Biotinyl 5'-adenylate: Corepressor role in the regulation of the biotin genes of Escherichia coli K-12

(in vitro regulation/operator-repressor-corepressor interaction/corepressor formation)

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ABSTRACT A DNA filter-binding technique was used to study the interaction of the biotin repressor and operator site. From a biotin saturation curve, the concentration for half-maximal binding ($K_{0.5}$) was calculated to be 1 $\mu$M. However, in a study with the in vitro coupled transcription-translation system in which biotin served as the corepressor, the $K_{0.5}$ for repression was 7.1 nM. This marked difference of over 2 orders of magnitude was attributed to the activation of biotin by the partially purified repressor preparation in the in vitro system. The activated product formed from biotin, ATP, and repressor preparation was identified as biotinyl 5'-adenylate by paper chromatography and hydroxamic acid formation. Synthetic biotinyl 5'-adenylate was as effective as biotin in the in vitro system ($K_{0.5}$, 10 nM) and much more effective than biotin in the DNA-binding assay ($K_{0.5}$, 1.1 nM versus 1 $\mu$M). These studies indicate that biotinyl 5'-adenylate has a more direct role in the regulation of the biotin genes than does biotin per se.

A cluster of five genes coding for the enzymes of the biotin biosynthetic pathway is located at min 17 on the chromosomal map of Escherichia coli (1). These genes are transcribed divergently (2) and their expression is coordinately regulated by biotin through repression (3). The repressive action of biotin is mediated through the bioR gene situated at min 89 close to the bfe locus (4, 5).

We have previously described an in vitro coupled transcription-translation system for synthesis of two of the enzymes of the biotin gene cluster, 2,8-diaminopelargonic acid aminotransferase and dethiobiotin synthetase, encoded by A and D genes on the l and r strands, respectively (6). Biotin was effective in repressing the synthesis of both enzymes only in the presence of a partially purified repressor preparation. The specificity for biotin was demonstrated by the inability of various biotin analogs, both natural and synthetic, to function as corepressors. In a preliminary study of the direct interaction of the biotin-repressor complex with the operator site on the DNA by using the DNA filter-binding technique (7, *), we obtained evidence that the true corepressor may not be biotin per se but rather some active form of biotin. In this communication, we present data which indicate that biotinyl 5'-adenylate (biotinyl-AMP) is involved in the regulation of the biotin genes.

MATERIALS AND METHODS

Chemicals. d-Biotin, purchased from Sigma, was recrystallized from water. Biotinyl-AMP was prepared and purified according to the procedure of Lane et al. (8). The preparation was estimated to be 85% pure on the basis of biotin hydroxamate formation, biotin content, and absorption at 260 nm. Paper chromatography in isopropanol/acetic acid/water/ammonium hydroxide, 50:28:20:2 (vol/vol), showed a single UV light-absorbing spot which gave a positive ferric chloride test for biotin hydroxamic acid. Biotin hydroxamic acid was prepared according to the method of Christner et al. (9). $^{32}$P[Orthophosphoric acid and $^{3}$H]ATP (25 Ci/mmol; 1 Ci = 3.7 x 10$^{10}$ becquerels) was obtained from New England Nuclear. $d$-[carboxyl-$^{14}$C]Biotin (59 mCi/mmol) was obtained from Amersham. All other chemicals were of the highest grades commercially available.

Preparation of DNA. $^{32}$P-Labeled $\lambda$ bio DNA was prepared from a heat-inducible phage, $\lambda c 1857b i o A B F C D S 7$, kindly supplied by Max Gottesman. A biotin deletion mutant of E. coli K-12, T5-2, was grown to a density of $4 \times 10^{8}$ cells per ml in a low-phosphate medium (pH 7.3) containing per liter: 10 g of neopeptone, 2.5 g of yeast extract, 5 g of NaCl, 2 g of glucose, 5 $\mu$g of biotin, 5 mg of thiamine, and 1.2 g of MgSO$_4$. The phage lysate was then added to give a multiplicity of infection of 3 and the incubation was continued until a cell density of $1.2 \times 10^{9}$ per ml was attained. After the addition of 10 mCi of $^{32}$P[Orthophosphoric acid, the temperature of the culture was shifted to 42°C for 15 min for phage induction and then returned to 34°C for an additional 3-hr incubation.

The cells were collected by centrifugation, resuspended in 40 ml of phage buffer (10 mM Tris-HCl, pH 7.3/2 mM MgSO$_4$/68 mM NaCl), and lysed with chloroform. After the cell debris was removed by centrifugation, the phage were precipitated with polyethylene glycol at a final concentration of 10% (wt/vol) and 0.1 vol of 5 M NaCl and sedimented by centrifugation at 8500 rpm for 15 min. The phage pellet was resuspended in 10 ml of phage buffer and further purified by centrifugation in a CsCl density gradient (10). The purified phage was extensively dialyzed for 20 hr against a number of changes of 0.015 M sodium citrate, pH 7.0, initially containing 2 M NaCl which was gradually reduced to 0.5 M to prevent osmotic lysis of the phage. The DNA was purified by the phenol extraction procedure and the residual phenol was removed by extensive dialysis against 1 mM Tris-HCl, pH 7.5/0.1 mM EDTA. The DNA concentrations were determined spectrophotometrically at 260 nm using an extinction coefficient of 0.02 cm$^{-2}$/mg. The samples were dissolved in Aquasol II (New England Nuclear) and radioactivity was determined in a Beckman scintillation counter. The total yield was 5–7 mg of DNA with a specific activity of 5–8 x 10$^{6}$ cpm/ug.

Unlabeled $\lambda$ DNA was used in the binding experiments to minimize the binding from protein impurities in the partially purified repressor preparations. The source for this DNA was the helper phage in the double lysogen H105 (c1857s7, c1857s7 rif$^{2+18}$) kindly furnished by J. B. Kirschbaum. This DNA was digested with the restriction enzyme EcoRI; the resulting 9–11-kb fragments were purified; the DNA concentration was determined spectrophotometrically at 260 nm; and the specific activity was determined as described above.


Abbreviations: biotinyl-AMP, biotinyl 5'-adenylate, $K_{0.5}$ half-maximal saturation.
lysogen was also the source for the partially purified repressor protein. The DNA was prepared in the same manner as described above after the helper phage was separated from the defective phage in a CsCl density gradient.

**Repressor Preparation.** We have previously described the preparation of a partially purified repressor protein (6). This preparation was purified an additional 10- to 12-fold by chromatography on a calf thymus DNA-cellulose column. This represents a 550-fold purification on the basis of the biotin binding assay. For the present studies a portion of the DNA-cellulose fraction was subjected either to gel filtration on Sephadryl 200 (Pharmacia) or to isoelectric focusing. The M, 44,000 fraction (preparation I) contained 0.085 mg of protein per ml and had a biotin binding activity of 529 pmol/ml. The pH 7.2 fraction (preparation II), whose protein concentration could not be determined because it was eluted with a buffer containing bovine serum albumin, had a biotin binding activity of 181 pmol/ml. Detailed results of the further purification of the repressor protein will be published elsewhere.

**DNA Filter-Binding Assay.** The membrane filter-binding technique of Riggs and Bourgeois (7) was adopted to measure the specific retention of λ biotin [32P]DNA in the presence of the biotin repressor protein and the corepressor. The reaction mixture contained in a final volume of 0.1 ml: binding buffer [1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM magnesium acetate, 10 mM KCl, 0.2 mM dithiothreitol, and 5 µg of bovine serum albumin], 0.1 µg of λ biotin [32P]DNA, 10 µg of λ DNA, and various concentrations of repressor protein and corepressor (biotin or biotinyl-AMP). In practice, 0.4 ml of the reaction mixture was incubated for 15 min at 25°C and 0.1 ml aliquots were filtered through Schleicher and Schuell 15-mm BA 54 membrane filters presoaked for 1 hr in wash buffer (binding buffer without dithiothreitol and bovine serum albumin). Triplicate samples were filtered simultaneously and each filter was washed with 50 µl of wash buffer. The filters were dried, Aqueous II was added, and radioactivity was determined by liquid scintillation counting. All values are averages of triplicate determinations and were corrected for nonspecific binding (minus biotin) which was about 10% of the total input counts.

**RESULTS**

The interaction of the biotin repressor protein with the operator site was studied with filter-binding technique in which DNA complexed with the repressor protein is retained on the filter, whereas free DNA passes through. The results shown in Fig. 1 indicate that, in the presence of a constant amount of λ biotin [32P]DNA and biotin, the amount of λ biotin [32P]DNA retained on the filter increased with increasing repressor concentration. A plateau was reached at about 40 µl of repressor and corresponds to about 11% of the total input counts above the background. In the absence of biotin, there was essentially little change in DNA retention with increasing repressor concentration. Thus, the repressor protein does not bind to DNA unless biotin is present. The biotin saturation curve shown in Fig. 2 indicates an increase in DNA binding with increasing concentration of biotin up to about 1.0 µM which then levels off gradually. The concentration of biotin giving half-maximal saturation (K0.5) as determined from a double-reciprocal plot (Fig. 2 inset) was 1 µM. When the repression of diaminopelargonic acid aminotransferase synthesis was studied as a function of biotin concentration in the in vitro system the results shown in Fig. 3 were obtained. The K0.5 value calculated from the double-reciprocal plot was 7.1 nM (Fig. 3 inset) which is more than two orders of magnitude lower than that observed in the DNA filter-binding assay.

![FIG. 1. DNA binding to filter as a function of repressor concentration in the presence (O) and absence (A) of 20 µM biotin. Indicated volumes of repressor preparation I were used after 1:100 dilution. The [32P]DNA concentration was 0.1 µg/0.1 ml (5000 cpm).](image1.png)

This marked discrepancy in the K0.5 value between the two assay systems suggested that biotin may have been converted in the in vitro system into a more active form such as biotinyl-AMP. The latter has been shown to be an intermediate in the formation of the biotin-dependent enzymes, converting the apoenzyme to the active holoenzyme by the covalent attachment of the biotin prosthetic group (9). This compound was synthesized and the above experiments were repeated. The results in Fig. 4 show that maximal DNA retention was attained at about one-eighth of the concentration of the repressor protein in the presence of biotinyl-AMP as compared with biotin (Fig. 1). In addition, the binding at the plateau represents 35% of the total input counts compared with 11% for biotin. The biotinyl-AMP saturation curve in Fig. 5 shows a plateau at about 3-4 nM and the concentration for K0.5, as calculated from a double reciprocal plot (Fig. 5 inset), was 1.1 nM. Because the results of the binding experiments suggested that the repressor–operator interaction is much more efficient in the presence of biotinyl-AMP than biotin, it was of interest to determine the effectiveness of biotinyl-AMP as a corepressor in the in vitro transcription–translation system. The data (not shown) were essentially identical with those shown for biotin in Fig. 3, yielding a K0.5 value of 10 nM compared to 7.1 nM for biotin.

**Activation of Biotin.** Although the above data suggested biotin activation in the in vitro system, it was essential to

![FIG. 2. DNA binding to filter as a function of biotin concentration. Repressor preparation II (2.5 µl) was used after 1:5 dilution. The [32P]DNA concentration was 0.13 µg/0.1 ml (2650 cpm). (Inset) Double-reciprocal plot of the data.](image2.png)
demonstrate the formation of biotinyl-AMP with the S-30 extracts in the in vitro studies. In a preliminary experiment, 0.16 μM biotin was incubated with an S-30 extract in the presence of 10 mM ATP and 10 mM Mg²⁺ and filtered through an Amicon PM10 filter. The filtrate when tested in the DNA filter-binding assay gave maximum DNA retention. The same concentration of biotin under identical conditions gave only the background binding. When the filtrate was chromatographed on paper in a number of solvent systems, biotinyl-AMP could not be detected by various methods. Even the use of [14C]biotin did not reveal any new radioactive compound. Because the activating enzymes frequently bind the acyl-adenylate intermediate rather tightly (11), we chromatographed the entire reaction mixture without filtration and again failed to detect biotinyl-AMP formation.

The only other external protein source in the in vitro system was the partially purified repressor preparation, which was absolutely required to demonstrate repression by biotin. The S-30 extract alone, prepared from a bioR⁺ strain, was unresponsive to biotin (6). To determine if the activation reaction was associated with this preparation we tested the effectiveness of ATP in the DNA filter-binding assay with the same biotin concentration used with S-30 extracts. Once again, maximum DNA retention was observed. Thus, we concluded that the filtrate from the S-30 preparation was only supplying the biotin and ATP for activation by the repressor preparation.

As indicated previously, many of the acyl-adenylates are tightly bound to the activating enzymes. When [14C]biotin, ATP, and the repressor preparation were incubated and the mixture was passed through a Sephadex G-25 column, a measurable amount of radioactivity emerged with the protein in the void volume (Fig. 6A). Very little bound [14C]biotin was found in the absence of ATP. When [3H]ATP was used as the marker in the presence of biotin, once again, the radioactivity emerged with the void volume (Fig. 6B). In the absence of biotin the radioactivity was markedly reduced. The DNA fil-

FIG. 3. In vitro repression of diaminopelargonic acid aminotransferase synthesis as a function of biotin concentration. The experimental conditions were as described (6) with the indicated concentrations of biotin. One milliliter of the synthesis mixture contained 16 μl of a repressor preparation purified through the DNA-cellulose stage. This preparation contained 0.53 mg of protein per ml and a biotin binding activity of 1.47 nmol/ml. (Inset) Double-reciprocal plot of the data.

FIG. 4. DNA binding to filter as a function of repressor concentration in the presence (O) and absence (△) of 8 nM biotinyl-AMP. Indicated volumes of repressor preparation I were used after 1:100 dilution. The [32P]DNA concentration and radioactivity were the same as in Fig. 1.

FIG. 5. DNA binding to filter as a function of biotinyl-AMP concentration. Repressor preparation II (2.5 μl) was used after 1:20 dilution. The [32P]DNA concentration was 0.1 μg/0.1 ml (4600 cpm). (Inset) Double-reciprocal plot of the data.

FIG. 6. (A) The reaction mixture in 0.3 ml contained 70 mM Tris acetate (pH 8.2), 20 mM magnesium acetate, 3 mM dithiothreitol, 1 mg of bovine serum albumin, 0.42 nmol of [14C]biotin, and 2.5 μg of repressor preparation (biotin binding, 6.8 pmol/μg) from DNA-cellulose chromatography. The mixture was incubated for 30 min at 30°C in the presence (O) and absence (△) of 3 mM ATP and applied to a Sephadex G-25 column (1 × 23 cm) previously equilibrated with 10 mM phosphate buffer, pH 7.2/0.5 mM dithiothreitol/0.5% glycerol. The column was eluted with the same buffer, 0.5-ml fractions were collected in the liquid scintillation vials, and the radioactivity was measured. (B) The reaction mixture excluding biotin was essentially as above except [3H]ATP (2 nmol) and 25 μg of repressor were used. In addition, an ATP regenerating system containing 70 mM phosphoenolpyruvate and 25 μg of pyruvate kinase was also included. The mixture was incubated for 30 min at 30°C in the presence (O) and absence (△) of 15 μM biotin and applied to a Sephadex G-25 column. Fractions of 0.25 ml were collected and radioactivity was determined as above. V₀, void volume.
The reaction mixture in 0.1 ml volume contained 1.8 mM Tris-HCl (pH 7.6), 36 mM KCl, 1.8 mM magnesium acetate, 1 mM mercaptoethanol, 20 μM EDTA, 0.34 nmol of [14C]biotin, 12 μg of repressor preparation (biotin binding 7.6 pmol/μg) from a DNA-cellulose column, and 40 mM ATP where indicated. After 30 min of incubation at 25°C, 200 μg of synthetic biotinyl-AMP was added and 50 μl of the reaction mixture was spotted on each of two Whatman No. 3 paper strips. The strips were chromatographed in solvent system I [isopropl alcohol/acetic acid/water/ammonium hydroxide (60:28:20:2, vol/vol)] and solvent system II [isolbutyric acid/ammonium hydroxide/water (66:1:33, vol/vol)].

Table 1. Identification of biotinyl-AMP by paper chromatography

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Solvent I</th>
<th>Solvent II</th>
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<tr>
<td></td>
<td>14C</td>
<td>UV</td>
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<tr>
<td>+ATP</td>
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<td>0.36</td>
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<td>−ATP</td>
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*DISCUSSION*

Our present studies on biotin operator–repressor interaction using the very sensitive and specific DNA-binding assay indicated that biotinyl-AMP rather than biotin per se is involved as the corepressor in the regulation of the biotin genes. That biotinyl-AMP is much more effective in complex formation is indicated from the K_{0.5} value, which is three orders of magnitude lower than biotin. In addition, a much lower concentration of repressor is required to saturate the DNA when biotinyl-AMP serves as corepressor. The DNA filter-binding efficiency with biotinyl-AMP is almost 24-fold greater than with biotin, indicating that the operator–repressor complex is much more stable in the presence of biotinyl-AMP.

A significant finding of our experiments is the demonstration that biotin is converted to biotinyl-AMP by the repressor preparation rather than by S-30 extracts. This activated form of biotin is firmly bound to protein because we have been able to isolate the complex on a Sephadex G-25 column. However, the binding is not covalent because trichloroacetic acid treatment of the complex results in no retention of biotin in the precipitated protein, suggesting that biotinylated protein is not involved in the regulatory process.

Our most purified repressor preparations still show biotinyl-AMP-synthesizing activity and reveal several protein bands on disc gel electrophoresis. We have, as yet, been unable to localize either the repressor activity or the activating activity in any of these bands. The simplest explanation for our data would be that biotinyl-AMP produced by the activating enzyme binds to the repressor protein and this complex in turn binds to the operator site. Because we have been unable to detect free biotinyl-AMP in reaction mixtures containing biotin, ATP, Mg^{2+}, and various repressor preparations, we suggest an alternate explanation, that biotinyl-AMP-activating enzyme complex may be serving the corepressor function. However, we cannot exclude the possibility that both functions, activation and repression, are due to the product of the bioR gene. Further purification of the repressor protein will be required to distinguish among these possibilities.

In view of the fact that the DNA filter-binding assay revealed that the activation process is essential in the regulation of biotin biosynthesis, it may be worthwhile to reexamine the question of the true nature of the corepressor in the amino acid regulatory systems with this technique.

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