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Lac repressor purification without inactivation of DNA binding activity

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ABSTRACT

A procedure has been developed which eliminates the commonly observed inactivation of the DNA binding activity of the lac repressor during purification. The operator binding activity of the repressor obtained by this method is 100 ±10%. The repressor can be stored frozen indefinitely without losing its affinity for DNA.

INTRODUCTION

The lac repressor-operator interaction is one of the primary model systems currently used to study DNA-protein interactions and the regulation of gene expression at the transcriptional level. A major difficulty limiting progress in this field has been inactivation of the DNA binding activity of the repressor protein on isolation by currently published methods (1-4). At best, only half of the material appeared to actively bind bacteriophage DNA containing the lac operator (4). This fact, which was generally recognized but not advertised, clearly complicated the interpretation of many experiments designed to determine the molecular basis of the repressor-operator interaction, and may also explain some of the difficulties experienced with crystallization of repressor. In this communication, we report a method which over a two-year period has routinely yielded fully active lac repressor protein, in which a one-to-one binding stoichiometry with operator-containing DNA (λplac or φ80dlac) is observed.

MATERIALS AND METHODS

The purification method used is a modification of the existing procedures (1-4).

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Bacterial Strains and Culture Conditions: Both E. coli strains M96 (i^{sq}, Ref. 5) and BMH74-14 (i^{ql}, Ref. 6) were grown in an inexpensive, modified M9 medium which contained per liter: Na_2HPO_4, 4.3 g; KH_2PO_4, 2.1 g; NaCl, 0.36 g; NH_4Cl, 0.71 g; casamino acids (Difco), 2.6 g; $CaCl_2$, 0.071 mM; $MgSO_4$, 0.71 mM; thiamine, 0.71 ng/ml; and glucose, 1.4 g. Both strains were grown at 32°C until the A_{600} was 1.2; then the medium was quickly heated to 45°C for fifteen minutes; growth was continued at 35°C for four hours; the medium was chilled to 10°C and the cells were harvested and frozen. The yield from 350 liters of culture ranged from 850 to 1000 grams of cell paste.

Materials: Phosphocellulose was fresh Whatman P-11, washed in 0.5 N NaOH and HCl. DNase was from Sigma or Calbiochem, and ammonium sulfate was Schwarz/Mann "ultra pure enzyme grade." The two stock buffers used were as follows: CSB: 0.2 M Tris (pH 7.75 at 4°C), 0.2 M KCl, 10 mM magnesium acetate, 0.3 mM DTT (dithiothreitol), 5% glycerol, 0.1 mM ONPF (orthonitro-phenylfucoside), and 50 μg/ml PMSF (phenylmethyl sulfonyl fluoride). KPG: potassium phosphate buffer to the indicated molarity of total phosphate (made with a 5:1 ratio of K_2HPO_4 to KH_2PO_4 to produce pH 7.4 at 4°C), 0.3 mM DTT, 0.1 mM EDTA (ethylenediamine tetraacetic acid), 5% w/v glucose and 50 μg/ml PMSF.

Repressor Isolation: Approximately 900 g of frozen cell paste were broken up and suspended in 500 ml of CSB, which resulted in spontaneous lysis upon thawing. At all times during this procedure, the temperature of repressor-containing solutions was kept between 0 and 5°C. Ten mg of DNase were added to the lysis suspension to reduce the viscosity, and the pH was adjusted to 7.5. Enough CSB was added to bring the volume up to 1500 ml and the cell debris was pelleted in a Beckman type 19 rotor (2 hours at 15,000 rpm). The supernatant was decanted, and solid ammonium sulfate was added slowly until the solution was 33% saturated (231 g/l of supernatant). Throughout the ammonium sulfate fractionation, the pH was maintained between 7.4 and 7.6 by continuous monitoring with a pH meter and addition of 1 N KOH as required.

The precipitated protein was pelleted in a Sorval GS3 rotor (2 hours at 8,000 rpm), and the pellet was resuspended in 250 ml of 0.075 M KPG.

This fraction was then dialyzed against 20 l of 0.075 M KPG using an Isco rapid dialyzer. Ribosomes and other solid matter were removed by pelleting in a Beckman type 35 rotor (3.5 hours at 35,000 rpm). The supernatant was applied to a 1600 ml phosphocellulose column (6.5 x 55 cm) which was washed with 0.075 M KPG until the A_{280} of the effluent dropped below 0.1. The repressor then was eluted with a 0.075 to 0.4 M KPG gradient (total volume 5.5 l). Elution was followed by measuring A_{280} and IPTG (isopropyl thiogalactoside) filter binding activity (1). In the typical elution profile of Figure 1, two distinct peaks of repressor activity can be seen.

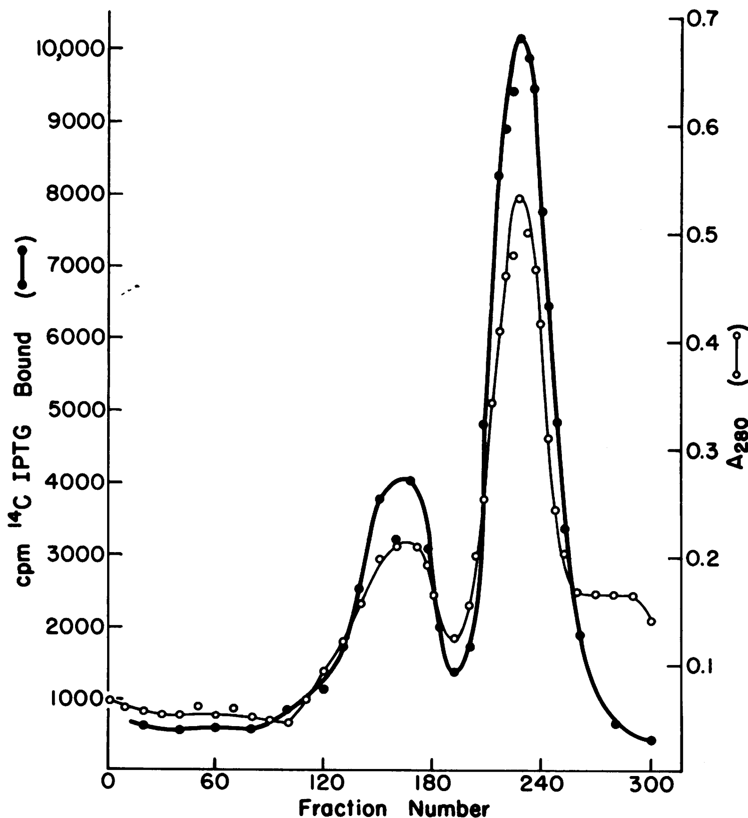

Figure 1. The phosphocellulose elution profile of the lac repressor. The details of the materials and methods are described in the text. The heavy line and closed circles represent the IPTG binding activity of the repressor, while the light line and open circles represent total protein.

In most preparations, fractions from the second (and much larger) peak were pooled, while the rest of the phosphocellulose fractions were discarded. (When there was sufficient material in the small peak, it was pooled separately and purified in parallel with the main peak.) A 0.075 M KPG solution, rendered saturated in ammonium sulfate at 5°C and pH 7.4, was added to the pool until the ammonium sulfate reached 40% saturation. The precipitated protein was pelleted in a Sorval GS3 rotor (1 hour at 8,000 rpm) and the pellet resuspended in 25 ml of 0.1 M KPG. This fraction was passed over a 1500 ml Sephadex G-200 column (5 x 75 cm, reversed flow). Non-IPTG binding material often appeared to pass through in the void volume, while the repressor peak followed. The pooled fractions from this column constituted the final product, which was rapidly frozen in liquid nitrogen (in 0.1 M KPG) and stored at -70°C.

Operator Binding Assays: Repressor-operator binding was measured by the membrane filtration method (7) using the buffers and concentrations of Lin and Riggs (8). Complete binding curves were obtained and the repressor concentration determined from the half-saturation point of the curve. DNA for binding assays was phenol-extracted from either φ80dlac or λplac bacteriophage (9,10). φ80dlac phage were separated from helper phage by banding in a CsCl gradient. Both ^{32}P and ^{3}H thymidine labeling was used at a specific activity of approximately 4 mCi/mg. The purity of the DNA was checked by sedimentation in sucrose gradients; only DNA giving a single, sharp peak was used.

RESULTS AND DISCUSSION

100% active repressor is defined as that which shows a 1:1 binding stoichiometry to pure φ80dlac or λplac DNA. Note that this definition is equivalent to assuming one λplac binding site per repressor tetramer and that one repressor tetramer per DNA molecule is sufficient to cause filter binding. The stoichiometry is calibrated from the A_{260} and A_{280} of the DNA and protein solutions respectively. Values of the extinction coefficient of the repressor, $E^{1\%}_{280}$, have been reported in the recent literature as ranging from 5.9 (11) to 6.9 (12). We have used the most recent value of 6.05 (13). (Using the older value of 6.9 would have given more than 100% activity.)

To date, ten preparations using the procedure outlined above or minor variations of it have been carried out in this laboratory, with eight of those preparations yielding 100 ±10% active repressor. Identifiable mishaps during the two other extractions resulted in approximately 50% active material, confirming the lability of this protein. An additional preparation using our procedure has been carried out independently in another laboratory, and it also resulted in 100% active repressor (K. Matthews and R. O'Gorman, Rice University, personal communication).

The principal modifications in the method which we think are important are: the addition of (A) PMSF and (B) glucose to the buffers. Glucose was chosen because it was a polyglycol that was also an effector of the repressor, i.e., an anti-inducer. (C) Avoiding extremes of repressor concentration, i.e., keeping it between 0.2 mg/ml and 30 mg/ml and preferably near 10 mg/ml. (D) Careful control of temperature and pH. (E) The gel filtration step after phosphocellulose chromatography.

The yield of repressor from one 350-liter culture varied from 350 to 1000 mg with the highest yield being correlated with maximum air flow during cell growth and induction. The purity of the protein was checked by SDS-polyacrylamide gel electrophoresis. Only a single band was seen, even with highly overloaded gels on which a 2% impurity should have been apparent.

Two peaks of repressor activity were usually seen (Figure 1), but their significance is unclear: re-chromatography on phosphocellulose of combined aliquots from both peaks yielded only a single peak. The operator binding activity and operator dissociation rate (14) were the same for repressor samples from both peak fractions. Experiments to determine the significance of these two peaks are in progress; one current working hypothesis is that of an octamer-to-tetramer equilibrium.

The stability of the operator binding activity depends upon the method of storing the repressor: when kept frozen at -70°C, the repressor is stable for a year or more. However, repressor appears to be metastable in solution, and under certain handling conditions its activity can fall rapidly, often plateauing at about 50%. For this reason, it is important to monitor the activity of repressor during the course of any experiment.

In conclusion, the method reported here has routinely yielded fully active lac repressor. It has been repeated many times in this laboratory and independently checked in another laboratory. The protein can be stored frozen indefinitely in this highly active form. The availability of fully active repressor should accelerate experiments which will lead to an understanding of its structure and function.

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