PDMP-DSD (B), PLPMe-DSD (C), and PLS-DSD (D) in 0.1 M potassium phosphate buffer, pH 7, at 25 °C over the spectral range 300–500. A, spectra were recorded in the absence (1) and in presence of 14 mM isoserine (2), 55 mM l-serine (3), 51 mM glycine (4), and 0.4 mM l-DAP (5). B, spectra were recorded in the absence (1) and in presence of 75 mM l-serine (2), 0.3 mM l-DAP (3), and 13 mM glycine (4). C, spectra were recorded in the absence (1) and in presence of 0.35 mM l-DAP (2), 102 mM l-serine (3), 16 mM isoserine (4), and 116 mM glycine (5). D, spectra were recorded in the absence (1) and in presence of 20 mM d-serine (2) and 0.53 mM l-DAP (3).
was confirmed by phosphorescence (16, 42) and time-resolved fluorescence (28, 43) studies on TRPS and OASS. Förster energy transfer is dependent on the distance between and orientation of donor and acceptor chromophores (44). In fact, TRPS has a single tryptophan, Trp-177 (45), which is 23 Å from the PLP cofactor, oriented properly, and thus is responsible for the energy transfer. OASS has two tryptophan residues per subunit (46), but the fluorescence of one of these, Trp-50, is completely quenched (42), whereas the indole ring of the second, Trp-161, is 23 Å from the second tryptophan residue and is properly oriented for energy transfer (47). TDA also has two tryptophan residues per subunit, Trp-153 and Trp-458 (35). The C-terminal residue, Trp-458, resides in the regulatory domain, which is far removed from the active site, whereas Trp-153 aligns with the same region of sequence containing Trp-177 of TRPS and Trp-161 of OASS. Upon formation of the external aldimine, an enhancement of the long wavelength band is observed for OASS (12, 42), TRPS (16), and TDA (33). It is documented for TRPS (32) and OASS (47) that enhancement of the long wavelength fluorescence is accompanied by cofactor rotation about the axis defined by the line between C-2' and the phosphate, improving orientation for energy transfer; this is also presumably true for TDA. Thus, changes in the intrinsic tryptophan fluorescence report on the environment around the tryptophans with contributions from the cofactor, whereas the long wavelength band reports specifically on the relative orientation between a tryptophan and the active site cofactor.

Circular dichroism spectra report on the differential absorption of left- and right-hand circularly polarized light, and as such is a particularly useful probe of protein structure. The presence of optically active chromophores, i.e., chiral compounds, gives rise to Cotton bands. Two types of chromophores can be differentiated, inherently dissymmetric chromophores producing intrinsic Cotton effects and inherently symmetric but dissymmetrically perturbed chromophores creating extrinsic or induced Cotton effects (48). Intrinsic Cotton effects, such as the amino acids that make up the sequence of the protein, reflect the conformation of the protein. Thus, far UV CD spectra are a measure of the secondary structural components in a given protein and are thus an indicator of the overall structure of the protein. On the other hand, induced Cotton effects are primarily manifestations of the specific structure of the cofactor-protein complex. Hence, for the achiral PLP, binding to a protein results in a signal induced by the asymmetric molecule, centered at the absorption maximum of the chromophore, providing information about the symmetry of binding of the cofactor in the asymmetric protein. Torchinsky (36) has suggested that induced CD data can best be interpreted in terms of an induced dissymmetry factor which is the ratio of the ellipticity of the induced CD signal obtained at a given protein concentration (Δε) to the extinction coefficient of the absorb-
Circular dichroism data in the far UV for OASS (12) and TRPS (49) are consistent with proteins that are composed of largely \( \alpha \) and \( \beta \) structure (47, 30). In the visible region, induced CD signals are observed for OASS as positive Cotton bands centered at the maxima for the internal aldimine and the external aldimine with L-cysteine (27). The molar ellipticities are identical for both internal and external aldmines, and the ratio of the molar ellipticities at 412 (or 418 for the external aldimine) and 330 nm is identical to the ratio of absorbencies at the two wavelengths. Data for TRPS are also very similar to those obtained for OASS (29, 50).

The above spectral properties of the native DSD are used in these studies to obtain information on the bound cofactor in free enzyme and the external Schiff base intermediate.

**Native \( \delta \)-Serine Dehydratase**—The prominent visible absorption band centered at 413–415 nm exhibited by DSD is indicative of the ketoenamine form of the internal Schiff base as shown in Scheme 1 (1, 6). In addition, the enzyme has some absorbance in the 330 nm range, indicating the enolimine form of the Schiff base. Addition of amino acids to DSD gives either an increase in the extinction coefficient at 415 nm or a shift to longer wavelengths with an increase in extinction coefficient (Fig. 1, Table II, (10)). In all cases, finite absorbance is observed at 330 nm, indicative of the presence of the enolimine.

The fluorescence emission spectrum of native DSD when excited at 280 nm is similar to that obtained for OASS, TRPS, and TDA (see above) with an emission maximum at 335 nm due to intrinsic tryptophan fluorescence and a maximum at 500 nm, suggestive of Förster resonance energy transfer from tryptophan to PLP. The observed emission maximum of 335 nm is located in the middle of the 324–350 nm range. Several options for the possible environment of the tryptophanyl residues can be envisioned. Some of the tryptophans may be buried in the hydrophobic interior of the protein, whereas others may be more or less exposed to aqueous solvent. The suggestion is consistent with data interpretation for OASS and TRPS, which have similar maxima, given the location of Trp-161 in OASS, which is partially exposed to solvent (12, 42, 47), and the location of Trp-177 for TRPS, at the \( \alpha/\beta \) subunit interface (16, 30). The long wavelength fluorescence of DSD is suggestive of Förster energy transfer as for TRPS, OASS, and TDA (see above). Consistent with the suggested energy transfer, a decrease in fluorescence emission at 335 nm is observed upon reconstitution of apoDSD with PLP, concomitant with the appearance of the emission band at 530 nm (8).

Although the sequence homologies between TRPS, OASS, TDA, and DSD are moderate, the structure of a monomer of TRPS (30), OASS (47), and TDA (35) in the same orientation (data not shown) suggest that the overall fold of the proteins in this family are very similar. In addition, the tryptophan residue of TRPS (Trp-177), OASS (Trp-161), and TDA (Trp-153) responsible for energy transfer to PLP is located between the internal Schiff base lysine (Lys-87, Lys-41, and Lys-61) and the glycine cluster (Gly-232–234, Gly-176–180, and Gly-188–191) engaged in binding of the phosphate group of PLP. Based on these data, it is possible to pinpoint the tryptophan residue that is most likely responsible for energy transfer in DSD. DSD contains 4 tryptophan residues, in positions 27, 197, 374, and 422 (7, 11), the internal aldimine lysine is in position 118 and the glycine cluster in positions 281–286. Only Trp-197 is in the correct sequence space in comparison to tryptophans responsible for Förster energy transfer in the other PLP enzymes.

Furthermore, tryptophan residues of DSD can be modified with 2-hydroxy-5-nitro-benzyl bromide at pH 6.5 after masking reactive \(-\)SH groups with \( p \)-mercuribenzoate. Tryptic digest of the modified enzyme and separation of the peptides revealed unequivocally the tripeptide, Ala-196—Trp-197—Lys-198.2

The effect of amino acids on the fluorescence emission spectrum of native DSD is striking (Fig. 1). Although the fluorescence at 335 nm is invariant with the identity or concentration of the amino acid, the intensity of the long wavelength band varies dramatically, with a ratio of \( F_{335}/F_{330} \) that ranges from 3 for free enzyme to 0.4 for enzyme in the presence of glycine or DAP, a factor of about 8-fold. As suggested above, data can be interpreted in terms of a change in the relative orientations of a tryptophan residue and the PLP cofactor upon amino acid binding. Federiuk and Shafer (10) have suggested the enhancement is due to a conformational change upon transamination to form the external Schiff base, mediated by the amino acid carboxylate. In agreement with this suggestion, addition of L-cysteine to native OASS at pH 9 causes a fluorescence enhancement of the long wavelength band accompanied by a blue shift in the \( \lambda_{\text{max}} \) from 500 to 490 nm (12).

Formation of an external Schiff base has been shown to cause a change in the orientation of the PLP cofactor (see above). For example, the PLP in TRPS undergoes a tilt of 20° upon formation of the \( \alpha \)-aminoacylate Schiff base (51), whereas formation of the methionine external Schiff base of OASS causes a 13° tilt of PLP compared with the internal Schiff base (55). Another indicator of a change in the position of the cofactor dependent upon the substrate analogue used is the difference in \( \lambda_{\text{max}} \) values for the long wavelength fluorescence emission. Values of \( \lambda_{\text{max}} \) will change dependent on the environment around the chromophore and/or the electronic structure of the chromophore. Given the structure of the amino acid analogues used, little if any change in the electronic structure of the chromophore is expected.

If it is assumed that formation of the external Schiff base with any amino acid generates the same conformation of the cofactor, then all amino acids should give the same fluorescence enhancement. The differences observed must then result from differences in the side chains of the amino acids. Enhancement does not correlate with affinity of enzyme for substrate analogues. The best example of this is the enhancement of about 8-fold obtained with both glycine and DAP which have apparent dissociation constants of about 5 and 0.05 mM, respectively. One does not necessarily expect a correlation of these two parameters since enhancement deals with orientation of the cofactor and affinity with the arrangement of functional groups around the \( \alpha \)-carbon. Thus, differences in affinity reflect how well the active site accommodates the amino acid side chain.

The far UV CD spectral data are similar to those obtained for the other PLP-dependent enzymes in this family, which is largely \( \alpha/\beta \) secondary structure. The visible induced CD spectra exhibit maximum ellipticity at the maximum wavelength observed in the absorbance spectrum. Thus, a band is observed for the ketoenamine at about 415 nm, and a shoulder is observed for the enolimine at about 330 nm. The band at 415 nm gives a diacymetry factor of \( 7 \times 10^{-4} \), and this is the value all others can be compared with, which is native enzyme in the presence of amino acids or cofactor-substituted enzymes.

Addition of amino acids gives a decrease in the diacymetry of the bound cofactor, with isoserine exhibiting the smallest change (27%) and all of the others giving a decrease of 80–90%. The decrease in disacmetry is almost certainly related to a rupture of the internal Schiff base linkage to Lys-118, and a

2 A. Bakardjieva and K. D. Schnackerz, unpublished observations.
resulting tilt of the cofactor upon formation of the external Schiff base (see above).

Taken together, there is an excellent correlation between the decrease in dissymmetry, increased fluorescence enhancement, and increase in the extinction coefficient of the visible chromophore upon formation of the external Schiff base. Glycine, DAP, and l-serine give similar values of changes in the above parameters: 80–90% decrease in dissymmetry, 7.5–13.3-fold increased fluorescence enhancement, and 1.25–1.33-fold increase in the extinction coefficient. L-isoserine, on the other hand, gives values of 27%, 1.7-fold, and 1.06-fold, respectively, for the three parameters. Thus, in agreement with the suggestion of Federiuk and Shafer (10), an α-amino acid that allows the α-carboxylate subsite of the active site to be filled gives a more symmetrically bound cofactor which is better oriented as an acceptor for tryptophan fluorescence.

Cofactor-substituted d-Serine Dehydratases—The far UV CD spectral data suggest that there are no gross changes in the overall structure of the protein, whatever cofactor analogue is bound in the active site of DSD, whether or not the internal or external Schiff base is present. Thus, as for native DSD, spectral data can be interpreted in terms of local changes in cofactor orientation within the active site. In comparing the visible CD spectra of DSD with different cofactor analogues, one must be aware that the spectrum depends on the chemical nature of the chromophore (4). At face value, a direct comparison of the substituted DSDs cannot be made; however, changes in the cofactor are only at the 5′-phosphate and thus represent minimal if any changes to the chromophore. The near identity of the visible CD band for native enzyme, PDMP-DSD, and PLS-DSD suggests the cofactor is bound almost identically in all three proteins. The PLPMe-DSD, however, shows a significant decrease in the intensity of the visible CD band, suggesting a difference in the orientation of the bound cofactor compared with the other three proteins. The induced CD data for PDMP-OASS and PLPMe-OASS are identical to those of the native enzyme but with a red shift in the λ\text{max} value for the former enzyme (26). In the visible CD, almost identical intensities were observed for native aspartate aminotransferase and PDMP-reconstituted aspartate aminotransferase (52).

Addition of amino acids results in a change in the visible absorption band with either an increase or decrease in absorbance with the same λ\text{max} or in most cases a shift in the λ\text{max} to the red (Table II). Data are consistent with formation of an external Schiff base between the cofactor (analogue) and the amino acid. In all cases, a mixture of tautomers similar to those shown in Scheme 1 must be present.

The PDMP-DSD exhibits significant activity compared with the native enzyme. The enzyme appears to have a similar extinction coefficient at 415 nm and is bound asymmetrically based on its Δε/є value. However, its long wavelength fluorescence emission is only 14% that of native DSD, suggesting that its orientation relative to Trp-197 is not optimal as an energy acceptor, accounting for its reduced activity. Addition of amino acid analogues parallels the changes observed with native DSD, an indicator that with the exception of the subtle change in orientation relative to Trp-197, the methylene at C-5′ is well accommodated.

Reconstitution with PLS results in an inactive enzyme. The PLS-DSD has higher extinction coefficients compared with native DSD, and the same relative fluorescence of its long wavelength band. However, it is, relatively speaking, symmetrically bound with a dissymmetry factor of only 0.4, 6% that of native DSD. Thus, although its orientation relative to Trp-197 is similar to that of the native enzyme, the protein sees it quite differently. The difference between PLP and PLS is the absence of a negative charge in the case of the latter, so that it is not anchored very tightly to the 5′-phosphate site. Addition of DAP has little effect on any of the parameters but does produce a fluorescence enhancement and a slight decrease in the dissymmetry factor, whereas L-serine causes an increase in the latter and a greater increase in fluorescence enhancement. Of note is the presence in the UV-visible spectrum of a significant 330 nm band representing the enolimine.

The PLPMe-DSD differs most significantly in its visible CD signal, which is decreased as discussed above compared with that of either native DSD or the other cofactor-substituted enzymes. In addition, its long wavelength fluorescence is also decreased. Addition of amino acid analogues has little effect on any of the spectral probes. The methyl ester group thus causes the cofactor to be bound in such a way that it is fixed in a suboptimum orientation, and such that it lacks the necessary mobility in the site to form a productive external Schiff base.

The absence or reduction in activity of the analogue-reconstituted enzymes is not a result of the inability to bind the amino acid and form an external Schiff base. As an example, PLS-DSD has an order of magnitude higher affinity for d-serine compared with native DSD, yet it cannot form the α-aminoacrylate intermediate. Likewise, PDMP-DSD has high affinity for Dl-DAP, and PLPMe-DSD forms an external Schiff base with glycine, and the former is active and the latter is not. All of these data are consistent with changes in the orientation of the cofactor analogue with respect to the general base that accepts the α-proton in the elimination reaction or the general acid that must protonate the leaving hydroxide or both. It is also possible that motion of the cofactor that accompanies the elimination reaction is restricted in the analogue-reconstituted enzymes, but this is less likely given the ability to form the external Schiff base that also requires motion of the cofactor (documented by enhancement of the long-wavelength fluorescence band in the presence of amino acids, Table II).

In some cases, PLP analogues incorporated into other PLP-dependent enzymes could induce enzymatic activity. Aposerine transhydroxymethylase can be completely reactivated by PLPMe, indicating that this enzyme can function normally with a monoanionic phosphate group of the cofactor (53). The incorporation of PLPMe is, however, much slower than that of the natural cofactor. The aldime of the monomethyl ester shows a spectral shift to shorter wavelengths by 15 nm suggesting some distortion in binding of this analogue. PDMP can substitute for PLP in cytosolic aspartate aminotransferase and exhibits 5–6% activity of the native enzyme (52, 54). The absorption maximum of the PDMP internal aldime is located at 363 nm at pH 8.3, identical to λ\text{max} of the corresponding PLP aldime but with a slight decrease in intensity (see also Table II). When PDMP is substituted for PLP in OASS, the reaction with 5-thio-2-nitrobenzoate as substrate showed a V/K\text{TNB} value of 31% that of the native enzyme. A slight decrease in the intensity of the internal aldime in addition to a small red shift is observed for PDMP-OASS in comparison to native OASS (26). Furthermore, the substitution of PLP by PLS yields partially active arginine decarboxylase and tryptophanase (4), but PLS is much less tightly bound than PLP, in agreement with the above assessment. External Schiff bases formed with DL-DAP exhibit no ellipticity in the visible range for native DSD, PDMP-DSD, or PLS-DSD. Results almost certainly are indicative of equal amounts of both the D- and L-isomers in the external Schiff base, in agreement with the almost identical binding constants for DL-DAP and L-DAP (Table I). The only enzyme that exhibits a positive ellipticity in the presence of DL-DAP is the PLPMe-DSD, suggesting a difference in binding of the D- and L-isomers and a difference in the orientation of the
cofactor in the internal and external Schiff bases of PLP-MeDSD and the other three enzymes, in agreement with the above discussion.

In summary, the internal and external Schiff bases for all DSD species are comprised of a mixture of tautomeric forms, the ketoenamine and the enolimine (Scheme 1). From the above studies, several general conclusions can be drawn. 1) Substitution with cofactor analogues gives no gross change in the overall structure of DSD, as indicated by the far UV CD spectra. 2) The $\lambda_{\text{max}}$ and intensity of the long wavelength fluorescence band is an indicator of the orientation of the cofactor (analogue) in the active site of DSD. 3) Formation of the external Schiff base for any of the cofactor-substituted enzymes also results in no gross change in the structure of the enzyme. The latter result is opposite those obtained with OASS, which catalyzes a similar reaction, and exhibits significant changes in its far UV CD spectrum upon formation of an external Schiff base with either L-cysteine or L-serine (27). A large conformational change occurs upon formation of the external Schiff base of OASS. 3) Data presented here suggest no such structural change occurs in the DSD-catalyzed reaction, likely related to the differences between $\beta$-replacement reaction catalyzed by OASS and the $\beta$-elimination reaction catalyzed by DSD. 4) For external aldimines, changes in symmetry of the bound cofactor and changes in intensity of the long wavelength band must be attributed to local conformational changes and/or changes in the orientation of the bound cofactor (analogue). Data reported here will serve as a frame of reference for local structural changes in site-directed mutagenesis experiments.

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