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N-Terminal Amino Acid Sequences of D-Serine Deaminases of Wild-Type and Operator-Constitutive Strains of *Escherichia coli* K-12

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The N-terminal amino acid sequences of the D-serine deaminases from strains of *Escherichia coli* K-12 that harbor wild-type and high-level constitutive catabolite-insensitive operator-initiator regions are identical: Met-Ser-GluNH_2-Ser-Gly-Arg-His-Cys. This result indicates that the operator-initiator region is probably distinct from the D-serine deaminase structural gene.

Evidence exists in several bacterial systems that the site of action of the regulatory gene product (operator-initiator region) is distinct from the first structural gene of the operon (5, 38). Mutations in these sites of action do not affect the N-terminal structures of the first enzymes. The D-serine deaminase (Dsdase) operon consists of an operator region, defined by *cis*-dominant constitutive mutations (23), and a single structural gene (26). We wished to determine whether the Dsdase operator was distinct from the structural gene. A procedure for purification of Dsdase was worked out previously by Dupourque et al. (12), and operator and regulator mutants exist which form high levels of Dsdase in the absence of inducer (23). We were therefore able to determine and compare the N-terminal amino acid sequences of Dsdase harboring a wild-type and a mutant operator, as described below. The first eight amino acids of the two Dsdases are identical.

MATERIALS AND METHODS

Strains. Strains used for preparation of Dsdase were EM1600 (*dsdC*$^+$*dsdO6 dsdA*$^+$) and EM20038 (*dsdC3 dsdO*$^+$ *dsdA*$^+$/F′ *dsdC3 dsdO*$^+$ *dsdA*$^+$). Both are derivatives of K-12 strain W3828, differing from it only in their mutant *dsd* alleles and the presence of the F′ in EM20038 (24). The abbreviations *dsdC*, *dsdO*, and *dsdA* indicate Dsdase regulatory, operator, and structural genes, respectively.

Chemicals. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone was purchased from Worthington Biochemicals Corp.; Triton X-100 was from Baker. Precoated thin-layer plates (cellulose and silica gel) (E. M. Merck brand) and chromatographic sandwich chambers were obtained from Brinkman. β-2-Thienyl-DL-alanine, 2,4-dinitrofluorobenzene, dimethylaminonaphthalene sulfonyl chloride, and standard dimethylaminonaphthalene sulfonyl amino acids were obtained from Calbiochem; standard dinitrophenyl (DNP) amino acids were from Schwarz/Mann; standard phenylthiohydantoin amino acids and *N*-ethylmorpholine were from Sigma Chemical Co.; phenylisothiocyanate was from Eastman Organic Chemicals; and trifluoroacetic acid, sequenation grade, was from Pierce. All reagents used for the Edman degradation were stored under N_2.

Cell cultivation. Cells from strain EM20038 were cultivated by the New England Enzyme Center to a density of 2 × 10^9/ml (late logarithmic phase of growth) in minimal medium (26) at 35 C and shipped as a frozen paste.

Purification of Dsdase. Crystalline Dsdase from strain EM20038 was prepared by the method of Dupourque et al. (12). Crystalline Dsdase from strain EM1600 was a gift of E. E. Snell.

Assay for Dsdase activity. For crude extracts, the dinitrophenylhydrazine method (26) was used, and for purified preparations the lactic dehydrogenase method (11) was used.

Protein. Protein was determined by the method of Lowry et al. (21).

Amino acid composition. Preparation and hydrolyses were carried out by the method of Moore and Stein (28). Amino acid anaylses were performed in a Beckman model 120 C amino acid analyzer, with β-2-thienyl-DL-alanine as the internal standard (37).

Performic acid-oxidized (27) samples were used for the determination of cysteine/cystine residues as cysteic acid and of methionine residues as methionine sulfone.

Tryptophan residues were determined by the method of Goodwin and Morton (16).

Correction for losses of threonine and serine was obtained by extrapolation to zero-time of hydrolyses. For isoleucine the value of the longest hydrolysis was taken.

Reduction with $NaBH_4$. Reduction with $NaBH_4$ was done by the method of Schirch and Mason (36).

Reduction and carboxymethylation. Reduction and carboxymethylation were done by the method of Crestfield et al. (9).

Tryptic digestion and peptide mapping. The $NaBH_4$-reduced Dsdases from strains EM20038 and EM1600 were subjected to reduction and carboxymethylation and then digested with trypsin (11). The resulting peptides were applied on cellulose thin-layer plates. The plates were subjected to electrophoresis in the first dimension and chromatography in the second dimension by the method of Burns and Turner (6). Electrophoresis was in glacial acetic acid-98% formic acid-water (170:50:2,800, vol/vol/vol; pH 2) (6) at 20 V/cm, with quinine sulfate as the marker (39). Chromatography was in isoamyl alcohol-pyridine-water-ethyl alcohol-acetic acid (70:70:60:20:5, by volume) (39). Detection and identification of peptides on thin-layer peptide maps were accomplished by the procedure of Dowhan and Snell (11), except chlorination, for which the chlorine-tolidine test (3) was substituted.

N-terminal amino acid determination. N-terminal amino acids were determined by the dinitrophenylation method (15, 34) on performic acid-oxidized (27) protein.

Thin-layer chromatography on cellulose plates (29) was used for identification of DNP amino acids. The following solvent systems were used: for ether-soluble DNP amino acids, *sec*-butanol-phtalate (pH 6) (31) in the first dimension and the "toluene" system (1) in the second dimension; for acid- and water-soluble DNP amino acids not extractable with ether, *n*-propanol-34% NH_4OH (7:3, vol/vol) or *n*-butanol-34% NH_4OH (80:20, vol/vol) (4).

N-terminal peptide. Performic acid-oxidized DNP protein was subjected to tryptic digestion (32) in 0.6% Triton X-100 (20). Isolation and purification of the DNP N-terminal peptide were as for DNP amino acids.

Dansylation reaction. Dansylation procedures, in conjunction with Edman degradation, hydrolysis, and identification by thin-layer chromatography, were carried out by the method of Gros and Labouesse (17).

Amino acid sequence analysis. A three-stage Edman degradation (13) with dansylation was employed according to the method of Percy and Buchwald (30), with the following modifications. The protein samples were previously oxidized by performic acid (27). After coupling, the mixture was extracted three times with two volumes of benzene and with one volume of ethyl acetate. The remaining aqueous phase was dried in vacuo. Cleavage with trifluoroacetic acid was repeated twice at room temperature. The resulting thiazolinones were separated by successive extraction with ethylene dichloride and 1-chlorobutane (three times each). Conversion of the thiazolinones into the corresponding phenylthiohydantoins and extraction of the phenylthiohydantoin amino acid were carried out as described by Edman and Berg (14). The residual protein was brought into solution and a portion was removed for dansylation. The residual protein was then subjected to the next degradative cycle. Identification of phenylthiohydantoin amino acids were accomplished by thin-layer chromatography on silica gel plates with fluorescence indicator, by the method of Jeppsson and Sjöquist (19). Ninhydrin-collidine staining (33) and the chlorine-tolidine test (3) were used either separately or in succession. Phenylthiohydantoin histidine and arginine were identified by specific color reactions (13).

RESULTS

Sources of Dsdase. The object of this work was to compare the N-terminal amino acid sequences of Dsdase from a *dsdO* and a *dsdO*$^+$ strain, to determine whether the *dsdO* mutation affected the N-terminal sequence of the enzyme. Preparation of the large amounts of enzyme necessary for sequence analysis is facilitated if one has available high-level constitutive mutants. As a source of enzyme from a strain with a wild-type operator, therefore, we used EM20038, a homogeneous merodiploid derivative of EM20032 (24), which has two copies of the wild-type operator and structural genes and also of the regulatory constitutive allele *dsdC3*. It forms Dsdase constitutively at a rate about 300 times that in the uninduced wild type. The enzyme was prepared and purified to homogeneity as described above. Pure Dsdase from the operator-constitutive strain EM1600 was prepared by Dupourque et al. (12).

Purity and sedimentation properties. Polyacrylamide gel electrophoresis (8, 10) of the crystalline Dsdase preparation from strain EM20038 gave a single band. Sedimentation velocity experiments were carried out, as described by Schachman (35), in a Beckman Spinco model E analytical ultracentrifuge with a standard double-sector cell and schlieren optics. Dsdase from strain EM20038 was found to sediment as a single symmetrical boundary (Fig. 1). The $s_{20,w}$ value (3.56 ± 0.10) was found identical with that of Dsdase from strain EM1600 (11), indicating identical molecular weight. Both results indicate purity and homogeneity of the preparation.

Amino acid composition. The results of amino acid analysis are shown in Table 1. Our analysis of Dsdase from strain EM20038 is nearly identical to that of Dowhan and Snell (11) from strain EM1600, showing only a small difference in the best whole numbers of glutamic acid, glycine, and alanine.

Tryptic peptide maps. Dsdases from strain EM1600 and EM20038 were treated prior to tryptic digestion as described above. Both enzymes yielded the same number of peptides (Fig. 2) by thin-layer electrophoresis and chromatography. Besides the presence of 36 peptides, two additional faint spots appeared on cadmium-ninhydrin-stained maps. The position of two peptides was different on the maps for the two Dsdases.

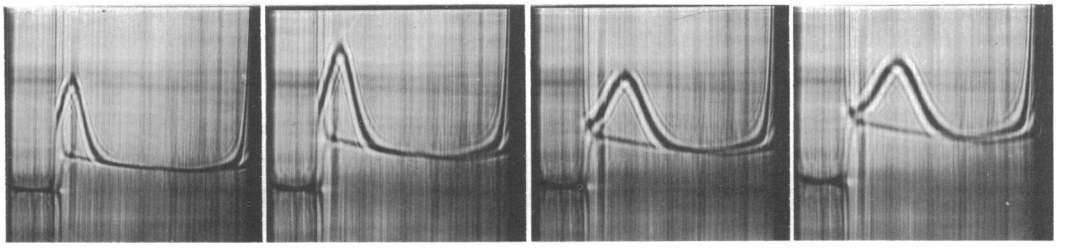

FIG. 1. *Sedimentation pattern of Dsdase from strain EM20038 at 42,040 rpm and 15 C; 0.7% protein in 0.1 M PO_4 buffer, pH 7.8. Direction of sedimentation is from left to right, and the exposures shown were taken after 16, 32, 80, and 112 min at bar angles of 60°, 50°, and 45°, respectively.*

TABLE 1. *Amino acid composition of Dsdase from strain EM1600 (11) and EM20038*

Amino acid	Best whole no.		Mol%	
	Dsdase, strain EM1600	Dsdase, strain EM20038	Dsdase, strain EM1600	Dsdase, strain EM20038
Lysine	19	19	5.35	5.31
Histidine	11	11	3.31	3.29
Arginine	17	18	5.83	6.13
Aspartic acid	31	31	7.83	7.78
Threonine	20	20	4.44	4.41
Serine	27	27	5.16	5.12
Glutamic acid	49	51	13.89	14.35
Proline	14	14	2.98	2.96
Glycine	45	43	5.64	5.35
Alanine	47	45	7.33	6.97
Cystine (half)	5	5	1.12	1.11
Valine	27	27	5.87	5.83
Methionine	12	12	3.46	3.43
Isoleucine	17	16	4.22	3.95
Leucine	46	47	11.43	11.59
Tyrosine	13	14	4.66	4.98
Phenylalanine	18	18	5.81	5.77
Tryptophan	4	4	1.63	1.62

N-terminal amino acid. By dinitrophenylation or dansylation, the N-terminal amino acid for Dsdase from strain EM20038 was identified as methionine, the same as was found for Dsdase from strain EM1600 (11).

N-terminal peptide. The performic acid-oxidized (27) DNP Dsdases were digested with trypsin as described above. The acidified mixture was extracted with ether, ethylacetate, and *n*-butanol. The extracts and the water phase were examined by thin-layer chromatography. The DNP N-terminal peptide was present in the ether extract. The chromatographic mobilities of the DNP N-terminal peptides from the two Dsdases were identical. The spots were removed, extracted, and acid hydrolyzed. The resulting amino acids were identified by thin-layer chromatography and staining as described above for peptide maps. Besides DNP methionine sulfone, serine, glycine, glutamic acid and, as a faint spot, arginine were found in both cases.

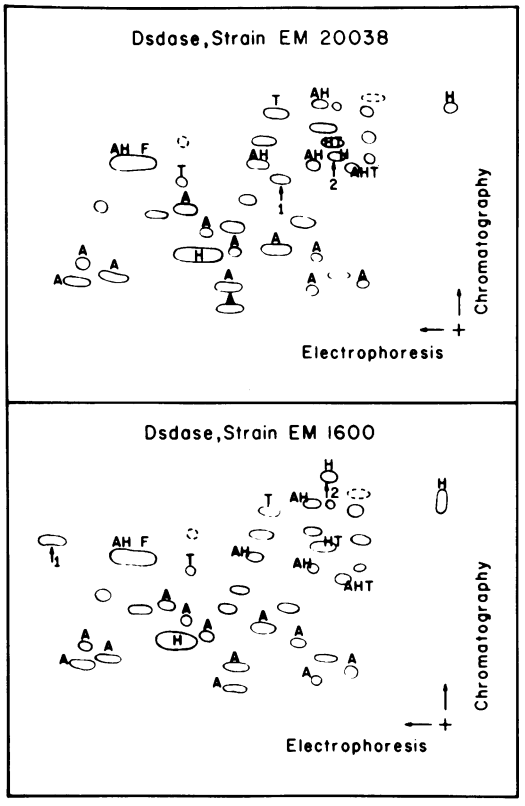

FIG. 2. *Tryptic peptide maps of Dsdases from strains EM20038 and EM 1600 on cellulose thin-layer plates. Peptides marked A, H, and T contained arginine, histidine, and tryptophan, respectively; those marked F were fluorescent under ultraviolet light. Peptides 1 and 2 appeared in different positions on the two maps.*

Amino acid sequence analysis. Edman degradation together with dansylation was used to determine the amino acid sequence of the two Dsdases from the N-terminal end. At each step

of the degradation a fraction taken from the residual protein was dansylated, purified by gel filtration, and hydrolyzed, and the resulting N-terminal dimethylaminonaphthalene sulfonyl amino acid was identified by thin-layer chromatography (Fig. 3). Simultaneously the residual protein was subjected to Edman degradation and the corresponding released phenylthiohydantoin amino acid was identified by thin-layer chromatography (Fig. 4). All extracts were checked by suitable chromatographic systems with standard amino acid derivatives.

The N-terminal amino acid sequence was found identical for both Dsdases as:

$$\text{Met-Ser-GluNH}_2\text{-Ser-Gly-Arg-His-Cys}$$

Methionine and cysteine/cystine were identified as methionine sulfone and cysteic acid, respectively. The third residue appeared as glutamic acid by dansylation, whereas the corresponding phenylisothiocyanate degradation resulted in phenylthiohydantoin glutamine.

DISCUSSION

Dsdase synthesis is probably regulated at the level of transcription, as its induction is mediated by the cyclic 3',5'-adenosine monophosphate (cAMP)-cAMP binding protein (CAP factor) system. The site of action of both controls is an operator-initiator region (*dsdO*) adjacent to the structural gene *dsdA* (25). There does not seem to be a clear distinction between

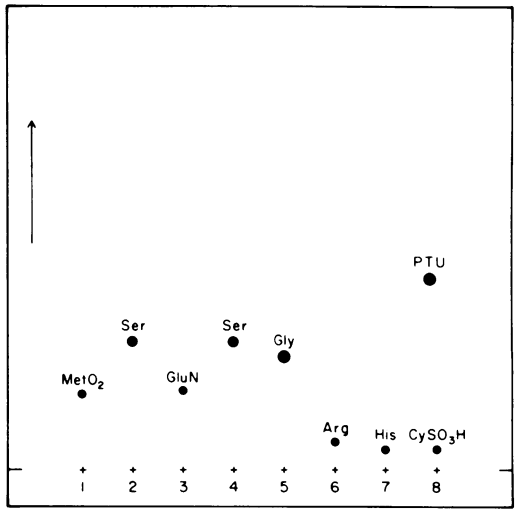

FIG. 4. *Chromatography of phenylthiohydantoin amino acids released at various steps of the Edman degradation on silica gel thin-layer plates with fluorescence indicator. Solvent systems V and IV (19) were used in succession in one dimension, except for phenylthiohydantoin glycine where solvent V was applied twice. Solvent V: heptane, 58 ml; propionic acid, 17 ml; and ethylene chloride, 25 ml. Solvent IV: heptane, 50 ml; n-butanol, 30 ml; and formic acid (75%), 9 ml.*

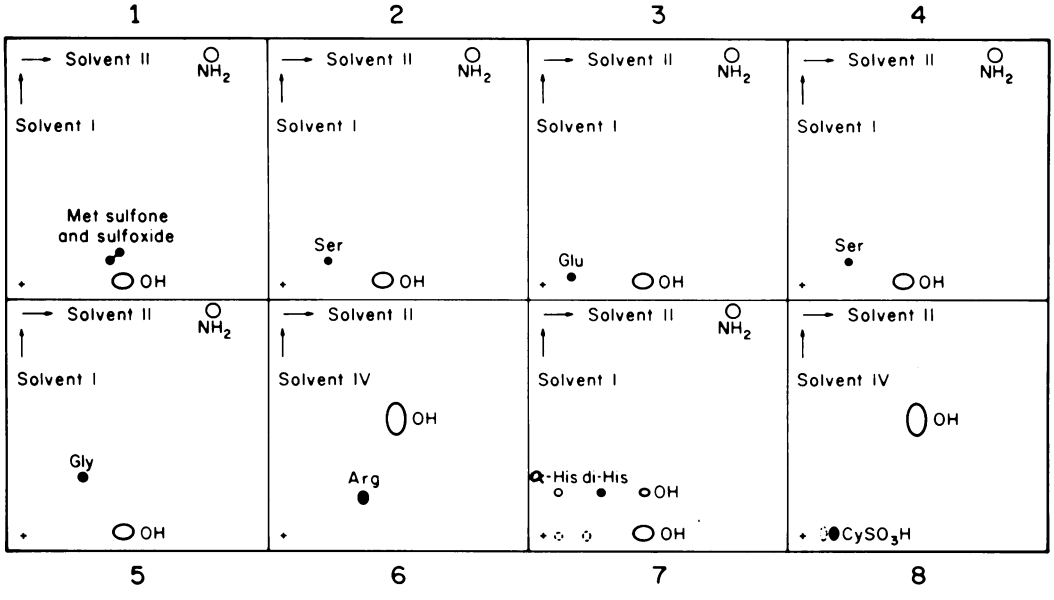

FIG. 3. *Chromatograms of dimethylaminonaphthalene sufonyl amino acids obtained at each step of the Edman degradation on silica gel thin-layer plates. Solvents used (17) were: (I) benzene-pyridine-acetic acid (80:20:5, vol/vol/vol); (II) toluene-monochloroethanol-25% ammonia (60:10:4, vol/vol/vol); (IV) toluene-monochloroethanol-25% ammonia (60:10:0.5, vol/vol/vol).*

operator and promoter function in the Dsdase operon, since *dsdO* mutations always result in a decreased cAMP-CAP requirement as well as in enhanced constitutivity (2), and the DS-*dsd* regulatory gene product complex can partially replace the cAMP-CAP complex in promoting induction (25). The question remained as to whether the Dsdase operator-initiator region and structural gene are separate entities. If they are not, they might be colinear for the length of the operator, or a portion of the operator might precede the structural gene.

The data presented above show that the Dsdases specified by operons with a normal and a mutant operator are identical in at least the first eight amino acids, Met-Ser-GluNH_2-Ser-Gly-Arg-His-Cys. The N-terminal amino acid in each case is L-methionine. L-Methionine is the normal initiating amino acid in bacterial proteins (7, 40). These results indicate that if there is overlap between the operator and structural gene it extends farther than the 24 nucleotides that code for the first eight Dsdase amino acids. This conclusion must of course be qualified because in some cases the same amino acid can be specified by multiple codons that differ in only a single nucleotide. It is therefore conceivable that the operator-constitutive mutation of strain EM1600 resulted in a codon change but not an amino acid change.

If the *dsdO* region is a linear deoxyribonucleic acid structure without loopouts or other perturbations, it should be very short. This conclusion is based on the fact that *dsdO* mutations always decrease or remove the cAMP-CAP requirement (2). The mutant operator in strain EM1600 is completely independent of the cAMP-CAP complex (25). Since this complex is required for efficient initiation of Dsdase synthesis in strains with wild-type operators, its site of action should be very close to the N-terminal end of the operon. Since there is no difference in the N-terminal amino acid sequences of Dsdases from the *dsdO*$^+$ and *dsdO6* strains, the site of the *dsdO6* mutation most probably precedes the Dsdase structural gene.

There is at least one amino acid difference in the Dsdases from strains EM20038 and EM1600 (Fig. 2), but it is not N-terminal and therefore probably does not affect regulation. Such a difference is not surprising, since the mutations to constitutivity in both strains resulted from ethyl methane sulfonate mutagenesis (18, 22). The amino acid change did not affect the enzyme's K_m for DS, but it did result in enhanced protease sensitivity. The primary Dsdase of *Escherichia coli* K-12 is unstable in vivo, being slowly converted to a tetrameric form in which each of the monomers is about half the size of the primary Dsdase monomer (Heincz, unpublished data). The primary Dsdase of strain EM1600 is somewhat more unstable than that of strain EM20038 or the wild-type parental strain (Heincz and E. McFall, manuscript in preparation); thus, the structural mutation must be in strain EM1600.

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