Purification and Characterization of D-Serine Deaminase Activator Protein

MARIA C. HEINCZ, SUSAN M. BORNSTEIN, AND ELIZABETH MCFALL*

Department of Microbiology, New York University School of Medicine, New York, New York 10016

Received 30 January 1984/Accepted 2 July 1984

We purified the *dsdC* gene product, the specific activator of *dsdA* (D-serine deaminase) gene expression, to about 25% homogeneity from a strain in which its expression was amplified 100-fold. The purification involved, successively: DNase and high-salt treatment of cell extracts, DNA-cellulose chromatography, and Dyematrix (Amicon Corp.) column chromatography. We identified the protein as a discrete spot on two-dimensional O'Farrell gels after the DNA-cellulose step and quantitated it by densitometry. The active form was found to be a dimer. We estimated that there were eight activator dimers per wild-type cell. The activator is a slightly basic protein, with an experimental K_m for its ligand D-serine of about 7×10^{-6} M. The low concentration of the activator in wild-type cells and its autorepression may explain the previously observed partial dominance of *dsdC*⁺ in *dsdC*^c/*dsdC*⁺ merodiploids.

Synthesis of D-serine deaminase in wild-type *Escherichia coli* K-12 is induced by the substrate D-serine. This induction is a transcriptional activation process; a specific activator protein, the product of the regulatory gene *dsdC*, and D-serine are absolutely required for expression of the structural gene *dsdA* (13, 17). The cyclic AMP-cyclic AMP binding protein complex is required for maximal expression but has no effect without the activator. The activator represses its own synthesis in the absence of D-serine (19) but apparently not that of the enzyme (13). Thus, as the primary macromolecule involved in control of *dsdA* expression, this activator protein should be a useful prototype with which to examine the mechanism of activation of gene expression.

We previously purified the D-serine deaminase activator sufficiently to demonstrate in vitro its role in induction of *dsdA* expression. We found it to be quite stable even in the absence of its ligand D-serine to freezing, thawing, dialysis, and ammonium sulfate precipitation. It is stable indefinitely when stored in liquid nitrogen (13). The activator monomer is a protein of about 34 kilodaltons as determined by gel electrophoresis of extracts of maxicell strains bearing *dsdC*⁺ plasmids (19). By amplifying expression of *dsdC*⁺ on multicopy plasmids, we were able to purify the activator sufficiently that we could see its subunit as a discrete, well-defined spot on two-dimensional O'Farrell gels, finally characterize it as a protein, make an estimate of its concentration, and define a reference activity.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are described in Table 1. Strain SB86, which harbors pSB8W1, was the source of activator for the procedure and characterization described below. Its chromosomal genotype is $\Delta(ds dAC)$. pSB8W1, a *dsdC*⁺ chimeric plasmid derived from pUC8 (26), was constructed as follows. The entire *dsdA*⁺*C*⁺ region is carried on a 4-kilobase (kb) *PvuII*-*Sall* fragment (8), with the *PvuII* site at the C-terminal end of *dsdC*. This *PvuII*-*Sall* *dsd* fragment from pAC221 (8) was cloned into the *EcoRI*-*Sall* site of pHUB2, creating pAC131 (Fig. 1). (The *EcoRI* site of pHUB2 had previously been filled in with T4 DNA polymerase and deoxynucleotides [14] and was recon-

stituted in the process.) We then attempted to clone a *SmaI*-*EcoRI* fragment of pAC131, which contains *dsdC*⁺ and the promoter and N-terminus of *dsdA*, into the *SmaI* or *HpaI*-*EcoRI* cloning sites of pUC9 (26) and pHUB2 (3). This would have positioned the N-terminal region of *dsdC*⁺ adjacent to *lacP* or λp_L . The fragment would not clone in that direction; apparently the *dsdA* or *dsdAp* material and the strong promoters are not congenial. It cloned easily in the opposite direction, into the *SmaI*-*EcoRI* site of pUC8, to form pSB8W1 (Fig. 1). The high copy number (about 200) of this plasmid, gave us a 5-fold greater yield of activator than we had obtained before and 100-fold greater than the haploid wild-type level. Strain SB85, which is identical to strain SB86 except that it harbors the parental plasmid pUC8, served as a $\Delta dsdC$ control strain in the experiments described below.

Growth of cells. For activator preparation, cells of strain SB86 were grown with vigorous aeration in Zubay DNA medium at 32°C (29). A starter culture was grown with 20 μ g of ampicillin per ml to a density of 1.5×10^8 cells per ml, then added to 12 volumes of fresh medium without antibiotic. Growth was continued to a final density of 10^9 to 1.5×10^9 cells per ml, then the cells were harvested. D-Serine to a final concentration of 200 μ g/ml was added at the beginning and at mid-log phase of growth to induce activator synthesis (19). D-Serine is a gratuitous inducer in strain SB86 *dsdA*. For assay of D-serine deaminase synthesis, strains SB87 and SB88 were grown in tryptone broth (25) with and without D-serine at 500 μ g/ml.

Recombinant DNA techniques. Restriction enzymes were used according to the specifications supplied by the manufacturer. Plasmid preparations, agarose gel electrophoresis of DNA, ligation, and transformation of competent cells were as described previously (8).

Chemicals and enzymes. DNase I and DNA-cellulose were purchased from Worthington Diagnostics. Sodium dodecyl sulfate (manufactured by British Drug House Chemical Ltd.) was purchased from Gallard-Schlesinger Chemical Manufacturing Co. All other chemicals used in electrophoresis were from Bio-Rad Laboratories. Standards used in two-dimensional electrophoresis were from Bio-Rad and Pharmacia Fine Chemicals. Ampholines were from LKB Instruments Inc., Nonidet P-40 was from Imperial Shell, and ultrapure urea was from Schwarz/Mann. Sephadryl S-200 superfine

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Relevant characteristics	Reference
EM6116	$\Delta dsdAC \Delta(gal-uvrB) thi$ HfrH	22
SB85	EM6116(pUC8 Ap ^r)	This work
SB86	EM6116(pSB8W1 Ap ^r <i>dsdC</i> ⁺)	This work
EM6177	EM6116($\lambda dsdA^+C^+$)	22
FB5020-7	<i>purF his dsdC dsdA</i> ⁺	8
SB87	FB5020-7(pSB8W1 Ap ^r <i>dsdC</i> ⁺)	This work
JM83	<i>dsdA</i> ⁺ <i>C</i> ⁺ <i>thi</i>	26
SB88	JM83(pSB8W1 Ap ^r <i>dsdC</i> ⁺) ^a	This work

^a Strain SB88 was formed by transformation of pSB8W1 into strain JM83.

and molecular weight standards for gel filtration were from Pharmacia, Dyematrix orange A and blue B were from Amicon Corp., Aquacide II was from Calbiochem-Behring, and silver nitrate and 50% glutaraldehyde were from Fisher Scientific Co. Components of the cell-free system were as described before (13). All other chemicals were of the highest-quality commercial grade.

Enzymes *Sall*, *PvuII*, T4 DNA polymerase, and T4 DNA ligase were from New England Biolabs. *SmaI* and *EcoRI* were from Bethesda Research Laboratories. Plasmids pUC8 and pUC9 were a gift from Bethesda Research Laboratories.

Activator and D-serine deaminase assays. The activator was

assayed by its ability to promote expression of *dsdA*⁺ in the coupled transcription-translation system developed by Zubay (29) exactly as described previously (13). The template DNA was $\lambda dsdA^+C^+$ from strain EM6177 (22) and was present at the previously defined standard concentration of 50 μ g/ml of synthesis mixture. In vitro D-serine deaminase synthesis was allowed to proceed for 1 h. It was linear for this period. The enzyme formed was measured in the standard assay described previously as nanomoles of pyruvate formed from D-serine per milliliter of synthesis mixture at 36°C per 20-min incubation (13, 19). No activation was found with preparations made from the $\Delta dsdC$ strain SB85.

Within the range of activator concentrations that we used in this work and previously, the response of the in vitro system to added activator was linear (13). We obtained an approximate quantitation of the activator (see below). We could thus define a reference molar activity for the activator based on the activity of D-serine deaminase programmed in vitro as a result of the action of a limiting amount of the DNA-cellulose preparation described below. We found that at an activator concentration of 5 nmol/ml, sufficient D-serine deaminase was programmed in vitro in 1 h per milliliter of synthesis mixture to generate 125 nmole of pyruvate in 20 min. One nanomole of activator therefore programs sufficient enzyme in 1 h to generate 75 nmol of

FIG. 1. Plasmids pHUB2, pAC131, pUC8, and pSB8W1. Plasmid pAC131 was constructed by inserting a *Sall*-*PvuII* segment of *dsdA*⁺*C*⁺ DNA into the *Sall*-*EcoRI* sites on vector pHUB2. Directional insertion of the *dsd* DNA was made possible by converting the *EcoRI* cut on pHUB2 DNA to a blunt end with T4 DNA polymerase (14) and dNTPs before digestion with *Sall* and then ligating the vector and insert DNA under standard conditions. The *EcoRI* site in the resulting plasmid, pAC131, was restored while the *PvuII* site was eliminated. The *SmaI*-*EcoRI* *dsdC*⁺ DNA segment from pAC131 was cloned into the unique *SmaI*-*EcoRI* sites on pUC8. After ligation, the DNA was used to transform strain JM103 with selection for Ap^r and screening for Lac⁻ on indicator plates by the method described by Viera and Messing (26).

pyruvate in 1 h under standard conditions for in vitro synthesis and assay of the enzyme.

Activator purification. The revised procedure involved DNA removal by DNase and high-salt treatment, followed by DNA-cellulose and Dyematrix column chromatography. The yield and enrichment of the activator were similar ($\pm 20\%$) in separate preparations from strain SB86.

All steps during harvesting and cell breakage were at 4°C. After cells were harvested by centrifugation, the cell paste was frozen overnight or longer at -70°C.

Frozen cells were allowed to thaw, suspended in buffer A (described by Williams et al. [28]) supplemented with 1 mM D-serine (50 g of cells per 200 ml of buffer), centrifuged, and resuspended in the same. They were broken by two passages in a French pressure cell, first at 6,000 and then at 2,000 lb/in².

The DNase and high-salt treatment was based on the procedures of Alberts and Frey and Williams et al. (1, 28). The divalent cation concentration of the cell extract was adjusted to 20 mM MgCl₂ and 4 mM CaCl₂ (final concentrations). Eight units of Worthington DNase I was added per milliliter of extract, followed by gentle stirring for 90 min. The NaCl concentration was then adjusted to 0.5 M, and gentle stirring was continued for 1 h. Cell debris and ribosomes were removed by successive low- and high-speed centrifugations: 30,000 $\times g$ for 30 min and 100,000 $\times g$ for 3.5 h. The clear supernatant was then poured into dialysis bags and covered with Aquacide II until the volume decreased about threefold (2 to 3 h). The extract was then dialyzed against modified Alberts and Frey (1) buffer A (0.02 M Tris-hydrochloride [pH 8.1], 5 mM EDTA [pH 8.0], 0.05 M NaCl, 0.5 M dithiothreitol, 5% glycerol, 1 mM D-serine) for 12 h with three buffer changes. This buffer A was also the equilibration buffer for the DNA-cellulose column. Sometimes a light precipitate formed; it was removed by low-speed centrifugation. The activator could not be assayed at this point because of the presence of EDTA, which is necessary to maintain DNase in an inactive state.

DNA-cellulose columns were prepared by the method of Alberts and Herrick (2); the extract was applied, and the columns were washed with equilibration buffer and eluted stepwise with increasing NaCl as described previously. We previously found that we routinely lose 20 to 30% of the activator at this step (13).

Amicon Dyematrix orange A and blue B columns were

prepared and used according to the specifications of the manufacturer. This included washing with 8 M urea in 0.5 M NaOH followed by equilibration with 0.02 M Tris-hydrochloride (pH 7.5) containing 0.25 M NaCl, 0.5 mM dithiothreitol, and 1 mM D-serine. For elution, the NaCl concentration was increased to 1.5 M. Column fractions in each case were concentrated with Aquacide II and dialyzed against the appropriate buffer for further use.

Two-dimensional O'Farrell gels. Two-dimensional electrophoresis was essentially as described by O'Farrell and Neidhardt (4, 20, 21). Three sets of conditions were used for the first dimension: nonequilibrium pH gradient electrophoresis at pH 3.5 to 10, equilibrium isoelectric focusing at pH 5 to 8, and equilibrium isoelectric focusing at pH 3.5 to 10. The second dimension was a 10 to 15% gradient sodium dodecyl sulfate slab gel for the equilibrium isoelectric focusing gels and 11.5% for the nonequilibrium isoelectric focusing gels.

Silver stain. Two-dimensional O'Farrell gels were stained with a silver stain method modified from that of Levy and Rifkin (J. Biol. Chem., in press). We found that the gels could be stained with Coomassie blue (20), destained, and subsequently silver stained. The stained gels were soaked in 10% glycerol-7% acetic acid for 1 h or longer before drying, covered with Bio-Rad cellophane membranes, and dried on a Bio-Rad gel drier at 85°C under high vacuum for 1.5 h.

Measurement of pH gradient. After isoelectric focusing to equilibrium, cylindrical gels were frozen at -70°C and sliced with a Bio-Rad gel slicer into 1-mm slices. Adjacent pairs of slices were placed in 1 ml of degassed distilled water and incubated at room temperature for 1 h with gentle shaking and then the pH of each set was measured.

Sephacryl chromatography. The activator preparation from a DEAE-cellulose column (13) was dialyzed against 0.01 M Tris-acetate buffer (pH 8.2; containing 0.5 mM dithiothreitol, 1 mM D-serine, 0.5 M NaCl, and 5% glycerol) before application to the column. Preparation of the Sephacryl S-200 column, calibration for molecular sieving according to appropriate molecular weight standards, and elution were done according to the specifications of the manufacturer. Fractions were dialyzed against buffer C (13) to reduce salt concentration and assayed in the cell-free system.

RESULTS

Purification of activator. We previously described a partial purification of the activator from a haploid *dsdC*⁺A strain, in

TABLE 2. Purification of *dsdC* activator^a

Step	Total protein (mg)	Total activity (10 ³ U) ^b	Sp act (10 ³ U/mg of protein)	Purification factor ^c
1. Whole cells	1,650	— ^d	— ^d	
2. DNase plus high-salt treatment followed by high-speed centrifugation, concentration, and dialysis	860	200 ^{c,d}	0.23 ^{c,d}	1 ^c
3. DNA-cellulose chromatography, pooled fractions concentrated and dialyzed	2.5	139	56	240
4. Dyematrix blue B eluate, concentrated and dialyzed	0.5	106	212	920

^a Purification data for 10 g (wet weight) of packed cells (strain SB86). Preparations were usually made from 50 to 60 g of cells.

^b To determine activity, activator preparations were assayed in the standard in vitro D-serine deaminase synthesis system described in the text. Activity units refer to nanomoles of pyruvate generated in 1 h as a result of the programming of D-serine deaminase synthesis by measured amounts of the activator.

^c Extrapolated from the DNA-cellulose value on the basis of previous recovery of the activator from DNA-cellulose columns (13).

^d —, Cannot be assayed at this stage.

FIG. 2. Two-dimensional gels of DNA-cellulose preparations of strains EM6116 (A) and SB86 (B). The first dimension was nonequilibrium gradient (Nephge) electrophoresis (pH 3 to 10) with Ampholines mixture (1,250 Vh); the second dimension was sodium dodecyl sulfate-acrylamide slab gel electrophoresis (11.5%). The approximate molecular weight scale was based on the Neidhardt grid and Bio-Rad molecular weight standards; 3 μ g of protein was used per gel. The arrow indicates the position of the activator.

which the primary steps were DEAE-cellulose chromatography and subsequent DNA-cellulose chromatography (13). After cloning *dsdC*⁺ into several multicopy vectors and transforming them into strain EM6116 Δ *dsdAC*, we attempted the same purification, expecting an enhanced yield, but found virtually nothing. The plasmid-borne *dsdC*⁺ genes were functional since they programmed activator synthesis in vitro as well per DNA molecule as did λ *dsdA*⁺*C*⁺ (data not shown). We tried several other purification techniques, but none improved the yield. The problem appeared to be that the activator remained tightly bound to plasmid DNA and was removed with it in the initial steps.

To recover the activator from plasmid-bearing strains, DNase treatment was essential. We found that the following protocol (Table 2) minimized activator loss while yielding a reasonable purification. All steps were carried out at 4°C in the presence of 1 mM D-serine (see above for details).

(i) **Treatment of cell extract with DNase followed by high-salt treatment.** DNase plus high-salt treatment served to remove DNA. It was analogous to the previous DEAE-cellulose chromatography and ammonium sulfate precipitation of cell extracts (13). The DNase broke down DNA, the high-salt concentration dissociated DNA-binding proteins from oligonucleotides, and Aquacide reduced the volume. Subsequent dialysis against a high concentration of EDTA inactivated DNase and removed salt. There was a 48% loss of total cell protein at this point as a result of cell washing and breakage, etc., which we assume to be nonspecific.

(ii) **DNA-cellulose chromatography.** The activator elutes from DNA-cellulose columns at 0.3 M NaCl. This step removed DNase and proteins that do not bind DNA and separated the activator from DNA-binding proteins that elute at other salt concentrations. We used stepwise elution with NaCl concentrations of 0.1M, 0.3M, 0.5M, and 1.0M. In vitro expression of *dsdA*⁺ DNA exposed to these preparations was linear with time, indicating that there was no residual DNase activity. This step was previously found to give about 300-fold purification (13).

(iii) **Dymatex orange A and blue B column chromatography.** The orange A column did not bind the activator, the blue B column did so with elution at 1.5 M NaCl. This step gave a further three-to fivefold purification. On the basis of densitometer scanning of O'Farrell gels of the DNA-cellulose preparation (described below), this product was about 25% pure.

Two-dimensional gel separation of activator. Paired DNA-cellulose column preparations from strains SB86 and SB85 were used to separate the activator protein on two-dimensional O'Farrell gels. The two strains are isogenic except for the presence of a 2-kb fragment carrying *dsdC*⁺ on plasmid pSB8W1 in strain SB86. Nonequilibrium pH gradient electrophoresis (pH 3.5 to 10) followed by 11.5% sodium dodecyl sulfate slab gel electrophoresis in the second dimension and silver staining gave good resolution of the activator (Fig. 2).

FIG. 3. Two-dimensional gels of DNA-cellulose preparations of strains EM6116 (A) and SB86 (B). The region of the activator pI is shown. The first dimension was isoelectric focusing to equilibrium (pH 3.5 to 10) with Ampholines mixture (7,800 Vh); the second dimension was sodium dodecyl sulfate-acrylamide slab gel electrophoresis (10 to 15% linear gradient). The pH gradient formed in the first dimension is indicated; 6 μ g of protein was used for each gel.

It yielded a large, clearly defined spot, well separated from other proteins. The subunit molecular weight was ca. 33,000, in good agreement with the value of ca. 34,000 obtained from maxicell gels (19). There was no trace of a protein in the same position on the companion gel of the control extract (Fig. 2).

The activator was not as well resolved in the pH range of 5 to 8 with equilibrium isoelectric focusing. By extending the pH range to 3.5 to 10 and the time of the run from 3,500 to 7,800 Vh, it was again well separated from other proteins (Fig. 3).

Calculation of activator pI. We estimated the activator isoelectric point (pI) to be 8.0 ± 0.5 (Fig. 3). The calculation was based on the measured pH gradient with the equilibrium isoelectric focusing (pH 3.5 to 10; see above) and the known position of other proteins. It is subject to a possible distortion of as much as 1 pH unit because of the presence of urea, whose effect varies from protein to protein (T. Phillips, personal communication).

Molecular weight of functional activator. To determine whether the functional form of the activator is a monomer or dimer, etc., we carried out Sephacryl gel filtration (Fig. 4). Activity was found in a sharp, symmetrical peak, corresponding to a molecular weight of about 65,000 (range, 63,000 to 68,000). The molecular weight range was calculat-

ed by using the reference standards and the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where K_{av} is the distribution coefficient of a given protein, V_e is the elution volume of that protein, V_0 is the void volume of the column, and V_t is the total volume of the column. The activator molecular weight was obtained from a plot of K_{av} for the standards versus their molecular weights (Fig. 4). Thus, the functional activator appears to be a dimer.

Activator-D-serine binding constant. We attempted to use the equilibrium dialysis or ammonium sulfate precipitation (6, 12) method to assay the activator, using D,L-[14 C]serine and DNA-cellulose activator preparations, without success. However, we calculated an experimental K_m for D-serine binding to the activator in terms of ability of the complex to promote cell-free D-serine deaminase synthesis (13) by measuring the amount of D-serine deaminase programmed per unit of time over an appropriate range of D-serine concentrations (Fig. 5). The value is 7×10^{-6} M at 36°C.

Lack of activation of chromosomal *dsdA*⁺ by plasmid-specified *dsdC*⁺. We previously observed a curious phenomenon when either *dsdA*⁺ or *dsdC*⁺ alone was cloned with the *dsd* control region onto multicopy plasmids. D-Serine deaminase was not inducible in *dsdA*⁺*dsdC*/p*dsdA dsdC*⁺ or *dsdA dsdC*⁺/p*dsdA*⁺*dsdC* arrangements, although a Δ (*dsdAC*)/p*dsdA*⁺*C*⁺ strain was highly inducible (8, 19). The *dsdAC*⁺

FIG. 4. Sephacryl S-200 gel filtration of native activator protein (DEAE-cellulose preparation [see the text]). Sample, 3.5 ml (15 mg of protein per ml); column bed, 1.6 by 90 cm; fraction volume, 1.4 ml; flow rate 20 ml/h. Symbols: ○, optical density at 280 nm (OD₂₈₀); ●, activity; inset ○, molecular weight calibration curve for column. The molecular weight (MW) standards for the calibration were: aldolase, 158,000; bovine serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen A, 25,000; RNase A, 13,700.

FIG. 5. Activator K_m measurement of DNA-cellulose preparation. A double-reciprocal plot of pyruvate generated versus D-serine (DS) concentration in a cell-free system is shown; 10 μ l of the DNA-cellulose preparation of activator (1.2 mg of protein per ml) was used for each data point. The activator preparation was previously dialyzed to remove DS.

plasmid programmed activator synthesis effectively in vitro. Plasmid SB8W1 has at least a fivefold higher copy number than pAC51, the *dsdA* Δ *dsdC*⁺ plasmid used previously, based on yield of plasmid DNA and activator from plasmid bearing strains. To see whether an increased amount of plasmid specified activator would promote expression of chromosomal *dsdA*⁺, or whether the presence of the plasmid would in some way interfere with activation on the chromosome, we constructed strains SB87 *dsdA*⁺ *dsdC*(pSB8W1) and SB88 *dsdA*⁺*C*⁺(pSB8W1). Upon exposure of pSB87 to D-serine, the result was the same as before, i.e., no increase in D-serine deaminase activity above the basal level. Strain SB88 was inducible to the same extent as its haploid parent (data not shown). We conclude that activator specified by multicopy plasmids does not act on the chromosomal *dsdA* promoter.

DISCUSSION

To define the properties of the D-serine deaminase activator protein and to obtain a measure of its concentration in our preparations, it was necessary to physically separate the activator from other proteins. This was difficult because of the very small amount of activator. However, by cloning *dsdC*⁺ on a high-copy-number plasmid and revising the purification scheme, we obtained sufficient material with which to answer our primary questions.

The activator subunit molecular weight obtained from the two-dimensional gels, 33,000, agreed well with the value previously estimated from maxicell preparations (19). On the basis of its Sephacryl elution pattern, the native protein had a molecular weight of about 65,000, indicating that it was a dimer, like the catabolite activator protein (27), *cro*, and the

λ repressor (23). With a pI of 8, it was a slightly basic protein. On the basis of its programming of D-serine deaminase synthesis in vitro, we calculated an approximate experimental activator K_m for D-serine of 7×10^{-6} M, which is indicative of tight binding. In contrast, the K_m of D-serine deaminase for D-serine is only 1.3×10^{-3} M (11).

We obtained an approximate quantitation of activator in our DNA-cellulose preparation by densitometer scanning (20) of the two-dimensional O'Farrell gels. Thus, repeated scans indicated that the preparation was 5% pure. By relating this value to the number of cells used to generate a unit amount of the material we were able to approximate the number of activator molecules per wild-type cell. This DNA-cellulose preparation contained 1.2 mg of protein per ml, and 2×10^{12} cells (7.2 g of cell paste) were used to generate 1 ml of it. The activator routinely survives DNA-cellulose chromatography with 20 to 30% loss of biological activity (13). We assumed a 30% loss of activator at the DNA-cellulose step, as we observed previously (13). There was a 48% loss of protein, presumably nonspecific, preceding the DNA-cellulose step, giving a total recovery of 0.36. The activator monomer had a molecular weight of about 33,000, and the functional form was apparently a dimer (66,000). The activator yield from strain SB86 was amplified 100 times over that from the wild type. The induced level of activator dimers in the wild type cell should then be approximately:

$$\frac{1.2 \times 10^{-3} \times 0.05 \times 6 \times 10^{23}}{2 \times 10^{12} \times 6.6 \times 10^4 \times 0.36 \times 100} = 8$$

per cell, a very small number. It is likely, in fact, that the uninduced level is significantly less, perhaps of the order of one per *dsdC*⁺ operator site, as we observed severalfold repression of β -galactosidase in *dsdC*::Mu d1 (Ap^r *lac*)/*dsdC*⁺ strains. We have qualitative evidence from maxicell preparations (19) that the level of activator in the cell is of this order. The intensity of the activator monomer bands specified by multicopy plasmids was similar to that of D-serine deaminase (uninduced). The uninduced level of D-serine deaminase in wild-type cells was barely detectable, corresponding to about 10 molecules per cell. In *dsdC-lacZ* and *dsdA-lacZ* operon fusions, the levels of β -galactosidase were also similar in the absence of D-serine (19).

Determination of the molar concentration of the activator in the DNA-cellulose preparation also allowed us to calculate a reference activity for the protein, based on the ability of that preparation to activate D-serine deaminase synthesis in vitro (see above). Since the amount of enzyme programmed per unit time under the defined assay conditions is a linear function of the amount of activator present, we could approximate the molar concentration of activator in other preparations, using the in vitro assay.

The finding that the amount of activator in wild-type cells is very small offers an explanation for the mixed dominance results that we previously observed with both *dsdA*⁺*C*⁺/*dsdA*⁺*C*⁺ and *dsdAC*⁺/*dsdA*⁺*C*⁺ F'. merodiploids (5, 15). We found that the presence of the *dsdC*⁺ allele in such merodiploids reduced the constitutive rate of D-serine deaminase synthesis 3- to 10-fold (13). We attributed these results to subunit mixing, as the activator does not seem to be a repressor of D-serine deaminase synthesis in the absence of inducer (13). However, the reduction was, in most cases, too great to be explained in this way, since the activator was a dimer. We also recently showed that activator synthesis is autoregulated (19); thus, it is likely that the number of molecules per uninduced cell is even less than

what we calculated. We now believe that in *dsdC^c/dsdC⁺* F' merodiploids, the *dsdC^c* product would be largely in the active form, and therefore would not repress its own synthesis. The *dsdC⁺* product would, however, repress both at its own operator site and at the *dsdC^c* operator site. Therefore, the constitutive level of enzyme would be low in *dsdC^c/dsdC⁺* strains because of repression of the *dsdC^c* activator, not repression of the enzyme.

It seems a little curious that activator should repress its own synthesis since the derepressed level is so low to begin with. The repression does have one perhaps useful effect, however, since the *dsdA* and *dsdC* initiation sites are adjacent (19; unpublished data), which may hold true for some other such systems as well. It positions the protein near the *dsdA* promoter site. If D-serine appears, the activator needs only to move from the *dsdC* operator to the *dsdA* initiator, a relatively few nucleotides, to promote *dsdA* expression. If there were no repression site, it would be free in the cytoplasm and would have to search out its target. Thus, it seems an efficient arrangement for the *dsd* system to have the structural and control genes adjacent and to have a very weak *dsdC* promoter, but also a repression site for *dsdC* adjacent to the *dsdA* activation site. The *araC* and *malT* activators are present in wild-type cells in considerably higher concentration, about 55 and 70 molecules per cell, respectively (9, 10), but they both must be able to act through the cytoplasm as they activate operons unlinked to their own.

There remains the problem of why the activator specified by multicopy plasmids such as pSB8W1 does not promote expression of chromosomal *dsdA⁺*. We observed *trans* activity of *dsdC⁺* in F' merodiploids and in λ *dsdC⁺* merodiploids, with λ integrated in the *dsd* region. Multicopy *dsdA⁺C⁺* plasmids specify very high rates of D-serine deaminase synthesis upon induction in a Δ (*dsdAC*) host (8; unpublished data), and purified activator functions well in vitro (8). Strain SB88, which is *dsdA⁺C⁺* on the chromosome as well as *dsdC⁺* on the plasmid, is inducible. It therefore seems that the activator specified by multicopy plasmids does not reach the chromosome. We observed a similar phenomenon previously with a *dsdC^c3A⁺/F' dsdC⁺A⁺* merodiploid (16) that could assume two configurations. In the normal configuration, with early transfer of only *dsdC⁺*, there was the usual *trans* effect of *dsdC⁺*. In the other, in which F' seemed to be integrated, there was no *trans* effect of *dsdC⁺*. We can suggest that the multicopy plasmid and aberrant F' were more sequestered from their chromosomal homologues than the normal F's. There may be secondary affinity sites for the activator near its primary target, as there are for the *lac* repressor (24), so that new molecules are quickly trapped in the parental or other immediately adjacent *dsdC-dsdAp* regions after or even during translation. Activator molecules added to the cell-free system, on the other hand, are not bound, and they must seek out the *dsd* control regions. Some support for this idea comes from previous measurements on the time required for transcription of *dsdA⁺* mRNA in the cell-free system (13). From the time of initiation it requires an average of 4 min, the same as for *lacZ⁺* mRNA, which is nearly three times as long. The extra 2 min for *dsdA⁺* transcription may be time that the average activator spends in finding *dsdAp*.

ACKNOWLEDGMENTS

We are most grateful to T. A. Phillips and F. C. Neidhardt for their hospitality, instruction in the O'Farrell gel techniques, and helpful discussions; to H. Levy for the silver staining technique; to

R. Celis for instruction in equilibrium dialysis; and to H. C. Kelker, Y. K. Yip, and A. W. Bernheimer for many helpful discussions.

This investigation was supported by Public Health Service grant GM11899 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Alberts, B., and L. Frey. 1970. T4 bacteriophage gene 32: a structural protein in the replication and recombination of DNA. *Nature* (London) **227**:1312-1318.
2. Alberts, B., and G. Herrick. 1971. DNA cellulose chromatography. *Methods Enzymol.* **21**:198-217.
3. Bernard, H.-U., and D. R. Helinski. 1979. Use of the phage promoter P_L to promote gene expression in hybrid cloning vehicles. *Methods Enzymol.* **68**:482-492.
4. Bloch, P. L., T. A. Phillips, and F. C. Neidhardt. 1980. Protein identifications on O'Farrell two-dimensional gels: location of 81 *Escherichia coli* proteins. *J. Bacteriol.* **141**:1409-1420.
5. Bloom, F. R., and E. McFall. 1975. Isolation and characterization of D-serine deaminase constitutive mutants by utilization of D-serine as sole carbon or nitrogen source. *J. Bacteriol.* **121**:1078-1084.
6. Bourgeois, S. 1971. Techniques to assay repressors. *Methods Enzymol.* **21**:491-500.
7. Carothers, A. M., M. C. Heincz, and E. McFall. 1980. Position effect on expression of *dsd* genes cloned onto multicopy plasmids. *J. Bacteriol.* **142**:185-190.
8. Carothers, A. M., E. McFall, and S. Palchaudhuri. 1980. Physical mapping of the *Escherichia coli* D-serine deaminase region: contiguity of the structural and regulatory genes. *J. Bacteriol.* **142**:174-184.
9. Casadaban, M. 1976. Regulation of the regulatory gene for the arabinose pathway, *araC*. *J. Mol. Biol.* **104**:557-570.
10. Debarbouille, M., and M. Schwartz. 1979. The use of gene fusions to study the expression of *malT*, the positive regulator gene of the maltose regulon. *J. Mol. Biol.* **132**:521-534.
11. Dupourque, D., W. A. Newton, and E. E. Snell. 1966. Purification and properties of D-serine dehydrase from *Escherichia coli*. *J. Biol. Chem.* **241**:1233-1238.
12. Gilbert, W., and B. Müller-Hill. 1966. Isolation of the *lac* repressor. *Proc. Natl. Acad. Sci. U.S.A.* **56**:1891-1898.
13. Heincz, M. C., and E. McFall. 1978. Role of the *dsdC* activator in regulation of D-serine deaminase synthesis. *J. Bacteriol.* **136**:96-103.
14. Maniatis, T., E. J. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. McFall, E. 1967. Dominance studies with stable merodiploids in the D-serine deaminase system of *Escherichia coli* K-12. *J. Bacteriol.* **94**:1982-1988.
16. McFall, E. 1967. "Position effect" on dominance in the D-serine deaminase system of *Escherichia coli* K-12. *J. Bacteriol.* **94**:1989-1993.
17. McFall, E. 1973. Role of adenosine 3',5'-cyclic monophosphate and its specific binding protein in the regulation of D-serine deaminase synthesis. *J. Bacteriol.* **113**:781-785.
18. McFall, E. 1975. *Escherichia coli* K-12 mutant forming a temperature-sensitive D-serine deaminase. *J. Bacteriol.* **121**:1074-1077.
19. McFall, E., and M. C. Heincz. 1983. Identification and control of synthesis of the *dsdC* activator protein. *J. Bacteriol.* **153**:872-877.
20. O'Farrell, P. H. 1975. High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
21. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High-resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**:1133-1142.
22. Palchaudhuri, S., E. McFall, and A. M. Carothers. 1976. Specialized transduction of D-serine deaminase genes: formation of lysogens that yield high λ -d-*dsd*/ λ ratios and formation of a dimeric λ -d *dsd*. *J. Bacteriol.* **127**:998-1014.
23. Ptashne, M., A. Jeffrey, A. D. Johnson, R. Maurer, B. J. Meyer, C. O. Pabo, T. M. Roberts, and R. T. Sauer. 1980. How the λ

- repressor and *cro* work. *Cell* **19**:1–11.
24. **Reznikoff, W. S., R. B. Winter, and C. K. Hurley.** 1974. The location of repressor binding sites in the *lac* operon. *Proc. Natl. Acad. Sci. U.S.A.* **71**:2314–2318.
 25. **Shimada, K., R. A. Weisburg, and M. E. Gottesman.** 1972. Prophage lambda at unusual chromosomal locations. I. Location of the secondary attachment sites and the properties of the lysogens. *J. Mol. Biol.* **63**:483–503.
 26. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mpat-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259–268.
 27. **Weber, K. J., D. B. McKay, and T. A. Steitz.** 1982. Two helix binding motif of CAP found in *lac* repressor and *gal* repressor. *Nucleic Acids Res.* **10**:5085–5102.
 28. **Williams, K. R., M. B. LoPresti, M. Sefoguchi, and W. H. Konigsberg.** 1980. Amino acid sequence of the T4 DNA helix-destabilizing protein. *Proc. Natl. Acad. Sci. U.S.A.* **77**:4614–4617.
 29. **Zubay, G.** 1973. *In vitro* synthesis of protein in microbial systems. *Annu. Rev. Genet.* **7**:267–287.