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Site-specific DNA-affinity chromatography of the lac repressor

Glenn Herrick

Department of Cellular, Viral and Molecular Biology, College of Medicine, University of Utah, Salt Lake City, UT 84132, USA

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ABSTRACT

To test the feasibility of site-specific DNA-affinity chromatography, E. coli lac repressor was bound to an operator-containing DNA column, and in parallel to a non-operator DNA column. Salt gradient elution shows: 1) elution from non-operator DNA was near 250mM KCl or NaCl; interpretation of this result suggests the usefulness of the procedure for studying salt-dependence of DNA-protein affinities; 2) elution from operator-containing DNA was delayed (average elution = 1000mM salt), demonstrating a feasibility of site-specific DNA-affinity chromatography, if one provides a sufficiently favorable ratio of specific to non-specific DNA binding sites; 3) repressor eluted from operator-containing DNA over a very broad salt range, which may represent chromatography-generated repressor heterogeneity.

INTRODUCTION

Site-specific DNA-affinity chromatography should be a useful tool for detecting, purifying and studying site-specific DNA-binding proteins. The procedure has not often been used, however, probably due to the complication of non-specific DNA binding: by analogy with well-known site-specific DNA-binding proteins, it might be assumed that in general any such protein will also show a weak affinity for non-site DNA, which in most situations is present in vast excess over the site. Because the lac repressor interactions with operator and non-operator DNA are so well characterized, I chose this system for test experiments of site-specific DNA-affinity chromatography.

The procedure is to apply protein to each of two columns, one with only non-specific weak sites (w) and the other with both w and specific sites (s), both in excess of the binding protein ($w \gg w_o$; $s \gg s_o$). The bound proteins are then eluted with identical salt gradients, and the eluants analyzed for proteins. Salt elution (KCl or NaCl) was chosen because most DNA-binding proteins do seem to bind at least in part electrostatically to the negative charges of the DNA-phosphate backbone (1). Both the operator and non-operator affinities of lac repressor are weakened dramatically by salt (2-6).

The success of the technique requires that the protein of interest elute at a higher salt from the w+s column than from the w column: $u^s > u^w$. A very simplified view of chromatography helps make this understandable in terms of binding constants. For a given set of conditions, the number of column volumes of elution required to bring a ligand to the bottom of a column (V_e) is related to the concentration of binding sites and the association constant (7): $V_e = 1 + K[sites]$. Hence, as the salt gradient flows through the column, binding weakens, V_e drops to 2 and below, and the protein elutes. The salt concentration where $K[sites] = 1$ and $V_e = 2$ only slightly underestimates the average salt concentration of the peak of the protein profile. For w-site binding at this salt concentration, u^w,

$$K_w = 1/[w]. \qquad (1)$$

For the protein to remain bound to the s-sites on the w+s column at u^w, $V_e > 2$ and $K_s[s] > 1$. Dividing by equation (1) and rearranging,

$$K_s/K_w > [w]/[s].$$

In other words, at u^w the protein will remain bound to the w+s column only if s-binding is stronger than w-binding by a factor larger than that by which the w-sites outnumber the s-sites. The protein should then elute later from the w+s column at u^s. This expectation has been achieved with the lac repressor, using pMB9 plasmid DNA on the w column and pHO-2 DNA for the w+s column. pHO-2 is a pMB9 plasmid (5500 base pairs) with a single effective operator inserted at the Eco RI site (8). If each nucleotide of pHO-2 is considered to be an independent w-site (9), then pHO-2 DNA has 1.1×10^4 w-sites for each s-site.

MATERIALS AND METHODS

DNA matrices: Plasmid DNAs were purified from cleared lysates (10) of chloramphenicol-induced (11) E. coli HB101 (pMB9) or (pHO-2) (8) obtained from J. R. Sadler. DNA-celluloses were prepared as described previously (1), but included ultraviolet irradiation in absolute ethanol (12); 30% of input DNA was bound, giving a DNA concentration of 2.0mM nucleotides, assuming 50% of the packed volume is liquid. DNA-sepharoses were prepared by the method of Arndt-Jovin et al. (13), using Sepharose CL-2B (Pharmacia); 50% of input DNA was bound to give 0.30mM nucleotides, assuming no dead volume.

Chromatography: Columns were poured in plastic pipets with nylon wool plugs. Columns were used repeatedly with no effect, despite the presence of Mg^{++} in the buffer, which was the binding buffer (BB) of Riggs et al. (14):

10mM tris-HCl, pH 7.4, 10mM KCl, 10mM magnesium acetate, 0.1mM Na_3EDTA, 0.1mM dithiothreitol, 5% v/v dimethysulfoxide, 50 ug/ml bovine serum albumin (Pentex, stock heated 30' 65°C to destroy nuclease). Columns were stored between uses in BB + 2.5M KCl. Prior to use the columns were rinsed with 5-10 column volumes of BB-no KCl. Repressor was chipped from the frozen stock and diluted in BB-no KCl just prior to loading. Following loading, columns were rinsed with at least 5 column volumes of BB-no KCl. Identical gradients of KCl in BB were simultaneously pumped onto the two parallel columns. Gradients were generated with a three-vein peristaltic pump, one vein pumping from the high salt reservoir to the mixing chamber, the other two from the mixing chamber to the columns. The gradients were monitored by conductimetry. All operations were at 4°C.

Repressor: E. coli DH9(pHIQ3) (15) was grown in a high-density fermentor (16). From 94 gm of cells 39 mg of repressor was obtained by the method of Rosenberg et al. (17). The preparation was judged > 95% pure by SDS gel electrophoresis. The fresh material was 30% active in binding operators (repressor excess) in the filter-binding assay, assuming the molar extention coefficient of Butler et al. (18). No endonuclease was detected when 1.4 ug of supercoiled pMB9 was incubated with 0.40 ug of repressor in 50 ul of BB, 6 hr 20°C. Repressor-operator complexes decayed with a single off-rate ($t_{1/2}$ = 120 min.), measured by exchange (19). The preparation was quick-frozen and stored at -80°C. Assay of this material 5 months later, at the end of chromatography studies, showed 22% operator binding activity and a faster but still single off-rate ($t_{1/2}$ = 48 min, linear between 0 and 92% decay).

Filter binding assay: The repressor assay of Riggs et al. (2) was used as described by Betz and Sadler (16), except 190 ul of 200 ul assays in BB were filtered through 24 mm Schleicher and Schuell BA85 disks. The operator concentration was 6×10^{-11} M, in the form of pHO-2, which was labeled with [^{3}H] thymidine in vivo in a thy$^-$ strain (1.60×10^6 cpm/pmole of pHO-2). More than 90% of the [^{3}H] pHO-2 can be bound by stoichiometric amounts of repressor. Introduction of salt in assaying column fractions did not interfere significantly with the assay (15% inhibition by 50mM KCl). Each set of assays included a control with excess repressor and 1mM isopropyl-thiogalactoside, which routinely reduced counts bound to background.

RESULTS AND DISCUSSION

The gradient elution profiles of lac repressor from pMB9 and pHO-2 DNA

cellulose columns are shown in Figure 1. Repressor elution from the pMB9 column peaks at 160mM KCl, with considerable tailing, reflected in a mean of 220mM KCl (u^w). This tailing is probably not an artifact of column geometry or imperfections, having been observed with a variety of columns. It may reflect non-specific sites with unusually high affinities for repressor; such sites have been detected by other techniques (20-22).

Other approaches have already been developed to study lac repressor's dependence of K_w on u (5,6,23). The current work was not designed to obtain such data (these gradients are too steep), but the results, while crude, do demonstrate that the gradient elution procedure could be used to study u-dependence of K_w in a "window" not readily attainable by the other procedures (higher u/lower K_w, varying [w]). In a variety of conditions (flow rate, gradient steepness, column geometry, column matrix - see Figure 2 below), u^w

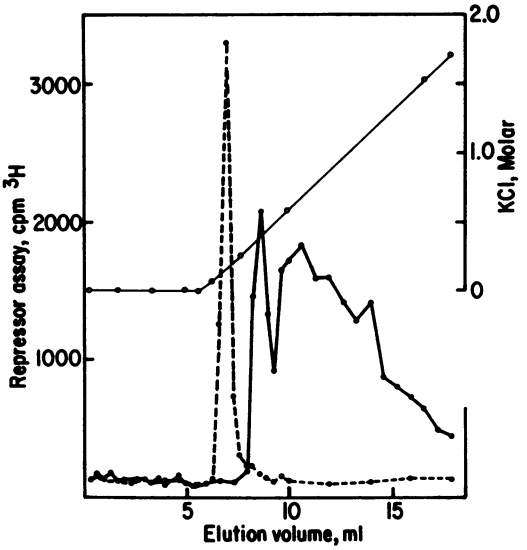

Figure 1. DNA-cellulose chromatography of lac repressor. Either pMB9 DNA-cellulose (dashed line) or pHO-2 DNA-cellulose (heavy line) was loaded with 2.0 pmoles of repressor (assayed by filter binding); the column volumes were 0.50 ml (7 cm tall), containing 0.50 umoles of DNA nucleotides and, for pHO-2 DNA, 46 pmoles of operator. After the rinse with BB-no KCl, identical KCl gradients were applied (thin line). The flow rate was 0.74 column volumes/hour. Aliquots of indicated fractions (3 ul for pMB9, 10 ul for pHO-2) were subjected to the filter binding assay for repressor. Total filter-bound counts are indicated. Recoveries of repressor were 17% (pMB9) and 32% (pHO-2); most of the activity loss occurred in the eluted fractions, the earliest of which were stored 4 days on ice prior to assay.

ranged from 180 to 360mM (average of six experiments = 245mM). This is consistent with statements in the literature (20,24) and with an extrapolation of the data of Lin and Riggs (3), where $K_w[w]$ is set = 1, yielding a predicted u^w = 280mM.

Record and collaborators (4) have shown that Mg^{++} is not necessary for repressor binding, and in fact competes with repressor for binding, especially at low ionic strength. In the conditions of Fig. 1, but using Record's T buffer, which lacks Mg^{++}, and NaCl elution, u^w = 250mM (not shown), which does not differ significantly from u^w = 220mM KCl in Binding Buffer, which contains 10mM Mg^{++}. This substantiates that Mg^{++} is not required for repressor binding. Extrapolation of the Record group's data on NaCl sensitivity of K_w in T buffer (4) to $K_w[w] = 1$ yields u^w = 230mM NaCl.

At u^w = 245mM, one can extrapolate from the data of Lin and Riggs (3) that $K_s = 1.4 \times 10^{11} M^{-1}$. (Extrapolation of Winter's data (25) to 245mM KCl (no Mg^{++}) gives $K_s = 8.2 \times 10^{11} M^{-1}$.) Taking $K_w[w] = 1$ gives $K_w = 5.0 \times 10^{2} M^{-1}$, or 2.8×10^{8} times weaker than K_s, which is much greater than the ratio of sites (1.1×10^{4}). Thus, repressor should remain bound to the pHO-2 column at u^w. Fig. 1 shows this is true and demonstrates that site-specific DNA-affinity chromatography does work in this particular case.

While repressor does elute later from pHO-2 than from pMB9 DNA, the elution profile is very broad and begins to rise soon after u^w and tails to very high salt (Fig. 1). The average repressor eluted at 980mM KCl. This phenomenon is insensitive to flow rate over a 13-fold range: at 0.09 column volumes per hour, u^s = 1080mM; at 0.44, 890mM, 1000mM, 1080mM, 1100mM; at 1.2, 900mM (average u^s = 1010mM). Operator saturation does not seem to explain the broad profile, since no difference was observed when repressor was loaded at either 1% or 10% saturation of operators on the column (not shown). Again in agreement with deHaseth et al. (4), Mg^{++} shows no effect, with u^s = 900mM NaCl in T buffer, but otherwise as in Fig. 1 (not shown).

These results suggest that the broad profile reflects some heterogeneity in the repressor-operator complexes found on the pHO-2 column. Heterogeneity of operators seems unlikely. First, while repressor binding can be affected by super-helicity of operator plasmids, pHO-2 DNA was chosen for this work because its operator-repressor complex is equally stable whether supercoiled or relaxed (8). However, it could be argued that operators are otherwise distorted or even UV-damaged on the pHO-2 DNA-cellulose. Therefore, pHO-2

(and pMB9) DNA-sepharose was prepared, since it is quite different in its method of preparation and the nature of the DNA matrix linkage (13). Nonetheless, the same broad elution profile was observed with u^s = 1080mM KCl (Fig. 2).

The possibility of repressor heterogeneity remains open. Macroscopic repressor aggregation is a noted problem with lac repressor preparations (e.g., 26), especially in Mg^{++} (6), and it might be argued that the broad elution is a result of slow solubilization of such aggregates and has no bearing on K_s; such an argument does not explain the normal elution from non-operator pMB9 DNA, nor the absence of a Mg^{++} effect.

As noted by Rosenberg et al. (17), most lac repressor preparations do not show full operator binding capacity. While careful precautions were taken to avoid repressor inactivation in the preparation of the repressor used in the current experiments, 22 to 30% of the repressor, taken as tetramers, was capable of binding pHO-2 DNA in the standard filter binding assay ($6x10^{-11}$M operators). However, unless the act of chromatography regenerates active repressor, the inactive repressors should not contribute to the elution profile, which was monitored by the filter binding assay. Examination of

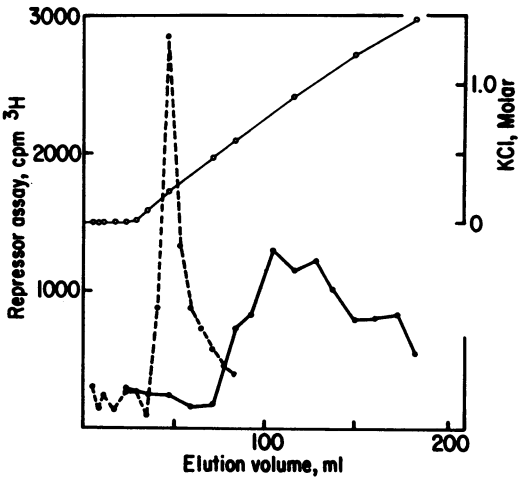

Figure 2. DNA-sepharose chromatography of lac repressor. Symbols and buffers as Fig. 1. Columns were 10 ml packed volume (20 cm tall), containing 3 umoles DNA nucleotides, and for pHO-2 DNA, 270 pmoles of operators, onto which 11 pmoles of repressor were loaded. The flow rate was 0.09 column volumes per hour. Assays were performed on 10 ul aliquots. Recoveries were 20% (pMB9) and 47% (pHO-2).

lifetimes of filter-bindable repressor/pHO-2 complexes shows only a single off-rate ($t_{1/2}$ = 48 min.) when the repressor stock was tested after the final chromatography experiment was performed. This argues against heterogeneity in the "active" fraction of the repressor prior to application to pHO-2 columns.

Possibly the chromatography procedure generates this heterogeneity. For example, some combination of moderate ionic strength and distortions of operator-bound tetramers might cause subtetramers to form. Riggs, Suzuki and Bourgeois (14) observed a subtetramer (dimer?) in old repressor preparations which eluted early from phosphocellulose. This was not filter-assayable, but it should be noted that even the tetramer formed relatively weak operator complexes ($t_{1/2}$ of complex = 19 min., ref. 2). Furthermore, paired subunits appear to repress *in vivo* (27). It seems possible that subtetramers generated from contemporary tetramers might indeed be filter-assayable and might contribute to early eluting fractions from pHO-2 columns; a distinct peak of varying prominence has been repeatedly observed at 400mM salt (a moderate example is seen in Fig. 1), which might represent some stable subtetramer repressor. Large scale columns and examination of properties of eluted repressor are suggested.

Despite the complication of apparent *lac* repressor heterogeneity, these experiments do demonstrate the feasibility of site-specific DNA-affinity chromatography. The procedure might be used in a variety of ways. First, given a cloned DNA sequence of interest, one should be able to search for proteins which bind it specifically. Second, alternate cycles of w+s and w chromatography should provide a powerful purification strategy for site-specific DNA-binding proteins. Finally, refinement of the procedure and its theoretical treatment should provide a useful complement to existing approaches to studying ionic strength dependence (or any other gradient parameter) of binding constants.

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