Requirement of Adenosine-3', 5'-Cyclic Monophosphate for L-Arabinose Isomerase Synthesis in Escherichia coli

TERUKO NAKAZAWA AND TAKEHI YOKOTA
Department of Bacteriology, School of Medicine, Juntendo University, Bunkyo-ku, Tokyo, Japan

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Adenosine-3', 5'-cyclic monophosphate (cyclic AMP) is essential for the synthesis of L-arabinose isomerase in Escherichia coli. Cyclic AMP appears to be required for the transcription of deoxyribonucleic acid into messenger ribonucleic acid (RNA), since the enzyme synthesis is not observed in induced cells to which cyclic AMP is added after messenger RNA synthesis is arrested by rifampin or after inducer removal.

Adenosine-3', 5'-cyclic monophosphate (cyclic AMP) is an important factor for intracellular regulation. In higher animals, it plays a role in the activation of certain enzymes, such as protein kinases, and has been assumed to be the common mediator for the action of various hormones (20). In bacteria, the nucleotide has been known to be an essential component for the initiation of transcription of several inducible enzymes (14, 18, 23).

Activation of the lactose operon as well as of the galactose operon requires the catabolite gene activator protein (CGA protein or CRP) and cyclic AMP (3, 7, 16). The CGA protein activated by cyclic AMP probably interacts at the promoter region of the lac operon (19). Thus the CGA protein-cyclic AMP complex acts as a general system of positive control for these catabolite-sensitive genes.

The protein product of gene araC (C protein) is required for normal expression of the L-arabinose operon in Escherichia coli (22). If C protein is absent, either because of deletion of the C gene (22) or a nonsense mutation within it (10), the arabinose operon cannot be induced. The C protein is, therefore, a positive regulator of the operon. In addition to its positive control properties, C protein also has repressing properties (6). Thus the control system of the arabinose operon is quite different from the well-known negative control of the lactose operon, where absence of its regulatory protein leads to full expression of the lactose gene (11).

To study the relationship between the specific positive control of C protein and the general positive control of the CGA protein-cyclic AMP complex on the arabinose operon, we examined the effect of cyclic AMP on the synthesis of L-arabinose isomerase, one of the three structural gene products of the arabinose operon. In this study an absolute requirement of cyclic AMP for the synthesis of L-arabinose isomerase will be described. Evidence will also be presented showing that cyclic AMP acts at the level of transcription of deoxyribonucleic acid (DNA) into messenger ribonucleic acid (RNA). Recently, two papers have appeared dealing with cell-free studies on the regulation of the arabinose operon where the requirement of cyclic AMP for the synthesis of L-aribulokinase, another structural gene product of arabinose operon, has been demonstrated in vitro (8, 25).

MATERIALS AND METHODS

Bacteria. E. coli CA7902 (cya, thi) isolated by J. Beckwith (21) and its cya+ revertant, CA7902 (cya+), were used.

Chemicals. L-Arabinose was purchased from Merck, Darmstadt, Germany; isopropyl-thio-β-D-galactopyranoside (IPTG) from Sigma Chemical Co., St. Louis, Mo.; α-nitrophenyl-β-D-galactopyranoside (ONPG) from Calbiochem, Los Angeles, Calif.; MacConkey agar from Eiken, Tokyo, Japan; and Casamino Acids from Difco Laboratories, Detroit, Mich. Cyclic AMP was a gift from Asahi Chemical Industry Co., Ltd., Tokyo, Japan, and rifampin from Daiichi Seikagaku Co., Ltd., Tokyo, Japan. All other chemicals were obtained commercially and were of analytical grade.

Cell growth and induction of L-arabinose isomerase. Single colonies grown on MacConkey plates were transferred into medium LC, a medium containing 0.2% sodium lactate, 0.1% Casamino Acids, and thiamine (5 μg/ml) in M-9 (1), in the presence of 0.1 mM cyclic AMP and incubated at 37°C with shaking for overnight. The rest of the colonies were streaked on MacConkey plates and were confirmed to be pure clones. A sample of the cell suspension was transferred into 20 volumes of fresh
medium LC without cyclic AMP, and cells were grown at 37°C. The preliminary incubation in medium LC with cyclic AMP was omitted when strain CA7902 (cya*) was used. Cells were harvested by centrifugation when the cell population reached about 10⁶ cells per ml. In experiments with strain CA7902, cells were confirmed to be adenyl cyclase-less mutants by growing on MacConkey agar plates, and those preparations which contained more than 5% of cya* revertants were discarded. If the preliminary culture in the presence of cyclic AMP was omitted, more than 30% of cya* revertants appeared when cells were tested on MacConkey agar plates.

The cells were washed once with 0.12 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.0) containing 1mM sodium phosphate, suspended in this buffer at a density of 10⁹ per ml, and then treated with sodium ethylenediaminetetraacetate (EDTA) by the method of Pastan and Perlman (17); the suspension was incubated at 37°C for 2 min with 10 mM EDTA and then diluted with 10 volumes of prewarmed medium LC. Experiments were begun within a few minutes after dilution. All experiments were performed at 37°C. To terminate enzyme synthesis, samples of cell suspension (0.2 ml) were pipetted into tubes containing 10 µg of chloramphenicol at 0°C. At the end of the incubation one drop of toluene was added to each tube, and the tubes were vigorously agitated with a Vortex mixer for 30 s and incubated for 5 min at 37°C with shaking.

Assay of L-arabinose isomerase. The activity of L-arabinose isomerase was determined by the modification method of Cribbs and Englesberg (2). The assay system contained 10 µmol of L-arabinose, 0.1 µmol of MnCl₂, 10 µmol of borate buffer (pH 8.0), and the enzyme, in a total volume of 0.4 ml. The reaction mixture, without the enzyme, in small test tubes was placed in an ice bath, the enzyme was added, and a 0.1-ml sample was removed and pipetted into 0.9 ml of 0.1 N HCl (zero time). The tubes containing the reaction mixture were then placed in a 37°C water bath and an additional 0.1-ml sample was removed after 20 min. Assays for ribulose were conducted on these samples directly employing the cysteine-carbazole test (5). The color produced was determined 30 min after the addition of reagents, using a Klett-Summerson colorimeter with a no. 54 filter. L-Ribulose-β-nitrophenyl hydrazone was employed as a standard, and under our conditions 21.0 Klett units were equivalent to 0.01 µmol of ribulose. The reaction was linear with respect to both time and amount of enzyme added. The unit of enzyme was defined as the amount that catalyzes the formation of 1 µmol of ribulose per min. Specific activities of the enzyme were expressed in terms of milligrams of cell dry weight, which was determined by its relationship to the Klett units of a cell suspension using a Klett-Summerson colorimeter with a no. 66 filter. Increase in cell density from the start to the end of the induction period was less than 10% in each case.

RESULTS

Effects of cyclic AMP and glucose on L-arabinose isomerase synthesis in E. coli

CA7902 (cya*). Cyclic AMP stimulates L-arabinose isomerase synthesis in Tris-EDTA-treated E. coli CA7902 (cya*). In one experiment shown in Fig. 1, enzyme synthesis induced by L-arabinose increased nearly twofold by the addition of 5 mM cyclic AMP. Figure 1 also demonstrates the effect of glucose on induction. When 10 mM glucose and 5 mM L-arabinose were added simultaneously to Tris-EDTA-treated cells, enzyme synthesis occurred in 10 min, after a 5-min lag, but then ceased completely. Cyclic AMP, however, completely overcame this glucose repression and restored enzyme synthesis to a similar extent to the nonrepressed, cyclic AMP-stimulated level. These results are consistent with those recently reported by Katz and Englesberg (12) and suggest a mechanism of control by cyclic AMP in L-arabinose isomerase induction similar to that of other cases of catabolite-sensitive enzyme synthesis.

Effect of cyclic AMP on L-arabinose isomerase synthesis in E. coli CA7902. For the purpose of studying the stimulatory effect of cyclic AMP more precisely, strain CA7902, an adenyl cyclase-less mutant, was used. Figure 2 shows the results of a typical experiment. When Tris-EDTA-treated cells were incubated with L-arabinose alone, no measurable enzyme synthesis occurred for at least 60 min. On the other hand, when the cells were incubated with
L-arabinose in the presence of cyclic AMP, the enzyme appeared after a 5-min lag period, increased gradually in the next 20 min, and then was synthesized at an approximately linear rate over the next 30 min. These results indicate that cyclic AMP is essential for the synthesis of L-arabinose isomerase.

In the next experiment, Tris-EDTA-treated cells were incubated at 37°C in medium LC in the presence of cyclic AMP prior to the addition of L-arabinose. In this case, the specific activity of the enzyme increased linearly after the lag period of 3 min (Fig. 3). On the other hand, when preincubation was performed without cyclic AMP as in the case of the non-preincubated culture, there was a lag of approximately 15 min, which accounts for the approximately twofold difference in specific activities at 40 min.

To examine whether these effects of cell preincubation with cyclic AMP (i.e., shortening of the lag period and the stimulation of the rate of enzyme synthesis) were specific for L-arabinose isomerase induction, experiments were conducted on β-galactosidase induction using the same Tris-EDTA-treated cells as in Fig. 3. As shown in Fig. 4, when cells were preincubated with cyclic AMP at 37°C for 30 min, and then IPTG was added, the time course of β-galactosidase induction was essentially the same in the preincubated and non-preincubated cultures, and it was approximately linear. Thus the effect of cyclic AMP in preincubation appears not to be the general phenomenon for the synthesis of inducible enzymes.

The effect of varying the concentration of cyclic AMP on L-arabinose isomerase synthesis is shown in Fig. 5. A half-maximal effect of cyclic AMP was observed at about 0.1 mM. We routinely used 5 mM cyclic AMP in later experiments to ensure a maximal effect. L-Arabinose isomerase production was also dependent on L-arabinose concentration (Fig. 6). In the presence of excess cyclic AMP, the half-maximal synthesis was produced by about 0.6 mM L-arabinose.

Site of cyclic AMP action. Cyclic AMP may be required for L-arabinose isomerase synthesis at the level of transcription of DNA into messenger RNA, translation of messenger RNA into protein, or the conversion of inactive precursor to active enzyme. Cyclic AMP may also act, either additionally or alternatively, by stimulating the synthesis of active C protein, at the level of transcription, translation, or protein conversion. To investigate the site of cyclic AMP action, Tris-EDTA-treated cells were incubated with L-arabinose for 20 min, rifampin (100 μg/ml) was added to inhibit new DNA-dependent RNA synthesis (15, 24), and the cells were incubated for another 30 min in the presence of cyclic AMP (Fig. 7). If cyclic AMP were required for translation of messen-
ger RNA into protein or the conversion of inactive precursor to active enzyme, the enzyme activity could have been detected even after the addition of rifampin. The fact that cyclic AMP does not produce any L-arabinose isomerase in rifampin-treated cells indicates that the nucleotide is required for messenger RNA synthesis.

In the next experiment, an attempt was made to calculate the half-life of L-arabinose isomerase-specific messenger RNA. Tris-EDTA-treated cells were incubated with L-arabinose and cyclic AMP for 20 min, rifampin was added, and the cells were incubated for another 30 min (Fig. 8). L-Arabinose isomerase production continued for about 4 min after the addition of rifampin and then ceased completely. The half-life of the messenger RNA calculated from a semilogarithmic plot was roughly 2 min.

Additional evidence that cyclic AMP acts on L-arabinose isomerase-specific messenger RNA synthesis comes from studies on the effect of inducer removal. As shown in Fig. 9, cyclic AMP had no effect on enzyme synthesis when added to cells which had been incubated with L-arabinose and cyclic AMP for 20 min and which were then washed to remove the inducer and the nucleotide. The results of this experiment agree well with the rifampin experiments and show that the inducer and cyclic AMP are essential for the synthesis of L-arabinose isomerase-specific messenger RNA.

**DISCUSSION**

Like other inducible enzymes (4), the syn-

![Graph 1](http://jb.asm.org/)

**Fig. 4.** Effect of preincubation on the lag period of β-galactosidase induction in E. coli CA7902. Tris-EDTA-treated cells (0.38 mg/ml) were preincubated for 30 min: (i) in medium LC in the presence of 5 mM cyclic AMP and then 1 mM of IPTG was added at time zero (○); (ii) in medium LC in the absence of cyclic AMP, and then 1 mM IPTG and 5 mM cyclic AMP were added simultaneously at time zero (●). Dashed line represents a culture without preincubation in which 1 mM IPTG and 5 mM cyclic AMP were added simultaneously at time zero (●). At the time indicated, a 0.5-ml portion was removed and toluidinized as described in Materials and Methods. β-Galactosidase activity was measured using ONPG as a chromogenic substrate (9).

![Graph 2](http://jb.asm.org/)

**Fig. 5.** Effect of cyclic AMP concentration on L-arabinose isomerase induction in E. coli CA7902. Tris-EDTA-treated cells (0.41 mg/ml) were incubated in medium LC containing 5 mM L-arabinose and varying concentrations of cyclic AMP. Samples were taken at 30 and 60 min after the start of induction to calculate the rate of enzyme synthesis.

![Graph 3](http://jb.asm.org/)

**Fig. 6.** Effect of L-arabinose concentration on L-arabinose isomerase induction in E. coli CA7902. Tris-EDTA-treated cells (0.43 mg/ml) were incubated in medium LC containing varying concentrations of L-arabinose and 5 mM cyclic AMP. The rate of enzyme synthesis was calculated as shown in Fig. 5.
Fig. 7. Effect of rifampin on the synthesis of L-arabinose isomerase in E. coli CA7902. Tris-EDTA-treated cells (0.38 mg/ml) were incubated from zero time in medium LC containing 5 mM L-arabinose (ARA). Rifampin (RF; 100 μg/ml) was added at 20 min (arrow), after which incubation was continued in the presence of 5 mM cyclic AMP (●). Open circles represent a control culture to which rifampin was not added.

Fig. 8. Effect of rifampin on the synthesis of L-arabinose isomerase during induction in E. coli CA7902. Tris-EDTA-treated cells (0.38 mg/ml) were incubated from zero time in medium LC containing 5 mM each of L-arabinose (ARA) and cyclic AMP. Rifampin (RF; 100 μg/ml) was added at 20 min (arrow), after which incubation was continued for 30 min (●). Open circles represent a control culture to which rifampin was not added.

Fig. 9. Effect of cyclic AMP on L-arabinose isomerase production after inducer removal in E. coli CA7902. Tris-EDTA-treated cells (0.38 mg/ml) were incubated in medium LC containing 5 mM each of L-arabinose (ARA) and cyclic AMP for 20 min, and were then filtered (fil) and washed on a membrane filter (47-mm diameter, 0.45-μm pore size, Millipore Corp.) After washing with M-9, cells were suspended in medium LC (0.36 mg/ml) and were incubated in the absence (O) and presence (●) of 5 mM cyclic AMP.

The effect of cyclic AMP could be at the level of transcription of DNA into messenger RNA, translation of messenger RNA into protein, or conversion of inactive precursor to active enzyme. To distinguish between these possibilities, we used rifampin, an antibiotic which blocks the initiation of messenger RNA synthesis without affecting its elongation or polypeptide synthesis (15, 24). No measurable enzyme synthesis occurred when cyclic AMP was added to cells which were already incubated with L-arabinose and in which new messenger RNA synthesis was arrested by rifampin (Fig. 6). Thus cyclic AMP does not appear to act at a translational step nor does it affect the conversion of an inactive precursor to an active enzyme, but rather seems to act at a site of transcription. Additional evidence, showing that the nucleotide acts at the transcription step, comes from experiments in which cells lower the intracellular level of cyclic AMP in E. coli (13), derepression by exogenous cyclic AMP could be explained by restoration of the nucleotide level within the cells which is indispensable for the enzyme synthesis. Moreover, the absolute requirement of the nucleotide for the synthesis of L-arabinose isomerase was demonstrated using strain CA7902, an adenyl cyclase-less mutant (Fig. 2).
were incubated with L-arabinose and cyclic AMP, and then washed free from the inducer (Fig. 8): cyclic AMP does not stimulate enzyme production if added immediately after the inducer was removed.

The synthesis of catabolite-sensitive enzymes so far examined requires cyclic AMP for the initiation of transcription (14, 18, 23). Evidence presented in this paper indicates that L-arabinose isomerase synthesis also requires the nucleotide at the level of messenger RNA synthesis, which is consistent with the recent studies on the in vitro synthesis of L-ribulokinase, another structural gene product of the arabinose operon (8, 25). In addition to the requirement for cyclic AMP, Greenblatt and Schleif also demonstrated the requirement of C protein for L-ribulokinase synthesis (8).

Although the biochemical basis for the control function of C protein remains unknown, evidence so far obtained indicates that the regulation of the arabinose operon by cyclic AMP is the same as that of the lactose operon. A different kinetic behavior of the induction of L-arabinose isomerase from that of β-galactosidase by preincubation with cyclic AMP, however, might suggest another function of the nucleotide specific for L-arabinose isomerase induction. When Tris-EDTA-treated cells of strain CA7902 were preincubated with cyclic AMP, the lag period was shortened and L-arabinose isomerase was produced linearly at a markedly stimulated rate (Fig. 3). On the other hand, these phenomena were not observed in the case of β-galactosidase synthesis in the same Tris-EDTA-treated cells (Fig. 4). Thus if the cells are depleted of cyclic AMP, they should be supplied previously by the nucleotide to give full induction of L-arabinose isomerase. Since this preincubation effect was observed only in L-arabinose isomerase induction and not in β-galactosidase, it seems that cyclic AMP controls C gene transcription. Whether this is the only site of action of the nucleotide in ara control cannot be determined from our experiments.

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LITERATURE CITED


