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# Cooperative Binding to DNA of Catabolite Activator Protein of *Escherichia coli*†

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ABSTRACT: Binding parameters are reported for the interaction of *Escherichia coli* catabolite activator protein (CAP) with nonspecific DNAs (primarily native calf thymus DNA, which presumably does not contain specific functional sites for CAP). The main experimental techniques used were circular dichroism and a thermodynamically rigorous centrifugation method which allows quantitative analysis of the binding. It was found that in the absence of cyclic adenosine 3′,5′-monophosphate (cAMP) CAP binds cooperatively to double-helical DNAs, including poly[d(A-T)] and poly[d(I-C)]. The presence of cAMP eliminates the cooperativity, while the overall strength of the binding is increased. Circular dichroism spectra imply that the interaction of CAP with DNA causes a shift from the DNA B form to the C form; this occurs regardless of whether cAMP is present. Any conformational changes in the protein which may underlie the cooperative effect are not detectable by circular dichroism. It appears that CAP does *not* tend to melt double-helical DNA. The data show that a molecule of CAP covers 13 base pairs when bound to DNA. Values for other parameters of the cooperative interaction are reported for a range of ionic conditions (50–80 mM NaCl) at $T = 22$ °C. The intrinsic affinity of the protein for DNA, $K$, decreases at higher [NaCl], while the cooperativity parameter, $\omega$ (which measures the probability that two CAP molecules on DNA will be bound at adjacent sites), has a value of about 100, independent of ionic strength. The data indicate that approximately six ionic interactions are involved in the binding of a CAP molecule to DNA. Extrapolation of the $K\omega$ results to more physiological ionic conditions implies that in vivo some CAP molecules may be nonspecifically bound to the *E. coli* chromosome even in the absence of cAMP. At the higher cAMP levels at which CAP actively promotes transcription it seems likely that nonspecific DNA binding may play an in vivo role in modulating the action of CAP.

The catabolite activator protein (CAP)[1] of *Escherichia coli* stimulates transcription at certain catabolite-sensitive operons (Zubay et al., 1970; Emmer et al., 1970). Studies with both intact cells and cell-free systems indicate that CAP promotes mRNA synthesis only in the presence of relatively high levels

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received August 10, 1978.* This work was supported in part by grants from the Research Corporation (7870), the National Science Foundation (PCM 75-23696), and the MSU General Research Support Fund. Portions of this research were submitted (by S.A.S.) to the Graduate School of Michigan State University in partial fulfillment of the requirements for the M.S. degree. A preliminary account of these studies was presented to the Biophysical Society (Washington, D.C., 1978).

[1] Abbreviations used: CAP, catabolite activator protein; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; 5′-AMP, adenosine 5′-monophosphate; PMSF, phenylmethanesulfonyl fluoride; PPO, 2,5-diphenyloxazole; $Na_2EDTA$, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate; GdnHCl, guanidine hydrochloride, CD, circular dichroism.

0006-2960/79/0418-0255$01.00/0 

of cyclic adenosine 3′,5′-monophosphate (cAMP) (Perlman et al., 1969; deCrombrugghe et al., 1971). The cAMP–CAP complex apparently binds at the promoter region and enhances initiation of RNA chains by RNA polymerase; in the absence of cAMP, CAP is not active. This hypothesis explains the phenomenon of catabolite repression, in which certain inducible enzymes (e.g., those needed to metabolize lactose or galactose) are not produced if cells are grown on glucose, even though the appropriate inducer is present (Epps & Gale, 1942; Magasanik, 1970). The level of cAMP in *E. coli* grown in glucose medium is low (Pastan & Perlman, 1970), and this presumably prevents CAP action and, hence, limits transcription from catabolite-sensitive promoters.

A variety of physical and chemical studies on CAP has been reported. The protein is a 45 000-dalton dimer, composed of two apparently identical subunits (Anderson et al., 1971). It undergoes a conformational change upon binding cAMP (Krakow & Pastan, 1973; Wu & Wu, 1974; Wu et al., 1974; Eilen & Krakow, 1977). As is generally true for DNA-binding proteins, CAP displays substantial affinity for DNA regions other than its specific functional site. This nonspecific binding of CAP to various DNAs has been studied semiquantitatively using nitrocellulose filter assays and band sedimentation in sucrose gradients (Riggs et al., 1971; Nissley et al., 1972; Krakow & Pastan, 1973; Majors, 1975). It has been shown that under certain ionic conditions CAP and DNA are relatively tightly associated at pH 6 regardless of whether cAMP is present, while at pH 8 under similar conditions the binding is strong in the presence of cAMP but weak in its absence (Krakow & Pastan, 1973). It has proved difficult to demonstrate directly *specific* CAP–DNA interactions in vitro. Majors (1975) has reported conditions under which some preferential binding to promoter regions can be detected using a filter assay. These data indicate that CAP has a 5- to 10-fold higher affinity for the promoter compared to nonspecific DNA binding, in contrast to *lac* repressor which binds to the operator about $10^6$ times better than to nonoperator DNA regions (Lin & Riggs, 1972; von Hippel et al., 1975).

We have undertaken a study of nonspecific CAP–DNA binding for several reasons. We expect our results to shed light on the specific association of CAP with the promoter, perhaps by suggesting the nature of the protein–DNA and protein–protein interactions which are occurring. Furthermore, it has been shown that *lac* repressor in vivo is virtually always bound to DNA, either to the operator region or to other parts of the chromosome (Kao-Huang et al., 1977). Thus nonspecific binding may function as a general modulator of transcription by controlling the *availability* of regulatory proteins for interactions with their particular sites of action. An eventual goal of this research is to quantify the distribution of CAP between specific and possible nonspecific DNA binding sites in vivo. Finally, the relatively well-characterized CAP protein can serve as a model to help elucidate molecular details of DNA–protein interactions in general. In this paper we describe the association of CAP with, mainly, double-helical DNA in the absence of cAMP. The binding was found to be highly cooperative, in the same sense that the gene-32 protein of phage T4 binds cooperatively to single-stranded DNA (Alberts & Frey, 1970). We have characterized this interaction by evaluating the binding site size (number of DNA base pairs covered by a bound CAP molecule), along with the values and the ionic strength dependences of the intrinsic DNA–protein association constant and of a parameter which describes the degree of the cooperativity.

## Materials and Methods

*Materials.* Catabolite activator protein (CAP) was purified from *E. coli* strain CR63, which was a gift from Dr. Loren Snyder. Lysozyme, ribonuclease, deoxyribonuclease, bovine serum albumin, casein, trypsin, calf thymus DNA, poly[d-(A-T)], poly[d(I-C)], poly(dC), 3′,5′-cAMP, 5′-AMP, 3′,-5′-cGMP, phenylmethanesulfonyl fluoride (PMSF), deoxycholic acid, guanidine hydrochloride (GdnHCl), bromphenol blue, and Coomassie brilliant blue R were obtained from Sigma Chemical Co. The [$^3$H]cAMP (26 Ci/mmol) was from Amersham/Searle Corp. Cellulose powder CF 11, cellulose phosphate (coarse fibrous P1), and DEAE-cellulose (DE-52) were purchased from Whatman, while Sephadex G-75 was from Pharmacia. Acrylamide and bis(acrylamide) were supplied by Bio-Rad Laboratories and 2,5-diphenyloxazole (PPO) by Research Products International Corp. All other chemicals were the highest grade available and were purchased from normal commercial sources.

Purity of the cAMP was checked by paper chromatography using the procedure of Smith et al. (1960). The cAMP was shown to be free of both cGMP [using the solvent system isobutyric acid–1 M ammonium hydroxide–0.1 M $Na_2EDTA$ (100:60:1.6)] and 5′-AMP [using the solvent system isopropyl alcohol–concentrated ammonia–water (7:1:2)]. Single-stranded calf thymus DNA was prepared by heating native calf thymus DNA in a boiling-water bath for 10 min. The DNA was in 10 mM Tris (pH 7.9 at 22 °C), 33 mM NaCl, 0.1 mM $Na_2EDTA$. DNAs were routinely phenol extracted before use.

*Purification of CAP.* A modification of the procedure of Anderson et al. (1971) was used. Cells were lysed as described by Burgess & Jendrisak (1975). In a typical purification 250 g of frozen *E. coli* was placed in 500 mL of 4 °C lysing buffer (10 mM Tris-OAc, pH 8.2, 10 mM $Mg(OAc)_2$, 60 mM KOAc, 0.05 mM dithiothreitol (DTT), 0.1 mM $Na_2EDTA$, 130 μg/mL of lysozyme, 23 μg/mL of PMSF). This was slowly mixed and allowed to sit for 20 min. Then 10 mL of 4% sodium deoxycholate was slowly blended in and the solution was again left for 20 min. The solution became quite viscous at this point. Approximately 2 mg each of ribonuclease and deoxyribonuclease was added with stirring and the solution was left overnight at 4 °C. (A preparation performed without adding ribonuclease yielded results similar to those in which ribonuclease was used.) This crude extract was centrifuged at 16 000*g* for 2 h. The supernatant was saved and dialyzed for 3 days vs. six 10-L changes of buffer A (10.0 mM potassium phosphate, pH 7.7, 0.05 mM DTT, 0.1 mM $Na_2EDTA$).

The dialyzed crude extract was batch-treated with 500 mL of wet-packed DE-52 equilibrated with buffer A. The mixture was slowly filtered through Whatman 41 filter paper on a Buchner funnel. The DE-52 was washed with three 100-mL rinses of buffer A and the rinses were added to the rest of the flowthrough.

The DE-52 flowthrough was then adjusted to pH 7.0 with 0.5 N acetic acid and loaded onto a phosphocellulose column (2.5 × 35.0 cm) equilibrated with buffer B (10 mM potassium phosphate, pH 7.0, 0.05 mM DTT, 0.1 mM $Na_2EDTA$). The column was washed with buffer B which contained, in addition, 0.3 M KCl until the effluent had an absorbance less than 0.1 at 280 nm. CAP was then eluted with a 1.0-L linear gradient, 0.3–1.0 M KCl in buffer B. Fractions were collected and monitored for absorbance, conductivity, and cAMP binding activity (see below). Active fractions were pooled, and the protein was precipitated by adding 390 mg/mL of solid

$(NH_4)_2SO_4$ and centrifuging at 16000*g* for 30 min. The precipitate was resuspended in 6.0 mL of buffer A + 0.1 M KCl and dialyzed overnight at 4 °C vs. 1.0 L of this buffer.

The dialysis was stopped and any aggregated material removed by a 2-min low-speed spin in a clinical centrifuge. The supernatant was diluted threefold with buffer A and loaded onto a DNA–cellulose column (0.9 × 13.5 cm) equilibrated with buffer A. The DNA–cellulose had been prepared according to the method of Alberts & Herrick (1971) using native calf thymus DNA. The column was washed with buffer A; under these conditions most of the CAP passed directly through the column. The fractions showing cAMP binding activity were pooled and an $(NH_4)_2SO_4$ precipitation was performed as before. The pellet was resuspended in 2.5 mL of buffer A + 0.5 M KCl and dialyzed overnight at 4 °C vs. 1.0 L of this buffer.

The sample was removed from dialysis and was loaded onto a Sephadex G-75 column (2.5 × 90.0 cm) equilibrated with buffer A + 0.5 M KCl. Elution was performed with the same buffer. Fractions were assayed for absorbance and cAMP binding activity. Active fractions were pooled and precipitated as before. The pellet was resuspended in 5.0 mL of buffer C (10 mM Tris-HCl, pH 7.9, 0.1 mM $Na_2EDTA$, 0.05 mM DTT, 0.1 M NaCl) and dialyzed overnight at 4 °C vs. 1.0 L of buffer C. The dialyzed sample was stored at −20 °C in 500-μL aliquots. Protein purified in this way was greater than 95% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

*Measurement of cAMP Binding to CAP.* Binding of cAMP to CAP was determined by a modification of the procedure of Anderson et al. (1971). Each assay consisted of 100 μL containing 10 mM 5′-AMP, 200 μg of casein, 10 mM potassium phosphate, pH 7.7, [$^3$H]cAMP (120000 cpm), and CAP. This was incubated in an ice–water bath for 5 min. Then 400 μL of cold, saturated $(NH_4)_2SO_4$ was added and the sample was centrifuged at 8000*g* for 10 min. The supernatant was removed and discarded. The pellet was resuspended in 10.5 mL of scintillation fluid [0.5% PPO in ethanol–toluene (1:3)] and counted. A background level was established by repeating the assay with 20 mM unlabeled cAMP added to the reaction mix.

*Gel Electrophoresis.* $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (1970). The acrylamide and bis(acrylamide) had been recrystallized from acetone. Twelve-percent gels were run, using crystallized trypsin as a molecular weight standard.

*Extinction Coefficients.* In our experiments the concentrations of CAP and of DNA were evaluated from absorbance measurements and are thus only as accurate as are values for the extinction coefficients, $\epsilon$. For the protein, values of $\epsilon_{230}$, $\epsilon_{265}$, and $\epsilon_{280}$ were therefore determined using a spectroscopic method based on a procedure developed by Edelhoch (1967). All absorbance measurements were made on a Gilford Model 250 spectrophotometer with the slit width held constant at 0.40 mm.

A stock solution of CAP in buffer C was prepared and its absorbance spectrum recorded. The solution was then diluted with concentrated GdnHCl (pH 7.5) to make the final solution 6 M GdnHCl. The absorbance of the denatured CAP solution was measured at 280 and 288 nm. Either of these values, along with knowledge of the numbers of tryptophan (2), tyrosine (5),[2] and cysteine (2) residues per subunit (Anderson et al., 1971), permits calculation of the concentration of protein in the stock solution, from which the extinction coefficients of native CAP can be computed. The average values found, per CAP subunit, are $\epsilon_{230} = 1.24 \times 10^5$ $M^{-1}$ $cm^{-1}$, $\epsilon_{265} = 1.51 \times 10^4$ $M^{-1}$ $cm^{-1}$, and $\epsilon_{280} = 1.99 \times 10^4$ $M^{-1}$ $cm^{-1}$. Results from data at 280 and 288 nm agreed within 5%. These values for the extinction coefficients were used throughout this work.

The $\epsilon_{280}$ result in in reasonable accord with a value of $\epsilon_{280} = 1.75 \times 10^4$ $M^{-1}$ $cm^{-1}$ which can be calculated from the absorbance spectrum reported by Anderson et al. (1971) for a CAP solution of specified concentration (as determined by the Lowry procedure). The $\epsilon_{280}$ value for native CAP is 11% greater than $\epsilon_{280}$ for denatured CAP; this is within the 0–20% hyperchromic effect usually seen for native compared to denatured proteins (Beaven & Holiday, 1952).

The extinction coefficients for native calf thymus DNA were taken to be, per base pair: $\epsilon_{230} = 5.6 \times 10^3$ $M^{-1}$ $cm^{-1}$, $\epsilon_{265} = 1.2 \times 10^4$ $M^{-1}$ $cm^{-1}$, and $\epsilon_{280} = 6.8 \times 10^3$ $M^{-1}$ $cm^{-1}$ (Felsenfeld & Hirschman, 1965).

*Buffers for Physical Measurements.* Unless otherwise noted, all buffer solutions contained 10 mM Tris, 0.1 mM $Na_2EDTA$, plus NaCl to give the indicated [$Na^+$], pH 7.9 at 22 °C.

*Circular Dichroism Measurements.* These were made on a Jasco spectropolarimeter which had been modified for circular dichroism (CD) by Sproul Scientific Co. Measurements were made at ~22 °C over the wavelength range 220–340 nm. After each aliquot of DNA or CAP was added during a titration, the absorbance spectrum of the solution was read in a Gilford Model 250 spectrophotometer, followed by recording of the CD spectrum.

*Sedimentation Experiments.* The binding of CAP to DNA was measured using the thermodynamically rigorous sedimentation velocity method of Jensen & von Hippel (1977), adapted for use with the Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics and a photoelectric scanner (Revzin & von Hippel, 1977). This permits the contents of a cell to be examined several times during a run. Solutions of CAP and of DNA were dialyzed overnight against the appropriate buffer and were mixed approximately 2 h prior to each run. Identical results were found using solutions which were prepared by overnight dialysis of DNA–CAP mixtures at the desired base pair–protein ratio. The sedimentation velocity runs were performed at 22 °C at speeds from 10000 to 40000 rpm.

Absorbance readings in the analytical ultracentrifuge were made with the slit fully open (2.0 mm). Because of the wide spectral band-pass, absorbance readings in the Model E differed somewhat from those measured with the Gilford spectrophotometer. Correction factors to account for this difference were determined by measuring the absorbances of CAP and of DNA first in the Gilford and then in the Model E. The ratios of the absorbances (corrected for different path lengths) were used as the calibration factors. (See also Revzin & von Hippel (1977).)

At any time during the sedimentation experiment concentrations of DNA and CAP at each point in the centrifuge cell can be evaluated by analysis of the absorbance readings at two wavelengths (265 and 230 nm were routinely used) (Revzin & von Hippel, 1977). We used this approach to determine the total concentration of each macromolecule in our solutions. This was done early in the run, when any large aggregates had sedimented out of solution but the DNA–CAP complexes had barely left the meniscus. The input concentrations of DNA and CAP found in this way agreed well with

[2] There is some uncertainty as to whether there are five or six tyrosine residues per subunit (Anderson et al., 1971). If there are six tyrosines, the calculated $\epsilon_{280}$ value would change by less than 5%.

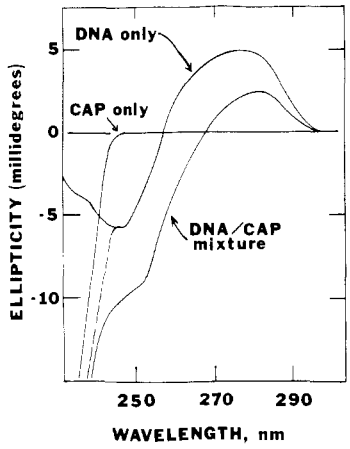


FIGURE 1: Circular dichroism spectrum of a CAP–native calf thymus DNA mixture in 5 mM $Na_2HPO_4$, 15 mM NaCl, 0.1 mM $Na_2EDTA$, 0.05 mM DTT, pH 7.7; $T$ = 22 °C; [CAP] = $1.5 \times 10^{-6}$ M (dimers), [DNA] = $2.8 \times 10^{-5}$ M (base pairs). Also shown are spectra for CAP alone and for DNA alone at the same concentrations. The dashed line is the sum of these spectra and coincides with the DNA-only curve at wavelengths above 245 nm (where CAP shows essentially no circular dichroism).

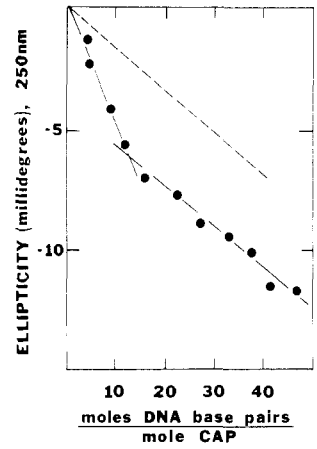


FIGURE 2: Titration of CAP with native calf thymus DNA, monitored by circular dichroism (see text). The lower curve (●) shows data for a solution with [CAP] = $1.1 \times 10^{-6}$ M (dimers), to which DNA was added to give various nucleic acid–protein ratios. All data were normalized to the same protein concentration. The dashed line is for DNA only; this was obtained under identical conditions, except that CAP was omitted from the solution. The buffer contained 5 mM $Na_2HPO_4$, 15 mM NaCl, 0.1 mM $Na_2EDTA$, 0.05 mM DTT, pH 7.7, $T$ = 22 °C.

the values expected from the amounts of DNA and protein stocks used in making the solutions. This method for evaluating the DNA and CAP concentrations circumvents any problems from the small amount of aggregation which tends to occur during solution preparation.

## Results

*Binding of CAP Causes a Conformational Change in DNA.* Circular dichroism spectra of CAP alone, of native calf thymus DNA, and of a CAP–DNA solution are shown in Figure 1. The ionic strength is sufficiently low that all protein molecules in the DNA–CAP solution will be bound to the DNA (see sedimentation results below). It is easily seen from Figure 1 that the CD spectrum of the complex differs from the sum of the individual protein and DNA spectra, i.e., a nonzero difference spectrum is observed in the wavelength region 245–300 nm. This implies that a conformational change occurs in either CAP or DNA or both. Since the CD of CAP is virtually zero at these wavelengths it seems likely that the DNA undergoes a change in conformation upon the binding of CAP. Similar experiments were performed in the presence of $5 \times 10^{-5}$ M cAMP; evidence presented below shows that the cyclic nucleotide interacts with CAP under these conditions. After correction for the circular dichroism of cAMP, the results were indistinguishable from those in the absence of cAMP. Thus the DNA conformational change reflected in the circular dichroism is the same whether cAMP is present or absent.

At lower wavelengths (210–230 nm), where the CD of the protein is much larger than that of the DNA, the difference spectrum is nil, and again cAMP has no effect (data not shown). Furthermore, the spectrum of a solution of CAP alone does not change significantly when cAMP is bound. Thus circular dichroism is not sufficiently sensitive to measure conformational changes in CAP due to cAMP binding nor can any such changes be detected when the protein associates with DNA.

*Binding Site Size for CAP is 13 Base Pairs per Bound Protein Molecule.* The CD spectral changes were used to evaluate the binding site size, $n$, defined as the number of base pairs covered by a bound CAP molecule. These results are shown in Figure 2. In this experiment a fixed amount of CAP was titrated with DNA and the ellipticity followed at 250 nm (where the difference spectrum is maximal). The ionic strength is low, so that binding is stoichiometric. The ellipticity at low values of the DNA/CAP ratio reflects the DNA conformational change due to CAP binding, since all DNA binding sites are filled under these conditions of CAP excess. At high values of the DNA/CAP ratio, all protein molecules in the solution are bound; hence the contribution to ellipticity from additional DNA is that of free DNA. The slope of the line at high DNA/CAP ratios is seen to be parallel to the DNA-only line in Figure 2. We interpret the rather sharp break in the curve as the point where all the DNA binding sites are just filled with protein molecules; at this point the solution contains about 13 base pairs of DNA per CAP molecule, so that $n$ = 13. Similar results are found when the titration is performed in the presence of $5 \times 10^{-5}$ M cAMP (data not shown). Furthermore, the value of $n$ = 13 is also obtained when a fixed amount of DNA is titrated with CAP, which confirms that the DNA can indeed be saturated with protein in these experiments.

*Sedimentation Technique for Measuring the Binding of CAP to Double-Helical DNA.* In this approach (Revzin & von Hippel, 1977), DNA and protein are uniformly distributed in the Model E analytical ultracentrifuge cell at the start of the run. Under appropriate ionic conditions the solution will contain DNA–protein complexes and some free (unbound) protein. In these studies of nonspecific binding each rather long DNA molecule contains many protein binding sites and may have several CAP molecules bound to it. Typical traces are shown in Figure 3 for the case where cAMP ($5 \times 10^{-5}$ M) is present in the solution. A two-phase boundary is seen. The spectrophotometer on the centrifuge had been calibrated so the absorbance readings from the traces can be converted into concentrations of DNA and CAP using a two-wavelength analysis (Revzin & von Hippel, 1977). The faster moving section of the boundary in Figure 3 has a sedimentation coefficient larger than that for either DNA or protein alone, and analysis of the absorbances at 265 and 230 nm confirms that this relatively rapidly sedimenting material consists of DNA–protein complexes. The slow-moving material, which had barely started to leave the meniscus when the scans in Figure 3 were taken, is identified as free CAP using its spectral characteristics and sedimentation coefficient (all of this

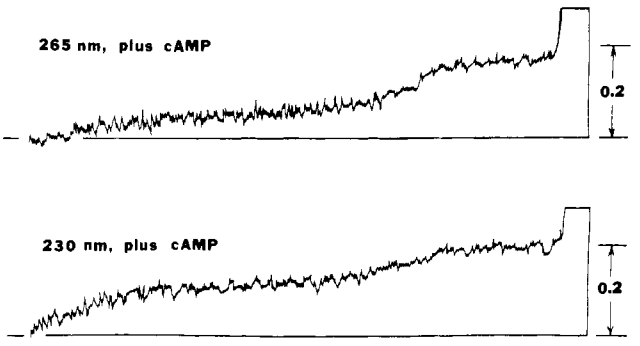


FIGURE 3: Scans from Model E ultracentrifuge experiments in which DNA–protein complexes and unbound CAP are seen in the presence of cAMP. The buffer contained 10 mM Tris-HCl, 100 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.9; $T$ = 22 °C; [cAMP] = 0.05 mM. Input concentrations: [CAP] = $4.3 \times 10^{-7}$ M (dimers), [DNA] = $1.7 \times 10^{-5}$ M (base pairs). Sedimentation was from left to right. Scans were taken 38 min (265 nm) and 36 min (230 nm) after the rotor reached a speed of 40 000 rpm. The relatively high noise level (even at 265 nm) seen in this figure and in Figure 4 arises because of the sizable absorbance of the cAMP, which is present in both the reference and sample sectors. The calibration factors relating absorbance readings in the centrifuge to those in the Gilford spectrophotometer are appreciably affected by the presence of cAMP.

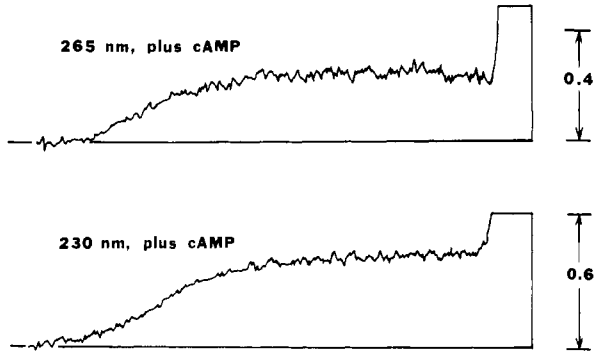


FIGURE 4: Scans from Model E ultracentrifuge experiment showing that all CAP molecules can bind to DNA noncooperatively in the presence of cAMP. The buffer contained 10 mM Tris-HCl, 25 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.9; $T$ = 22 °C; [cAMP] = 0.05 mM. Input concentrations: [CAP] = $5.8 \times 10^{-7}$ M (dimers), [DNA] = $6.8 \times 10^{-5}$ M (base pairs). Sedimentation was from left to right. The single boundary has a sedimentation coefficient of 22 S compared with 16 S for the DNA alone. Scans were taken 27 min (265 nm) and 29 min (230 nm) after the rotor reached a speed of 32 000 rpm.

material does leave the meniscus at later times). Figure 3 shows that the cAMP-containing solution contains no DNA molecules which are completely devoid of protein—free DNA regions exist between the CAP molecules which are bound to a given DNA molecule.

Data of this type can be used to determine an association constant for DNA–CAP binding. To evaluate this quantity it is necessary to determine the concentration of free protein (see below). In these sedimentation runs the DNA–protein complexes are always in equilibrium with free CAP, and the concentration of unbound CAP is determined from the absorbance of the slow-moving free protein after the CAP–DNA complexes have sedimented to the bottom of the cell.

*All CAP Molecules Are Active in Nonspecific Binding.* If an experiment similar to that shown in Figure 3 is performed at sufficiently low ionic strength, a single-phase boundary of DNA–protein complexes is observed (Figure 4). No free protein remains as slow-moving material, indicating that every CAP molecule is capable of binding to DNA.

*Pressure Effects Are Not Important in the CAP–DNA Interaction.* Since the pressure on a solution in the ultracentrifuge cell is very large, one must consider whether this will affect the DNA–protein equilibrium. Such effects can in theory be enormous (Harrington & Kegeles, 1973). We have tested for pressure effects by changing the rotor speed during the run. Increasing the angular velocity from 10 000 to 40 000 rpm did not change the amount of unbound CAP. Thus, a 16-fold variation in pressure had no noticeable effect on the equilibrium. This indicates, though it does not conclusively prove, that the binding constants measured in the ultracentrifuge are not very different from those at atmospheric pressure. There are to our knowledge no previous reports in the literature of measurements of association constants for CAP–DNA binding at atmospheric pressure.

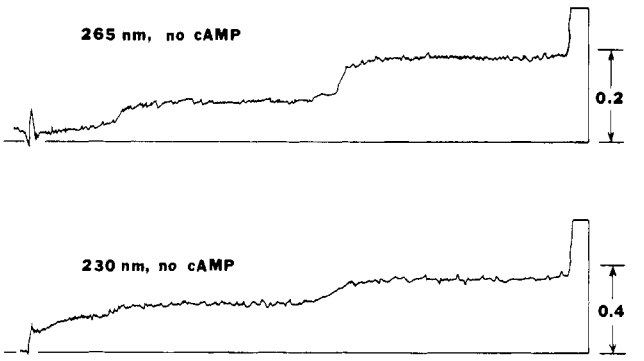


FIGURE 5: Scans from Model E ultracentrifuge experiment in which all three species (DNA–protein complexes, free CAP, and free DNA) are seen in the absence of cAMP. The buffer contained 10 mM Tris-HCl, 69.8 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.9; $T$ = 22 °C. Input concentrations: [CAP] = $1.8 \times 10^{-6}$ M (dimers), [DNA] = $2.8 \times 10^{-5}$ M (base pairs). Sedimentation was from left to right. Scans were taken 55 min (265 nm) and 57 min (230 nm) after the rotor reached a speed of 20 000 rpm.

*Free CAP Does Not Self-Associate.* Sedimentation studies of CAP alone were performed over the entire range of protein concentrations relevant to the CAP–DNA experiments, with and without cAMP. These runs yielded $s_{20,w} = 3.3 \pm 0.4$, which agrees well with the previously reported value of 3.5 (Anderson et al., 1971) and is what would be expected for a roughly spherical protein of molecular weight 45 000. Thus there is essentially no self-association of unbound CAP in our experiments.

*In the Absence of cAMP, CAP Binds Cooperatively to Double-Helical DNA.* When experiments similar to those shown in Figure 3 were performed in the absence of cAMP, *three-phase* boundaries were seen (Figure 5). In this case sedimentation and absorbance characteristics identify the fastest portion of the boundary as DNA–protein complexes, the slowest moving part as free CAP, and the intermediate section as free DNA molecules to which no protein molecules are bound. We interpret these results to mean that CAP binds cooperatively to DNA, i.e., protein molecules preferentially bind next to already bound CAP molecules.

This interpretation is strengthened by the observation that at low ionic strengths a two-phase boundary is seen, consisting of a rather broad array of fast-moving DNA–protein complexes and slower moving free DNA molecules (Figure 6). As was the case when cAMP was present all CAP molecules bind DNA, but without cAMP the protein binds in clusters. As the CAP/DNA ratio is increased in these solutions, the ratio of protein to DNA in the complexes is found to level off at approximately 1 CAP molecule per 15 base pairs, which is reasonably consistent with a binding site size of 13.

*Evaluation of Equilibrium Constants for CAP–DNA Binding.* McGhee & von Hippel (1974) derived the following

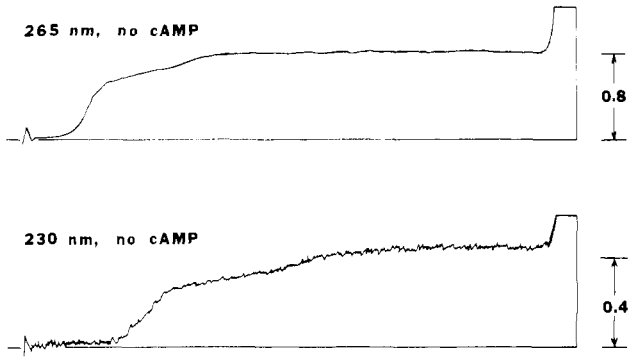


FIGURE 6: Scans from Model E ultracentrifuge experiment in which DNA–protein complexes and free DNA are seen in the absence of cAMP. The buffer contained 10 mM Tris-HCl, 40 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.9; $T$ = 22 °C. Input concentrations: [CAP] = $4.65 \times 10^{-7}$ M (dimers), [DNA] = $6.75 \times 10^{-5}$ M (base pairs). Sedimentation was from left to right. Scans were taken 43 min (265 nm) and 82 min (230 nm) after the rotor reached a speed of 20000 rpm.

Table I: Effect of Ionic Strength on Binding Parameters of CAP + Native Calf Thymus DNA

| $[Na^+]$ (mM)[a] | $K$ ($M^{-1} \times 10^{-4}$)[b] | $\omega$ | $K\omega$ ($M^{-1} \times 10^{-6}$) |
|---|---|---|---|
| 50 | 5.96 | 70 | 4.17 |
| 60 | 1.98 | 110 | 2.18 |
| 70 | 0.65 | 175 | 1.14 |
| 80 | 0.73 | 60 | 0.44 |

[a] Solutions contained 10 mM Tris-HCl, 0.1 mM $Na_2EDTA$, and NaCl to give the stated $Na^+$ concentration; pH 7.9; $T$ = 22 °C; no cAMP. [b] Intrinsic association constants, $K$, are in units of $M^{-1}$, with concentrations molar in CAP (dimer of two subunits) and DNA base pairs.

equation to describe the cooperative binding of large ligands (e.g., proteins) to long lattices (e.g., DNA),

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

where $\bar{\nu}$ = degree of binding (moles of protein bound per mole of DNA base pairs), $L$ = concentration of free (unbound) protein, $n$ = binding site size (number of DNA base pairs covered by a bound molecule of protein), $K$ = intrinsic protein–DNA association constant, $\omega$ = cooperativity parameter = (association constant for protein binding adjacent to a bound protein)/(association constant for protein binding to an isolated region of DNA), and $R = [[1 - (n + 1)\bar{\nu}]^2 + 4\omega\bar{\nu}(1 - n\bar{\nu})]^{1/2}$. Equation 1 permits analysis of binding data by means of a Scatchard (1949) plot. A single centrifuge run yields one point on such a plot. The value of $L$ is the concentration of slowly sedimenting (free) protein, and the value of $\bar{\nu}$ is determined from knowledge of $L$ and of the total DNA and protein concentrations. Several runs, each using different amounts of input DNA and CAP, generate the entire Scatchard plot. The data are then fitted to eq 1 and, in principle, the parameters $K$, $\omega$, and $n$ can be evaluated.

*Ionic Strength Dependence of CAP–DNA Binding.* Figure 7 is a Scatchard plot for the cooperative binding of CAP to native calf thymus DNA in 10 mM Tris-HCl, 50 mM $Na^+$, pH 7.9. The large "hump" is characteristic of strongly cooperative binding. The data were fitted to eq 1 using a nonlinear method of least-squares computer program to determine the optimal values for $K$ and $\omega$, assuming $n$ = 13.

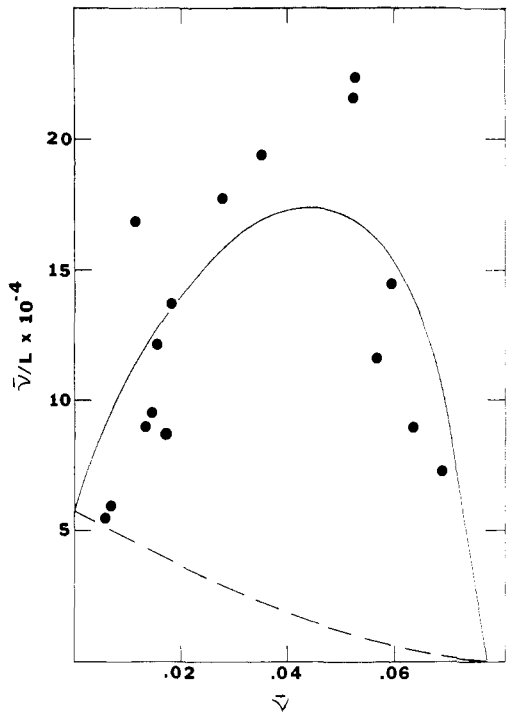


FIGURE 7: Scatchard plot for the cooperative binding of CAP to native calf thymus DNA in the absence of cAMP. The buffer was 10 mM Tris-HCl, 49.8 mM NaCl, 0.1 mM $Na_2EDTA$, pH 7.9; $T$ = 22 °C. The theoretical curve which gives the best fit to the data (using a nonlinear method of least-squares approach) was evaluated from the theory of McGhee & von Hippel (1974), assuming a value of $n$ = 13 for the binding site size. The dashed line is the predicted Scatchard plot for a noncooperative system having the same $K$ and $n$ values, but for which $\omega$ = 1.

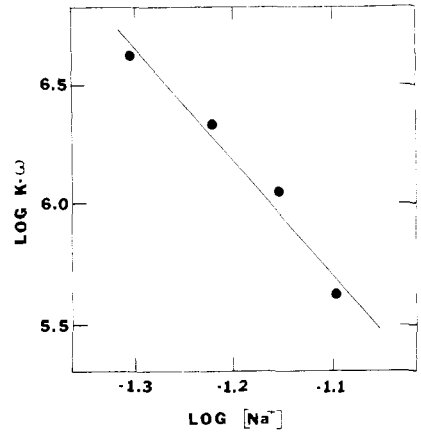


FIGURE 8: Variation of $K\cdot\omega$, a measure of the overall affinity of CAP for native calf thymus DNA, as [NaCl] was varied from 49.8 to 79.8 mM in the absence of cAMP. The buffers were at pH 7.9 and also contained 10 mM Tris-HCl, 0.1 mM $Na_2EDTA$; $T$ = 22 °C. The units of $K$ are $M^{-1}$ while $[Na^+]$ is in mol/L.

Similar experiments were performed using solutions of higher ionic strength, and the parameters $K$ and $\omega$ were evaluated from Scatchard plots. The results are presented in Table I and Figure 8. Since the base sequence of calf thymus DNA is heterogeneous, the intrinsic association constants in Table I are average values. These $K$ values should give an accurate representation of CAP–DNA binding since, as noted below, the strength of the interaction seems to be rather independent of base sequence.

It is difficult to obtain precise data in these experiments at high values of $\bar{\nu}$ because there is a tendency for some aggregation to occur as the negative charge of the DNA is

neutralized by bound protein. It is therefore gratifying that the experimentally determined values of $\bar{\nu}/L$ do approach zero as $\bar{\nu} \rightarrow 1/n = 0.077$, $n$ having been evaluated independently from circular dichroism results. It should be noted that even in the absence of good data at high $\bar{\nu}$ values, the parameters $K$ and $\omega$ can be extracted with confidence from the theoretical equation using results at lower $\bar{\nu}$ along with the value $n = 13$.

Accurate binding data in the presence of cAMP are more difficult, though not impossible, to generate because the optical measurements in the Model E ultracentrifuge must be made against the background of cAMP absorbance. Such studies are currently in progress. We report here that in the presence of cAMP the binding of CAP to DNA is very tight when $[Na^+] < 75$ mM (i.e., no unbound protein molecules can be detected), and there is no detectable interaction at our DNA and CAP concentrations when $[Na^+] > 125$ mM. In contrast, the overall binding is weaker in the absence of cAMP—very tight association is seen only for $[Na^+] < 40$ mM, while the binding disappears in our solutions at $[Na^+] > 90$ mM. These results confirm the findings of other workers, who used entirely different techniques and concluded that cAMP can enhance the affinity of CAP for DNA (Riggs et al., 1971; Nissley et al., 1972; Krakow & Pastan, 1973).

*Other Aspects of the Cooperative CAP–DNA Interaction.* The presence of $Mg^{2+}$ at low concentrations (3–10 mM) in CAP–DNA solutions containing 15 mM $Na^+$ had no effect on the cooperative characteristics of the binding. However, at 25 mM $Mg^{2+}$ (and 15 mM $Na^+$) no binding at all was detected by our technique. Thus the action of $Mg^{2+}$ seems to arise primarily from its contribution to the ionic strength of the solution; no specific effects are apparent.

To check the effects of base sequence on the CAP–DNA interaction in the absence of cAMP, experiments were performed using the alternating copolymers poly[d(A-T)] and poly[d(I-C)]. Cooperative binding was seen with both of these double-helical synthetic DNAs. Furthermore, although sufficient data for Scatchard plots were not obtained, we have done experiments which indicate that the strength of binding is independent of base sequence. Thus, at 50, 60, and 70 mM $Na^+$, centrifuge runs were performed using three solutions, each of which contained the same amount of CAP and the same concentration of base pairs of either calf thymus DNA, poly[d(A-T)], or poly[d(I-C)]. At a given $[Na^+]$, approximately the same amount of free protein was found in all three runs, which implies that the effects of base sequence on the binding are not pronounced.

If a solution of CAP plus DNA at 25 mM $Na^+$ is titrated with cAMP, the cooperative effect disappears. The concentration of protein in this experiment was $5.4 \times 10^{-7}$ M (dimers) and the DNA was at $1.6 \times 10^{-5}$ M (base pairs). Full cooperativity was seen at $1 \times 10^{-6}$ M cAMP, no cooperativity at $1 \times 10^{-5}$ M cAMP, and the midpoint of the titration was at roughly $6 \times 10^{-6}$ M cAMP. Cyclic GMP does not behave in the same way—the cooperative character of the CAP–DNA interaction is retained even when cGMP is present at $1 \times 10^{-4}$ M.

Some preliminary centrifugation studies were made on the binding of CAP to single-stranded DNA (denatured calf thymus DNA or poly(dC), at 50 mM $Na^+$). In comparable solutions of native DNA plus protein most of the CAP is bound to the DNA. However, in the single-stranded DNA runs the bulk of the protein was free in solution and sedimented separately from the DNA. Thus CAP shows less affinity for single-stranded than for double-helical DNA. A small cooperative effect was seen in experiments at 25 mM $Na^+$ using denatured calf thymus DNA, but this result is ambiguous since the DNA may contain double-helical regions (hairpin loops). No cooperativity was evident in the CAP–poly(dC) solutions tested at 25 mM $Na^+$. Further experiments are in progress to quantitate the interaction of CAP with single-stranded DNA.

Finally, we have considered whether the fast-sedimenting species observed in solutions containing CAP plus double-helical calf thymus DNA might in fact be a complex of CAP plus single-stranded DNA. This would imply that CAP can, in a cooperative manner, completely melt the native DNA. This possibility is rendered unlikely by the single-stranded DNA studies just described and also by the following experiment. Native calf thymus DNA was mixed with CAP at a DNA–CAP ratio near saturation (17 DNA base pairs/protein molecule) in low ionic strength solution so that virtually all the protein is bound. Little change in ultraviolet absorbance is seen, while a rather large hyperchromicity is likely if the protein could denature the DNA. (Increases in $A_{280}$ and $A_{260}$ of less than 9% *are* observed, but the solution shows a small amount of aggregation, which makes it difficult to separate the difference spectrum into contributions from scattering and from true absorbance phenomena.) Addition of NaCl to make the solution 200 mM $Na^+$ dissociates the complexes and results in an absorbance spectrum which is exactly the sum of the corresponding individual native DNA and CAP spectra. This is *not* what would be found if CAP had melted the calf thymus DNA to separate strands; in that case the DNA would not reanneal to the native structure and a substantial hyperchromic effect from the DNA would be observed.

## Discussion

Our circular dichroism studies show that when a CAP molecule is bound to DNA it covers about 13 base pairs. Within experimental error the same value for the binding site size is found in the presence or absence of cAMP. This is a reasonable result for a globular protein, such as CAP, having a molecular mass of 45 000 daltons (Anderson et al., 1971). The molecular diameter of ovalbumin, which is similar to CAP in molecular mass and frictional coefficient, is about 55 Å as determined by diffusion measurements (Lamm & Polson, 1936; Tanford, 1961). So a slightly asymmetric CAP molecule could easily be accommodated by a 13 base-pair region of DNA (about 44 Å long).[3]

Figure 1 shows that the CAP–DNA interaction causes appreciable CD changes in the wavelength region where the protein shows virtually no circular dichroic effect. In contrast, no changes were detected at lower wavelengths, where the protein shows a large CD spectrum. It thus seems reasonable to assign the observed circular dichroism effects to conformational changes in the nucleic acid. Comparison of the spectral changes with spectra for A-, B-, and C-form DNA (Tunis-Schneider & Maestre, 1970) implies that the binding of CAP results in a shift from the B-form DNA structure to the C form. This, in turn, implies that the protein causes compacting of the DNA helix along the helix axis (Marvin et al., 1961). The same CD effects are observed when cAMP is present in the solution; thus both CAP and the cAMP–CAP complex cause the same structural changes when bound to DNA.

[3] These data do not fully rule out the possibility that a bound CAP molecule actually covers ~26 base pairs and that two CAP molecules can share the same DNA segment by binding to both sides of the helix. The *lac* repressor may in fact interact with DNA in this manner (Butler et al., 1977; Zingsheim et al., 1977).

As indicated above, no CD changes were seen which could be attributed to DNA-induced conformational changes in the protein. Furthermore, no appreciable circular dichroism effects were observed when cAMP was added to a solution containing only CAP. Since it has been shown by several approaches that binding of cAMP indeed causes conformational transitions in CAP [Krakow & Pastan, 1973; Wu & Wu, 1974; Wu et al., 1974; Eilen & Krakow, 1977], we conclude that CD is not sensitive enough to detect the structural alterations which are occurring in this particular protein.

In the absence of cAMP, the binding of CAP to double-helical DNA is cooperative; that is, when a protein molecule binds to the nucleic acid it creates a favorable binding site for another CAP molecule, and the proteins are found in clusters on the DNA. The sedimentation results indicate that these clusters probably contain on the order of perhaps a few hundred CAP molecules, since if short clusters were formed one would not expect to find a situation in which a substantial fraction of the rather long DNA molecules are completely devoid of bound protein.[4] The cooperativity is apparently not a strong function of base sequence; the effect is seen with poly[d(A-T)] or poly[d(I-C)] as well as with calf thymus DNA, and the overall binding affinity is the same for these three DNAs. Preliminary studies imply that CAP association with single-stranded DNA is noncooperative.

Comparison of sedimentation data at various ionic strengths for CAP–DNA binding with and without cAMP shows that the binding of cAMP to CAP very much reduces the cooperative effect[5] while simultaneously increasing the strength of the DNA–protein interaction. Titration of CAP–DNA complexes with cAMP (see Results) shows that the cooperativity is about "half gone" at a cAMP concentration of $6 \times 10^{-6}$ M. From this result one can estimate the binding constant for cAMP to CAP–DNA complexes. This requires an assumption as to how many cAMP molecules bound per CAP are required to eliminate the cooperativity. For instance, if one cAMP per CAP is necessary, then the dissociation constant for the interaction of cAMP with CAP–DNA complexes is $6 \times 10^{-6}$ M. This result will be useful for a full thermodynamic analysis of this system as more thermodynamic data are acquired in future studies.

It is of interest to ask whether the cooperative effect arises from direct protein–protein interactions, from alterations in DNA structure which make favorable the binding of a CAP molecule next to an already bound CAP, or from a combination of these effects. While circular dichroism does not reveal any structural changes in the protein upon DNA or cAMP binding, the DNA conformational change seen by CD is probably not solely responsible for the cooperativity, since the same effect on DNA is seen in the presence of cAMP. It is possible that cAMP could reduce the cooperativity simply if its binding sites on CAP are such that electrostatic repulsions between cAMP–CAP complexes on DNA would become significant. However, since cAMP binding to CAP does alter the protein conformation (resulting, among other things, in heightened DNA affinity), it seems likely that a conformational change in CAP upon binding to DNA is involved in the cooperative effect. This is currently being investigated using several approaches, including fluorescent probes and proteolytic digestion experiments.

The data in Table I show that, as is usual for DNA–protein interactions, the strength of the binding diminishes at higher ionic strengths. Analysis of the Scatchard plots showed that the statistical fit of the theoretical equation to the data is quite sensitive to the value of the $K\omega$ product but is less sensitive to changes in the individual values of $K$ and $\omega$ as long as these are varied so as to keep $K\omega$ constant. Thus, the uncertainties in the values of $\omega$ in Table I are such that one can say only that the cooperativity parameter has a value of about 100 and is approximately independent of ionic strength. Most of the ionic strength dependence lies, as expected, in $K$, the intrinsic affinity of CAP for DNA.

The number of electrostatic interactions involved in CAP–DNA binding may be deduced from the ionic strength dependence of the association constant using the equation of Record et al. (1976) and de Haseth et al. (1978)

$$-\frac{\text{d log } K_{\text{obsd}}}{\text{d log } [\text{M}^+]} = m'\psi + k \qquad (2)$$

where $K_{\text{obsd}}$ is the observed association constant, $[\text{M}^+]$ is the concentration of monovalent cations, $\psi = 0.88$ for double-helical DNA, $m'$ is the number of ionic interactions between the protein and the DNA (which equals the number of monovalent cations released from DNA when the complex is formed), and $k$ is the number of ions released from the protein when the complex is formed. Assuming that most of the ionic effects arise from the very strong polyelectrolyte DNA, then $k$ is negligible with respect to $m'\psi$ and $m'$ can be evaluated. If $\omega$ indeed does not depend on ionic strength, then

$$-\frac{\text{d log } K\omega}{\text{d log } [\text{M}^+]} = -\frac{\text{d log } K}{\text{d log } [\text{M}^+]} = 0.88m' = 4.85$$
$$m' = 5.5$$

Thus about five or six positively charged groups on the protein interact with DNA in the CAP–DNA complex (no cAMP present); this is a reasonable result in view of the 13 base-pair site size for CAP binding. Studies of the ionic strength dependence of CAP–DNA binding in the presence of cAMP are under way. It will be of interest to compare the number of ionic interactions with and without cAMP to see how much of the increased affinity of cAMP–CAP for DNA arises from electrostatic effects.

To a limited extent these results for the nonspecific CAP–DNA interaction may be pertinent to the specific functioning of CAP at the promoter region. Since cAMP is needed for CAP to stimulate transcription in vivo, the cooperative effect reported here may be irrelevant per se. However, one cannot rule out the possibility that a change in CAP conformation upon specific binding to the promoter leads to an interaction between one or more CAP molecules and RNA polymerase which facilitates the initiation process. In any case, some aspects of the CAP–CAP cooperativity may apply to possible CAP–RNA polymerase interactions at the promoter. Finally, our data imply that CAP is not a "melting protein" when it interacts nonspecifically with DNA. If this is also the case for specific binding, then it seems likely that

[4] The calf thymus DNA used here has an average $s_{20,w}$ of 16, which corresponds to a molecular weight of about $4 \times 10^6$, or about 6000 base pairs (Eigner & Doty, 1965). Thus this DNA could accommodate clusters of CAP containing as many as 450 molecules. Our data show that at low input CAP/DNA ratios the DNA molecules are *not* saturated with protein. As the CAP/DNA ratio increases we find a higher degree of saturation of the complexes (they sediment faster) as well as more complexes formed (fewer totally free DNA molecules). It may be worth noting that the work of Ruyechan & Wetmur (1975) implies that the average cluster size should be on the order of $\omega^{1/2} \approx 10$–15; if this were the case we would not observe a biphasic sedimentation boundary. The reasons for this apparent discrepancy between theory and experiment are under study.

[5] We do not yet have sufficiently accurate Scatchard plots for cAMP-containing solutions to unequivocally state that there is no cooperativity at all. All indications, however, are that cAMP–CAP complexes bind to DNA in a noncooperative manner.

the CAP function is not mediated through melting of the DNA. Its action may involve a change in DNA structure from the B to C form, perhaps in conjunction with a direct CAP–RNA polymerase interaction.

An important question in these studies is whether CAP may be bound nonspecifically to the bacterial chromosome in vivo. It appears from both in vitro and in vivo experiments that virtually all *lac* repressor molecules in the cell are bound to DNA, either at the operator region or at nonspecific sites (von Hippel et al., 1974; Kao-Huang et al., 1977). From the data in Table I and Figure 8 we can estimate whether CAP too will be nonspecifically bound in vivo. This involves assuming cellular concentrations for DNA of $\sim 1.7 \times 10^{-2}$ M [$\sim 10^7$ base pairs/cell; each base pair represents a potential CAP binding site (McGhee & von Hippel, 1974)] and for CAP of $2.2 \times 10^{-6}$ M [about 1300 molecules/cell (Anderson et al., 1971)]. Extrapolation of the data in Figure 8 to a physiological ionic strength of 0.17 M $Na^+$ equivalents (Kao-Huang et al., 1977) implies that on the order of 20% of the CAP molecules will be DNA bound in the absence of cAMP. This arises from the high degree of cooperativity which compensates for the relatively low intrinsic protein–DNA affinity at this ionic strength. Furthermore, in the presence of cAMP there will likely be considerably more nonspecific binding of CAP in the bacterium. Uncertainties as to true physiological conditions mean that calculations such as these may not be terribly accurate but they are suggestive enough that we are undertaking experiments with *E. coli* minicells of the type performed with *lac* repressor (Kao-Huang et al., 1977) to test directly the possibility that CAP is nonspecifically bound to DNA in vivo. We note that in vitro experiments to date imply that the specific and nonspecific association constants for CAP–DNA binding are not very different (Riggs et al., 1971; Majors, 1975). If this result is upheld by further studies of CAP–promoter interactions, then nonspecific binding will play an important role in controlling the biological function of CAP.

Acknowledgments

We thank Mildred Martin and Gail McDole for expert technical assistance.

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