Interaction of Catabolite Activator Protein of *Escherichia coli* with Single-Stranded Deoxyribonucleic Acid†

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ABSTRACT: The interaction of the catabolite activator protein (CAP) of *E. coli* with single-stranded polydeoxynucleotides has been characterized by physical studies. Circular dichroism (CD) reveals that binding of CAP alters the conformation of poly(dA). The accompanying spectral change was used to monitor the titration of poly(dA) with CAP, which showed that a bound CAP molecule covers 13 bases. This result was confirmed and was shown to apply also to CAP–poly(dT) binding by means of a thermodynamically rigorous sedimentation method. The centrifugation data show that the binding of CAP to poly(dA) and poly(dT) is highly cooperative. In the absence of adenosine cyclic 3′,5′-phosphate (cAMP), the cooperativity parameter, ω (which measures the probability that two CAP molecules on DNA will be found at adjacent sites), has a value of at least 800 for poly(dT) and 600 for poly(dA), independent of ionic strength. The interaction is base specific in that the change in the intrinsic affinity of the protein for DNA, K, with [NaCl] is much larger for CAP–poly(dA) binding than for CAP–poly(dT) binding. Furthermore, addition of cAMP to CAP–poly(dT) solutions reduces ω to 260 while simultaneously increasing K 5-fold; adding cAMP to CAP–poly(dA) solutions does not affect the value of ω but does cause a substantial increase in K. Fluorescence studies established that in all cases cAMP was bound to the CAP–single-stranded DNA complexes. Thus, cooperativity is seen in both the absence and the presence of cAMP. The binding of CAP to single-stranded DNA is much weaker than its binding to native DNA; therefore, CAP is not a generalized DNA melting protein. This finding does not support models of CAP action which require that CAP unwind DNA as it binds to the promoter region. The data are more consistent with models in which DNA-mediated protein–protein interactions are a crucial factor in the mechanism whereby CAP stimulates the initiation of transcription by RNA polymerase at catabolite-sensitive operons.

The catabolite activator protein (CAP) of *E. coli* mediates the action of cyclic AMP in increasing the transcription of "catabolite-sensitive" operons, presumably by enhancing the binding of RNA polymerase and initiation of mRNA synthesis at promoter regions of DNA [for a review, see Pastan & Adhya (1976)]. The molecular mechanism of CAP action has not yet been elucidated. One model (Gilbert, 1976) for this mechanism involves an alteration in DNA structure upon CAP binding which is somehow transmitted to the RNA polymerase binding site in the promoter region. This conformational change (perhaps an unwinding of the DNA) would facilitate the "melting in" of RNA polymerase and initiation of transcription from the appropriate DNA strand. An alternative model involves direct interactions between polymerase and CAP as the crucial part of the mechanism. This view is consistent with recent experiments comparing the chemical modification patterns of specific promoter regions in the presence or the absence of the regulatory proteins. The data show that for both the *lac* (Simpson, 1979, 1980) and the *gal* (Taniguchi et al., 1979) promoters the CAP and RNA polymerase binding sites are in close proximity, so that protein–protein interactions are feasible.

A number of physical studies have shown that CAP undergoes a substantial conformational change upon binding cAMP (Wu et al., 1974; Kumar et al., 1980). Two distinct domains in the protein have been identified; one, which is carboxyl proximal, is involved in DNA binding while the amino-proximal region interacts with cAMP (Eilen et al., 1978). Early studies of the binding of CAP to DNA by using a nitrocellulose filter assay (Riggs et al., 1971) did not demonstrate specific binding of CAP to promoter-containing DNA. Majors (1975) eventually established that a 200 base-pair restriction fragment containing the *lac* promoter has about a 10-fold higher affinity for CAP than do other, non-promoter-containing DNA regions. Thus, CAP, like most DNA-binding proteins, displays a high degree of nonspecific as well as specific binding.

Studies of nonspecific binding help to elucidate the major characteristics of DNA–protein interactions, such as whether there is base-sequence or base-composition specificity, whether the affinity is higher for single-stranded or double-helical DNA, etc. Results of such studies may also provide insight into the specific CAP–*promoter* interaction, which will surely share common features with nonspecific binding.

It has been shown that in the absence of cAMP CAP binds cooperatively to double-helical DNA (Saxe & Revzin, 1979). Addition of cAMP diminishes the cooperativity while increasing the overall affinity of CAP for DNA (Saxe & Revzin, 1979; Takahashi et al., 1979). The binding affinity is rather insensitive to base composition. Recently, Takahashi et al. (1979) reported data on the nonspecific interactions of CAP with native and denatured DNAs. They concluded that the binding strengths are about equal for single-stranded and double-helical DNAs. They also indicated that the degree of cooperativity is neither affected by whether the DNA is native or denatured nor dependent on the presence or absence of cAMP. However, interpretation of their data is complicated by their choice of single-stranded DNAs, denatured calf thymus DNA and ϕX 174 DNA, neither of which has a simple structure in solution. The presence of large numbers of (perhaps imperfect) hairpin loops makes it difficult to determine the extent to which CAP is interacting with single-stranded or double-helical regions.

As part of our overall effort to elucidate the mechanism of CAP action, we have characterized the nonspecific binding of CAP to poly(dA) and poly(dT). These synthetic polydeoxynucleotides are truly single-stranded in solution; their

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structures are not complicated by hairpin-loop formation. We show that, in fact, CAP binds much less tightly to these "simple" single-stranded DNAs than to double-helical DNA. We also find that under certain conditions CAP–CAP cooperative interactions on single-stranded DNA are not diminished by cAMP. The implications of these results for models of CAP action will be discussed.

Materials and Methods

Materials. CAP was isolated from *E. coli* strain CR63 by a method previously described (Saxe & Revzin, 1979). Poly(dA), poly(dT), adenosine 3′,5′-phosphate (cAMP), and chymotrypsin were all from Sigma Chemical Co. The polydeoxynucleotides were further purified by extracting twice with phenol which had been saturated with 0.010 M Tris–HCl, pH 8.1, and 0.010 M Na_2EDTA; the aqueous phase was then dialyzed against four changes of 0.020 M Tris–HCl and 0.001 M Na_2EDTA to remove any residual phenol. The cAMP had been checked for purity by paper chromatography by use of published solvent systems (Smith et al., 1960). All other chemicals were ACS reagent grade or better from commercial sources.

The poly(dT) was found to have a sedimentation coefficient of $s_{20,w} = 4.4$ while the poly(dA) had $s_{20,w} = 8.4$. Using these values in the appropriate equation of Studier (1965) yields molecular weights for poly(dT) of 5.1×10^4 and for poly(dA) of 3.2×10^5.

Buffers. Unless otherwise noted, all buffer solutions contained 0.010 M Tris-HCl and 0.0001 M Na_2EDTA, plus NaCl to give the indicated [Na^+], pH 8.1, at 22 °C. In all experiments involving cAMP, the cyclic nucleotide was at 2×10^{-5} M.

Circular Dichroism. These measurements were made on a Jasco Model ORD/UV5 spectropolarimeter which had been modified to perform circular dichroism (CD) measurements by Sproul Scientific Co. The concentrations of DNA and protein in each solution used were monitored by reading the absorbance spectrum of the solution in the CD cell prior to doing any CD measurements. All CD spectra were taken at room temperature (22 °C).

Ultracentrifugation. Sedimentation studies were done in a Beckman Model E analytical ultracentrifuge equipped with absorption optics and a photoelectric scanner, using a thermodynamically rigorous sedimentation velocity method previously described (Revzin & von Hippel, 1977; Saxe & Revzin, 1979). The primary data from a centrifugation experiment are the ultraviolet absorbance distributions throughout the cell at various times during the run. This permits evaluation of the concentration of each component at all points in the cell, provided that the extinction coefficients, ϵ, are known. Since the model E optical system uses a fully open monochromator slit which leads to a wide spectral band-pass, extinction coefficients in the centrifuge differ somewhat from those in our Gilford Model 250 spectrophotometer. Correction factors to account for these differences were determined by measuring the absorbance of a CAP or DNA solution in the Gilford, then transferring the solution to an ultracentrifuge cell, and reading the absorbance in the model E before any significant portion of the macromolecules had moved away from the meniscus. In binding experiments, concentrations of CAP and DNA were determined by analysis of the absorbances at 230 and 260 nm, using the equations

$$A_{260} = \epsilon_{260}^{DNA}[DNA] + \epsilon_{260}^{CAP}[CAP] \quad (1)$$

$$A_{230} = \epsilon_{230}^{DNA}[DNA] + \epsilon_{230}^{CAP}[CAP] \quad (2)$$

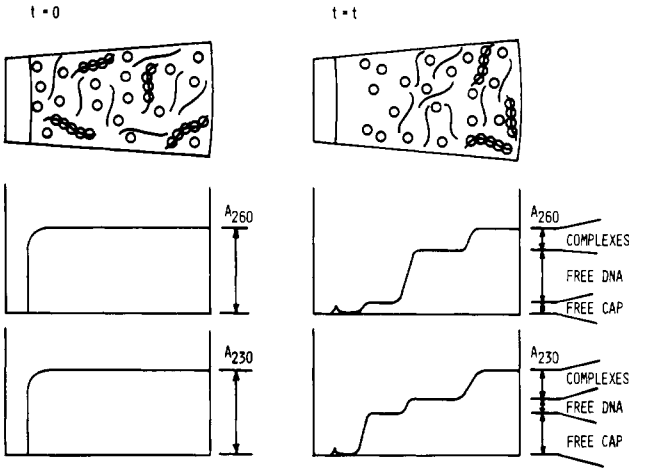

FIGURE 1: Schematic diagram of a sedimentation experiment showing scans at two wavelengths at the start of an experiment ($t = 0$) where the DNA–protein solution is uniformly distributed throughout the cell, and at a later time ($t = t$) at which centrifugation has resolved the components into a three-phase boundary. Also indicated is the composition of each section of the boundary as deduced from its spectral and sedimentation characteristics.

These equations are applicable since we found that there is no appreciable difference spectrum between CAP–DNA complexes and equivalent amounts of the free components (data not shown).

Data from a hypothetical centrifuge run are shown in Figure 1. For the case in which there is cooperative binding of CAP to the single-stranded DNA, a three-phase boundary may be observed. The species corresponding to each section of the boundary can be identified by its sedimentation and absorbance characteristics. Thus, the faster moving material is seen to be DNA–protein complexes, the slowest moving absorbance represents unbound CAP, and the intermediate section of the boundary in Figure 1 corresponds to DNA to which no protein is bound. Since the essence of our centrifugation method involves separating the components of an *equilibrium* mixture, it is necessary to establish that the three-phase system represented in Figure 1 is indeed at equilibrium. The observation that the unbound CAP and free DNA molecules do not interact after the complexes have sedimented away is consistent with a highly cooperative system; DNA and CAP at these lower concentrations are below the "threshold" limit of the cooperative binding. Indeed, when DNA and CAP were mixed at these concentrations in a separate control experiment, no interaction was seen even after waiting up to 6 h following mixing. Further evidence that the three-phase CAP–DNA system is truly at equilibrium derives from two additional observations: (1) the centrifugation pattern was unchanged after the solution had been left for 44 h at room temperature, and (2) a three-phase sedimentation boundary was obtained whether the solution was prepared by directly mixing DNA and CAP at the relatively low ionic strengths used in binding experiments or by mixing the DNA and protein at high salt where there is no interaction and dialyzing to the final (low) NaCl concentration.

The CAP–DNA binding equilibrium was analyzed by using the theory of McGhee & von Hippel (1974) for the binding of large ligands to infinite DNA lattices. In this approach, the data are plotted following the method of Scatchard (1949); thus, it is necessary to evaluate the quantities ν, the binding density (moles of CAP bound per total moles of DNA bases), and L, the concentration of unbound protein. Each solution yields one point on a Scatchard plot. A series of centrifuge runs at various input concentrations of CAP and DNA gen-

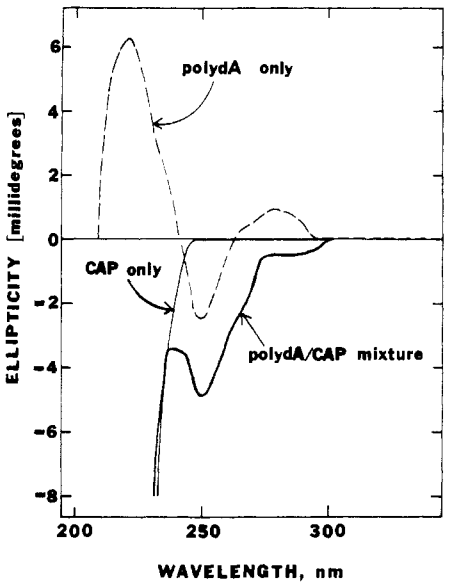

FIGURE 2: Circular dichroism spectrum of a CAP–poly(dA) mixture in 0.010 M NaCl, 0.010 M Tris-HCl, and 0.0001 M Na_2EDTA (pH 8.1, T = 22 °C); [CAP] = 1.2×10^{-6} M, [poly(dA)] = 1.7×10^{-5} M (bases). Also shown are spectra for CAP alone and for poly(dA) alone at the same concentrations. The complexes show a CD spectrum different from the sum of the spectra of the individual components; this is particularly clear from data at wavelengths longer than 250 nm, where the protein shows no circular dichroism.

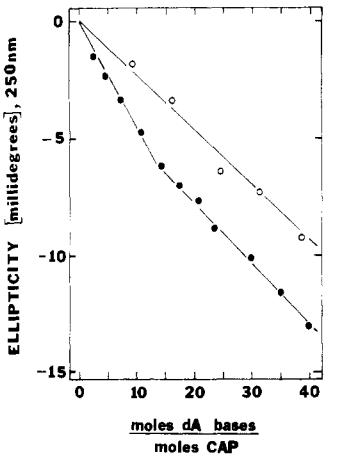

FIGURE 3: Titration of CAP with poly(dA), monitored by circular dichroism as described in the text. The lower curve (●) shows data for a solution with [CAP] = 1.2×10^{-6} M, to which poly(dA) was added to give various nucleic acid–protein ratios. All data were normalized to the same protein concentration. The upper curve (○) is for poly(dA) only; this was obtained under identical conditions except that CAP was omitted from the solution. The buffer contained 0.010 M NaCl, 0.010 M Tris-HCl, and 0.0001 M Na_2EDTA, pH 8.1 (T = 22 °C).

erates the entire plot. The concentration of unbound protein is determined directly from the absorbance of the slow moving CAP, or by two-wavelength analysis in the case of poly(dT) where the sedimentation coefficient of the DNA was too low to allow separation of CAP and poly(dT) into two distinct phases. The amount of CAP which is complexed to DNA can be determined by two-wavelength analysis of the faster moving material or simply by difference, using the input concentration of CAP and the measured amount of free protein. Both approaches give consistent results. The binding density is then the concentration of bound CAP divided by the total amount of poly(dA) or poly(dT) in the solution. With the sedimentation technique, association constants in the range 10^5–10^7 M^{-1} can be measured.

Fluorescence. An Aminco-Bowman D223 spectrofluorometer was used for fluorescence measurements. The excitation wavelength was held constant at 295 nm while the emission spectrum was scanned from 250 to 550 nm. The ultraviolet absorbance spectrum of each solution was determined prior to any fluorescence measurements. To monitor inner filter effects (reduction in the fluorescence intensity of CAP due to addition of absorbing species), a solution of *N*-acetyltryptophanamide was prepared having the same fluorescence intensity as that of the CAP solution under consideration. Any additions of cAMP or DNA to the protein solution were also made to the *N*-acetyltryptophanamide solution. In no case was the fluorescence intensity of the amino acid analogue reduced by more than 5%; hence, the observed quenching of the protein fluorescence in the presence of cAMP reflects conformational changes in the CAP.

Results

Circular Dichroism. Figure 2 shows that there is a nonzero difference spectrum between the sum of the individual CD spectra for CAP and poly(dA) and that for CAP–poly(dA) complexes. These results are similar to those for complexes of CAP and double-stranded DNA (Saxe & Revzin, 1979) in that most of the difference appears in the region where the protein shows no circular dichroism, suggesting that the effect arises from structural changes in the DNA. For determination of the number of DNA bases covered by a bound CAP molecule, titrations of a fixed amount of CAP with poly(dA) were performed at 0.010 M NaCl by monitoring the ellipticity at 250 nm as a function of the ratio of poly(dA) to CAP concentration. At this ionic strength, sedimentation results show that essentially all CAP molecules are bound to poly(dA) (see below). The titration curve generated in this manner, Figure 3, has a break at 13 (±1) bases per CAP molecule, which is the point at which all CAP molecules in solution have become bound to DNA. Past this point there is an excess of poly(dA) over CAP, and the changes in ellipticity become equal to those observed when poly(dA) is added to an identical solution without protein. Titration of a fixed amount of poly(dA) with CAP gives the same results, indicating that the system is indeed at equilibrium and that poly(dA) can be completely saturated with CAP under these conditions. Addition of cAMP to CAP–poly(dA) solutions does not alter the CD difference spectrum. The CD signal for poly(dT) shows only a very small change upon CAP binding.

Sedimentation. At sufficiently low ionic strength in the presence of excess poly(dA) or poly(dT) (more than 13 bases per CAP molecule), centrifugation experiments show that all molecules of CAP are found in DNA–CAP complexes. Thus, every CAP molecule in the solution is capable of binding to single-stranded DNA. Furthermore, experiments with the protein in the absence of DNA, over the entire ionic strength range of interest here, showed no self-association of CAP molecules; that is, the sedimentation coefficient obtained, $s_{20,w}$ = 3.3, agrees quite well with the previously reported value of $s_{20,w}$ = 3.5 (Anderson et al., 1971). Binding experiments were performed at rotor speeds from 16 000 to 36 000 rpm with no detectable variations in the results, indicating that the very large pressures generated in the ultracentrifuge do not appreciably perturb the equilibrium.

Scatchard plots for the binding of CAP to poly(dT) or poly(dA) are shown in Figure 4. The pronounced "hump" in these plots is diagnostic of a highly cooperative system. The data in Figure 4 were analyzed by the equation of McGhee & von Hippel (1974):

$$\frac{\bar{\nu}}{L} = K(1 - n\bar{\nu}) \times \left[\frac{(2\omega - 1)(1 - n\bar{\nu}) + \bar{\nu} - R}{2(\omega - 1)(1 - n\bar{\nu})}\right]^{n-1}\left[\frac{1 - (n + 1)\bar{\nu} + R}{2(1 - n\bar{\nu})}\right]^2 \quad (3)$$

where K = the intrinsic protein–DNA association constant, ω = the cooperativity parameter, n = the number of bases covered by a bound CAP molecule, and $R = [[1 - (n + 1)\bar{\nu}]^2 + 4\omega\bar{\nu}(1 - n\bar{\nu})]^{1/2}$. The experimental data were fit to this equation by using a nonlinear method of least-squares approach in which K and ω are varied, taking $n = 13$, based on our circular dichroism results.

The Scatchard plots in Figure 4 have a dearth of experimental points on the downward slope of the curve at high values of $\bar{\nu}$. In practice, it is exceedingly difficult to obtain such points since high concentrations of DNA and protein are required and the complexes often precipitate out of solution under these conditions. It should be noted, however, that the curve intersects the horizontal axis at the known value of $\bar{\nu} = 1/n = 0.077$.

Although eq 3 is strictly applicable only to infinitely long DNAs, we have applied it to our studies of CAP interactions with poly(dT) having an average length of 190 bases (which can accommodate about 15 CAP molecules). If the binding of CAP were noncooperative, "end" effects due to the relatively short DNA would result in at most a 7% error in our calculations (McGhee & von Hippel, 1974); we can expect a similar error for cooperative systems. The major consequence of having short DNA molecules will be to underestimate the degree of cooperativity (ω), especially in CAP–poly(dT) interactions where the average number of CAP molecules in a cooperative cluster would tend to be much greater than 15 (no cAMP). In this case, the short poly(dT) chain length results in fewer protein–protein interactions than would occur if the polynucleotide lattices were very long. The same considerations apply to the CAP–poly(dA) system where, as is seen below, the cluster length may be larger than the 74 CAP molecules which can be simultaneously bound to a typical poly(dA) chain (960 bases long) in our solutions. Thus, the values of ω extracted from the Scatchard plots in Figure 4a,b,d may be much lower than the true values. Epstein (1978) has derived a theory which shows that under most conditions the infinite lattice approximation is valid even for unexpectedly short chains, except for the situation where $K\omega L \simeq 1$. Experimental uncertainties in the values for K and ω preclude accurate application of Epstein's theory to our data. However, this theory predicts that for short lattices the average cluster length will be *smaller* than it would be for an equivalent amount of longer DNA molecules. As seen below, our data imply that the cluster lengths can be considerably larger than expected from the theory of McGhee & von Hippel (1974).

The values of the binding parameters obtained from the plots in Figure 4 are given in Table I. The results show that in the absence of cAMP CAP binding to poly(dT) is highly cooperative ($\omega \geq 800$) and that the degree of cooperativity appears to be independent of ionic strength. The intrinsic affinity, K, of CAP for poly(dT) decreases at higher ionic strengths, as is normally the case for DNA–protein interactions. At 0.0175 M NaCl, addition of cAMP to CAP–poly(dT) solutions reduces the cooperativity ($\omega = 260$) while simultaneously increasing K; the overall binding affinity, $K\omega$, is increased. Under our experimental conditions, the cAMP–CAP–poly(dT) system always showed a two-phase boundary [no uncomplexed poly(dT)] rather than a three-phase boundary. This occurs because the several-fold reduction in ω lowers the average number of CAP molecules in a cooperative cluster and increases the probability that every poly(dT) molecule will have some CAP bound to it. At 0.025 M NaCl, CAP–poly(dT) interactions are much reduced (data not shown). We were able to detect a small amount of binding at 0.025 M NaCl in the presence of cAMP; a two-phase boundary was observed under these conditions.

The binding of CAP to poly(dA) is also highly cooperative. In the absence of cAMP, a Scatchard plot could be obtained only at 0.0175 M NaCl (Figure 4d). At 0.010 M NaCl, the binding was so tight that no unbound protein could be seen by our technique; hence, reliable data for a Scatchard plot could not be generated. This observation permits us, however, to estimate that the association constant for CAP–poly(dA) binding at 0.010 M NaCl has a value of at least 8×10^6 M^{-1}. If the affinity were lower than this, we would expect to see

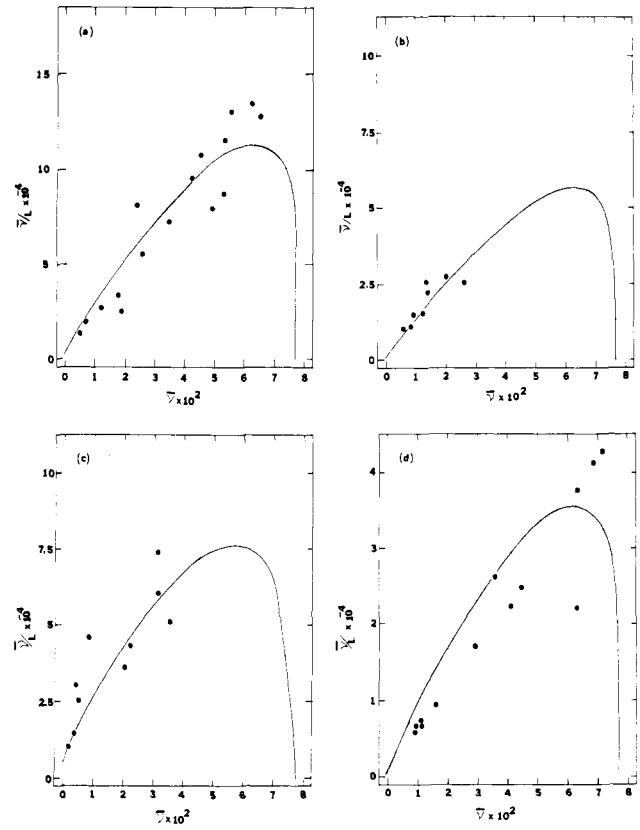

FIGURE 4: Scatchard-type plots for the binding of CAP to single-stranded DNA. All solutions contained 0.010 M Tris-HCl and 0.0001 M Na_2EDTA, pH 8.1, plus NaCl at the indicated concentration, T = 20 °C. The theoretical curves were fit as described in the text. (a) CAP–poly(dT), 0.010 M NaCl; (b) CAP–poly(dT), 0.0175 M NaCl; (c) CAP–poly(dT), 0.0175 M NaCl plus 2×10^{-5} M cAMP; (d) CAP–poly(dA), 0.0175 M NaCl.

Table I: Binding Parameters for CAP–Single-Stranded DNA Interactions

solution	K (M^{-1})[a]	ω[b]	$K\omega$
CAP–poly(dT), 0.010 M NaCl	2800	>800	2.2×10^6
CAP–poly(dT), 0.0175 M NaCl	1200	>800	1.0×10^6
CAP–poly(dT), 0.0175 M NaCl, + 2×10^{-5} M cAMP	6500	260	1.7×10^6
CAP–poly(dA), 0.0175 M NaCl	1600	>600	1.0×10^6

[a] Units of K are molar in [CAP] and in DNA *bases*. [b] Comparison of the experimental data points with the theoretical curves in Figure 4 by using a method of least-squares approach indicates that uncertainties in the values of K and ω are about ±15%. Note, however, that for reasons discussed in the text values of ω in the absence of cAMP may be severely underestimated.

some free protein in our solutions; the binding strength may in fact be greater than this estimate. Conversely, at 0.025 M NaCl, no binding of CAP to poly(dA) was detectable by the centrifugation method; from this, we estimate that the overall binding constant at 0.025 M NaCl must be less than 4×10^5 M^{-1}.

Addition of cAMP to CAP–poly(dA) solutions at 0.010 M NaCl caused no change in the sedimentation pattern; a two-phase boundary persisted, in which the amounts of free poly(dA) and of DNA–protein complexes were not altered. This result gives us no information about the effect of cAMP on K, but it does imply that there is little or no change in the value of ω. At 0.0175 M NaCl, adding cAMP results in more protein molecules becoming bound to DNA, although there remains a three-phase boundary. Furthermore, the sedimentation coefficient of the CAP–poly(dA) complexes is not affected by the presence of cAMP or by the [NaCl]. In each of these experiments, there were on average 54 CAP molecules bound per poly(dA) molecule. Thus, the degree of cooperativity is the same in the presence or the absence of cAMP and is not a function of salt concentration. It follows then that at 0.0175 M NaCl the value of K must increase to account for the increase in overall affinity ($K\omega$) upon cAMP binding. At 0.025 M NaCl, no binding of CAP to poly(dA) is seen even in the presence of cAMP.

The interaction of CAP with poly(dA) and poly(dT) can be further characterized by analysis of the composition of the cooperative protein–DNA complexes. For instance, absorbance data for the fast moving poly(dA)–CAP complexes at 0.0175 M NaCl in the absence of cAMP reveal that at low binding density ($\bar{\nu} = 0.01$) each poly(dA) molecule is about $^2/_3$ saturated with protein. On average, 54 CAP molecules are bound to each 960 base polymer, which corresponds to about 18 bases per CAP molecule in the complexes. In solutions in which the value of $\bar{\nu}$ is higher, the ratio of poly(dA) bases complexed to CAP molecules levels off at 13 [74 CAP molecules per poly(dA) molecule]. This confirms the value for the binding site size determined from CD data. The increase in the level of complexed CAP is also reflected in a parallel rise in the sedimentation coefficient of the complexes from 53 S at $\bar{\nu} = 0.01$ to 70 S for $\bar{\nu} \geq 0.04$. *Since these CAP–poly(dA) solutions show a three-phase boundary, including some uncomplexed poly(dA), there must be on average only one cooperative cluster of proteins on any poly(dA) molecule.* Thus, it follows that the length of the cooperative cluster ranges from 54 at $\bar{\nu} = 0.01$ to greater than 75 for $\bar{\nu} \geq 0.04$. These values are much larger than those calculated by the theory of Ruyechan & Wetmur (1975), which for $\omega = 600$ predicts cluster lengths of about 13 CAP molecules at $\bar{\nu} = 0.01$ and 25 at $\bar{\nu} = 0.04$. An analysis of the McGhee–von Hippel (1974) theory by S. Kowalczykowski (unpublished experiments) reveals that a cluster length of 54 would require that ω be about 6000! It is clear that cooperativity in the CAP–single-stranded DNA system is even more pronounced than indicated by the results in Table I. Cluster lengths much longer than those predicted by the McGhee–von Hippel theory are also observed for CAP binding to double-stranded DNA (Saxe & Revzin, 1979; A. Revzin, unpublished experiments); in these experiments, the DNAs are quite large, so the infinite lattice approximation surely holds. This phenomenon is currently under investigation by using centrifugation and electron microscopy to measure cluster lengths for CAP binding to native plasmid and phage DNAs of known molecular weight. As discussed above, the sedimentation method yields values for the cluster size under conditions where a three-phase equilibrium exists, as well as providing estimates of K and ω in both cooperative and noncooperative systems. Perhaps CAP–DNA binding has some additional feature not included in the McGhee–von Hippel model; for example, the cooperativity might arise from more than simply nearest-neighbor interactions. Even if this should be the case, however, it would not affect the qualitative conclusions presented here (e.g., the CAP–single-stranded DNA interaction is highly cooperative etc.).

For poly(dT) at 0.010 M Na^+ in the absence of cAMP, the DNA–protein ratio in the complexes again levels off at about 13 bases per CAP molecule at high $\bar{\nu}$ values. Therefore, the binding site size for CAP on poly(dT) is also $n = 13$. We could not determine the cooperative cluster length for the relatively short poly(dT) chains since those poly(dT) molecules in complexes were completely covered with protein, even at low $\bar{\nu}$ values. In the presence of cAMP, however, the complexes at $\bar{\nu} = 0.035$ are found to contain about 6–7 CAP's bound to every poly(dT) molecule. This is reflected in the observed change in the sedimentation pattern upon addition of cAMP from a three-phase to a two-phase boundary in which there is no molecule of poly(dT) without protein bound to it. In this case ($\omega = 260$), the Ruyechan–Wetmur theory predicts an average cluster length of about 15 molecules while the McGhee–von Hippel approach (S. Kowalczykowski, unpublished experiments) predicts a result very near to the value of 6–7 that we observe.

Fluorescence. Excitation of CAP at 295 nm results in an emission maximum at 350 nm, the magnitude of which is dependent on NaCl concentration. Adding cAMP at 2×10^{-5} M to solutions of CAP over a NaCl concentration range from 0.010 to 0.100 M causes a 20–25% quenching of the emission peak. This demonstrates that cAMP is indeed binding to CAP at the low ionic strengths used in the sedimentation studies. Furthermore, addition of cAMP to solutions of CAP–poly(dA) or CAP–poly(dT) complexes yields an equivalent quenching, indicating that cAMP is binding to the DNA-bound CAP.

Preliminary studies showed that increasing the NaCl concentration from 0.010 to 0.100 M results in a very large (60%) decrease in the intrinsic fluorescence of CAP. This led us to consider whether the protein was undergoing a profound conformational change at low ionic strengths. We investigated this question by means of limited chymotryptic digestion of CAP. Krakow and co-workers (Krakow & Pastan, 1973; Eilen & Krakow, 1977) showed that CAP is resistant to chymotrypsin in the absence of cAMP while addition of cAMP resulted in partial proteolysis to generate a chymotrypsin-resistant "core" which binds cAMP but not DNA. We found exactly this digestion behavior at both low (0.010 M) and high (0.100 M) NaCl concentrations. Thus, the conformational change which leads to the fluorescence quenching is not severe enough to alter the susceptibility of the protein to proteolytic cleavage.

Discussion

The circular dichroism results imply that the binding of CAP to poly(dA) is accompanied by a conformational change in the polynucleotide. The spectral changes are somewhat unusual—the negative lobe at 250 nm is enhanced, but the positive band centered around 280 nm disappears and changes sign upon CAP binding. It is difficult to assign these effects to specific shifts in base alignment or to interactions between the bases and particular amino acid residues in the protein. It is clear, however, that a substantial change in the environment of the bases occurs when CAP binds to poly(dA). On the other hand, no effect on the CD is observed upon CAP–

poly(dT) binding, indicating that CAP induces little change in poly(dT) conformation. Thus, the circular dichroism results imply that CAP interacts with poly(dA) and poly(dT) in significantly different ways. This is reflected in the differences seen in the magnitudes and ionic strength dependences of the binding parameters for CAP interactions with poly(dA) and poly(dT) as discussed below.

A value for n of 13 bases covered by a bound protein molecule was derived from circular dichroism titrations and was confirmed by detailed analysis of sedimentation data. This result agrees well with the value $n = 15$ reported by Takahashi et al. (1979) for CAP–ϕX174 DNA interactions and is a reasonable value for a 45000-dalton, roughly spherical protein such as CAP (Anderson et al., 1971). We note that the binding site size for CAP–double-helical DNA interactions is 13 base pairs (Saxe & Revzin, 1979). While it is tempting to speculate that the protein has the same configuration whether bound to single-stranded or double-helical DNAs, confirmation of this awaits further data on the interbase distances in CAP–single-stranded DNA complexes and on details of the DNA–protein contacts involved in the binding of CAP to native and denatured DNAs.

It is of interest that CAP displays base specificity in binding to the single-stranded homopolymers used in these studies. The interaction with poly(dT) is weaker than that with poly(dA) at [NaCl] < 0.0175 M and is much less sensitive to the NaCl concentration. Furthermore, cAMP causes a reduction in the cooperativity parameter for CAP–poly(dT) binding but apparently has little or no effect on CAP–poly(dA) cooperativity.

The ionic strength dependence of the protein–DNA interaction can be analyzed by the theory of Record et al. (1976), which relates the change in association constant with monovalent cation concentration to the number of ionic interactions involved in the binding process:

$$\frac{\partial \log K\omega}{\partial \log [M^+]} = -m'\psi + a \qquad (4)$$

where M^+ = the concentration of monovalent cations, ψ = the ionic parameter for DNA ($\psi = 0.71$ for single-stranded DNAs), m' = the number of ionic interactions, and a = the contribution from anion effects. Since we find little change in ω with NaCl concentration for either poly(dT) or poly(dA), the entire ionic strength dependence resides in K. From the two values of K for poly(dT) in Table I, we find that $-\partial \log K/\partial \log [M^+] = 1.5$. If anion effects are negligible, then we compute $m' = 2$ (± 1), which means that about two ionic interactions are involved in CAP–poly(dT) binding. To obtain a value of m' for the poly(dA) case, we applied eq 4 to the estimated and measured association constants for poly(dA) at various [NaCl]; this yields $m' \simeq 5$ (± 2) ionic interactions upon CAP–poly(dA) binding, provided that ω is independent of ionic strength and ignoring possible anion effects. While this value for m' is to some extent based on approximate values for K, our estimate of K for CAP–poly(dA) binding at 0.010 M NaCl is a minimum value while that at 0.025 M NaCl is a maximum value. Errors in these estimates thus will likely understate the difference in ionic strength dependences for binding of CAP to the two single-stranded DNAs. At this time, we can only speculate as to the causes for the different $\partial \log K/\partial \log [M^+]$ values. If there are in fact no anion effects, then the conformation of the protein when on poly(dA) must differ sufficiently from that when bound to poly(dT) that three additional positively charged residues are in close proximity to the poly(dA) phosphodiester backbone. Alternatively, there may be anions binding to CAP when it interacts with poly(dT) or anions released from the protein when it binds to poly(dA), or both. An extensive investigation by von Hippel and coworkers has shown that anion release is an important factor in the interaction of the gene 32 protein of phage T4 with single-stranded polynucleotides (P. H. von Hippel, unpublished experiments). Further experiments are needed to clarify the situation in the case of CAP binding to single-stranded DNA.

The affinity of CAP for poly(dA) or poly(dT) is much weaker than that for double-helical DNAs. This is manifested in the low ionic strengths (<0.025 M NaCl) needed to observe the binding by our sedimentation technique. While association constants for the CAP–calf thymus DNA system were measured at about 0.070 M NaCl in the absence of cAMP and at 0.100 M NaCl in the presence of cAMP (Saxe & Revzin, 1979), at these higher salt concentrations the affinity of CAP for poly(dA) or poly(dT) is so diminished that no interaction at all can be detected. This is in contrast to the results of Takahashi et al. (1979), who report that CAP has approximately the same affinity for single-stranded and native DNA. Although their system is complicated by the presence of hairpin loops in denatured calf thymus and single-stranded ϕX 174 DNA, their results may reflect the presence of particular single-stranded DNA sequences having very high affinities for CAP. If this is the case, then the differences in binding parameters for the CAP–poly(dA) and CAP–poly(dT) interactions could be minor compared to variations among natural DNA regions, especially promoter sequences. However, in the absence of other evidence, it must be emphasized that our data and those of Takahashi et al. (1979) imply that CAP is *not* a generalized DNA melting protein, and thus do not support models of CAP action which require the protein to unwind DNA.

Our results are more consistent with models which postulate that protein–protein interactions play a primary role in the stimulation of transcription by CAP. The cooperativity parameters for both poly(dA) and poly(dT) are very large; thus, the binding of a CAP molecule to single-stranded DNA creates an environment favorable for the binding of additional protein molecules. Adding cAMP has no effect on the CAP–poly(dA) interaction while it reduces but does not eliminate the cooperativity in CAP–poly(dT) binding. This is consistent with the data of Takahashi et al. (1979), who in addition reported cooperativity in the CAP–native DNA system in the presence of cAMP. Thus, CAP–cAMP complexes can participate in DNA-mediated protein–protein interactions. The relationship of this observation to the mechanism by which CAP enhances initiation of transcription by RNA polymerase is currently being investigated by physical studies of complexes of CAP and of RNA polymerase with specific promoter regions.

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Measurement of Ribonucleotide Pool Specific Activities by an in Vivo Method: Comparison with an in Vitro Method†

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ABSTRACT: A biological assay is described for the specific activity of the nucleotides incorporated into nuclear ribonucleic acid (RNA). Under labeling conditions in which the pool specific activity reaches steady state by a process described by a continuous function, this method allows the determination of pool specific activity at all times of labeling. In *Drosophila* cultured cells incubated with [^{3}H]uridine, the incorporation of radioactivity into transfer RNA (tRNA) and the absolute rate of tRNA synthesis were used to obtain an absolute value for the average specific activities of the uridine 5′-triphosphate (UTP) and cytidine 5′-triphosphate (CTP) precursor pools. The average specific activity of the UTP and CTP pool obtained by this in vivo method was 2–3-fold higher than the specific activity of the whole-cell UTP and CTP pools as assayed by an in vitro method. This suggests that the ribonucleotide pools in *Drosophila* cultured cells are compartmentalized. Use of the in vivo assay of ribonucleotide pool specific activities described here should avoid underestimates or overestimates which may arise when absolute rates of RNA synthesis are calculated from measurements of whole-cell ribonucleotide pool specific activities. The rate of labeling of tRNA was used to determine the rate at which the combined UTP and CTP pools equilibrate. This rate was then used to determine that the rate of processing of pre-tRNA to mature tRNA is 0.21 min^{-1}; this corresponds to a half-life of 3.3 min.

An important problem in understanding the control of eukaryotic gene expression is the relative contribution of transcription, posttranscriptional processing, and turnover to the level of expression of a particular gene. In order to assess the relative transcriptional efficiencies of the promotors of different genes, it is necessary to measure the absolute rate of synthesis of particular RNA[1] species.

Absolute rates of synthesis are frequently determined by measuring the rate of labeling of RNA after the addition of radioactive precursor. Knowledge of the behavior of the specific activity of the whole-cell ribonucleotide triphosphate pool during the labeling period is then used to convert the rate of labeling to a rate of synthesis. An underlying assumption of this method is that the ribonucleotide precursor pool is not compartmentalized, i.e., that the specific activity of the total ribonucleotide triphosphate pool extracted from the cell is identical with the specific activity of the ribonucleotide pool used for nuclear RNA synthesis.

There is controversy in the literature as to whether the specific activity of the whole-cell ribonucleotide pool is identical with the specific activity of the ribonucleotide precursor pool utilized for nuclear RNA synthesis. In HeLa cells, the steady-state specific activity attained in mRNA was equal to that attained in the total UTP pool (Kramer et al., 1973; Wiegers et al., 1976); furthermore, the ratio of uridine to cytidine specific activity was the same in hnRNA, pre-rRNA, polio virus RNA (which is synthesized in the cytoplasm), and the total acid-soluble pools (Wu & Soiero, 1971; Soiero & Ehrenfeld; 1973; Puckett & Darnell, 1977). These various lines of evidence are consistent with the hypothesis that ribonucleotide pools are not compartmentalized.

Studies on a number of other cell lines, however, support the concept that ribonucleotide pools are compartmentalized. Rates of labeling of nuclear RNA and Mengo virus RNA (which is synthesized in the cytoplasm), in cells which had undergone prior pool expansion, were compared to their re-

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[1] Abbreviations used: RNA, ribonucleic acid; mRNA, messenger RNA; tRNA, transfer RNA; UTP, uridine 5′-triphosphate; hnRNA, heterogeneous nuclear RNA; rRNA, ribosomal RNA; MEM, minimum Eagle's medium; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CTP, cytidine 5′-triphosphate; Cl_3CCOOH, trichloroacetic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; BSA, bovine serum albumin; UMP, uridine 5′-monophosphate.

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