Regulation of argA Operon Expression in Escherichia coli K-12: Cell-Free Synthesis of Beta-Galactosidase Under argA Control

NORMAN KELKER* and THOMAS ECKHARDT
Department of Microbiology, New York University School of Medicine, New York, New York 10016

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Regulation of argA operon expression in Escherichia coli K-12 was studied in a cell-free, deoxyribonucleic acid-dependent, enzyme-synthesizing system. λAZ-7 deoxyribonucleic acid, which carries a fusion of the lacZ structural gene to the argA operon so that β-galactosidase synthesis is under argA regulation, was used as the template. To eliminate extraneous readthrough from λ promoters, λ repressor was introduced into the synthesis mixtures by preparing the S-30 component from a strain (514X5a-12-29) that carries a multicopy hybrid plasmid (pKB252) containing the λcI gene. Under these conditions β-galactosidase synthesis was repressed 90% by the arginine repressor when a sufficient concentration of L-arginine was present. This repression could be overcome by escape synthesis when the λAZ-7 deoxyribonucleic acid concentration in the synthesis mixtures was increased. Guanosine 3′-diphosphate-5′-diphosphate stimulated β-galactosidase synthesis from this template.

The nine genes of the arginine biosynthetic pathway constitute five single gene operons and a four-gene bipolar operon, the arginine cluster (Fig. 1). All of the pathway enzymes are regulated by repression mediated by the arginine repressor, the protein product of the argR gene. Evidence from in vivo (16) and in vitro studies (5) indicates that the arginine repressor, in the presence of arginine, represses transcription at each of these operons. In this work regulation of the argA operon was studied.

Acetylglutamate synthase, the product of gene argA, is regulated by end-product inhibition as well as by repression. Acetylating activity and regulation of this activity by arginine were first demonstrated by measuring acetylglutamate accumulation in resting cells of a strain defective in argB and argR (23). Subsequent attempts in several laboratories to detect activity in cell extracts were unsuccessful because of the instability of the enzyme. However, Leisinger and Haas (9, 13) recently stabilized the enzyme sufficiently to carry out an in vitro assay and studies of end-product inhibition (6), and Marvil and Leisinger have purified the enzyme to homogeneity (14).

Since the argA operon has a high repression ratio (6), it is well suited for in vitro gene regulation studies, especially as an assay system for the argR gene product. However the instability of the enzyme would seem to preclude its use in cell-free enzyme synthesis studies such as those carried out for acetylornithinase (argE) (21) and for argininosuccinase (argH) (11). Such difficulties arising from loss of enzyme activity under the conditions of cell-free enzyme synthesis have been eliminated in the studies reported here by using as the template λ deoxyribonucleic acid (DNA) carrying a fusion of the lacZ structural gene to argA (7). β-Galactosidase synthesis from this template is under arginine control and provides a convenient method for cell-free studies of regulation of the argA operon.

MATERIALS AND METHODS

Bacterial strains. The S-30 preparation was made from strain 514X5a-12-29(pKB252) [F− lac relA trp rpsL ΔargA/CoIE1 Tet+ λcI+]. This strain was constructed from strain 514X5a (F− lac relA trp rpsL argR) by first selecting spontaneous thyA mutants by resistance to trimethoprim (15). The thyA derivative of 514X5a was transduced with the P1-like phage 363, which had been prepared on strain 4100-A2, a Mu-sensitive, λ-sensitive, arginine-requiring, heat-resistant survivor of heat induction of Mu lysogen 4100A (F− araD139 Δlac169 rpsL thy− argA::− Mu cts) (7). Selection was made for Thy+ in the presence of arginine. One of the argA transductants, designated 514X5a-12-29, was further modified by introduction of a multicopy hybrid plasmid, pKB252 (3, 18), carrying the cI gene of λ and genes for tetracycline resistance. Strain 514X5a-12-29 was transformed with pKB252 DNA, and selection was made for tetracycline resistance. The presence
of the plasmid was confirmed by testing for λ immunity.

The arginine repressor was extracted from strain EC113(pLC32-38) (F-argD argR aroE Δlac-pro rpsL/ColE1 argR+) (4).

**Bacteriophage.** λAZ-7 (7), which carries a fusion of lacZ to the argA operon, and λpac5 (24) were used as sources of template DNA for cell-free β-galactosidase synthesis.

**DNA preparation.** λpac5 was grown and prepared as described by Zubay (26). λAZ-7, which lacks an attachment site and, therefore, cannot form lysogens, was propagated by infection of a susceptible host. Strain MC4100(F-Δlac thi ara) was grown at 37°C in 10 liters of TYE medium which contains (per liter): 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 1 g of yeast extract, 1 g of glucose, 8 g of NaCl, and 20 mg of thymine. When the cell density reached approximately 2 × 10^9/ml, λAZ-7 was added at a multiplicity of infection of 0.01, and incubation was continued for 8 h. CHCl₃ (400 ml) was added and the lysate was placed at 4°C overnight. Deoxyribonuclease (1.0 μg/ml) was added, and the lysate was gently stirred for 2 h at 4°C. The CHCl₃ was removed by allowing it to settle, and the supernatant phage suspension was transferred to another vessel. The phage suspension was adjusted to 10% (wt/vol) polyethylene glycol and 0.5 M NaCl and left at 4°C for 2 h. The precipitate containing the phage was collected by centrifugation at 4°C at 9,000 rpm in a Sorvall GSA rotor for 10 min. The phage were extracted from the sediment by suspension in a small volume of suspension buffer (0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.3), 2 mM MgSO₄, 1 mM CaCl₂, 0.4% NaCl) followed by centrifugation. The supernatant was saved, and the sediment was again extracted by suspending it in suspension buffer followed by centrifugation. The two phage-containing supernatants were pooled, and all further steps of phase concentration and purification by CsCl centrifugation and of phenol extraction of DNA were done as described by Zubay (26).

**S-30 preparation.** S-30 was prepared by the method of Zubay (26) with some modifications. Cells of strain 514XSa-12-29 (pKB252) were grown at 28°C with vigorous aeration in a New Brunswick Microferm laboratory fermentor in 11 liters of medium containing (per liter): KH₂PO₄, 5.6 g; K₂HPO₄, 28.9 g; yeast extract, 10.0 g; thiamine, 15 mg; glucose, 10.0 g; and 1.0 M magnesium acetate, 2.0 ml. Growth was initiated by inoculation of 1 liter of overnight culture grown in the same medium but containing 15 μg of tetracycline per ml to minimize plasmid segregation. The cells were harvested in the late log phase, and S-30 preparation was begun immediately. Except for preincubation, all further steps were done at 4°C. A 40-g amount of wet cell paste was suspended in about 400 ml of S-30 buffer I (0.01 M Tris-acetate (pH 8.2), 0.014 M magnesium acetate, 0.06 M potassium chloride, and 0.006 M 2-mercaptoethanol). The cell suspension was centrifuged for 30 min at 10,000 rpm in a Sorvall GSA rotor. The cell sediment was resuspended in the same buffer and centrifuged again. The cells were suspended in 52 ml of S-30 buffer II (S-30 buffer I with 0.001 M dithiothreitol in place of 2-mercaptoethanol). The suspended cells were lysed at 4,000 lb/ln² in an Aminco pressure cell, and 1 μmol of dithiothreitol per ml was added to the broken-cell suspension. After one 30-min centrifugation at 16,500 rpm in a Sorvall SS-34 rotor, the supernatant was mixed with 5.6 ml of preincubation mixture prepared by the Zubay method (26). After 80 min of preincubation at 36°C without agitation, the S-30 extract was dialyzed against 500 ml of S-30 buffer III (S-30 buffer II with 0.06 M potassium acetate replacing potassium chloride) with a buffer change made each hour. After 4 h the S-30 preparation was distributed in 0.8-ml portions to nitrocellulose tubes; the portions were then frozen and stored in liquid nitrogen.

**Conditions for cell-free β-galactosidase synthesis and assay.** Except for slight modifications, which are noted here, β-galactosidase was synthesized and assayed as described previously (11, 26). Each 0.2-ml synthesis mixture contained per milliter: Tris-acetate (pH 8.2), 44 μmol; dithiothreitol, 1.37 μmol; potassium acetate, 55 μmol; ammonium acetate, 27 μmol; magnesium acetate, 14.7 μmol; calcium acetate, 7.4 μmol; 20 amino acids, 0.22 μmol each; adenosine 5′-triphosphate, 2.2 μmol; guanosine, cytidine, and uridine 5′-triphosphates, 0.55 μmol each; potassium phosphoenolpyruvate, 21 μmol; cyclic adenosine 3′,5′-monophosphate, 0.5 μmol; pyridoxine hydrochloride, 27 μg; nicotinamide adenine dinucleotide phosphate, 27 μg; flavine adenine dinucleotide, 27 μg; p-aminobenzoic acid, 11 μg; folinic acid, 27 μg; polyethylene glycol (Carbowax 6000, Union Carbide Corp., New York, N.Y.), 16 mg.

The mixture was incubated for 3 min at 38°C on a reciprocal shaking water bath before 2.6 mg (0.06 ml) of S-30 extract was added. Incubation with shaking was continued for 50 min, and then the tubes were removed to an ice bucket. A 1.5-ml volume of prewarmed assay buffer containing 0.53 mg of O-
nitrophenyl-β-D-galactoside, 0.1 M sodium phosphate (pH 7.3), and 0.14 M 2-mercaptoethanol was added to each tube, and the assay mixtures were incubated at 39°C. The assay incubation time varied from 10 to 120 min depending on the template used, the concentration of template DNA, and the conditions of the experiment. The assay was terminated by adding 1 to 2 drops of glacial acetic acid, and the tubes were chilled in an ice bucket for at least 10 min. The precipitated protein was removed by centrifugation, and 0.5 ml of supernatant was mixed with 0.5 ml of 1.0 M Na₂CO₃. The absorbance at 420 nm (A₄₂₀) of each sample was read against a water blank using quartz cuvettes with 1-cm light paths.

Preparation of arginine repressor extract. Arginine repressor extract was prepared as described previously (11). Cells of strain EC113(pLC32-38) were grown at 37°C in a New Brunswick Microferm laboratory fermentor in 11 liters of TYE medium from which NaCl was omitted but 5 mM magnesium acetate and 100 mg of L-arginine hydrochloride per liter were added. The cells were harvested in the late log phase, frozen at −70°C, and left overnight. All steps in repressor preparation were done at 4°C. The frozen cell paste (50 g) was placed at 4°C for 30 to 60 min. The softened paste was suspended in about 400 ml of repressor buffer I (0.01 M Tris-acetate [pH 8.2], 0.01 M magnesium acetate, 0.06 M KCl, 0.01 M 2-mercaptoethanol, 5% glycerol, and 1 mM L-arginine hydrochloride). The suspension was centrifuged for 20 min at 10,000 rpm in a Sorvall GSA rotor, the supernatant was discarded, and the cells were suspended in repressor buffer I and centrifuged again.

The sedimented cells were suspended in 85 ml of repressor buffer I and broken in an Amicon pressure cell at 6,000 lb/in². The broken-cell suspension was centrifuged for 30 min at 15,000 rpm in a Sorvall SS-34 rotor. The supernatant was centrifuged for 3.5 h in a Spinco 30 rotor at 27,000 rpm and dialyzed overnight against repressor buffer II (0.01 M potassium phosphate [pH 7.7], 5% glycerol, 0.7 mM dithiothreitol, and 2 mM L-arginine hydrochloride).

The dialyzed supernatant was placed on a 300-ml diethylaminoethyl-cellulose column (Schleicher & Schuell Co., Keene, N.H.) previously equilibrated with repressor buffer II, and 1.2 liters of repressor buffer II was then passed through the column. The adsorbed protein was removed with repressor buffer II containing 0.25 M NaCl. The yellow protein-containing fractions were pooled, and 2 volumes of saturated ammonium sulfate was added to precipitate the bulk of the protein. The precipitated protein was collected by centrifugation for 10 min at 10,000 rpm in a Sorvall GSA rotor. The protein was dissolved in 25 ml of repressor buffer III (0.01 M potassium phosphate [pH 7.2], 5% glycerol, 0.5 M dithiothreitol, and 1 mM L-arginine hydrochloride repressor.

A 2-ml amount was dialyzed overnight against repressor buffer IV (0.01 M Tris-acetate [pH 8.2], 14 mM magnesium acetate, 50 mM potassium acetate, 0.7 M dithiothreitol, and 5% glycerol) so that the repressor activity could be determined, and the remainder was dialyzed overnight against 1 liter of repressor buffer III with one buffer change. This fraction was referred to as the crude diethylaminoethyl extract.

A 65-mg amount of crude DEAE extract protein in repressor buffer III was added to a 3.5-ml double-stranded calf thymus DNA-cellulose column prepared by the method of Alberts and Herrick (1) and previously equilibrated with repressor buffer III. The column was washed with 30 ml of repressor buffer III to remove any weakly adsorbed protein, and the protein was eluted first with repressor buffer III containing 0.15 M NaCl and then with repressor buffer III containing 0.4 M NaCl. The fraction eluting between 0.15 and 0.4 M NaCl, which contained all of the recovered repressor activity, was dialyzed overnight against repressor buffer IV. This preparation was then frozen and stored in 200-μl portions in nitrocellulose tubes in liquid nitrogen. This fraction was 65-fold enriched in repressor activity compared with the crude DEAE extract.

RESULTS

Lambda repressor was introduced into the synthesis mixtures to prevent transcription of argA-lacZ initiated from λ promoters. In initial experiments with an S-30 component prepared from strain 514X5a-12-29, addition of arginine repressor extract to synthesis mixtures containing 0.2 mM L-arginine produced a maximum of 50% repression (N. Kelker and T. Eckhardt, unpublished data). When the experiment was done with an S-30 component prepared from strain 514X5a-12-29(pKB252), a maximum of 90% repression was observed. Arginine repressor extract had no effect on β-galactosidase synthesis in either S-30 preparation with λlac5 as the template. These results were interpreted to mean that, in the absence of the λ repressor, there was significant λ-initiated transcription continuing through argA-lacZ that was not subject to regulation by the arginine repressor. This was consistent with the direction of transcription of the fused gene and its location on the λ chromosome, i.e., in the same reading direction as leftward transcripts and distal to gene N (7). To eliminate any extraneous readthrough and obtain a precise measurement of argA promoter-initiated β-galactosidase synthesis, all further experiments were done in the presence of the λ repressor, i.e., with an S-30 component prepared from 514X5a-12-29 (pKB252).

Under these conditions, cell-free β-galactosidase synthesis from the λAZ-7 DNA template was easily detected even though it was approximately one-third that from the λlac5 DNA template (see legend of Fig. 7). After a lag period of 10 to 15 min, λAZ-7 DNA-dependent β-galactosidase synthesis proceeded until 50 min after initiation of synthesis (Fig. 2). It was directly dependent on the λAZ-7 DNA concentration (Fig. 3).

As noted above, β-galactosidase synthesis from the λAZ-7 DNA template was repressed
Repression brought about by the arginine repressor and 2.0 mM L-arginine was overcome by increasing the λAZ-7 DNA concentration (Fig. 6). Synthesis mixtures were prepared containing either arginine repressor extract, 2.0 mM L-arginine and increasing concentrations of λAZ-7 DNA, or 2.0 mM L-arginine and increasing concentrations of λAZ-7 DNA. In the absence of the arginine repressor, synthesis was directly dependent on λAZ-7 DNA concentration, whereas, in the presence of the arginine repressor, synthesis was reduced at low DNA concentrations and increased to the rate up to 90% by the arginine repressor, whereas λplac5 DNA-directed synthesis was unaffected (Fig. 4).

The effect of L-arginine, the presumed co-repressor (5, 16), on arginine repressor activity was studied by preparing synthesis mixtures containing varying concentrations of arginine and a constant amount of the arginine repressor (Fig. 5). Surprisingly, the amount of β-galactosidase synthesis was nearly normal in the absence of added arginine, indicating the presence of endogenous arginine available for protein synthesis. The arginine repressor was inactive at this low arginine concentration, but, as the arginine concentration increased, β-galactosidase synthesis was increasingly repressed up to a maximum of 90%.

FIG. 2. Time course of β-galactosidase synthesis with λAZ-7 DNA as the template. The DNA concentration was 5.3 μg/0.2 ml of synthesis mixture.

FIG. 3. β-Galactosidase synthesis as a function of λAZ-7 DNA concentration.

FIG. 4. β-Galactosidase synthesis as a function of added arginine repressor extract. Each synthesis mixture contained 2 mM L-arginine hydrochloride and either repressor buffer IV or repressor buffer IV containing repressor extract. Maximal enzyme synthesis, i.e., the activity of β-galactosidase synthetized in the absence of the arginine repressor, for λplac5 DNA (□) was A405/60 min = 0.774 and for λAZ-7 DNA (○) was A405/60 min = 0.239. The DNA concentration for both templates was 2.65 μg/0.2 ml of synthesis mixture.

FIG. 5. Effect of L-arginine concentration on repressor activity. Synthesis mixtures were prepared as described in the text, except that the L-arginine concentration was varied. Symbols: ○, repressor extract (3.3 μg/0.2 ml of synthesis mixture) added; ●, repressor extract omitted. The λAZ-7 DNA concentration was 2.65 μg/0.2 ml of synthesis mixture. The synthesis mixtures containing all of the components except the S-30 component were incubated, with shaking, for 3 min before synthesis was initiated by adding S-30.
obtained in the absence of the repressor as the DNA concentration increased.

λAZ-7 DNA-directed β-galactosidase synthesis was stimulated by guanosine 3′-diphosphate-5′-diphosphate (Fig. 7), with maximum synthesis at a 150 μM concentration.

DISCUSSION

The use of a phage carrying the lacZ fusion provides a means for studying regulation of synthesis of acetylglutamate synthase, a labile enzyme that very likely would not survive the conditions required for cell-free synthesis. The synthesis of β-galactosidase from the λAZ-7 DNA template is clearly under arginine control since, when extraneous readthrough from λ promoters was eliminated by the addition of the λ repressor, β-galactosidase synthesis was markedly repressed by the arginine repressor and a sufficient concentration of arginine.

The requirement for arginine for repression is in agreement with previous in vivo (16) and in vitro studies (5). In the S-30 system used here, it is not possible to determine other proteins that may be involved, since most, or perhaps all, cell proteins are present. However, Cunin et al. (5), using a purified transcriptional system (17), found that arginine and partially purified repressor, prepared as described in this paper, were sufficient to give 70% repression of argCBH messenger ribonucleic acid. Since the system was free from arginyl-transfer ribonucleic acid and arginyl-transfer ribonucleic acid synthetase, it appears that these are not required for a significant amount of repression.

β-Galactosidase synthesis in the absence of added arginine is apparently due to an endogenous source. Whether this results from arginine remaining in the S-30 component after dialysis to remove components of the preincubation mix, from proteolysis, or from arginine contamination of the other amino acids has not been determined.

Guanosine 3′-diphosphate-5′-diphosphate has been reported to inhibit cell-free synthesis of acetylornithinase (argE) (25) and stimulate synthesis of argininosuccinase (argH) (19). The effect on argA operon expression is clearly stimulatory (Fig. 7). So, in addition to the negative control exerted by the arginine repressor and arginine, argA is subject to a positive control by guanosine tetraphosphate. The nature of this positive control can only be inferred from hypotheses generated from studies in other systems (19, 20, 25), and the relationship, if any, to the negative control observed on argE expression is unclear at this time.

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LITERATURE CITED