An Equilibrium Study of the Cooperative Binding of Adenosine Cyclic 3′,5′-Monophosphate and Guanosine Cyclic 3′,5′-Monophosphate to the Adenosine Cyclic 3′,5′-Monophosphate Receptor Protein from *Escherichia coli*†

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ABSTRACT: The binding of adenosine cyclic 3′,5′-monophosphate (cAMP) and guanosine cyclic 3′,5′-monophosphate (cGMP) to the adenosine cyclic 3′,5′-monophosphate receptor protein (CRP) from *Escherichia coli* was investigated by equilibrium dialysis at pH 8.0 and 20 °C at different ionic strengths (0.05–0.60 M). Both cAMP and cGMP bind to CRP with a negative cooperativity that is progressively changed to positive as the ionic strength is increased. The binding data were analyzed with an interactive model for two identical sites and site/site interactions with the interaction free energy $-RT \ln \alpha$, and the intrinsic binding constant K and cooperativity parameter α were computed. Double-label experiments showed that cGMP is strictly competitive with cAMP, and its binding parameters K and α are not very different from that for cAMP. Since two binding sites exist for each of the cyclic nucleotides in dimeric CRP and no change in the quaternary structure of the protein is observed on binding the ligands, it is proposed that the cooperativity originates in ligand/ligand interactions. When bound to double-stranded deoxyribonucleic acid (dsDNA), CRP binds cAMP more efficiently, and the cooperativity is positive even in conditions of low ionic strength where it is negative for the free protein. By contrast, cGMP binding properties remained unperturbed in dsDNA-bound CRP. Neither the intrinsic binding constant K nor the cooperativity parameter α was found to be very sensitive to changes of pH between 6.0 and 8.0, at 0.2 M ionic strength and 20 °C. For these conditions, the intrinsic free energy and entropy of binding of cAMP are $\Delta H° = -1.7$ kcal·mol^{-1} and $\Delta S° = 15.6$ eu, respectively.

After it had been demonstrated that adenosine cyclic 3′,5′-monophosphate (cAMP)[1] could antagonize catabolite repression in *Escherichia coli* (Ullmann & Monod, 1968; Perlman & Pastan, 1968), a specific cAMP binding protein was isolated and purified, and it was shown to participate in the derepression effect. This protein was named "catabolite gene activator protein" (CAP) (Zubay et al., 1970) or "cAMP receptor protein" (CRP) (Emmer et al., 1970), and both Beckwith's and Pastan's groups reported that it could stimulate transcription and increase the synthesis of β-galactosidase, in the presence of cAMP, in the cell-free system of Chambers & Zubay (1969). From a classical view, the cAMP–CRP complex is presently expected to bind to the promoter region of certain catabolite-sensitive operons, thereby increasing the frequency of initiation of specific mRNA synthesis (Perlman et al., 1969; Zubay et al., 1970). However, CRP could well have a more general role for it was recently observed that natural polarity in polycistronic units is modulated by cAMP in a CRP-dependent interaction at sites apparently distinct from the promoter region (Ullmann et al., 1979).

In vitro studies showed that only the nonphysiological compound tubercidin cyclic 3′,5′-monophosphate (cTuMP) could mimic cAMP in activating the protein, whereas a few cyclic nucleotide analogues, and particularly guanosine cyclic 3′,5′-monophosphate (cGMP), were antagonists of cAMP actions on CRP. These include the following properties of the protein that respond to cAMP by large modifications which cGMP is able to release, stimulation of mRNA synthesis in vitro (de Crombrugghe et al., 1971; Parks et al., 1971) and binding to poly[d(A-T)] and to DNA at pH 8 (Krakow & Pastan, 1973; Riggs et al., 1971) and enhancement of the susceptibility to proteolytic cleavage leading to the α-core CRP (Krakow & Pastan, 1973; Krakow, 1975). All of these effects were ascribed to a particular conformational state of the protein that the effector cAMP could either select by stabilization or induce specifically. In a study based on the temperature-jump fast perturbation technique, it could be observed that a covalently labeled fluorescent derivative of CRP (AENS-CRP) exists in two interconvertible states, and only one of these binds cAMP efficiently (Wu & Wu, 1974; Wu et al., 1974). Small-angle X-ray scattering measurements were recently reported that provide direct physical evidence for contraction of the CRP molecule on binding cAMP (Kumar et al., 1980).

Although both physical–chemical and sequencing investigations showed that the CRP molecule is comprised of two identical polypeptide chains, initial studies on cAMP binding suggested that only one receptor site for the cyclic nucleotide was present per dimer (Anderson et al., 1971). This could have resulted from either of the two following causes: (1) each subunit contributes a part of the unique binding site and this implies, a priori, an asymmetrical spatial arrangement of the otherwise identical chains in the dimer, or (2) there exists a second binding site that escaped detection because of a low intrinsic affinity or because negative cooperativity exists between the binding sites within the dimeric protein.

Since we initiated a detailed physical–chemical investigation of the cAMP-modulable interactions of CRP with DNA (Takahashi et al., 1979), with DNA-dependent RNA polymerase (Blazy et al., 1980), and with other components of the

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[1] Abbreviations used: cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; CRP, adenosine cyclic 3′,5′-monophosphate receptor protein; AENS, *N*-[2-(acetylamino)-ethyl]-5-amino-1-naphthalenesulfonate; butyl-PBD, 2-(4-biphenylyl)-5-(*p-tert*-butylphenyl)-1,3,4-oxadiazole; $NaDodSO_4$, sodium dodecyl sulfate; dsDNA, double-stranded or native deoxyribonucleic acid.

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transcription machinery, it appeared highly desirable to study the details of the mechanism and thermodynamics of the association reactions of cAMP and cGMP with CRP. The existence of more than one binding site per dimeric protein was suggested by our recent observation that the molar absorptivity coefficient, as employed previously to calculate the CRP concentrations, was incorrect (B. Blazy et al., unpublished results).

A thorough investigation was thus undertaken by the equilibrium dialysis technique of the binding of cAMP and cGMP to pure CRP under various conditions of ionc strength, pH, and temperature. This study was extended to CRP bound nonspecifically to DNA (double stranded) and to the fluorescent covalently labeled derivative AENS-CRP. The results reported herein show conclusively that two binding sites do exist in dimeric CRP for the cyclic nucleotides cAMP and cGMP. Cooperativity exists between the sites that is progressively changed from negative at low ionic strength to positive at higher salt. Both the derivative AENS-CRP and the native and DNA-bound proteins were observed to bind cAMP with positive cooperativity in conditions of low ionic strength where cooperativity is negative with the free native protein. In all cases a good fit was achieved when the binding data were interpreted with a simple model in which the binding sites are intrinsically equivalent and the free energy of binding is changed when the ligand binds near an already filled site of the same dimeric molecule. Arguments will be presented that support the proposal that such an ordered interactive model may reflect local ligand/ligand interactions. These results will be discussed in connection with the conformational and functional properties of CRP and compared with binding data reported for other cAMP binding proteins.

Materials and Methods

CRP. We shall report elsewhere the details of our purification procedure together with additional or revised data on the basic chemical and physical–chemical properties of the protein (B. Blazy et al., unpublished results). When this procedure was used, all preparations obtained so far were observed to bind cAMP and to stimulate in vitro transcription of λp*gal* DNA correctly in the assays described by Pastan and co-workers (Anderson et al., 1971). Samples of CRP with an electrophoretic purity of the order of 99% ($NaDodSO_4$ polyacrylamide gel electrophoresis) were repeatedly obtained with a specific activity of 10 000 ± 1000 units/mg in the cAMP binding assay of Anderson et al. (1971). This high figure was only partly due to the use of a revised value of the molar absorptivity coefficient to calculate the concentrations of CRP. Quantitative amino acids analyses, refractive index increment measurements, and optical absorption at 230 nm (Jensen et al., 1976) were all found consistent with the value $\epsilon_{1\,cm}^{1\%}(\lambda_{max}) = 9.2$ or, based on the molecular weight of 45 000, $\epsilon = 2.04 \times 10^4\ M^{-1}\ cm^{-1}$ at the same wavelength (λ_{max} 278 nm) (B. Blazy et al., unpublished results), a value that compares well with the figure $\epsilon = 1.99 \times 10^4\ M^{-1}\ cm^{-1}$ recently reported by others (Saxe & Revzin, 1979).

AENS-CRP. The fluorescent reagent *N*-[2-((iodoacetyl)-amino)ethyl]-5-amino-1-naphthalenesulfonate (1,5-I-AENS) was synthesized according to Hudson & Weber (1973), and the protein was coupled with this probe[2] as described by Wu et al. (1974). The samples of AENS-CRP contained 2.0 ± 0.2 fluorescent groups/mol (on the basis of the revised absorptivity coefficient of the protein mentioned above). Stimulation of λp*gal* DNA transcription in vitro and change of the fluorescence of the probe on binding cAMP (Wu et al., 1974) were both correct with our preparation of the labeled protein.

Cyclic Nucleotides. cAMP was purchased from Schwarz/Mann (catalog no. 907405), and [^{3}H]cAMP, [^{14}C]cAMP, and [^{3}H]cGMP were obtained from Amersham, France. The latter were checked for chemical and radiochemical purity by thin-layer chromatography on Merck precoated PEI–cellulose F plates and with both 0.3 M lithium chloride and 0.2 M ammonium bicarbonate as developing solvents. UV-light examination and surface radioactivity measurements (thin-layer scanner RTLS 1A, Panax Equipment Ltd, Redhill, Surrey) did not reveal significant contaminations or degradation products, and this was also the case when solutions of the labeled nucleotides were controlled after having been exposed for 20 h at 20 °C to the working conditions employed in the binding experimentation.

Equilibrium Binding Measurements. Most binding measurements were conducted with the equilibrium dialysis technique in the apparatus EMD 101 from Hoefer (Hoefer Scientific Instruments, San Francisco) by using either EMD 103 membranes (Hoefer) or regenerated cellulose membranes (catalog no. SM 11533) from Sartorius (Göttingen). Before use the membranes were routinely boiled for 5 min in 5% (w/v) sodium bicarbonate containing 50 mM EDTA and extensively rinsed with distilled water. The solutions containing the protein or the ligand were introduced into the chambers with precision syringes (Hamilton), and volumes of 0.20 mL were employed in each compartment. The time needed to reach equilibrium to better than 1% was evaluated quantitatively from the experimentally determined permeability of the membranes. To that end, theoretical time profiles of the change in concentration of the ligand were computed in cases where protein and ligand were initially placed in the same compartment or separated in the twin chambers. The influence of the concentrations of protein and ligand on these profiles was analyzed, and within a series of working conditions the longest predicted time was chosen for the actual experiment (M. Takahashi, unpublished results).

Some measurements, and particularly all the experimentation with dsDNA-bound CRP, were conducted by the rapid-filtration technique described by Paulus (1969) in an apparatus constructed from altuglass according to the original description of the author. The filtrate was recovered from the pressurized cells with the use of microliter capillaries (Drummond) as suggested by Cantley & Hammes (1973). The upper chambers were filled with 0.20 mL of the mixtures of protein and ligand, and two 10-μL filtrate samples were recovered successively. Provided that the membranes (Sartorius SM 11533), which are assembled in place wet with water, be carefully dried by a stream of nitrogen gas just before use, we have observed that the counts measured in the first and second filtrate samples were identical to within better than 5%.

Samples of the dialysates (30 μL) or of the filtrate (10 μL) were diluted to 1.0 mL with water, thoroughly mixed with 10 mL of Triton/toluene/butyl-PBD (0.3 L:0.7 L:5 g), and counted for radioactivity in a liquid scintillation counter (Intertechnique SL 4000). The response was linear over the range of concentrations of the cyclic nucleotides used in the experiments. In double-label counting, we used as controls (Freifelder, 1976) mixtures of [^{14}C]cAMP and [^{3}H]cGMP of known composition and determined that the preset adjustments of the manufacturer were correct; thus, these were used routinely. Recovery of the total input counts was quantitative in all cases, which showed that nonspecific adsorption of the ligands was negligible.

[2] This compound is presently available commercially.

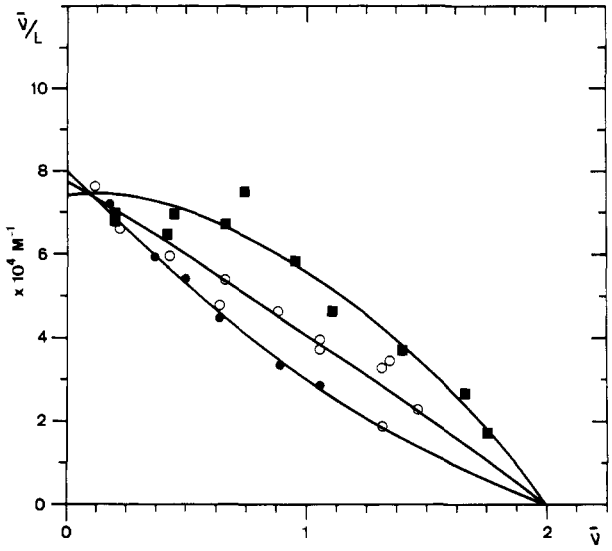

FIGURE 1: Binding of cAMP to CRP at different ionic strength. Scatchard plot of the equilibrium dialysis data observed at pH 8.0 and 20 °C. The concentrations used were within the ranges 2×10^{-6} M to 4×10^{-4} M for cAMP and 5×10^{-6} M to 6×10^{-5} M for CRP, and the time required to reach equilibrium to better than 1% was 8.5 h at this temperature. The theoretical curves were computed for the ordered interactive model presented in the text with the following best fit values of the parameters: at μ = 0.05 M (●), $K = (4.0 \pm 0.2) \times 10^4$ M^{-1} and $\alpha = 0.6 \pm 0.1$; at μ = 0.40 M (○), $K = (3.9 \pm 0.7) \times 10^4$ M^{-1} and $\alpha = 1.1 \pm 0.4$; at μ = 0.60 M (■), $K = (3.7 \pm 0.4) \times 10^4$ M^{-1} and $\alpha = 2.3 \pm 0.5$.

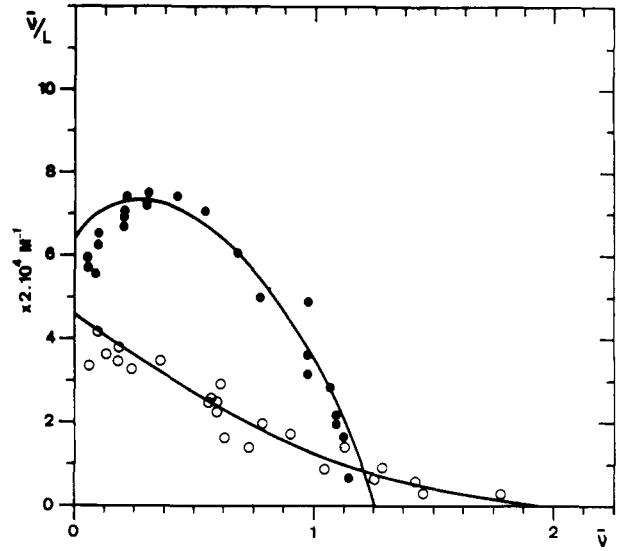

FIGURE 2: Binding of cAMP to CRP and to AENS-CRP. Scatchard representation of the equilibrium dialysis measurements at pH 8.0, 20 °C, and 0.2 M ionic strength. The best fit values of the parameters used to compute the theoretical binding curves were as follows: for CRP (○) $K = (4.6 \pm 0.5) \times 10^4$ M^{-1} and $\alpha = 0.30 \pm 0.06$, and for AENS-CRP (●) $K = (10.0 \pm 0.9) \times 10^4$ M^{-1} and $\alpha = 3.9 \pm 0.7$, with an apparent number of binding sites n = 1.25.

Buffers. The buffers used in the binding experimentation contained 50 mM (total concentration) of either Tris or Bis-Tris adjusted to the pH indicated (HCl) and supplemented with the required amounts of KCl to obtain the different values of the working total ionic strength ($^1/_2\sum c_i z_i^2$). In addition all buffers used contained 2 mM 2-mercaptoethanol and 1 mM EDTA.

Results

Binding of cAMP to CRP. Two molecules of cAMP bind to CRP at pH 8.0 and 20 °C in a range of concentration of the cyclic nucleotide varying between approximately 2×10^{-6} and 0.5×10^{-3} M. Some cooperativity is observed when the equilibrium binding data are displayed in the usual form of a Scatchard plot (Figure 1). Though not very strong, the cooperativity is rendered more evident when the ionic strength is changed, being negative at low salt and becoming progressively positive as the ionic strength is increased. Such an inversion of cooperativity is hardly explainable in terms of simple electrostatic interactions. This, together with the known identity of the polypeptide chains in the dimeric protein, led us to prefer a simple interactive model for the least-squares analysis. In this model (Saroff & Yap, 1972; Ferguson et al., 1975), it is assumed that the two sites are equivalent with an intrinsic binding free energy of $-RT \ln K$ that is changed to $-RT \ln (K \cdot \alpha)$ when the second molecule of the ligand binds to a dimeric protein molecule with an already filled site. Doing this, we introduce the free-energy contribution to binding $-RT \ln \alpha$ that originates from site/site interactions and results in cooperativity being either negative ($\alpha < 1$) or positive ($\alpha > 1$). For this model, when n, K, α, and L are used for the number of sites, intrinsic association constant, cooperativity parameter, and free ligand, respectively, the fractional saturation $\bar{\nu}$ is

$$\bar{\nu} = \frac{nKL(1 + \alpha KL)}{1 + 2KL + \alpha K^2 L^2}$$

from which the best fit (least-squares) values of K and α were obtained and were used to compute the theoretical binding curves shown in the figures.

Any contribution to the cooperativity that could have arisen from changes in the quaternary structure of the protein was excluded, since the sedimentation coefficient of CRP and of the cAMP–CRP complex remained almost unchanged within the range of protein concentration employed in this experimentation (data not shown). Furthermore, most equilibrium dialysis measurements were performed with different concentrations of the protein ranging from 2×10^{-6} M to 60×10^{-6} M without sizable influence of this factor on the binding properties; more precisely, identical ($\bar{\nu}$,$\bar{\nu}/L$) couples of values could be generated for different protein concentrations.

In Figure 2 are compared the binding properties of CRP and of the covalently labeled derivative AENS-CRP (Wu et al., 1974), both at 0.2 M KCl, pH 8.0, and 20 °C. The binding data for AENS-CRP could be fitted satisfactorily to the preceding model provided the value n = 1.25 be chosen for the number of binding sites. Though it cannot be excluded that additional sites with very low affinity exist, this strongly suggests that the labeled protein is not homogeneous with respect to cAMP binding. The prominent features in its behavior are increased intrinsic binding constant and cooperativity changing from negative to strongly positive.

Competitive Binding of cGMP and cAMP to CRP. Clearly, CRP binds two molecules of cGMP at pH 8.0 and 20 °C, and again cooperativity exists between the sites. Figure 3 shows the Scatchard plot observed at 0.2 M KCl where binding is negatively cooperative. By contrast, binding either at 0.05 M or at 0.4 M KCl was almost noncooperative.

That cGMP effectively binds to the same site as cAMP in CRP was demonstrated in a double-label experiment by using [^{14}C]cAMP and [^{3}H]cGMP. The measurements were conducted in such a way that various fractional saturation levels could be produced with respect to both of the cyclic nucleotides so as to ensure a complete exploration of the binding isotherm. Figure 4 shows the results displayed in the form of Scatchard plots appropriate to analyze competitive binding to one and the same site (Englund et al., 1969). Superimposed on the

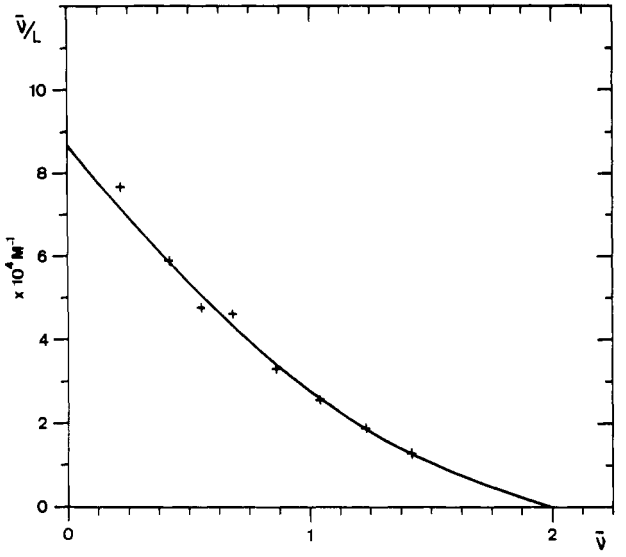

FIGURE 3: Binding of cGMP to CRP at pH 8.0, 20 °C, and 0.2 M ionic strength. The best fitting values of the binding parameters used to compute the theoretical curves were $K = (4.3 \pm 0.2) \times 10^4$ M^{-1} and $\alpha = 0.41 \pm 0.03$.

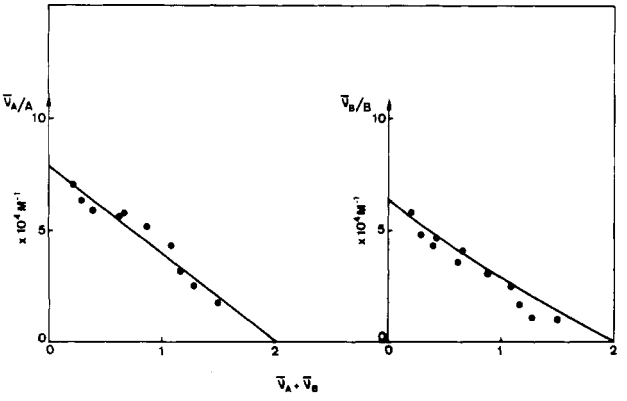

FIGURE 4: Mutually exclusive binding of cAMP and cGMP to CRP. Double-label equilibrium dialysis measurements at pH 8.0, 20 °C, and 0.4 M ionic strength. (A) stands for cAMP, and (B) for cGMP. Superimposed to the experimental points are the theoretical binding curves computed for competitive binding and using the best fit values of K and α obtained from independent experiments with each of the cyclic nucleotides alone in the same milieu: for cAMP $K = (3.9 \pm 0.4) \times 10^4$ M^{-1} and $\alpha = 1.1 \pm 0.2$; for cGMP $K = (2.9 \pm 0.3) \times 10^4$ M^{-1} and $\alpha = 1.0 \pm 0.2$).

experimentally determined values are the theoretical binding curves computed for that mechanism, using the best fit values of the binding parameters obtained from direct experiments with either cAMP or cGMP alone. Mutually exclusive, or competitive, binding is evident.

Modulation by Ionic Strength of the Binding Parameters. From Figure 1, it could be predicted that the intrinsic binding free energy and the interaction free energy contribution are both changed as the ionic strength is varied. This point is made evident and analyzed in Figure 5 which presents the change with KCl concentration of the binding parameters K and $K\alpha$ for cAMP and cGMP. Though quite similar, the behavior of the two ligands shows some distinctive features. First, we observe that the intrinsic binding constant for cGMP is much more sensitive to the variation in KCl concentration than is that for cAMP. Second, we note that the cooperativity almost disappears for cGMP binding at 0.05 M KCl, a condition where cAMP binding is negatively cooperative. Further, it is observed that almost no difference exists for either the absolute values of the binding free energy or for the cooperativity parameter α when CRP binds either cGMP or cAMP at 0.2 M KCl, i.e., an ionic strength that is considered representative of the in vivo conditions for *E. coli* (Kao-Huang et al., 1977).

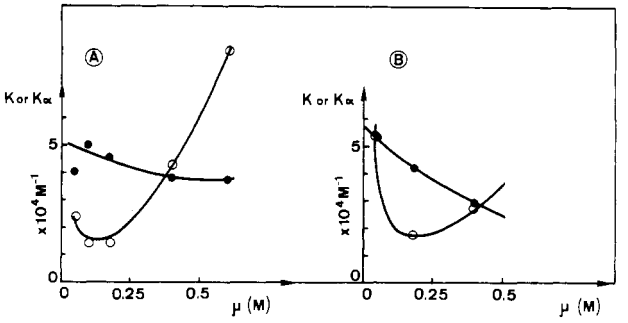

FIGURE 5: The effect of ionic strength on the binding of cAMP (A) and cGMP (B) to CRP. Binding parameters K (●) and $K\alpha$ (○) were determined by equilibrium dialysis at pH 8.0, 20 °C, and at the ionic strengths indicated (μ).

Binding of cAMP and cGMP to dsDNA-Bound CRP. CRP was recently shown to bind nonspecifically to both single-stranded or denatured DNA (ssDNA) and to native double-stranded DNA (dsDNA). This reaction is cooperative and modulated distinctively by the ionic strength and by cAMP (Takahashi et al., 1979). Here, we have explored the binding properties with respect to cAMP and cGMP of CRP bound quantitatively to dsDNA from calf thymus. This study was conducted at 0.05 M KCl, pH 8.0, and 20 °C, and the filtration technique described by Paulus (1969) was employed. For these ionic conditions, cAMP is known not to change significantly the affinity of CRP for dsDNA and, further, we could predict that the protein should be bound quantitatively to the DNA (Takahashi et al., 1979). The latter point could also be verified in a few control filtration experiments with membranes that were made permeable to CRP by treatment with zinc chloride (Seymour, 1940), for no protein was observed to be present in the filtrates. Control experiments also showed that the binding data obtained in the filtration technique were indistinguishable from that of equilibrium dialysis for either cAMP or cGMP binding to free CRP.

Figure 6 compares the binding data for cAMP obtained from the filtration experiments with dsDNA-bound CRP with the results of equilibrium dialysis measurements performed under the same conditions with the free protein. Clearly, binding of CRP to dsDNA results in an increased affinity for cAMP together with the reversal of the cooperativity from negative to positive. Surprisingly, the binding properties of dsDNA-bound CRP with respect to cGMP did not show any perturbation as compared to that of the free protein in the same conditions (Figure 7).

Effect of Temperature and pH on the Binding of cAMP to CRP. Equilibrium dialysis measurements were conducted at different temperatures between 5 and 37 °C in a medium buffered at pH 8.0 and with an ionic strength of 0.2 M at all of the temperatures indicated. The binding parameters K and α were calculated with the procedure presented in the first section, and the resulting values are given in Table I. When considering the binding of cAMP to an isolated site of the unliganded protein with an intrinsic binding constant K, we observed a good linearity of the relevant van't Hoff plot of ln K vs. T^{-1}. From this plot (not shown) we could compute the van't Hoff enthalpy change of binding, $\Delta H° = -1.7$ kcal·mol^{-1}, and the entropy change of binding, $\Delta S° = 15.6$ eu (at 20 °C). The rather low accuracy with which the interaction parameter α is defined precluded any definitive conclusion concerning

Table I: Effect of Temperature on cAMP Binding to CRP[a]

T (°C)	5	11	20	32	37
K (10^4 M^{-1})	5.1 ± 0.7	5.0 ± 0.2	4.6 ± 0.5	4.0 ± 0.2	3.8 ± 0.5
α	0.33 ± 0.08	0.39 ± 0.04	0.30 ± 0.05	0.39 ± 0.04	0.60 ± 0.18

[a] Experiments were conducted at pH 8.0 and 0.2 M ionic strength.

Table II: Effect of pH on cAMP Binding to CRP[a]

pH	6.0	6.5	7.0	7.5	8.0
K (10^4 M^{-1})	4.5 ± 0.4	5.2 ± 0.5	3.9 ± 0.4	4.1 ± 0.7	4.6 ± 0.5
α	0.17 ± 0.05	0.32 ± 0.05	0.27 ± 0.10	0.41 ± 0.10	0.31 ± 0.05

[a] Experiments were conducted at 20 °C and 0.2 M ionic strength.

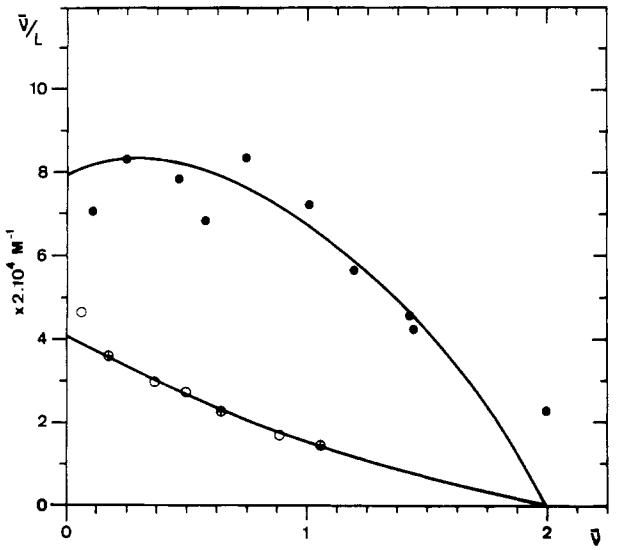

FIGURE 6: Binding of cAMP to dsDNA-bound CRP. The binding measurements were performed at pH 8.0, 20 °C, and 0.05 M ionic strength by the rapid filtration technique of Paulus (1969). Concentrations of CRP (dimer) and dsDNA (base pairs) were either 3 × 10^{-6} M and 5 × 10^{-5} M or 5 × 10^{-6} M and 1 × 10^{-4} M respectively, and cAMP was varied from 5 × 10^{-6} M to 5 × 10^{-5} M. For dsDNA-bound CRP (●) the theoretical binding curve was computed with the following best fit values of the parameters: K = (7.9 ± 3.7) × 10^4 M^{-1} and α = 3.0 ± 2.8. For comparison, binding data obtained with free CRP either by rapid filtration (⊕) or by equilibrium dialysis (○) are given together with a theoretical curve calculated with the best fit parameters presented in Figure 1 (μ = 0.05 M).

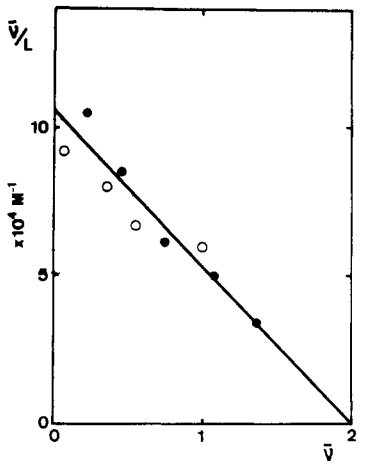

FIGURE 7: Binding of cGMP to dsDNA-bound CRP. Experimental conditions were identical with those in Figure 6. The theoretical binding curve (●) was computed with the following best fit parameters obtained for free CRP: K = (5.3 ± 0.5) × 10^4 M^{-1} and α = 1.0 ± 0.2. Experimental values for dsDNA-bound CRP (○) are superimposed.

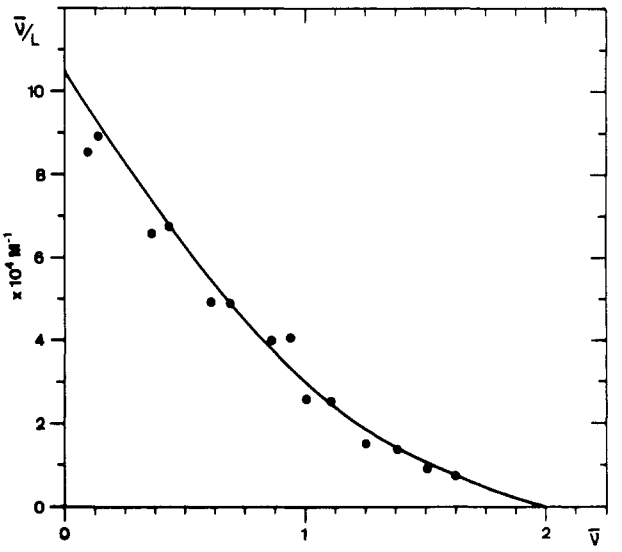

FIGURE 8: Binding of cAMP to CRP at pH 6.5. Equilibrium dialysis measurements at 20 °C and 0.2 M ionic strength. The best fit values of parameters used to compute the theoretical binding curve are included in Table II.

the relative weight of enthalpy and entropy contributions to the interaction energy, and further work is needed to clarify this point. However, the data shown in Table I would possibly suggest the existence of a minimum for α at a temperature near 20 °C.

A preliminary investigation of the effect of the pH on the binding of cAMP was conducted between pH 6.0 and pH 8.0 at 0.2 M ionic strength and 20 °C. The observations are reported in Table II and show conclusively that neither K nor α changes appreciably with pH in the range investigated. Figure 8 shows the Scatchard plot for the binding of cAMP at 20 °C, 0.2 M ionic strength, and pH 6.5; typical negative cooperativity is observed. An important finding is that the binding of cAMP to CRP is almost as efficient at pH 6.0 as it is at pH 8.0. Interest in this point originated from the reported fact that CRP binding to DNA or to poly[d(A-T)] at pH 8.0 strictly depends on the presence of cAMP, whereas binding is independent of cAMP at pH 6.0 (Riggs et al., 1971; Krakow & Pastan, 1973).

Discussion

Renewed interest in the study of cAMP binding to CRP was stimulated both by our success in reproducibly preparing the pure and active protein on a large scale and by the challenging observation reported by Pastan and co-workers that the dimeric protein could have only one binding site for its specific ligand (Pastan et al., 1974). Our recent studies of CRP binding properties with respect to DNAs (Takahashi et al., 1979) and to DNA-dependent RNA polymerase either free or DNA-

bound (Blazy et al., 1980) also demanded that the mechanism and thermodynamics of cAMP and cGMP binding to the protein be elucidated, for we could observe that these interactions are distinctively modulated by the cyclic nucleotides.

No doubt remains that there are indeed two binding sites for the cyclic nucleotides in dimeric CRP. Preliminary work on this problem failed to reveal the second binding site, but this is a quite trivial situation for, working at an ionic strength where cooperativity is negative, inaccuracy in the extinction coefficient of the protein together with crude binding studies contributed to suggesting that only one site existed (Anderson et al., 1971). It is almost certain that the CRP dimer is comprised of polypeptide chains with an identical sequence (Anderson et al., 1971; de Crombrugghe & Pastan, 1978), and demonstration of the existence of a second site for cAMP and cGMP rules out the possibility that the two otherwise identical chains could have contributed complementary domains in building a unique receptor site for the cyclic nucleotides.

Cooperativity in binding cAMP or cGMP to CRP appears to be complex in origin, for with the native protein it is reversed from negative at low ionic strength to positive at higher salt concentrations. Complexity is also perceptible from the observation that neither the pH (between 6.0 and 8.0) nor the temperature seriously affects the cooperativity parameter α (Tables I and II), and this suggests that multiple factors could compensate for their effects. To what extent any conformational change in the protein contributes to the binding properties of the cyclic nucleotides cannot be decided at present. Particularly puzzling is the observation that the binding parameters K and α and their response to the ionic strength variation are so closely comparable (Figure 5) for cAMP and cGMP, notwithstanding the fact that only the former is known to elicit a series of distinctively modified properties reminiscent of a two-state conformation behavior of the protein. The rather strong positive cooperativity observed in binding cAMP at low ionic strength to dsDNA-bound CRP, and to the chemically modified AENS-CRP as well, illustrates further this complexity and could also suggest some conformational flexibility of the protein. It is thus tempting to speculate that changes either in the solvent composition or in the local conformation of the protein promote one or the other of two antagonistic classes of interaction forces, the one being electrostatic and the other presumably arising from stacking contributions. Such stacking interactions are a well-established property of nucleotides, especially for GMP and AMP, and it has been reported that stacking can result in cooperative binding of the nucleotides to basic polyamino acids (Wagner, 1969). It could well be that the two binding sites are close enough so that repulsive electrostatic forces show up when cAMP or cGMP binds to CRP at low ionic strength that are eventually overcome by attractive stacking contributions at higher salt.

Modulable cooperativity might be a quite general behavior of the nucleotides as has been reported in the case of cyclic nucleotide-dependent protein kinases. In certain cases cooperativity also involves coupling of ligand binding with changes of the quaternary structure, and various models have been discussed by Ogez & Segel (1976) for cAMP-activated protein kinase from skeletal muscle. No dissociation of dimeric CRP molecules, or association of these, could be detected in our investigation, and this was also noted by Saxe & Revzin (1979) in another context. Relevant to the discussion is the example of cGMP binding to cGMP-dependent protein kinase from bovine lung (McCune & Gill, 1979). This dimeric protein does not dissociate on binding cGMP, and temperature- (and pH-) modulable cooperativity is observed for the binding of the ligand which the authors attributed to site/site interactions.

We have proved here that the binding domains of cAMP and cGMP overlap largely, which results in the association of these nucleotides being mutually exclusive, and this is in agreement with earlier semiquantitative observations (Anderson et al., 1971). Both the binding free energies and the site/site (or ligand/ligand) interaction forces are nearly equivalent. This is not surprising, for various enzymes that have different nucleotides for substrates show moderate specificity with respect to binding as exemplified by 3′-nucleotidase from mung bean sprouts (Loring et al., 1966) or by DNA polymerase of *E. coli* (Englund et al., 1969). However, as concerns CRP, cAMP is unique (with the nonphysiological compound cyclic tubercidin) in being able to produce the specific function of stimulation of transcription, and this is almost certainly related to some particular conformational state of the protein which cGMP cannot produce, or stabilize. The most striking difference between the two cyclic nuclotides is that which is elicited on binding CRP to dsDNA nonspecifically and results in a strongly positive cooperativity for cAMP binding at low ionic strength, whereas cGMP binding properties remain strictly unaltered. This specific change is worthy of interest with reference to the in vivo conditions. Though there is not a definitive agreement with the absolute values of the concentrations of the cyclic nucleotides in wild-type *E. coli* when grown in the absence of catabolite repression, it is generally accepted that the cAMP/cGMP ratio could exceed 100 (Botsford, 1975; Epstein et al., 1975; Bernlohr et al., 1974). When the binding properties of these cyclic nucleotides that we have characterized here are considered, it is virtually impossible to imagine that cGMP could antagonize cAMP action on CRP, especially if the protein happened to be bound to dsDNA. However, this does not exclude any possibility for cGMP to act as a modulator of the CRP-dependent regulations in *E. coli*. Subtly induced, or constitutive, readjustments in the presumably flexible conformation of the protein could change the situation as suggested by the observation reported by Sanders & McGeoch (1973) that a mutated CRP was activable not only by cAMP but also by cGMP.

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Proton Nuclear Magnetic Resonance Studies of Human Immunoglobulins: Conformation of the Hinge Region of the IgG1 Immunoglobulin†

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ABSTRACT: The conformation of the hinge region of the human IgG1 immunoglobulin has been investigated by making use of His-224 in the hinge region as a *built-in* proton nuclear magnetic resonance (NMR) probe. Human myeloma IgG1(κ) proteins Ogo and Yot and human polyclonal IgG were used along with their Fab and F(ab')$_2$ fragments for the assignment of the His-224 signals. The titration behavior of His-224 of the intact IgG and the fragments was compared. It was shown that the titration curves for the intact IgG and the F(ab')$_2$ fragments are identical and quite similar to those for the histidine residue in small peptides. By contrast, the Fab fragments give titration curves which are quite different from those for the intact IgG and the F(ab')$_2$ fragments. Conclusions derived may be summarized as follows: (1) in the intact IgG1, the hinge peptide is fully exposed to the solvent and exhibits internal motion which is much more rapid than the Fab segmental motion with respect to Fc; (2) at the loss of the Fc portion of the IgG, the conformation of the hinge peptide in the F(ab')$_2$ fragments remains unchanged; (3) the heavy–heavy interchain interactions involving the two disulfide bridges do not play the primary role in determining the conformation of the hinge region in the intact IgG as well as in the F(ab')$_2$ fragments; (4) the existence of a small stretch of peptide fragment Thr-225–Leu-234 is essential in maintaining the conformation of the hinge region of the intact IgG and the F(ab')$_2$ fragments; (5) in the Fab fragments, as a result of cleavage of a major portion of the hinge peptide, the C-terminal part of the heavy chain including His-224 is partially folded back toward the globular portion of the polypeptide chains; and (6) the hinge peptide in the Fab fragments still retains a degree of flexibility which is similar to that in the intact IgG and the F(ab')$_2$ fragments.

Proteins of the human immunoglobulin G (IgG)[1] class can be differentiated into four subclasses (IgG1 through IgG4), each with a distinctive heavy chain, γ1, γ2, γ3, and γ4; light chains can be either of λ or κ type, regardless of its heavy chain subclass.[2,3] The γ chains consist of four homology units, V_H, C_H1, C_H2, and C_H3, whereas the light chains are divided into

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[1] Abbreviations used: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; IgG, immunoglobulin G; NMR, nuclear magnetic resonance.

[2] The nomenclature for immunoglobulin G and its fragments is as recommended in *Bull. W.H.O.* (1964).

[3] The numbering system used in the present paper is based on protein Ag for the κ chain (Putnam, 1969) and on protein Eu for the γ1 chain (Edelman et al., 1969).

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