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D-Serine Dehydratase from Escherichia coli

DNA SEQUENCE AND IDENTIFICATION OF CATALLYTICALLY INACTIVE GLYCINE TO ASPARTIC ACID VARIANTS

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We have identified two glycy1 residues whose integrity is essential for the catalytic competence of a monomeric pyridoxal 5'-phosphate-requiring enzyme, D-serine dehydratase from Escherichia coli. This was accomplished by isolating and sequencing the structural gene from wild type E. coli and from two mutant strains that produce inactive monomeric dehydratase. DNA sequencing indicated the presence of a single glynine to aspartic acid replacement in each variant. The amino acid replacements lie in a glycine-rich region of D-serine dehydratase well removed from pyridoxal 5'-phosphate-binding lysine 118 in the primary structure of the enzyme. The striking effect of these two glycine to aspartic acid replacements on catalytic activity, the conservation of the glycine-rich region in several pyridoxal 5'-phosphate-dependent enzymes that catalyze αβ-eliminations, and the placement of similar glycine-rich sequences in well-characterized active site structures suggest that the glycine-rich region interacts with the cofactor at the active site of the enzyme.

D-Serine dehydratase (DSD) from Escherichia coli is one of the few monomeric pyridoxal 5'-phosphate-requiring enzymes (1), and as such provides an attractive target for studies of the mechanism of action of PLP-requiring enzymes. The enzyme interacts strongly with its cofactor via both covalent and noncovalent interactions (1-16) and catalyzes the conversion of D-serine, D-threonine, and D-allo-threonine to the corresponding α-keto acid and ammonia. The nearly complete amino acid sequence and 44% of the DNA sequence for D-serine dehydratase have been reported (17, 18), and the single PLP-binding lysine (K-118) has been identified (17). Little is known regarding the identity of other aminoacyl residues important for catalytic competence, however. The present study (i) establishes the complete DNA and deduced protein sequence of the wild-type enzyme and (ii) identifies two G → D replacements that virtually inactivate DSD but do not prevent the binding of cofactor. The replacements are shown to lie in an unusually glycine-rich region that resembles the glycine-rich sequence found in other enzymes that bind phosphorylated ligands.

MATERIALS AND METHODS

Chemicals, Antibodies, and Enzymes—Tryptone and yeast extract for E. coli (19) were purchased from Difco. DTT was obtained from Behring Diagnostics or Boehringer Mannheim. Reagents for polyacrylamide gel electrophoresis were purchased from IBI, and Tween 20 and 4-Chloro-1-naphthol for Western blotting from Bio-Rad. Polymin P and goat anti-rabbit horseradish peroxidase conjugate (61-202-3) were ordered from Miles. [γ-32P]ATP (>1000 mCi/mmol) and [35S]thio-ATP (>1000 mCi/mmol) were supplied by Amerham Corp. Other chemicals were analytical grade. Restriction enzymes and enzymes used for labeling of DNA were purchased from Bethesda Research Laboratories or New England BioLabs and were used according to directions. Previously prepared rabbit antiserum to wild-type DSD (20) was purified by sodium sulfate precipitation and DEAE-cellulose chromatography as described by Mage (21) prior to use in immunoblots.

Genetic Nomenclature, Bacterial Strains, and Plasmids—The inability to convert D-serine to pyruvate (DSD- phenotype) may result from alteration of one or more of three genetic loci known to be specific for DSD expression in E. coli K-12: (i) the structural gene of the enzyme, designated dsdA, (ii) the structural gene of a dsdA activator protein, dsdC, or (iii) the control region that lies between the two structural genes and contains the dsdA and dsdC promoters (dsdAp and dsdCp), and transcription start sites (22-24). The symbol dsdA or dsdA+ denotes the wild-type structural gene encoding DSD. Mutations in this gene that result in an inability to grow in the presence of D-serine are designated either dsdA- or dsdA(EM1201), dsdA(AC6082), etc., to denote a specific dsdA mutation. Mutations in the regulatory loci are designated dsdC-, dsdCp-, or dsdCp-.

Phenotypically DSD- strains were obtained from wild-type parent strains using methyl-N'-nitro-N-nitrosoanidine mutagenesis, penillicin selection, and selection in D-serine-containing media (25, 26). The locus of mutation was tentatively assigned as dsdC, dsdCp, dsdAp, or dsdA by complementation analysis and plaque transduction using the tester strains and procedures described by Bloom and McFall (27). Six strains that appeared to be dsdA- were chosen for further screening by protein and Western blot analyses. The E. coli K-12 strains and plasmids used in these studies are described in Table I. All cultures were grown at 37 °C in LB media (19). Where appropriate, ampicillin or tetracycline was added at a concentration of 50 μg/ml or 25 μg/ml, respectively. Bacteriophage M13mp19 was purchased from Bethesda Research Laboratories.

Preparation of Cell Extracts for Western Blotting—DSD- strains and strain C600 were grown overnight in 5 ml of LB broth ± p-serine (0.5 mg/ml). One ml of each overnight culture was centrifuged (14,000 x g for 5 min) and the cell pellet resuspended in 25 μl of S. cerevisiae lysis buffer (20% sucrose, 20 mm EDTA, 30 mm potassium phosphate, 0.5 mg/ml lysozyme, pH 7.8) and incubated at room temperature for 15 min. Cells were lysed by gently mixing the suspension with 275 μl of water (containing 1 mg/ml each of the protease inhibitors leupeptin,
pepsstatin, and aprotinin) and incubating the mixture on ice for 15 min. An aliquot (40 μl) of Buffer A (1 M potassium phosphate, 40 mM PLP, 50 mM EDTA, 10 mM DTT), adjusted to pH 7.5 with concentrated HCl, was then added and the mixture centrifuged to remove cell debris (14,000 × g for 10 min at 4 °C). Aliquots of the supernatant were removed for assay of DSD activity and for gel electrophoresis in preparation for Western blotting.

Assays of DSD Activity—Enzyme activity assays monitored the increase in absorbance at 220 nm (Δε = 1000 m^-1 cm^-1) associated with the conversion of D-serine to pyruvate. The standard assay solution contained 990 μl of 0.1 M potassium phosphate, pH 7.8, and 10 μl of crude protein extract (or 10 μl of a dilute solution of purified enzyme). Fifty μl of 0.5 M D-serine was added to start the reaction. Assays were performed at 25 °C in a EU700 GCA Photometer.

Screening of Cell Extracts by Western Blotting—Aliquots (2 μl) of each crude extract were electrophoresed on 9% SDS-miniPAGE and transferred to nitrocellulose (Millipore GSWP 304FO) by electroblotting using a Bio-Rad TransBlot apparatus. The subsequent steps represent a modified version of the Bio-Rad Immun-Blot protocol. The nitrocellulose was rinsed with H2O, then shaken for 4 h in 0.5% Tween 20, 20 mM Tris, 0.5 M NaCl, pH 7.5 (Tw/TBS) to block nonspecific protein-binding sites. The filter was then incubated overnight with anti-type DSD (75 μl of rabbit antiserum in 4 ml of Tw/TBS). After four washes in Tw/TBS to remove excess antibody, the filter was incubated for 2 h in 75 ml of Tw/TBS containing 10 μl of goat anti-rabbit horseradish peroxidase conjugate. The nitrocellulose was developed to detect the presence of DSD cross-reactive material with a solution prepared by combining (a) 60 μg of 4-Cl-1-napthol dissolved in 30 ml of methanol (ice-cold) and (b) 60 μl of 30% H2O2 dissolved in 100 ml of 0.1 M MES, pH 6.0. Following development for 5–10 min, the blot was rinsed several times with water, dried at room temperature, and photographed. All steps were carried out at room temperature in glass dishes.

Construction and Screening of Genomic Libraries—Unless specified, the techniques outlined by Maniatis et al. (19) were employed for plasmid DNA isolation, electrophoration of DNA fragments, and transformations. Chromosomal DNA was prepared (30) from strains EM1201 and AC6082 and digested with HindIII. Following electrophoresis on 0.8% agarose (FMC Biochemicals), the 4-5 kb region was removed for assay of DSD activity and for gel electrophoresis in preparation for Western blotting.

In situ gel hybridization, a simplified form of Southern blotting, was performed on 0.8% agarose (FMC Biochemicals), the 4-5 kb region was excised and transferred to nitrocellulose (Millipore GSWP 304FO) by electroblotting using a Bio-Rad TransBlot apparatus. The subsequent steps represent a modified version of the Bio-Rad Immun-Blot protocol. The nitrocellulose was rinsed with H2O, then shaken for 4 h in 0.5% Tween 20, 20 mM Tris, 0.5 M NaCl, pH 7.5 (Tw/TBS) to block nonspecific protein-binding sites. The filter was then incubated overnight with anti-type DSD (75 μl of rabbit antiserum in 4 ml of Tw/TBS). After four washes in Tw/TBS to remove excess antibody, the filter was incubated for 2 h in 75 ml of Tw/TBS containing 10 μl of goat anti-rabbit horseradish peroxidase conjugate. The nitrocellulose was developed to detect the presence of DSD cross-reactive material with a solution prepared by combining (a) 60 μg of 4-Cl-1-napthol dissolved in 30 ml of methanol (ice-cold) and (b) 60 μl of 30% H2O2 dissolved in 100 ml of 0.1 M MES, pH 6.0. Following development for 5–10 min, the blot was rinsed several times with water, dried at room temperature, and photographed. All steps were carried out at room temperature in glass dishes.

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**TABLE 1**

| Strain       | Genotype          | Source*   |
|--------------|-------------------|-----------|
| EM1201       | supE  strR  lacZ-  | EM1200 (28)|
| AC6082       | thr  nepL+  dsd-  | C600 (29, 23)|
| Transformations |                   |           |
| JM101b       | supE  thi-  proAB  F'  | BRL       |
|              | traD36 lacIqZ  M15 |           |
| Plasmids     | Size  Vector/Markers Insert*  dsd DNA present |
| pUC13        | 2.7  ampR          |           |
| ptacl2       | 2.6  lac ampR      |           |
| pAC 13       | 13.8  pMB9 tetR    |           |
| pTC-21       | 4.3  ptacl2 ampR   |           |
| pTC-28       | ptacl2 ampR       |           |
| pEM1201.35   | 4.7  ptacl2 ampR   |           |
| pAC6082.35   | 7.1  pUC13 ampR    |           |
|              | HD/4.4             |           |

* Parental E. coli K-12 strain C600 is dsdC+A+. Parental strain EM1200 (dsdC+Ap2A+) is constitutive for synthesis of wild-type DSD and is derived from dsdC+A+ strain W3828 (28). BRL, Bethesda Research Laboratories.

**FIG. 1.** Restriction map of the dsdA region and strategy for sequencing. The procedures used for subcloning and sequencing are described under "Materials and Methods." Arrows indicate the length and direction of selected fragments used for sequencing. Dashed arrows represent fragments derived from deletion subclones; solid arrows represent fragments derived from H/AI or S digestion. X indicates the site of the G → A transition in dsdA(EM1201) or dsdA(AC6082). Abbreviations used for restriction enzyme sites are: A, AvaI; Al, AluI; B, BstEII; H, HindIII; R, Rsal; S, Smal. 4.42-kb HindIII insert present in pEM1201.1 and pAC6082.35; □, dsdA-coding region.
was performed with the universal sequencing primer, modified 77 phosphoramidites (S. Biochemical Corp.), and the sequencing protocol supplied with the enzyme. A portion of each gene was also sequenced after cloning the intact 1.5-kb dedA fragment into HindIII-digested, dephosphorylated M13mp19. Following transformation of JM101, clones containing the insert in opposite orientations were isolated and used to prepare a series of overlapping deletion clones as described by Dowhan and Snell (34). Since the two clones was isolated and sequenced as described earlier. Sequence data obtained by both cloning strategies was used to assemble the complete sequence of each gene in both directions.

Subcloning of DNA into Expression Vector ptauc12—The 1.5-kb BamHI fragment containing either the wild-type or mutant structural gene was ligated into Psul-digested, dephosphorylated ptauc12. Following transformation of E. coli JM101, plasmid DNA was prepared from ampicolon colonies and screened for the presence and orientation of the insert by restriction analysis. To verify that the recombinant plasmids could express DSD under the direction of the tac promoter, JM101 containing either pTc-1, pTc-35, or wild-type expression plasmid pTc-21 was grown in 5 ml of LB up to an absorbance at 660 nm (A600) of 0.4. Isopropyl 
-1-thiogalactoside was added to a 1 mm final concentration, and cultures were allowed to grow for an additional 8 h before harvesting. Crude extracts were prepared as described earlier. The tac-promoter DSD activity assays. Western blotting were used to verify the expression of wild-type or mutant DSD.

Expression and Purification of DSD Proteins—DSD expression strains were grown for 5-6 h in 10 ml of LB, and used to inoculate flasks containing 1 liter of LB. After an overnight growth, the cells were harvested after an additional 7-8 h by centrifugation, and the cell paste was stored at -20 °C. A typical yield of cell paste from 15 liters of medium was 40 g in which yields 300-400 mg of purified wild-type or mutant protein.

Purification steps were performed at 4 °C unless otherwise specified. Protein concentration was determined from the absorbance of holoenzyme at 280 nm in 0.1 M potassium phosphate buffer, pH 7.8, using E280 = 10.5 (1). All buffers except the SPE lysis buffer (described earlier) contained 100 mM potassium phosphate, 1 mM DTT, 1 mM EDTA, pH 7.2. The ammonium sulfate fraction was taken to dryness, washed with 1 ml of 0.1 M formic acid (as an internal standard) in 0.1 ml of 0.2 M N-ethylmorpholine acetate, pH 8.5. Following a 2-h incubation at room temperature, the reaction was quenched by adding 0.9 ml of 0.1 M formic acid and passed through a small column (0.2 ml) of Dowex 50W-X8 (100-200 mesh, Bio-Rad) pre-equilibrated in 0.1 M formic acid. The column was rinsed with 1 ml of 0.1 M formic acid, 1 ml of water, and finally with 1 ml of 0.5 M ammonium hydroxide to release bound amino acids. The ammonium hydroxide solution was neutralized with 1 M potassium hydroxide. Western blotting was repeated twice with 0.1 ml of water, twice with 20 ml of 95% ethanol/triethylamine/water (4:2, 14,000 g, 14,000 g), and then to dryness. The residue was taken up in 20 ml of 95% ethanol/triethylamine/water (6:2:2), derivatized with 10% phenyl isothiocyanate in 95% ethanol, and analyzed by high performance liquid chromatography as previously described.

Deconvolution of the PLP Content of DSD—DSD (225 µM) was dialyzed for 18 h against three changes of Buffer D containing 250 µM PLP (stoichiometric with enzyme concentration plus 10%). Free and protein-bound PLP were then separated by the centrifuge column procedure of Penevsky (37). Sephadex G-50 resin was used for column matrices were pre-equilibrated in Buffer D without PLP. DSD samples were loaded onto the 1-ml syringe columns in a total volume of <100 µl and allowed to filter into the resin for 2-3 s prior to centrifugation at 5,000 g × 4 min at room temperature. Following two successive column centrifugations to remove unbound PLP, the sample was transferred to a 1.5-ml Eppendorf tube and centrifuged briefly (3 min, 14,000 g) to remove any traces of Sephadex. The ratio of apo/holoDSD in the supernatant was determined spectrophoto metrically.

Absorbance spectra were recorded at 25 °C on a Cary 219 spectrophotometer. Enzyme solution from the previous step (50-100 µl) was added to 900 µl of quenching buffer (6 mM guanidine hydrochloride, 0.5 M 2-hydroxyethylamine, 0.1 M KPi, adjusted to pH 7.8 with HCl) and the spectrum recorded against a reference solution of 50-100 µl of buffer D (without PLP) in 900 µl of quenching buffer. The high concentration of guanidine hydrochloride ensured that enzyme-bound PLP was released to form a Schiff base with 2-hydroxyethylamine.

Concentrations of PLP ([PLP]) and protein ([DSD]) in the assay were determined with the following equations:

\[ [DSD] = (\text{A}_{390} - \text{E}_{390} \times [\text{PLP}]) / \text{E}_{390} \tag{2} \]

where the molar absorbivities of the PLP-2-hydroxyethylamine Schiff base \((\text{E}_{390} = 5225 \text{ M}^{-1} \text{ cm}^{-1})\) and \((\text{E}_{390} = 4126 \text{ M}^{-1} \text{ cm}^{-1})\) at the subscripted wavelengths were determined from the spectra of solutions containing a known concentration of PLP in quenching buffer. The molar absorbivity of denatured apoDSD, \((\text{E}_{390} = \text{E}_{390}) (9,900 \text{ M}^{-1} \text{ cm}^{-1})\) was determined by diluting 0.1 ml of a stock solution of wild-type DSD (containing a known concentration of native enzyme in phosphate) into 0.9 ml of quenching buffer and substituting the following in Equation 2: the concentration of the diluted DSD ([DSD]), the molar absorbivity of the quenching buffer, the value of \(\text{E}_{390}\) and the value of [PLP] obtained from Equation 1. The concentration of DSD in the original stock solution was determined by the method of Dowhan and Snell (1) using a value of \(\text{E}_{280} = 10.5\) for native wild-type DSD. The molar absorbivity for the denatured apoDSD variants was determined in quenching buffer, as assumed to be equivalent to that of denatured wild-type apoencezyme.
RESULTS

Several strains of E. coli that could not grow in the presence of minimal medium containing d-serine were produced by NMNG mutagenesis (25-27). Presumably these strains produced insufficient active DSD to destroy D-serine, a growth inhibitor of E. coli. (38). SDS-PAGE and Western blotting allowed identification of strains likely to be expressing full fragment of (Table I and Ref. 23). The DNA fragment coding for wild-type DSD and the ddsA genes from the two mutant strains were isolated and further analyzed.

Previously prepared PAC 13 containing a 7.4-kb BstEII encased, and the site of mutation in EM1201 and AC6082 is indicated. The sequence CGTTTCAACGCAGCATCGC-ACCCCCTCCTTTT follows the second TAA end codon and may contain terminator signals of interest.

The sequence of ddsA from EM1201 and AC6082 disclosed the presence of a single glycine to aspartic acid replacement in DSD at position 279 and position 281, respectively (boxed area of Fig. 2). Interestingly, G-279 and G-281 reside in a glycine-rich region of DSD wherein glycine comprises 6 of 8 residues (279-286). The predicted secondary structure of DSD (Fig. 3) suggests that the G → D replacements reside in an

![Fig. 2. Nucleotide and amino acid sequence of ddsA. Numbering of amino acids and nucleotides begins with methionine 1. Nucleotide numbers are given over the sequence and correspond to the base aligned with the last digit. The ribosome-binding site in the (-10) region is overlined and the Real site used for isolation of ddsA fragments is marked. PIP-binding K118 is underlined. The glycine-rich sequence from position 279-286 is enclosed, and the site of mutation in EM1201 and AC6082 is indicated. The sequence CCGTTCACCGAGCATGC-AATCCCTTCCCTGGGTGAGCGATGGCGAGCTTTAAAGATCCGCCGCTTACCGCTATA-ACCCCCTCCTTTT follows the second TAA end codon and may contain terminator signals of interest.](image-url)
Extended turn between the carboxyl terminus of a β-sheet and the amino terminus of an α-helix.

Wild-type and mutant proteins were produced in transformants of JM101 that contain the DSD structural gene linked to the powerful tac promoter of plasmid ptacl2 (Table I). DSD accounts for 7–8% of the total bacterial protein in these expression strains, or about 10 times the amount of DSD produced by the single copy of genomic dsdA in strain EM1600 (dsdApdA+), a commonly used source of DSD (1, 42). The E. coli JM101 host strain expresses little or no DSD unless induced with L-serine. In control experiments, isopro pyl β-D-thiogalactoside-induced cultures of JM101 produced <0.1% of the DSD expressed by transformant strains. Enzyme was purified from crude bacterial extracts using a modified version of previously described procedures (1, 35, 42). Because of the possibility that the DSD variants might bind pyridoxal 5'-phosphate less tightly than wild-type DSD, they were purified in the presence of excess cofactor. Wild-type DSD, DSD(G279D) and DSD(G281D) were isolated in greater than 95% purity as judged by 9% SDS-PAGE (Fig. 4). Purified DSD(G279D) and DSD(G281D) displayed specific activities less than 0.3% that of wild-type DSD. The lack of activity of the variant enzymes could not be attributed to a failure to bind PLP. Determination of the cofactor content yielded values of 1.18, 0.97 and 0.99 mol bound cofactor/mol protein for DSD, DSD(G279D), and DSD(G281D), respectively. Furthermore, the activity of the variant proteins and wild-type DSD was unaffected by varying the concentration of PLP in the assay medium from 1.5- to 100-fold molar excess over enzyme.

Five cycles of Edman degradation indicated the sequence MENAK, and digestion with carboxypeptidase B liberated only MNQYLAKGR for all three proteins. These observations support the conclusion that the wild-type and variant proteins were isolated with their polypeptide chains intact. Thus, the inactivity of the variant proteins could not be attributed to proteolysis.

**DISCUSSION**

The DNA sequence of dsdA (Fig. 2) agrees with that previously reported (18) for nucleotides 1–585 with the exception of a nucleotide in the codon for A-192. GCT was observed rather than GCC. The deduced amino acid sequence agrees with that determined by direct peptide sequence analysis (17) as corrected by McFall and Runkel (18) except for the present assignment of T rather than S at position 204. This assignment was confirmed by Maxam and Gilbert sequencing (43) of a Smal/HAEIII fragment encoding residues 195–264. ACG (T-204) and GCT (A-192) were also observed in the dsdA sequence of EM1201, AC6082, and EM1502, a dsdC+ApdA+ constitutive strain.

It came as a surprise that the loss of catalytic activity seen in DSD(G279D) and DSD(G281D) was not due in either case to loss of a functional side chain, since a minimum of six functional groups have been implicated in catalysis or alignment of cofactor or substrate (1, 2, 10, 11, 14, 42, 44, 45). Introduction of an aspartyl side chain at position 279 or 281 may have perturbed the orientation of one or more of these groups, although gross perturbations of protein conformation are unlikely. The G → D replacements reside in what is predicted (39, 41) to be an extended β-turn in the most flexible region of the molecule (Fig. 3). Aspartic acid is frequently found in β-tu
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Table II
Glycine-rich sequences in PLP enzymes

| Enzyme (ligand) | Glycine-rich sequence | Mutation | Effect(s) |
|-----------------|-----------------------|----------|-----------|
| DSD (AMP)       | GGGGGGGG             | G279D    | Inactivation |
| AK (ADP)        | GGGGGGGG             | G279D    | Inactivation |
| Phosphatase A2 (ATP) | GGAGGCGGR         |          |           |
| LDH (NAD)       | GGAGGGAG             |          |           |
| GPD (NAD)       | GGGGRGGR             |          |           |
| ADH (NAD)       | GGGGGGGG             | G14D     | Inactivation and NAD affinity ↓ 100 ×* |
| GR (FAD)        | GGAGGGGG             |          |           |
| PBHA (FAD)      | GGAGGGGG             |          |           |
| Nitrogenase (ATP)| GGAGGGGG             |          |           |
| Phosphatase (ATP)| GGAGGGGG             |          |           |
| PK-C (ATP)      | GGGGGGGG             | G281D    | Inactivation |
| PK-cAMP (ATP)   | GGGGGGGG             | G281D    | Inactivation |
| v-ATPase (ATP)  | GGGGGGGG             | G281D    | Inactivation |
| EGFR (ATP)      | GGGGGGGG             | G281D    | Inactivation |
| Myosin (ATP)    | GGGGGGGG             | G281D    | Inactivation |
| CheB methyltransferase | GGGGGGGG         | G281D    | Inactivation |
| t-RNA nucleotidyl transferase (CTP) | GGGGGGGG | G281D    | Inactivation |
| p21 (c-Ha-ras-1) (GTP) | GGGGGGGG | G281D    | Inactivation |

*The abbreviations used in this table are: AK, adenylate kinase; LDH, lactate dehydrogenase; GPD, glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; PBHA, phosphohexose isomerase; PK-C, protein kinase C; PK-cAMP, cAMP-dependent protein kinase; EGFR, epidermal growth factor receptor; CheB, chemotaxis B.

2 The single residue flexibility coefficients for G-279, D-279, G-281, and D-281 are 0.973, 0.975, 1.043, and 1.055, respectively. Average flexibilities for the 8 residues of the glycine-rich region (279-286) are 1.046 (wild-type DSD), 1.047 (DSD(G279D)), and 1.052 (DSD(G281D).

3 Conserved regions (including the glycine-rich region) in PLP-requiring αβ-eliminases have been noted previously (17, 49).

Table III
Glycine-rich sequences in DSD and nucleotide-binding proteins

| Enzyme (ligand) | Glycine-rich sequence | Mutation | Effect(s) |
|-----------------|-----------------------|----------|-----------|
| DSD (PLP)       | GGGGGGGG             | G279D    | Inactivation |
| AK (AMP)        | GGGGGGGG             | G279D    | Inactivation |
| Phosphatase A2 (ATP) | GGAGGCGGR         |          |           |
| LDH (NAD)       | GGAGGGAG             |          |           |
| GPD (NAD)       | GGGGRGGR             |          |           |
| ADH (NAD)       | GGGGGGGG             | G14D     | Inactivation and NAD affinity ↓ 100 ×* |
| GR (FAD)        | GGAGGGGG             |          |           |
| PBHA (FAD)      | GGAGGGGG             |          |           |
| Nitrogenase (ATP)| GGAGGGGG             |          |           |
| Phosphatase (ATP)| GGAGGGGG             |          |           |
| PK-C (ATP)      | GGGGGGGG             | G281D    | Inactivation |
| PK-cAMP (ATP)   | GGGGGGGG             | G281D    | Inactivation |
| v-ATPase (ATP)  | GGGGGGGG             | G281D    | Inactivation |
| EGFR (ATP)      | GGGGGGGG             | G281D    | Inactivation |
| Myosin (ATP)    | GGGGGGGG             | G281D    | Inactivation |
| CheB methyltransferase | GGGGGGGG         | G281D    | Inactivation |
| t-RNA nucleotidyl transferase (CTP) | GGGGGGGG | G281D    | Inactivation |
| p21 (c-Ha-ras-1) (GTP) | GGGGGGGG | G281D    | Inactivation |

*The abbreviations used in this table are: AK, adenylate kinase; LDH, lactate dehydrogenase; GPD, glyceraldehyde-3-phosphate dehydrogenase; ADH, alcohol dehydrogenase; GR, glutathione reductase; PBHA, phosphohexose isomerase; PK-C, protein kinase C; PK-cAMP, cAMP-dependent protein kinase; EGFR, epidermal growth factor receptor; CheB, chemotaxis B.

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3 Conserved regions (including the glycine-rich region) in PLP-requiring αβ-eliminases have been noted previously (17, 49).
that glycine-rich sequences are found in many unrelated proteins including IgG light chain, keratin, collagen, ribosomal proteins, enolase, and heat shock proteins (62). Thus, the presence of glycine-rich sequences in distantly related PLP enzymes (63) does not in itself imply functional significance. The conservation of glycine at three or more homologous positions in PLP-requiring α/β-eliminases, however, together with the striking effect of glycine replacement in DSD at two of these conserved positions strongly suggests that the glycine-rich region plays a structural and/or functional role in this group of enzymes.

In many of the nucleotide-binding proteins, a highly conserved glycine-rich sequence is an important structural determinant of the nucleotide-binding site, as indicated by x-ray diffraction analyses (64–70) and the effect of G to X replacement on activity (Table III). The flexible glycine-rich loop connects the carboxyl edge of a β-sheet with the NH₂-terminal end of an α-helix and is usually juxtaposed with a phosphoryl group of the bound nucleotide cofactor or substrate (65, 67–72). Although insufficient information is available to generalize about the presence of glycine-rich regions at PLP-binding sites, a precedent for PLP attachment to a nucleotide-like binding fold clearly exists in the case of glycogen phosphorylase. The PLP-binding site of glycerokinase is well removed from any subunit interface (82) in contrast to that for aspartate aminotransferase (see below). Crystallographic studies of phosphorylase (82–84) show that residues 133–139 in the nucleotide-binding domain form a glycine-rich loop that separates the phosphates of enzyme-bound PLP and glucose-1-phosphate. PLP is intimately associated with this loop, G-134 being in van der Waals contact with C6 and N1 of PLP and G-135 interacting with C5' (83). Additionally, G-135(NH) is linked via two hydrogen-bonded water molecules to O22 of the highly solvated phosphate group of PLP (84). The glycerokinase phosphorylase sequence from positions 130 to 137 (GLGNGLGLG) is similar to the DSD sequence between residues 279 and 286 (GVGGGPGG). If the glycine-rich region of DSD were similarly juxtaposed with the bound PLP, G to D replacement at position 279 or 281 might inactivate DSD by sterically or electrostatically altering the orientation of the enzyme-bound cofactor.

The x-ray crystal structures of cytoplasmic and mitochondrial aspartate aminotransferases (85–89) indicate that these PLP enzymes lack glycine-rich sequences at their active centers, illustrating that a glycine-rich loop is not prerequisite for formation of a PLP-binding site. In this regard, it is important to note that most PLP enzymes are multimeric. The two PLP-binding sites of the isologous aspartate aminotransferase dimer reside at the subunit interface, where residues from both subunits contribute to cofactor binding (85–89). Such a structural arrangement is necessarily different from the PLP-binding site of DSD, a monomer, and may also contrast with the PLP-binding sites of multimeric PLP enzymes in which the bound cofactor is well removed from any subunit interface.

In summary, we have identified two point mutants of disA from E. coli that encode virtually inactive enzymes. The mutations do not alter residues with functional side chains capable of participating in catalysis, but instead affect two conserved glycerol residues that lie in an unusually glycine-rich region of the protein. This glycine-rich sequence, like the conserved glycine-rich sequences in nucleotide-binding proteins and glycogen phosphorylase, may constitute part of the cofactor binding site of DSD.

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