**In Vitro Interaction of the *Escherichia coli* Cyclic AMP Receptor Protein with the Lactose Repressor**

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Sedimentation equilibrium studies show that the *Escherichia coli* cyclic AMP receptor protein (CAP) and lactose repressor associate to form a 2:1 complex *in vitro*. This is, to our knowledge, the first demonstration of a direct interaction of these proteins in the absence of DNA. No 1:1 complex was detected over a wide range of CAP concentrations, suggesting that binding is highly cooperative. Complex formation is stimulated by cAMP, with a net uptake of 1 equivalent of cAMP per molecule of CAP bound. Substitution of the dimeric lacI-18 mutant repressor for tetrameric wild-type repressor completely eliminates detectable binding. We therefore propose that CAP binds the cleft between dimeric units in the repressor tetramer. CAP-lac repressor interactions may play important roles in regulatory events that take place at overlapping CAP and repressor binding sites in the lactose promoter.

The *Escherichia coli* lactose (lac) operon has long served as a paradigm of bacterial transcription regulation and is the source of protein-protein and protein-DNA interactions that are useful as models of transcription-regulatory processes in *Archaea* and *Eukarya* as well. The lac promoter region contains an array of binding sites positioned to allow bound proteins to interact in limited and specific ways. Within a 120-base pair sequence are located two binding sites for the lac repressor, two for CAP, and two for RNA polymerase (Fig. 1). Proteins occupying these sites can affect the affinities of other proteins for neighboring sites and can influence, via protein- or DNA-allostery, the catalytic activities of RNA polymerase. For example, under some *in vitro* conditions, lac repressor bound to operator 1 stimulates the binding of RNA polymerase to P1 (1) but appears to inhibit promoter clearance and thus, mature RNA synthesis (2). Under other higher [salt] solution conditions, the binding of lac repressor and RNA polymerase in the P1-operator 1 region appears to be mutually exclusive (Ref. 3 and references cited therein).

Little is known about the interactions of lac repressor and CAP in this regulatory system. The DNA surfaces occupied by CAP and repressor in the CAP site 2/operator 1 region of the promoter are coincident, whereas those in the CAP site 1/operator 1 region appear to be mutually exclusive (Ref. 3 and references cited therein).

EXPERIMENTAL PROCEDURES

**Proteins**—CAP was prepared as previously described (5, 7). It was homogeneous as judged by SDS-PAGE, 45% active in camp-dependent binding to lac promoter CAP site 1 and >90% active in nonspecific DNA binding. Samples of two preparations of wild-type lac repressor and two preparations of lacI-18 mutant repressor were kindly provided Dr. Kathleen Matthews. The wild-type repressor was >95% pure as judged by SDS-PAGE and >50% active in lac operator binding. The lacI-18 mutant repressor was homogeneous by SDS-PAGE and was ~30% active in lac operator binding (5, 8). CAP and lac repressor concentrations were determined spectrophotometrically using ɛ\text{CAP \(280 \text{ nm} \)} = 3.5 \times 10^9 \text{ M}^{-1} \text{ cm}^{-1} (9) and ɛ\text{Lac repressor \(280 \text{ nm} \)} = 2.2 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1} (10).

**Sedimentation Equilibrium Assays**—Samples were brought to dialysis equilibrium with 10 mM Tris, pH 7.8 at 4 °C, 150 mM KCl, 5 mM MgCl₂, 10 μM dithiothreitol, 1 μM leupeptin, supplemented where indicated with cAMP. Samples were centrifuged to equilibrium in a Beckman XL-A analytical ultracentrifuge equipped with an AN-60 rotor. Absorbance values were measured at 280 nm as functions of radial position. Five scans were averaged for each sample at each rotor speed. The approach to equilibrium was considered to be complete when replicate scans separated by ≥ 6 h were indistinguishable. Solvent densities were measured using a Mettler density meter.

At sedimentation equilibrium, the absorbance at a specified wavelength and position in the solution column is given by Equation 1 (11, 12).

\[
A(r) = \sum_n \alpha_n \exp (\sigma_n (r^2 - r_0^2)) + \zeta
\]

(Eq. 1)

Here \(A(r)\) is the absorbance at radial position \(r\), the summation is over all species \(n\); \(\sigma_n\) is the absorbance of the \(n\)th species at the reference position \(r_0\); \(\sigma_n = M_n (1 - v_p \omega^2)/RT\) with \(M_n\) the molecular weight of the \(n\)th species, \(v\) its partial specific volume (0.737 for CAP, 0.732 for repressor), \(\rho\) the solution density, \(\omega\) the rotor angular velocity, \(R\) the gas constant and \(T\) the absolute temperature. The baseline offset term \(\zeta\) compensates for slight position-independent differences in the optical properties of different cell assemblies. Absorbance scale equilibrium

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1 The abbreviations used are: CAP, cyclic AMP receptor protein; PAGE, polyacrylamide gel electrophoresis.

2 M. Fried, unpublished results.
FIG. 1. Map of the lactose promoter binding sites of CAP, lac repressor, and RNA polymerase. Data from Majors (28), Galas and Schmitz (29), Schmitz (30), and Malan and McClure (31). The repressor binding sites are designated operator 1 and operator 3, respectively. The starting points for transcripts from promoters P1 and P2 are indicated by the arrows. To improve clarity, the P2 binding site of RNA polymerase, located 22 base pairs upstream from P1, is offset vertically. The scale gives residue numbers with respect to the start of transcription of promoter P1.

RESULTS AND DISCUSSION

Sedimentation Analysis Shows that CAP and lac Repressor Form a 2:1 Complex in the Absence of cAMP—Representative sedimentation profiles of lac repressor and CAP, obtained at 4 °C and 18,000 rpm, are shown in Fig. 2A. The solid curves through the data are global least-squares fits of the expression for a single species (Equation 1 with n corresponding to lac repressor or CAP, as appropriate) to six data sets (2 concentrations, 3 rotor speeds) for each protein, obtained using the program NONLIN (34). The small, symmetrically distributed residuals demonstrate the compatibility of the single-species model with the data. The molecular weights returned by these analyses were 48,580 ± 1,760 for CAP and 153,498 ± 6,950 for lac repressor. The agreement with the molecular weights derived from sequence (Mc (CAP dimer) = 47,238; Mc (lac repressor tetramer) = 154,520) indicates that neither protein is significantly degraded nor aggregated, under our experimental conditions.

Solutions containing both CAP and lac repressor contained additional species that gave weight-average molecular weights significantly greater than those expected for noninteracting proteins. Shown in Fig. 2B is data acquired with such a mixture. The smooth curve represents the fit of Equation 1 (with terms for free CAP, free repressor, and a CAP-repressor complex) to the data. In this fit, Mc values of CAP and repressor were fixed but that of the third species was allowed to float, returning a value of 240,820 ± 14,140. This is within error equal to the value (248,996) expected for a 2:1 CAP/repressor complex. The small residuals attest to the compatibility of this model with the data, although the upward deviation of residuals at the bottom of the cell suggests that higher molecular weight species can form at very high [protein]. This result is the first demonstration, to our knowledge, of a direct interaction of CAP with lac repressor, in the absence of DNA.

Other models for the complex were tested, including a 1:1 CAP/repressor complex, a 3:1 CAP/repressor complex, self-association of CAP (with no repressor binding), and self-association of lac repressor (with no CAP binding). All of these models fit the data significantly less well than the 2:1 CAP/repressor model, and all gave larger residuals with nonrandom distributions (results not shown). In addition, a model with terms for

quotients (K) were evaluated by fitting datasets using Eq. 1 with $\frac{a_{cap}}{K a_{cap}}$. Concentration scale values were calculated using $K_{cap} = 1.44K' a_{cap}/2a_c + a_c$, where $a_c$ and $a_r$ are the molar extinction coefficients of CAP and repressor, respectively, and the value 1.44 is the square of the optical path length of the centrifuge cell.

FIG. 2. CAP and lac repressor associate in a 2:1 molar ratio in the absence of cAMP. A, representative data for CAP (C) and wild-type repressor (R), centrifuged to equilibrium at 18,000 rpm and 4 °C. The smooth curves are nonlinear least squares fits to the single-species form of Equation 1. The initial protein concentrations used were [CAP dimer] = 3.9 × 10^{-6} M; [lac repressor tetramer] = 1.4 × 10^{-6} M. B, data for a mixture of CAP and lac repressor centrifuged to equilibrium at 8,000 rpm and 4 °C. The smooth curve is the least squares fit to Equation 1, with Mc values of CAP and repressor fixed and $v_{complex}$ given by the weight average of the partial specific volumes of the constituent CAP and repressor proteins. The molecular weight of the complex was allowed to float. This analysis returned $M_c$ (complex) = 240,820 ± 24,143. The initial protein concentrations used were [CAP dimer] = 2.4 × 10^{-6} M; [lac repressor tetramer] = 1.2 × 10^{-6} M. C, lower: continuous variation (Job) plot (13) showing that the optimal combining ratio for complex formation is 2CAP:repressor. The total protein concentration was fixed ([CAP] + [repressor] = 3.4 × 10^{-6} M), but the mole fraction was allowed to vary as indicated. Upper panel, graph of $M_c$ of the CAP-repressor complex as a function of CAP mole fraction. The horizontal line through the data indicates the value of $M_c$ (248,996) expected for a 2:1 CAP/repressor complex.
Lac Repressor-CAP Protein Interactions

free CAP, free lac repressor, a 1:1 complex, and a 2:1 complex fit the data as well as the 3 species model, but returned values of the concentration term for the 1:1 complex \( (\alpha_{1,1,0}) \) that were within error equal to zero. Whereas this does not rule out the presence of a 1:1 complex in these reaction mixtures, it indicates that it does not accumulate to significant levels.

The continuous variation (Job) method (13) was used to further test the 2:1 complex model (Fig. 2C). Eight samples prepared with total protein fixed \((|\text{CAP}| + |\text{lac repressor}|) = 3.4 \times 10^{-6} \text{ M} \), but ranging in mole-fraction of CAP from 0.2 to 0.9, gave values of \( M_r \) (complex) compatible with a 2:1 molar ratio (mean ± S.D. = 243,600 ± 8,150; Fig. 2C, upper panel). In addition, the amount of complex observed depended on the mole-fraction of CAP, giving a maximum near \( X(\text{CAP}) = 0.7 \) (equivalent to a molar ratio of 2.3 CAP/repressor). These results confirm that the dominant complex has a 2:1 stoichiometry, and the preferential formation of 2:1 complex and the absence of detectable 1:1 complex at low CAP/repressor ratios strongly suggests that the binding of CAP to lac repressor is cooperative.

Complex Formation Is cAMP-dependent—A general mechanism for CAP-repressor binding in the presence of cAMP is shown in Reaction 1,

\[
K = \frac{n \text{cAMP} + 2\text{CAP} + \text{lac repressor} \leftrightarrow (\text{CAP}_2 \cdot \text{cAMP}_n) \cdot \text{lac repressor}}{[\text{CAP}] + [\text{lac repressor}]} \tag{1}
\]

for which \( K = [(\text{CAP}_2 \cdot \text{cAMP}_n) \cdot \text{lac repressor}] / ([\text{CAP}]^2 \cdot [\text{lac repressor}] + [\text{cAMP}]^n \cdot [\text{lac repressor}]) \). The cAMP stoichiometry\(^3\) can be evaluated from Equation 2 (14).

\[
\frac{\partial \log K_{obs}}{\partial \log [\text{cAMP}]} = n \tag{2}
\]

A graph of \( \log K_{obs} \) as a function of \( \log [\text{cAMP}] \) is shown in Fig. 3. The slope \((1.8 \pm 0.3)\) indicates that 2 equivalents of cAMP are bound for each CAP\(_2\)-repressor complex formed. Although these data do not specify the distribution of cAMP within the complex, in the absence of repressor, native CAP dimers bind one equivalent of cAMP over the concentration range \( 0 < [\text{cAMP}] < 20 \mu \text{M} \) spanned in this experiment (15). We therefore speculate that each molecule of CAP in the \( \text{CAP}_2 \)-repressor complex binds one molecule of cAMP. It is striking that the increased affinity of CAP for lac repressor with increasing [cAMP] parallels that of CAP for DNA, observed over the same [cAMP] range (7, 16). Thus, conditions that promote transcription-regulatory CAP-DNA interactions \textit{in vivo} may also promote CAP-repressor interactions.

\text{CAP Does Not Bind Dimeric lac Repressor}—A parallel analysis was carried out with the lacI-18 mutant lac repressor. This protein lacks 18 C-terminal residues that mediate tetramer formation in the wild-type protein (17, 18). The result is a dimeric repressor protein that binds one equivalent of DNA but cannot bridge between two DNA segments in the fashion observed with wild-type repressor (19–22). Shown in Fig. 4 are sedimentation data for the lacI-18 repressor alone and for a representative sample containing lacI-18 repressor and CAP. The data for repressor alone are well fit by a single-species model, with a weight-average molecular weight of 74,280 ± 3,780, in close agreement with that predicted for the dimer (M\(_r\) = 77,260). However, inclusion of CAP in the solution does not result in a detectable concentration of complex. The data are consistent with a two-species model, which returns \( M_r \) values of 50,030 ± 3,740 for CAP and 76,140 ± 4,350 for repressors. These values are in good agreement with the individual molecular weights of CAP and the lacI-18 repressor. The small, asymmetrical residuals indicate that the data for the R + C mixture is fully consistent with the independent sedimentation of these proteins.

\text{A Model of the CAP\(_2\)-lac Repressor Complex}—Because our preparations of lacI-18 repressor appeared homogeneous and of correct molecular weight by SDS-PAGE and sedimentation equilibrium criteria and were active in DNA binding (5, 8), it...
seems unlikely that their inability to bind CAP is a consequence of either proteolytic degradation or gross misfolding of the protein. We therefore hypothesize that the difference in CAP-binding activity of dimeric and tetrameric repressors is a functional difference of the native proteins. The repressor tetramer structure (Fig. 5) reveals two features not present in the dimer that we propose as candidates for CAP-interaction sites. These are the C-terminal structures that organize repressor as a dimer of dimers (and which are disrupted by the lacI-18 mutation), and the cleft between adjacent dimeric repressor assemblies. The approximate 2-fold symmetry of the tetramer implies that two copies of these structures are available to bind two equivalents of CAP, as was observed. Also shown in Fig. 5 is a schematic model of a CAP-repressor complex in which two molecules of CAP are depicted interacting with the cleft between adjacent dimeric repressor assemblies. Current models of lac promoter regulation (see Refs. 23–26) focus on the independent functions of CAP and lac repressor at their primary binding sites (CAP site 1 and operator 1). These models reflect the absence of clear evidence for regulatory cross-talk between CAP and repressor in the extensive literature dedicated to this system. One might well ask why such cross-talk is not seen, given the overlap of binding sites in the lac promoter region (Fig. 1), and the in vitro data indicating that CAP interacts with lac repressor (described above). The results presented in this paper may be a step toward the answer to that question. As a working hypothesis, we propose that formation of energetically favorable CAP-repressor contacts may compensate for the loss of preferred CAP-DNA contacts when CAP is displaced from operator 1 by lac repressor. These interactions could reduce the CAP potential to interfere with the regulatory repressor-operator 1 interaction. By the same token, favorable CAP-repressor interaction may reduce the overall energetic cost of the isomerization of the repressor-operator 3 complex that occurs when CAP binds CAP site 1 (6). These interactions could reduce repressor’s potential to interfere with the regulatory interaction of CAP with CAP site 1. These considerations predict new classes of CAP and lac repressor mutations that should allow testing of this hypothesis in vivo. Such mutations would disrupt the protein-protein interaction but not the corresponding DNA binding activities. Analogous mutations affecting CAP-RNA polymerase- and CAP-CyTR repressor-interactions have been identified (25, 27). If this hypothesis is correct, destabilization of the CAP-repressor complex should result in lac promoter repression that depends on the absence of cAMP and CAP, and transcription activation by CAP + cAMP that depends on the absence of lac repressor.

Finally, this is the first demonstration, to our knowledge, of a direct interaction of CAP with lac repressor, in the absence of DNA. Little is known about the location or functions of these proteins when they are not bound to their regulatory DNA sequences. The results presented here raise the possibility that CAP and lac repressor may be associated when not performing their transcription-regulatory functions.

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