Biochemistry

Volume 15, Number 20 October 5, 1976

Recovery of Operator DNA Binding Activity from Denatured Lactose Repressor†

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ABSTRACT: Lactose repressor monomer subunits, prepared with sodium dodecyl sulfate, have been renatured–reassociated to yield tetrameric repressors active in binding to operator DNA. The majority of molecules in renatured repressor populations have operator binding properties indistinguishable from the stock, undenatured repressors from which they were prepared. Renaturation of a mixture of monomers from wild-type (Q) and a tight binding repressor yields a population of active repressors containing molecules with operator binding characteristics intermediate to those of the "parental" repressor types.

Weber and Kuter (1971) demonstrated several years ago that the inducer binding activity of the lactose repressor could be recovered following denaturation with sodium dodecyl sulfate. The renaturation procedure involved removal of sodium dodecyl sulfate with an ion-exchange resin in the presence of 6 M urea. More recently Horiuchi and colleagues (Hamada et al., 1973) have shown that, in the presence of low concentrations of sodium dodecyl sulfate, the 7.2S tetrameric repressor (Müller-Hill et al., 1971) dissociates into 2.8S subunits.

We present evidence here that repressor subunits, obtained by sodium dodecyl sulfate treatment, can reassociate to form tetramers which are active with respect to operator DNA binding.

Materials and Methods

Sources of wild-type (Q) and the tight-binding repressor (QX86) were the λh80d*lac* prophage strains BMH-461 and QX86/P90, respectively, which have been described elsewhere (Jobe et al., 1974). The λh80d*lac* prophage carrying the tight-binding repressor allele QB11 (Betz and Sadler, 1976) was constructed by Joan L. Betz.

The purification procedure for repressor starting with 85 g of cells was similar to that employed by Laiken et al. (1972) with the two following exceptions. The resuspended ammonium sulfate precipitate (20–35% saturation) was desalted by use of a Sephadex G-100 (Pharmacia) column, and two phosphocellulose (Schleicher and Schuell) column steps were used. The repressor-containing fractions eluting at 0.18 M phosphate from the first phosphocellulose column were pooled, precipitated with $(NH_4)_2SO_4$, dissolved in 10 ml, dialyzed overnight against 0.16 M potassium phosphate containing 10^{-3}M dithiothreitol and applied to a 0.5 × 25 cm phosphocellulose column, which was eluted with 10 ml volumes of 0.16, 0.17, 0.18, 0.19, and 0.20 M phosphate buffers. Repressor-containing fractions (0.18 M phosphate) were pooled again, precipitated with $(NH_4)_2SO_4$, and redissolved in 1.0 ml of 0.5 M Tris[1] (pH 7.8 at 4 °C) containing 20% (v/v) glycerol and 10^{-2} M β-mercaptoethanol, and frozen at −70 °C. The final yield was about 10 mg of repressor from 85 g of cells for the three repressors alike (Q, QX86, QB11). The material was at least 85% pure by polyacrylamide–sodium dodecyl sulfate electrophoresis.

The procedure of Hamada et al. (1973) was employed for the dissociation of the repressor into its monomer subunits. Aliquots of stock repressors (ca. 300 μg of protein) were diluted with distilled water to a final concentration of 5% (v/v) glycerol and brought to 0.03% sodium dodecyl sulfate, with a 3% sodium dodecyl sulfate solution (with a final volume of about 600 μl). The molecular weight markers, *Escherichia coli* alkaline phosphatase (Worthington) and horseradish peroxidase (Worthington), were added to the final concentrations of 12 and 1.2 μg/ml, respectively. After about 30 min at room temperature these mixtures were placed on 12.1-ml glycerol gradients (10–30% v/v), made up in 0.5 M Tris (pH 7.5 at 5 °C), 10^{-3} M dithiothreitol, and 0.03% sodium dodecyl sulfate. The gradients were centrifuged for 24 h at 40 000 rpm at 12 °C in a Spinco SW41 rotor. Fractions (0.5 ml) were collected from the bottom of the tubes and analyzed for absorbance at

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[1] Abbreviations used: EA, ethylenediaminetetraacetate; IPTG, isopropyl thio-β-D-galactoside; Tris, tris(hydroxymethyl)aminomethane.

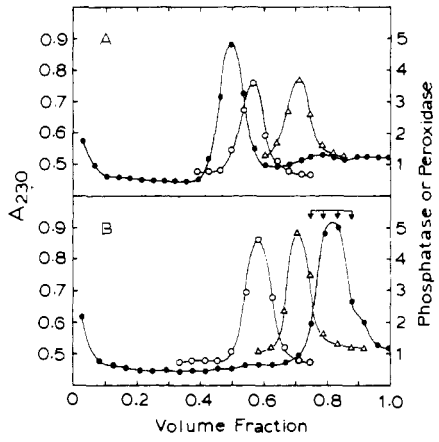

FIGURE 1: Sedimentation of wild-type repressor in the presence and absence of sodium dodecyl sulfate. Gradients were set up and run as described in Materials and Methods. Volumes and absorbancies at 230 nm (●) were measured for all fractions and then aliquots assayed for alkaline phosphatase (○) and horseradish peroxidase (△). Arrows indicate repressor fractions pooled and reserved. A displays the results from a control gradient which received no sodium dodecyl sulfate, while B is that from a gradient containing 0.03% (w/v) sodium dodecyl sulfate.

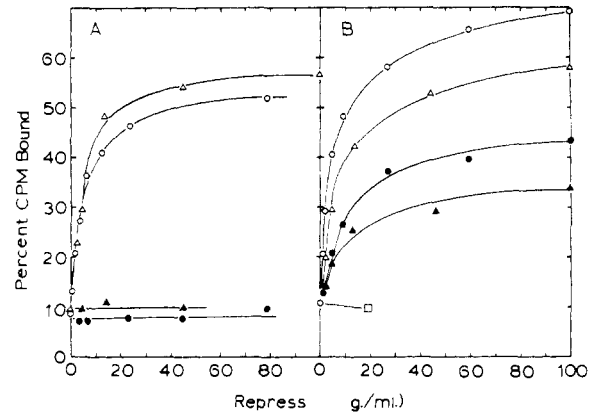

FIGURE 2: Operator binding by renat nd stock repressors. Repressor monomers were prepared and purified igure 1) and then reassociated as given in Materials and Methods. ator binding assays used ^{32}P-labeled O$^+$ operator DNA at 2 × 10⁻ ..l (about 3600 cpm applied per filter). Results are expressed as the percentages of the input DNA bound to filters. (A) Wild-type (Q) repressor. Closed and open symbols show respectively DNA binding in the presence and absence of mM IPTG. Triangles and circles represent respectively DNA binding by the renatured repressor and by the stock repressor from which it was prepared. (B) B11 repressor. Symbols are as in A. The square symbol shows the percentage of labeled DNA retained on the filter in the presence of a 20-fold molar excess of unlabeled λh80d*lac* (O$^+$) DNA plus 10 mM IPTG.

230 nm (repressor) and assayed for alkaline phosphatase (Garen and Levinthal, 1960) and horseradish peroxidase activities (Nakane and Kawai, 1974). Sodium dodecyl sulfate was omitted from the gradient and the repressor sample in control gradients.

Renaturation of repressor from sodium dodecyl sulfate gradients followed the procedure of Weber (Weber and Kuter, 1971). Peak fractions of repressor monomer from the gradients were pooled, brought to 6 M with solid urea, and 10^{-2} M with β-mercaptoethanol. To this was added 200 mg of packed, well-washed 200–400 mesh Dowex AG-1X2 resin. After 10–15 min incubation at room temperature, the resin was removed by filtration through a Millipore NCWP01300 nylon filter. Thereafter the filtrate was iced and dialyzed overnight at 4 °C against 1000 volumes of 0.5 M Tris (pH 7.5 at 4 °C), 10^{-2} M β-mercaptoethanol to remove urea, brought to 20% (v/v) glycerol and stored at −70 °C.

The source of [^{32}P]-λh80 d*lac* (O$^+$) DNA was the strain RV/80 (F$^-$Δ*lac*(x74); λh80CI$_{857}$S$_{t68}$; λh80CI$_{857}$S$_{t68}$ d*lac* (*i*$^+$*o*$^+$*z*$^+$*y*$^+$)). Growth of this strain and the purification of λh80 d*lac* DNA followed the procedures of Betz and Sadler (1975). DNA preparations employed had specific activities of about 10^6 cpm/μg DNA. Between 40 and 60% of input DNA cpm was filter bound in the presence of saturating concentrations of repressor, while less than 10% of input DNA cpm was filter bound in the absence of repressor.

Repressor–operator binding experiments used the nitrocellulose membrane filter procedure of Bourgeois and colleagues (Riggs et al., 1970) including their standard-binding buffer (10 mM Tris, pH. 7.4, 10 mM KCl, 10 mM Mg acetate, 0.1 mM EA, 0.1 mM dithiothreitol, 5% (v/v) dimethyl sulfoxide, and 50 μg/ml bovine serum albumin). Filters, either Schleicher and Schuell B6 or Millipore HAWP (47-mm diameter), were treated as described by Lin and Riggs (1972) before use.

Results

We have found the conditions of Hamada et al. (1973) quite satisfactory for the preparation of monomer subunits of repressor. In the presence of 0.03% sodium dodecyl sulfate, we obtain reproducibly the quantitative dissociation of repressor (wild-type, QX86, or QB11) as exemplified in Figure 1B, while the marker enzymes *E. coli* alkaline phosphatase and horseradish peroxidase appear not to be affected by sodium dodecyl sulfate at this concentration (see Figures 1A and 1B). The dissociated repressor sediments homogeneously as 2.7–2.8S material, this sedimentation coefficient agreeing with that published for the repressor monomer (Müller-Hill et al., 1971).

Weber and Kuter (1971) demonstrated that the inducer binding activity of repressor could be recovered following sodium dodecyl sulfate treatment by removal of the sodium dodecyl sulfate by Dowex AG-1X2 in the presence of 6 M urea. This prompted us to test the recovery of operator DNA binding activity by the same procedure, starting with (purified) repressor monomers. In early experiments, utilizing equal volumes of (packed) Dowex resin and protein solutions, recovery of repressor was low, on the order of 2–10%. Later use of less than 200 mg of resin per ml of protein solution improved the recovery to consistently at least 50% of the protein. In glycerol gradients (without sodium dodecyl sulfate) such as those shown in Figure 1, the renatured material sedimented at 7 S. Operator binding activities of the renatured and stock repressor were assayed simultaneously by titration against labeled operator DNA (Figure 2). The renatured material usually had at least 50% of the stock repressor's operator binding activity and on several occasions it was the same.

The purified QB11 repressor binds substantial amounts of operator DNA even in the presence of 10 mM IPTG (Figure 2B). This property, first indicated by studies on partially purified QB11 repressor (Betz and Sadler, 1976), complicates the measurement of background binding of labeled operator DNA to filters. We determined this background by adding an excess of unlabeled λh80 d*lac* *O*$^+$ operator DNA, thereby following the procedure of Jobe and Bourgeois (1972). In the presence of 10 mM IPTG, a 20-fold excess of unlabeled operator DNA reduces the retention of labeled DNA to the same values as found for the wild-type repressor (Figure 2B). It should be noted that a 50-fold excess of chicken erythrocyte

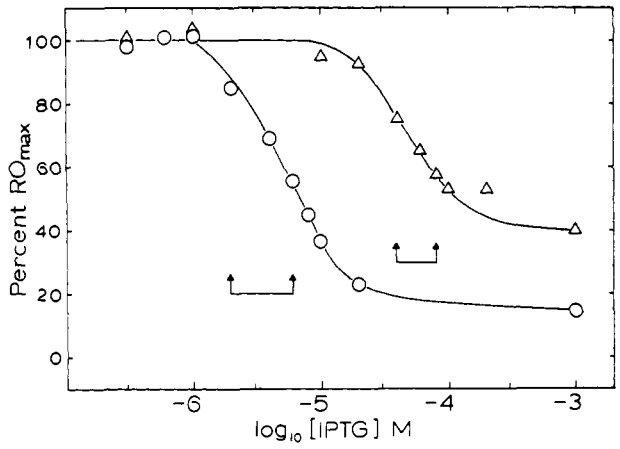

FIGURE 3: IPTG equilibrium release of renatured repressors from operator DNA. Renatured (Q) and (QX86) repressors were prepared as given in Materials and Methods. Sufficient quantities of renatured (Q) (○) or (QX86) (△) repressors were added to 2×10^{-12} M ^{32}P-labeled O^+ operator DNA to give 50–55% retention of label on filters. Volumes (1.0 ml) of this mixture were immediately distributed to tubes and brought to the indicated IPTG concentrations. After at least 40 min incubation, two aliquots were filtered from each tube. Results are expressed as percentages of DNA retention in the absence of IPTG (cpm in the absence of repressor, 14% of RO_{max} has not been subtracted). Arrow brackets give the range of $[IPTG]_{1/2}$ values found separately for stock (Q) ($2–6 \times 10^{-6}$ M IPTG) and QX86 ($4–8 \times 10^{-5}$ M IPTG) repressors.

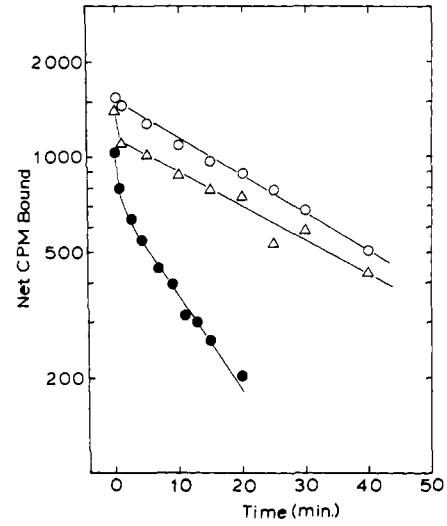

FIGURE 4: Lifetime of renatured repressors on operator DNA. Renatured (△) and stock (○) wild-type (Q) repressors were added, to final concentrations of about 7 ng/ml, to reaction mixtures in BB containing 2×10^{-12} M labeled O^+ operator DNA. At zero time a 50-fold excess of unlabeled operator DNA was added to each of these mixtures and duplicate samples were filtered at the indicated times. Filled circles show the lifetime of renatured B11 repressor on the O^+ operator in the presence of 1 mM IPTG. Background retention of labeled DNA on filters in the absence of repressor has been subtracted (290 cpm per filter).

or λh80 DNA, not carrying the operator, has little or no effect on B11 repressor binding to operator.

Titrations of renatured repressors against operator DNA, such as those in Figure 2, do not show whether the renatured materials have the same operator binding properties as the stock repressors, since these titration experiments are done at operator concentrations ($1–2 \times 10^{-12}$ M) at least 20-fold above the equilibrium dissociation constant of wild-type (Q) repressor ($K_D = 2–5 \times 10^{-14}$ M; Jobe et al., 1972). To test for such operator-binding alterations we measured two properties of the renatured repressors, exchange lifetimes on operator DNA and concentrations of IPTG required to dissociate half of the RO complexes at equilibrium $[IPTG]_{1/2}$.

Figure 3 shows representative IPTG-mediated equilibrium RO dissociation curves for renatured Q and QX86 repressors. Comparison of these curves with the ranges of $[IPTG]_{1/2}$ values found for stock repressors indicates that the renatured repressors do not differ significantly in this respect. It can be seen from the figure that pure QX86 repressor (like B11 repressor) is not completely dissociated from operator by saturating concentrations of IPTG (Jobe and Bourgeois, 1972).

While the data of Figure 3 indicate that the preponderance of the renatured Q and QX86 repressors are normal, this type of experiment would not reveal the presence of small fractions of repressors with altered operator binding properties. Operator binding lifetimes offer a more sensitive test of this possibility. As shown in Figure 4, both stock and renatured Q repressors show 25–28-min exchange half-lives. However, a small fraction (ca. 17%) of the renatured material dissociates from operator much faster. Also shown in the figure is the dissociation of renatured B11 repressor from operator in the presence of mM IPTG. The major half-life seen, of 10 min, is not significantly different from that for stock B11 repressors (Betz and Sadler, 1976), but again a fraction of the repressor (ca. 28%) dissociates more quickly. From these results it appears that a small fraction of repressor molecules obtained by renaturation may sustain some damage. We must note that not all renatured repressor preparations obtained have shown such detectable biphasic dissociation kinetics; moreover, we have seen such biphasic kinetics occasionally in untreated Q repressor stocks. Therefore the damaging event (or events) does not appear to be a necessary or unique concomitant of the denaturation–renaturation process.

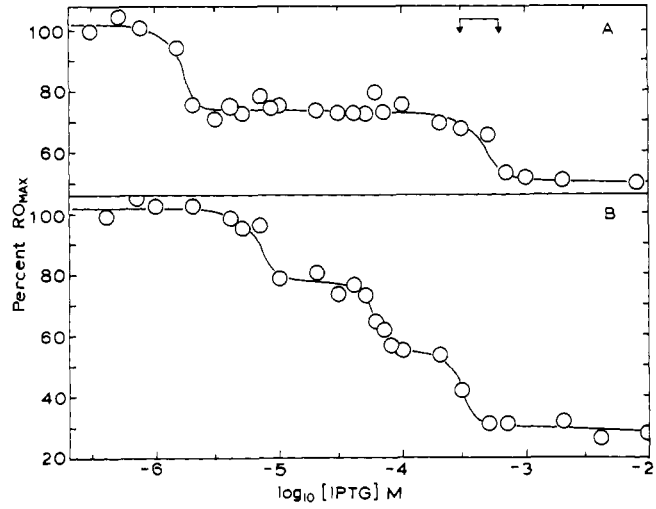

FIGURE 5: IPTG equilibrium release of mixed renatured repressors from operator DNA. (A) Wild-type and B11 monomers were prepared and renatured separately as given in Materials and Methods. The two were then added, in the ratio of approximately 1:1.5 to a single reaction mix containing 2×10^{-12} M labeled operator DNA in sufficient amounts to bind about 50% of the DNA to filters. This mixture was immediately distributed among a series of tubes and these were brought to the indicated IPTG concentrations. After at least 1.5 h incubation, two aliquots were filtered from each tube. Labeled DNA bound to filters is expressed as percentages of that bound at zero IPTG concentration. Background retention of labeled DNA (ca. 18% of RO_{max}) has not been subtracted from the data. Arrows indicate the range of midpoint values for IPTG release found for stock B11 repressors in separate experiments. (B) Similar preparations of wild-type and B11 monomers as used in A were mixed at approximately 2:1 and renatured together. The resulting mixed repressor preparation was then utilized for operator binding as described in A above.

Figure 5A shows the IPTG-mediated equilibrium release from operator of a mixture of separately renatured Q and B11 repressors. This profile serves as the control for the results obtained when Q and B11 monomers are mixed and renatured together, as shown in Figure 5B. Clearly the joint renaturation of mixtures of two kinds of monomers results in active repressors with new operator binding properties, intermediate to those of the "parental" repressor types. It also provides an independent demonstration that we are, in fact, obtaining repressor subunits by the procedure employed here; beyond that, the data presented in Figure 2 suggest the presence of three repressor types, with respect to operator binding, in the mixed tetramer population. Similar results were obtained with Q: QX86 mixed tetramer populations.

Discussion

In the present work we have found that the operator binding activity of lactose repressor can be recovered in excellent yields from repressor monomers purified in sodium dodecyl sulfate–glycerol gradients. Renatured–reassociated repressor tetramer populations generally show the same operator binding characteristics as the untreated stock repressors from which they were prepared. This technique offers a useful tool in the analysis of the repressor–operator interaction, particularly with regard to mixed tetramers, composed of normal and mutationally or chemically altered subunits.

As shown in Figure 5, the joint renaturation of two types of repressor subunit (Q and B11) leads to active repressor with operator binding properties intermediate to those of the parental repressor species. This outcome suggests that more than one repressor subunit is involved in the operator interaction.

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Fidelity of Chromatin Transcription in Vitro†

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ABSTRACT: Chromatin and DNA from Schneider's *Drosophila melanogaster* cell line 2 were transcribed in vitro with *Escherichia coli* RNA polymerase. Using mercurated UTP as precursor, the newly synthesized RNA could be separated from DNA and endogenous RNA by affinity chromatography on sulfhydryl-Sepharose 6B. Characterization of the transcription products with complementary DNA (cDNA) made from polyadenylated nuclear RNA and with fractionated cDNA probe demonstrated a fair quantitative fidelity in the in vitro transcript from chromatin which was not evident when DNA was transcribed. However, as shown by hybridization to total nuclear RNA, *E. coli* RNA polymerase transcribed both DNA strands from chromatin in vitro. We conclude that *E. coli* polymerase is able to distinguish sections of chromatin at which rapid synthesis of RNA occurs in the cell.

Early attempts to measure the fidelity with which chromatin is transcribed in vitro used relatively crude hybridization competition methods. It was concluded that the RNA made in vitro differed according to the source of the chromatin transcribed and resembled that in the cell or tissue from which it was prepared (Paul and Gilmour, 1968; Smith et al., 1969; Huang and Huang, 1969; Bekhor et al., 1969; Tan and Miyagi, 1970). However, since hybridization conditions employed allowed only the reaction of repetitive sequences, no precise conclusions could be drawn concerning the overall fidelity of in vitro transcription.

A more sensitive approach measures the concentration of a specific sequence in the in vitro transcript by hybridization to a radioactive probe (cDNA[1]) complementary to the se-

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‡ Recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

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[1] Abbreviations used: cDNA, complementary DNA; R_0t, product of the total RNA concentration (R_0) in molarity of nucleotides and time (t) in seconds; EDTA, ethylenediaminetetraacetate disodium salt; SSC, standard saline–citrate; Tris, tris(hydroxymethyl)aminomethane; poly(A), poly(adenylate); poly(U), poly(uridylic acid); PBS, 0.14 M NaCl–3 mM KCl–9.5 mM Na_2HPO_4–1.5 mM KH_2PO_4; TNE, 10 mM Tris-HCl (pH 7.4)–100 mM NaCl–1 mM EDTA.