

Proton Nuclear Magnetic Resonance Studies on Cyclic Nucleotide Binding to the *Escherichia coli* Adenosine Cyclic 3',5'-Phosphate Receptor Protein[†]

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ABSTRACT: A ¹H NMR study on the *Escherichia coli* adenosine cyclic 3',5'-phosphate receptor protein (CRP) alone and in its complexes with adenosine cyclic 3',5'-phosphate (cAMP), guanosine cyclic 3',5'-phosphate (cGMP), and a number of analogues is presented. Five sets of imidazole proton resonances are seen, corresponding to the five histidines per subunit found by amino acid analysis [Anderson, W. B., Schneider, A. B., Emmer, M., Perlman, R. L., & Pastan, I. (1971) *J. Biol. Chem.* 246, 5929-5937]. Four of the histidine residues, A, B, C, and D, are characterized by imidazole proton resonances with unusually narrow line widths (3-5 Hz) for a protein of molecular weight 45 000 and pKs in the range 6-7, suggesting that they lie in mobile regions of the protein. In contrast, the fifth histidine residue, E, has a broad C(2) proton resonance (~15 Hz) and a very low pK (≤5), indicating that it is buried in the deprotonated state in a rigid portion of the protein. The addition of cyclic nucleotides to CRP does not lead to any drastic changes in the protein spectrum. The most prominent changes are associated with the addition of cAMP and are characterized by an overall broadening of all the proton resonances of the protein and marked changes at the high-field end of the aromatic region. Following the addition of cAMP

and cGMP, a slow conformational change in the structure of CRP can be detected. This conformational transition occurs subsequent to the binding of a cyclic nucleotide molecule to one of the binding sites and is only complete when both cyclic nucleotide binding sites are almost completely saturated. For the cGMP-CRP complex, the upper limit for the interconversion rate between the two conformations is 18 s⁻¹, and for the cAMP-CRP complex, the interconversion rate lies in the range 60-120 s⁻¹. The conformations about the glycosidic bond of cyclic nucleotides bound to CRP were investigated by the measurement of transferred nuclear Overhauser enhancements, which showed that cAMP and its analogues are bound in the syn conformation with values for the glycosidic bond torsion angle χ [O(4')-C(1')-N(9)-C(4)] in the range 45-55° and that cGMP and its analogue inosine cyclic 3',5'-phosphate (cIMP) are bound in the anti conformation with values of χ of 225° and 240°, respectively. This contrasts to the situation in free cyclic nucleotides which exist in a syn/anti equilibrium mixture, cAMP being predominantly in the anti conformation and cGMP predominantly in the syn conformation. The implications and possible mechanism of this conformational selection by CRP are discussed.

The cyclic AMP receptor protein (CRP)¹ mediates the regulation of a number of catabolite-sensitive operons in *Escherichia coli*, cAMP acting as an allosteric effector (Zubay et al., 1970; Epstein et al., 1975; de Crombrughe & Pastan, 1978). The cAMP-CRP complex interacts with specific DNA sites at or near promoters, stimulating the initiation of mRNA synthesis (de Crombrughe et al., 1971; Majors, 1975; Taniguchi et al., 1979; Odgen et al., 1980).

CRP is a dimer of molecular weight 45 000 composed of two apparently identical subunits each of molecular weight 22 500 (Anderson et al., 1971; Riggs et al., 1971). Recently, the X-ray crystal structure of the cAMP-CRP complex has been determined at 2.9-Å resolution and has shown that each subunit consists of two structural domains (McKay & Steitz, 1981). The larger amino-terminal domain contains the cAMP binding site; the conformation of bound cAMP, however, could not be determined. The smaller carboxy-terminal domain was presumed to contain the DNA binding site on the basis of model building.

At present, the details of the interactions involved in the action of CRP at the molecular and atomic levels are almost entirely unknown and will require extensive structural studies of all the complexes involved. In this paper, we present a study on the solution structure of CRP by ¹H NMR spectroscopy and determine by means of the transferred nuclear Overhauser effect (Clore & Gronenborn, 1982a) the conformation about

the glycosidic bond of a number of bound cyclic nucleotides including cAMP and cGMP. We show that whereas cAMP and its analogues are bound in the syn conformation, cGMP and its analogues are bound in the anti conformation. This contrasts with their conformations in free solution where cyclic nucleotides exist in a syn/anti equilibrium (Davies, 1978), cAMP being predominantly anti and cGMP predominantly syn (Oida, 1977; Yathindra & Sundaralingam, 1974; Frazier et al., 1977). A preliminary report of part of this work has appeared (Gronenborn et al., 1981).

Experimental Procedures

Materials. CRP was purified from *E. coli* KLF 41 by the method of Takahasi et al. (1980) and was greater than 99% pure as judged by NaDodSO₄-polyacrylamide gel electrophoresis. cAMP, cGMP, cIMP, 8-Br-cAMP, and dibutyryl-cAMP were obtained from P-L Biochemicals and used without further purification. The S_p isomer of cAMPS was a gift from Dr. Eckstein (Max-Planck-Institut für Experimentelle Medizin, Göttingen). All other chemicals used were of the highest purity commercially available. Selective deu-

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¹ Abbreviations: CRP, cyclic AMP receptor protein; cAMP, adenosine cyclic 3',5'-phosphate; d8-cAMP, cAMP deuterated in the 8 position of the adenine ring; cGMP, guanosine cyclic 3',5'-phosphate; cIMP, inosine cyclic 3',5'-phosphate; cAMPS, S_p isomer of adenosine cyclic 3',5'-phosphorothioate; 8-bromo-cAMP, 8-bromoadenosine cyclic 3',5'-phosphate; dibutyryl-cAMP, N⁶,O²-dibutyryladenosine cyclic 3',5'-phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; TRNOE, transferred nuclear Overhauser enhancement; NaDodSO₄, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

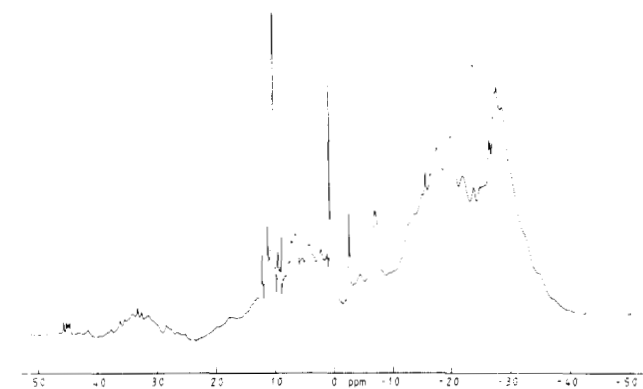


FIGURE 1: ^1H NMR spectrum at 270 MHz of native CRP. Experimental conditions: 0.28 mM CRP in D_2O containing 50 mM potassium phosphate, $\text{pH}^* 6.5$, 500 mM KCl, 1 mM EDTA, and 1 mM dioxane. The chemical shifts are expressed relative to internal dioxane (3.71 ppm downfield from 4,4-dimethyl-4-silapentane-1-sulfonate). Conditions of NMR spectroscopy are given under Experimental Procedures.

teration of the H(8) proton of the purine ring of the cyclic nucleotides was carried out by heating solutions of cyclic nucleotides in D_2O at 80°C for 7 h.

Sample Preparation. Samples for ^1H NMR were prepared by dialyzing 0.56 mM CRP 6 times over a period of 48 h against D_2O containing 50 mM potassium phosphate, $\text{pH}^* 6.5$ (meter reading uncorrected for the isotope effect on the glass electrode), 500 mM KCl, and 1 mM EDTA.

The pH of the samples was adjusted by the addition of microlitre volumes of 0.1–1.0 M KOD or DCl [>99 atom % D; CIBA (ARL) Ltd.] and measured by using a combination glass/reference electrode and a Radiometer Model 26 pH meter.

NMR Spectroscopy. ^1H NMR spectra were recorded at 270 MHz on a Bruker WH 270-MHz spectrometer operating in the Fourier-transform mode. A total of 2000 transients, obtained by quadrature detection for a spectral width of 4.2 kHz by using 8192 data points and an acquisition time of 0.974 s, were averaged for each spectrum. Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 1 Hz. Chemical shifts are expressed relative to internal 1 mM dioxane (3.71 ppm) downfield from 4,4-dimethyl-4-silapentane-1-sulfonate. The NOE and transferred NOE (TRNOE) experiments were carried out by using the pulse sequence $(t_1-t_2-\pi/2-\text{AT})_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0.5 s), t_2 is a short delay (2 ms) to allow for electronic recovery after removal of the selective irradiation, and AT is the acquisition time (0.487 s for a spectral width of 4.2 kHz with 4096 data points). Systematic NOE and TRNOE measurements irradiating at 20-Hz intervals were carried out over the entire aromatic and sugar proton regions of the cyclic nucleotides in the presence and absence of CRP. For the NOE experiments, 200 transients were averaged for each spectrum, and prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. All ^1H NMR spectra were recorded at 20°C .

Results

CRP Alone. Figure 1 shows the ^1H NMR spectrum of native CRP at $\text{pH}^* 6.5$ which shows highly resolved resonances in both the aromatic and aliphatic regions. The most striking features are extremely sharp lines in the histidine region (4–4.7 ppm) [with four C(2) proton resonances having a natural line width of 3–5 Hz, and equally sharp signals in the methyl region

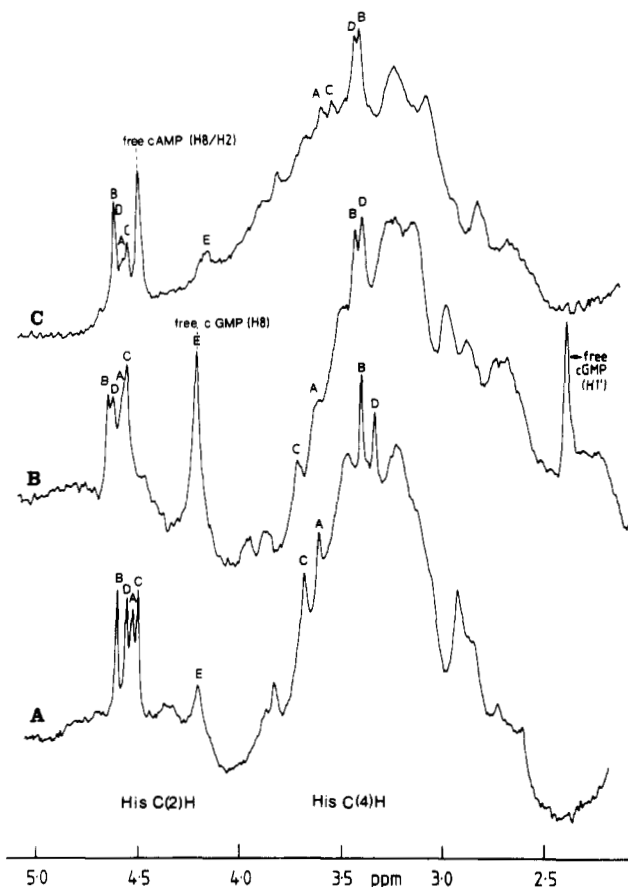


FIGURE 2: Aromatic region of the 270-MHz ^1H NMR spectrum of (A) native CRP, (B) the cGMP-CRP complex, and (C) the cAMP-CRP complex. Experimental conditions are as in the legend to Figure 1 except that the pH^* in (C) is 6.45 instead of 6.5 as in (A) and (B); the concentration of cGMP in (B) and cAMP in (A) is 1.3 mM.

(e.g., at -2.73 , -2.75 , and -1.63 ppm)]. Lines of histidine imidazole protons and other protons such as the $\text{S}-\text{CH}_3$ protons of methionine are in general sharper than the majority of lines in a protein spectrum due to their larger T_2 values. In this case, however, their extreme narrowness compared to the overall appearance of the spectrum requires an additional contribution, possibly from internal motion.

cAMP-CRP and cGMP-CRP Complexes. In Figure 2, the low-field regions of the CRP spectrum alone (Figure 2A) and in its complexes with cGMP (Figure 2B) and cAMP (Figure 2C) are shown. The well-resolved histidine C(2) and C(4) proton resonances are labeled A–E. The assignments of the C(4) proton resonances with their corresponding C(2) proton resonances are based on the pH titration data and intrasidue NOE measurements presented in the following paper (Clare & Gronenborn, 1982b).

In CRP alone, the imidazole proton resonances of four of the histidine residues, A–D, are sharp, whereas that of the C(2) proton resonance of histidine E has a considerably larger line width ($\Delta\nu_{1/2} \sim 15$ Hz). In addition, the C(2) proton resonance of histidine E is shifted to higher field than the other C(2) proton resonances at $\text{pH}^* 6.5$ due to its low pK (Clare & Gronenborn, 1982b).

The histidine region of the cGMP-CRP complex is similar to that of CRP alone, with the same four histidine residues (A–D) showing sharp signals while the C(2) proton resonance of histidine E is buried underneath the signal of the H(8) proton of the guanine ring of cGMP. The C(2) proton resonances of histidines C and D are shifted 0.015 ppm downfield,

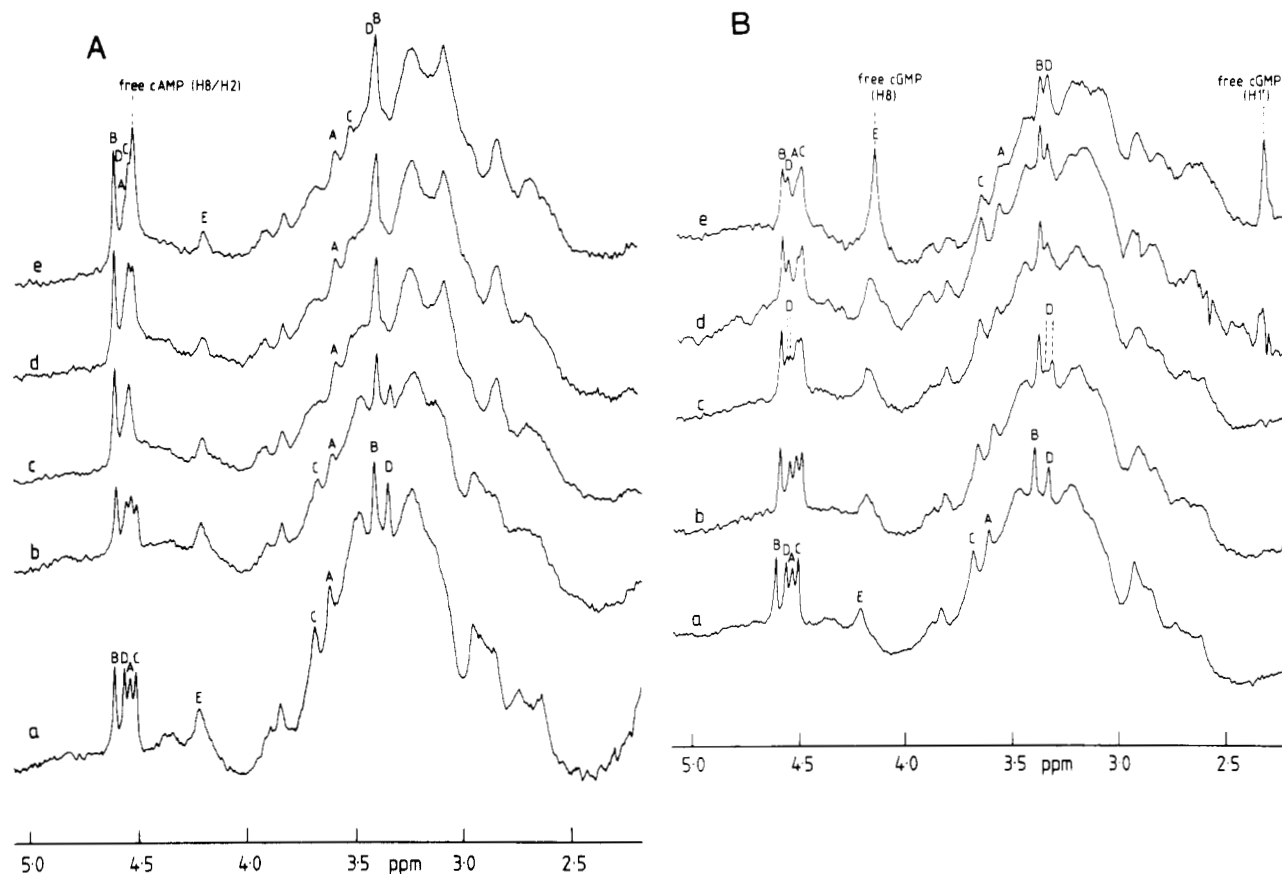


FIGURE 3: Effects of increasing concentrations of (A) cAMP and (B) cGMP on the histidine C(2) and C(4) proton resonances of CRP at pH* 6.5. Experimental conditions: (A) the total CRP concentration is 0.28 mM; the total concentration of cAMP, the concentration of free cAMP, and the fractional saturation are respectively (a) 0 mM, 0 mM, and 0; (b) 0.3 mM, 0.03 mM, and 1.19; (c) 0.65 mM, 0.15 mM, and 1.79; (d) 0.95 mM, 0.41 mM, and 1.92; (e) 1.25 mM, 0.70 mM, and 1.96. (B) The total CRP concentration is 0.25 mM; the total concentration of cGMP, the concentration of free cGMP, and the fractional saturation are respectively (a) 0 mM, 0 mM, and 0; (b) 0.63 mM, 0.21 mM, and 1.75; (c) 0.89 mM, 0.42 mM, and 1.87; (d) 1.03 mM, 0.57 mM, and 1.90; (e) 1.29 mM, 0.81 mM, and 1.93. The concentration of free ligand and the fractional saturation ν were calculated from the values of the binding constants and cooperativity parameters obtained by Takahashi et al. (1980): $K_a(\text{cAMP}) = 3.5 \times 10^4 \text{ M}^{-1}$, $\alpha(\text{cAMP}) = 1.7$; $K_a(\text{cGMP}) = 2.5 \times 10^4 \text{ M}^{-1}$, $\alpha(\text{cGMP}) = 1.4$ at an ionic strength of approximately 550 mM.

and the C(4) proton resonance of histidine D is shifted 0.03 ppm downfield, with respect to their positions in the free protein. The other histidine signals, however, are not affected by cGMP binding. Furthermore, the overall appearance of the aromatic region of the cGMP-CRP complex is very similar to that of CRP alone, suggesting that no major changes in the protein structure occur.

In the case of the cAMP-CRP complex, the differences are more substantial. The C(2) proton resonances of two histidines, C and D, are broader than in the spectrum of CRP alone, and their positions are slightly different. The corresponding C(4) proton resonances are shifted more substantially; the resonance of histidine C is shifted 0.141 ppm to higher field while that of histidine D is shifted 0.144 ppm to lower field. In addition, there are obvious changes in the aromatic region, especially at the high-field end, accompanied by a generalized increase in line width over the entire spectrum of approximately 10%. An overall broadening of the ^1H NMR spectrum would be expected if cAMP, upon binding to CRP, would tighten up the flexible protein structure into a more rigid one. This proposal is consistent with the results obtained by small-angle X-ray scattering experiments, which show a decrease in the radius of gyration due to an overall contraction of the CRP molecule upon binding cAMP (Kumar et al., 1980).

Figure 3 shows the effects of increasing amounts of cAMP (Figure 3A) and cGMP (Figure 3B) on the histidine reso-

nances of CRP. As both cyclic nucleotides bind relatively weakly to CRP [$K_a(\text{cAMP}) = 3.5 \times 10^4 \text{ M}^{-1}$, $\alpha(\text{cAMP}) = 1.7$; $K_a(\text{cGMP}) = 2.5 \times 10^4 \text{ M}^{-1}$, $\alpha(\text{cGMP}) = 1.4$ at an ionic strength of about 550 mM, where α is the cooperativity parameter in a two-state Adair equation; Takahashi et al., 1980] and the measured on rates are approximately diffusion limited ($k_{\text{on}} \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$; Wu & Wu, 1974), exchange between bound and free states would be expected to be intermediate or fast on the chemical shift scale. During the course of both cAMP and cGMP additions, no shifts in the positions of the free ligand resonances of the purine ring and of the H(1') proton of the sugar ring could be detected. If exchange between the free and bound states of the ligand were fast on the chemical shift scale, then shifts of ≥ 10 Hz would be detected. If, on the other hand, exchange between the free and bound states of the ligand were in slow exchange on the chemical shift scale, the transfer of saturation experiment (Forsén & Hoffman, 1963) should be able to locate the positions of the bound resonances by finding the irradiation frequency at which transfer of saturation to the corresponding resonance of the free ligand is observed, providing $k \gtrsim \rho_F$ (where k is the chemical exchange rate and ρ_F the spin-lattice relaxation rate of the observed nucleus in the free state) and the chemical shift difference between the free and bound ligand resonances is ≥ 40 Hz (Cayley et al., 1979). No bound ligand resonances could be detected in this manner. We were, however, able to demonstrate negative proton-proton transferred nuclear Ov-

erhauser effects (TRNOE; Clore & Gronenborn, 1982a) from the H(1') proton to the H(8) proton and vice versa in the case of cAMP which were maximal on irradiation at positions corresponding to those of free cAMP (see below). As one of the components involved in the TRNOE is the basic mechanism of transfer of saturation, namely, magnetic exchange between the free and bound states of the ligand, and as a negative TRNOE is only observed when $k \gtrsim 10\rho_F$ (Clore & Gronenborn, 1982a), we conclude that the positions of the bound H(1') and purine proton resonances of cAMP and cGMP are very close (≤ 10 Hz \approx 0.04 ppm) to the positions of the corresponding signals in free cAMP and cGMP and that bound and free cyclic nucleotides are in fast exchange on the chemical shift scale.

With respect to the protein spectrum, virtually no changes at all could be observed upon the addition of either cAMP or cGMP up to a value of the fractional saturation ν of 1 [where ν is defined as the number of moles of ligand bound per mole of CRP (i.e., per CRP dimer), and there is one cyclic nucleotide binding site per subunit]. Thereafter, significant changes in the protein spectrum could be detected.

In the case of the cAMP addition (Figure 3A), as ν increases above 1, selective broadening of the two histidine C(2) proton signals C and D occurs until three histidine C(2) proton signals, A, C, and D, merge into one broad peak centered around 4.5 ppm; a similar effect is seen for the corresponding C(4) proton resonances which initially disappear from the aromatic region. Addition of excess cAMP ($[\text{cAMP}_{\text{free}}]/[\text{cAMP}_{\text{bound}}] > 1.5$, $\nu > 1.95$) leads to a sharpening of the broadened histidine resonances which, as a result, become visible again in the spectrum but at different positions. The C(2) proton resonances of histidines A–D are all shifted 0.03–0.04 ppm to lower field in the fully saturated cAMP-CRP complex, while the C(4) proton resonance of histidine C is shifted 0.141 ppm to higher field and that of histidine D is shifted 0.144 ppm to lower field than in CRP alone. This behavior of initial broadening and later sharpening of the signals is characteristic of intermediate ligand exchange on the chemical shift scale.

In the case of the cGMP addition (Figure 3B), as ν increases above 1.8, one observes a decrease in the intensity of the C(2) proton signal of histidine D with a new signal appearing 0.015 ppm to lower field, the two signals trading intensity upon further increase in the concentration of cGMP until the old signal completely disappears at a value of $\nu > 1.9$. A decrease in the intensity of the corresponding C(4) signal is also observed with the new signal appearing 0.03 ppm to lower field of it. There is also an effect on the C(2) proton resonance of histidine C, namely, a decrease in intensity and a final position 0.015 ppm downfield.

Complexes of CRP with Related Cyclic Nucleotides. (A) *8-Bromo-cAMP*. Addition of 8-bromo-cAMP leads to small downfield shifts of 0.026 and 0.030 ppm for the C(2) proton resonances of histidine B and D, respectively, with similar effects on the corresponding C(4) proton resonances, which are shifted 0.015 and 0.022 ppm upfield, respectively. In general, the spectrum of the 8-bromo-cAMP-CRP complex is very similar to that of CRP alone.

(B) *cAMPS*. No shifts of any of the histidine resonances are seen, and the spectrum of the cAMPS-CRP complex is virtually identical with that of CRP alone.

(C) *Dibutyryl-cAMP*. There is a small downfield shift of 0.026 ppm in the position of the C(2) proton resonance of histidine A, very small downfield shifts of 0.011 and 0.004 ppm in the position of the C(2) proton resonances of histidines B and C, respectively, and no shifts in the positions of the C(2)

proton resonances of histidines D and E. The ligand H(8) resonance shifts 0.004 ppm to lower field while the H(2) resonance is initially broad and then shifts to higher field over a range of 0.03 ppm. A similar but downfield shift is observed for the H(1') and H(2') ligand signals. This is the only cAMP analogue that shows obvious fast exchange behavior of the ligand signals upon binding to CRP.

(D) *cIMP*. Small shifts of the histidine resonances (~ 0.007 ppm) can be seen, and again fast exchange behavior for the ligand is observed, with the H(2), H(8), and H(1') ligand signals moving upfield over a range of 0.044–0.048 ppm.

pH Dependence of the Histidine Resonances. The pH dependence of the chemical shifts of the histidine C(2) and C(4) proton resonances in CRP showed some abnormalities, and not all the pH titration curves could be fitted satisfactorily to the Henderson-Hasselbach equation. However, four of the five histidine residues, namely, histidines A–D, have pK values in the range 6–7.5 while histidine E has a very low pK value of ≤ 5 . The following paper discusses the pH titration behavior of the histidine residues in CRP alone and its N-terminal core in detail (Clore & Gronenborn, 1982b).

Addition of cyclic nucleotides has only small, if any, effects on the pK values of the histidine residues. In the case of the cAMP-CRP complex, the protonated shift of the C(4) proton resonance of histidine D is 0.15 ppm to lower field than in CRP alone, and there is possibly a small difference (≤ 0.1 pH unit) in the pK of histidine C. The cGMP-CRP complex, on the other hand, shows virtually overlapping titration curves with those of CRP alone, except for the curve of the C(4) proton resonance of histidine D, most probably due to a small downfield shift in its protonated form.

Determination of the Conformations of Bound Cyclic Nucleotides by the Measurement of Transferred Nuclear Overhauser Enhancements. The most powerful and potentially the most direct NMR method of conformational analysis of molecules in solution is the use of the proton-proton nuclear Overhauser enhancement (NOE) to demonstrate the proximity of two protons in space and to determine their separation (Noggle & Schirmer, 1971; Redfield & Gupta, 1971; Kuo & Gibbons, 1980; Poulson et al., 1980; Wagner et al., 1981; Kumar et al., 1981). In the case of ligands binding to proteins, particularly large ones ($M_r > 20,000$), it is usually not feasible to carry out direct NOE measurements as it is very difficult to observe the resonance of the bound ligand directly: if chemical exchange between the free and bound states is fast on the chemical shift scale, as in the case of cyclic nucleotide binding to CRP, only a single set of averaged ligand resonances will be seen; if, on the other hand, chemical exchange is slow on the chemical shift scale, the bound ligand resonances will usually be obscured by line-width and overlap problems. To overcome these problems, we have made use of the transferred nuclear Overhauser effect (TRNOE) to analyze the conformations of cyclic nucleotides bound to CRP. The TRNOE was initially observed between protein and ligand resonances and used to demonstrate the proximity of a bound ligand to a particular residue(s) of the protein (Bothner-By & Gassend, 1972; Balaram et al., 1972a,b; James & Cohn, 1974; James, 1976). The TRNOE involving bound ligand resonances was first reported by Albrand et al. (1979) and Cayley et al. (1979). The theory and applications of the TRNOE have recently been dealt with in detail by Clore & Gronenborn (1982a), and only the pertinent points will be summarized here. The basis of the proton-proton TRNOE involves the transfer of magnetic information concerning cross-relaxation between two bound ligand protons from the bound to the free state by

Table I: NOE's Observed for Free Cyclic Nucleotides and Proportions of the Syn Conformation about the Glycosidic Bond^a

irradiated resonance	obsd resonance	% NOE					
		cAMP	cAMPS	8-bromo-cAMP	dibutyl-cAMP	cGMP	cIMP
H(8)	H(1')	+10	+11			+8	+7
H(1')	H(8)	+11	+12			+16	+9
H(2')	H(8)	+13	+7			+2	+8
% syn conformation		30-40 ^{b,c}	~40 ^d	>90 ^e		>90 ^c	30-40 ^{c,d}

^a Experimental conditions: 3.3 mM cyclic nucleotide in a D₂O solution containing 50 mM potassium phosphate buffer, pH* 6.5, 500 mM KCl, and 1 mM EDTA; sample temperature 20 °C. NOE's to H(8) and H(2) purine resonances were distinguished by using cyclic nucleotides deuterated in the 8 position in those cases where the H(8) and H(2) resonances overlapped. No NOE's could be detected on the H(8) resonance following irradiation of the H(3') and H(5') sugar resonances. No NOE's could be detected on the H(2) resonance following irradiation of any of the sugar proton resonances. ^b Based on the computer simulations of Oida (1977) carried out on the basis of NOE measurements. The NOE's observed by Oida (1977) on cAMP were almost identical with those we observed. ^c Based on the theoretical conformational energy calculations of Yathindra & Sundaralingam (1974). ^d Estimated by comparison of the magnitude of the NOE's with those obtained for cAMP. ^e Davies (1978). The bulky bromine atom at the 8 position prevents formation of the anti conformation by steric hindrance.

chemical exchange so that negative TRNOE's on the easily detectable free or averaged ligand resonances may be seen following irradiation of other ligand resonances (free, bound, or averaged), thus conveying information on the proximity in space of bound ligand nuclei. In the presence of a protein,

for which $\omega\tau_c \gg 1$, a negative TRNOE on either the free or the averaged resonance of proton *i* will be observed following irradiation of either the free, bound, or averaged resonance of proton *j*, providing only the two following conditions are met: (1) $k > 10\rho_{iF}$ and (2) $|a\sigma_{iF/j}| < |(1-a)\sigma_{iB/j}|$, where *k* is the chemical exchange rate between the free and bound states of the ligand, ρ_{iF} is the total spin-lattice relaxation rate (including cross-relaxation terms) of the free ligand proton *i*, *a* is the mole fraction of free ligand, and $\sigma_{iF/j}$ and $\sigma_{iB/j}$ are the cross-relaxation rates between protons *i* and *j* in the free and bound states, respectively.

The results of systematic NOE and TRNOE measurements on the free cyclic nucleotides and the cyclic nucleotide-CRP complexes obtained by systematic irradiation at 20 Hz (≈ 0.074 ppm) intervals throughout the entire aromatic and sugar proton regions of the spectrum are summarized in Tables I and II, respectively. The TRNOE experiments in Table II were carried out with a 10-fold molar excess of free over bound cyclic nucleotide; under these conditions, the positions of the averaged ligand resonances will be approximately at the positions of the corresponding resonances in the free ligand, as free and bound cyclic nucleotides are in fast exchange on the chemical shift scale. Figure 4 shows the effects of selective irradiation of the H(1') and H(5') sugar resonances on the H(2) and H(8) resonances of cAMP and on the H(2) resonance of cAMP deuterated in the 8 position (d8-cAMP) in the presence of CRP. As the H(2) and H(8) resonances of cAMP are superimposed, effects on the H(2) and H(8) resonances can be distinguished by using d8-cAMP. Thus, irradiation of the H(1') resonance leads to a decrease in the intensity of the H(2)/H(8) resonance which is abolished when d8-cAMP is used, indicating that the TRNOE from the H(1') proton is solely to the H(8) proton. Irradiation of the H(5') resonance also leads to a decrease in the intensity of the

Table II: TRNOE's Observed for Cyclic Nucleotides in the Presence of CRP and Conformations about the Glycosidic Bond^a

irradiated ^b resonance	obsd ^c resonance	% TRNOE					
		cAMP	8-bromo-cAMP	cAMPS	dibutyl-cAMP	cGMP	cIMP
H(8)	H(1')	-20		-13			
H(1')	H(8)	-21		-17			
H(3')	H(2)	-6	-4	-12	-11		
H(5')	H(2)	-20	-18	-20	-12		
H(3')	H(8)					-3	-26
H(5')	H(8)					-10	-24
conformation of bound cyclic nucleotide		syn	syn	syn	syn	anti	anti
$r_{H(2)-H(5')}/r_{H(2)-H(3')}$ ^d		0.82 ^{+0.08} _{-0.11}	0.78 ^{+0.13} _{-0.18}	0.92 ^{+0.06} _{-0.06}	0.99 ^{+0.09} _{-0.09}		
$r_{H(8)-H(5')}/r_{H(8)-H(3')}$ ^d						0.82 ^{+0.15} _{-0.14}	1.01 ^{+0.05} _{-0.04}
$\chi[O(4')-C(1')-N(1)-C(4)]$ (deg) ^e		~45	~45	~50	~55	~225	~240

^a Experimental conditions: 3.3 mM cyclic nucleotide in the presence of 0.14 mM CRP (corresponding to an approximately 10-fold molar excess of free over bound cyclic nucleotide) in a D₂O solution containing 50 mM potassium phosphate buffer, pH* 6.5, 500 mM KCl, and 1 mM EDTA; sample temperature 20 °C. Under these conditions, the positions of the observed cyclic nucleotide resonances are at the positions of the corresponding resonances in the free cyclic nucleotides. ^b In all cases, no TRNOE's could be detected on the H(1') sugar resonance following irradiation of the H(2) purine resonance and on either the H(2) or the H(8) purine resonances following irradiation of the H(2'), H(4'), and H(5') sugar resonances. With respect to the H(4') and H(5') protons, it should be noted that there is no conformation about the glycosidic bond for which the H(4') and H(5') protons are close enough (<4 Å) to either the H(2) or the H(8) protons for TRNOE's to be observed. [Note the H(5') is axial to the H(4') proton whereas the H(5'') proton is equatorial to the H(4') proton]. ^c TRNOE's could not be observed on the H(2'), H(3'), or H(5') sugar resonances as they are obscured by the HOD peak and the proton resonances of the protein backbone, except in the case of dibutyl-cAMP where the H(2') and H(3') resonances are significantly downfield from the HOD peak. In the case of dibutyl-cAMP, a small negative TRNOE (-4%) was observed on the H(3') resonance following irradiation of the H(2) but not the H(8) resonance. ^d The distance ratios were determined by using the equation $r_{ij}/r_{ik} = \{N_i(k)/[N_i(j)]\}^{1/6}$ [where $N_i(x)$ refers to the normalized magnitude of the TRNOE observed on the resonance of proton *i* following irradiation of proton *x*]. The justification of this equation for the calculation of distance ratios from TRNOE's is discussed in the text (see Discussion). The errors on the distance ratios are calculated on the basis of an estimated error of $\pm 3\%$ in the values of the TRNOE's. ^e The convention used for defining χ is the standard one adopted by Davies (1978). The values of χ were estimated by model building based on the distance ratio $r_{H(2)-H(5')}/r_{H(2)-H(3')}$ for cAMP and its analogues and the distance ratio $r_{H(8)-H(5')}/r_{H(8)-H(3')}$ for cGMP and its analogue, cIMP. These ratios are only consistent with a 3'-endo conformation for the ribose and a gauche-trans conformation about the C(4')-C(5') bond, in agreement with findings on free 3',5'-cyclic purine nucleotides (Davies, 1978). The error in the estimation of χ is about $\pm 5^\circ$ with the exception of that for cGMP and 8-bromo-cAMP, which is about $\pm 10^\circ$.

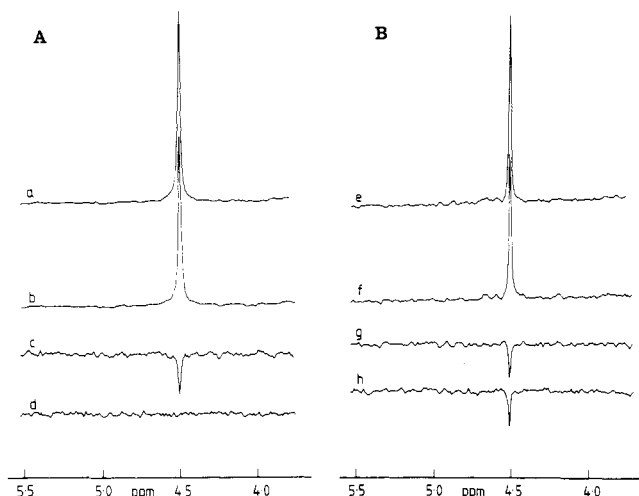


FIGURE 4: Effects on the H(2) and H(8) resonances of the adenine ring following irradiation of (A) the H(1') and (B) the H(5') sugar resonances of cAMP (3.3 mM) in the presence of CRP (0.14 mM). In cAMP, the H(2) and H(8) resonances are superimposed so that the use of d8-cAMP is required to determine which of the two protons is involved in the TRNOE's. (a) cAMP control spectrum with irradiation at 1.64 ppm; (b) cAMP spectrum with irradiation at 2.3 ppm [H(1') resonance]; (c) spectrum b minus spectrum a; (d) same as (c) but with d8-cAMP; (e) d8-cAMP control spectrum with irradiation at -0.32 ppm; (f) d8-cAMP spectrum with irradiation at 0.68 ppm [H(5') resonance]; (g) spectrum f minus spectrum e; (h) same as (g) but with cAMP. The amplitude scale is the same for spectra c-h and is double that for spectra a and b.

H(2)/H(8) resonance which, however, remains unaltered when d8-cAMP is used, indicating that the TRNOE from the H(5') proton is solely to the H(2) proton. The high degree of specificity from the observed TRNOE's excludes a nonspecific spin-diffusion mechanism (Clore & Gronenborn, 1982a; Gronenborn & Clore, 1982). In the case of cAMP, cAMPS, 8-bromo-cAMP, and dibutyl-cAMP in the presence of CRP, specific negative TRNOE's were observed on the H(8) resonance following irradiation of the H(1') resonance but not of the other sugar proton resonances, and on the H(2) resonance following irradiation of the H(3') and H(5') sugar resonances but not of either the H(2') or the H(1') sugar resonances. In contrast, in the case of cGMP and cIMP in the presence of CRP, we observed negative TRNOE's on the H(8) resonance following irradiation of the H(3') and H(5') sugar resonances but not of the other sugar proton resonances, and no TRNOE's were observed on the H(2) resonance of cIMP following irradiation of any of the sugar proton resonances.

We have also examined the dependence of the magnitudes of the TRNOE's on the molar ratio of free to bound cyclic nucleotide, $[L_F]/[L_B]$. This is illustrated in Figure 5 for the TRNOE's observed, in the presence of CRP, with cAMP on the H(2) and H(8) signals following irradiation of the H(5') and H(1') signals, respectively, and with cGMP on the H(8) signal following irradiation of the H(5') signal. The observed behavior is that predicted by the theory and calculations of Clore & Gronenborn (1982a), namely, an initial rapid increase in the value of $1 + N_i(j)$ [where $N_i(j)$ is the normalized magnitude of the TRNOE observed on the resonance of proton i following irradiation of the resonance of proton j] as the value of $[L_F]/[L_B]$ increases from 0 to 40, followed by a slow, almost linear, increase in the value of $1 + N_i(j)$, which rises asymptotically to its value for the free ligand in the absence of protein, as the ratio $[L_F]/[L_B]$ is further increased.

Discussion

Histidine Residues. Amino acid analysis has shown that

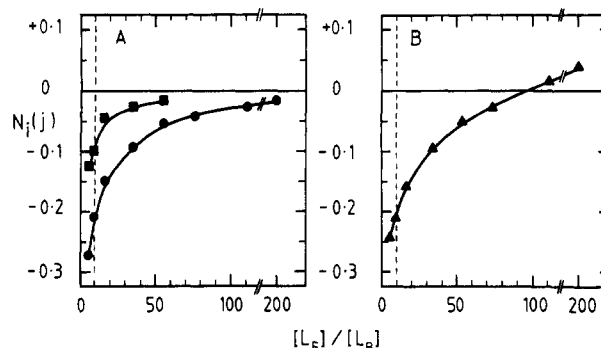


FIGURE 5: Observed dependence of the normalized magnitudes of the TRNOE's, $N_i(j)$, on the molar ratio of free to bound cyclic nucleotide ($[L_F]/[L_B]$) for complexes of cAMP and cGMP with CRP: (A) $N_{H_2}(H_5')$ for cAMP (●) and $N_{H_8}(H_5')$ for cGMP (■); (B) $N_{H_8}(H_1')$ for cAMP (▲). [The notation $N_i(j)$ refers to the normalized magnitude of the TRNOE observed on the resonance of proton i following irradiation of the resonance of proton j .] The dashed line is at $[L_F]/[L_B] = 10$, the value used for the TRNOE's given in Table II.

CRP contains five histidine residues per subunit (Anderson et al., 1971; Aiba & Krakow, 1981), four of which, residues A-D, give rise to very sharp imidazole proton signals with line widths at half-height ($\Delta\nu_{1/2}$) of 3-5 Hz, and one, residue E, gives rise to a broad C(2) proton resonance ($\Delta\nu_{1/2} \sim 15$ Hz). While the four sharp histidine C(2) proton resonances have chemical shifts in the protonated form which are very similar to those found for histidine residues in small peptides (Markley, 1975), and pK values in the range 6-7, histidine E shows a downfield shift of its C(2) proton resonance in the deprotonated state. The chemical shift of the C(2) proton resonance of histidine E is only marginally dependent on pH in the pH range studied (5.0-8.0), showing protonation shifts only at pH values below 6, thus indicating a pK value of ≤ 5 .

The line widths of the C(2) and C(4) proton resonances of histidines A-D are comparable to those seen in the much smaller protein dihydrofolate reductase (Wyeth et al., 1980), which has a molecular weight of 18 300, and are 3 times narrower than those seen in the protein phosphoglycerate mutase (G. C. K. Roberts, personal communication), which has a comparable molecular weight (52 000) to that of CRP (45 000). We therefore suggest that the narrow line widths of the imidazole proton resonances of histidines A-D are due to their location in flexible regions of CRP. In contrast to this, the markedly increased line width of the C(2) proton resonance of histidine E suggests that this amino acid lies in a rigid structure inside the protein and has therefore lost its internal mobility. This is consistent with the finding that the imidazole ring of histidine E only starts to become protonated at pH values below 6, indicating that this residue must be buried in the deprotonated ionization state at physiological pH values.

The observation of only five sets of histidine resonances for CRP alone and in its complexes with cyclic nucleotides leads to the suggestion that the conformations of the two subunits are probably identical in solution, at least as far as can be judged by the imidazole protons of the histidine residues, which have to be in an identical environment in both subunits.

Conformational Changes in CRP upon Cyclic Nucleotide Binding. The addition of cyclic nucleotides to CRP does not lead to drastic changes in the protein spectrum; the histidine C(2) proton region is hardly altered, and only with cAMP does one observe an overall broadening of all the proton resonances of the protein and marked changes at the high-field end of the aromatic region. This implies that the conformational changes in the structure of CRP upon the binding of cyclic nucleotides

are probably of a rather subtle nature and not as gross as was originally proposed (McKay & Steitz, 1981).

Nevertheless a slow conformational transition occurs from one distinct state to another during the course of the binding of both cAMP and cGMP to CRP. This conformational transition is only seen when the fractional saturation ν exceeds 1 and is only complete when both cyclic nucleotide binding sites are almost completely saturated ($\nu > 1.9$).

In the case of cGMP, two distinct sets of resonances are seen for the C(2) and C(4) protons of histidine D, the second set first becoming visible at $\nu \sim 1.8$ and the first set disappearing completely at $\nu > 1.9$. The two sets of signals are in slow exchange on the NMR time scale, the C(2) resonances being separated by 4 Hz and the C(4) resonances by 8 Hz, thus setting an upper limit on the interconversion rate between the two conformers of $<18 \text{ s}^{-1}$ [i.e., $<4(2^{1/2})\pi \text{ s}^{-1}$].

A similar conformational change is seen in the case of cAMP, but the two sets of resonances are in intermediate exchange on the NMR time scale. Initial broadening of the C(2) and C(4) proton resonances of histidines C and D occurs as ν exceeds 1, followed by a later sharpening of the signals which now appear in new positions as ν approaches 2. The final positions of the C(2) proton resonances of histidines C and D are 9–10 Hz downfield from their initial positions, that of the C(4) proton resonance of histidine C is 38 Hz to low field of its initial position, and that of the C(4) proton resonance of histidine D is 39 Hz to high field of its initial position. Thus, the interconversion rate between the two conformers must lie in the range 60–120 s^{-1} .

In the case of both cAMP and cGMP, the interconversion rates between the two conformers are much smaller than the expected value for the chemical exchange rate between free and bound cyclic nucleotides of the order of 10^3 s^{-1} , based on the relatively weak binding constants ($K_a < 10^5 \text{ M}^{-1}$; Takahashi et al., 1980) and the diffusion-limited on rates ($k_{on} \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$; Wu & Wu, 1974). It therefore seems likely that the observed slow conformational transition in the structure of CRP occurs subsequent to the binding of the second cyclic nucleotide molecule to CRP.

Conformations of Cyclic Nucleotides Bound to CRP. In free solution, most cyclic nucleotides exist as an equilibrium mixture of syn and anti conformations (Davies, 1978), which was confirmed by our NOE measurements as positive NOE's were observed on the purine H(8) resonance following irradiation of both the H(1') and H(2') sugar resonances (see Table I). Positive NOE's on the H(2) resonance following irradiation of the sugar proton resonances could not be detected for any of the free cyclic nucleotides. cAMP, cAMPS, and cIMP exhibited sizable and approximately equal positive NOE's, in the range +7 to +13%, on the H(8) resonance following irradiation of both the H(1') and H(2') resonances, indicating a slight predominance of the anti conformer, with approximately 60–70% of the molecules in the anti conformation (Oida, 1977). cGMP, on the other hand, exhibited a large positive NOE (+16%) on the H(8) resonance following irradiation of the H(1') resonance but only a small positive NOE ($\leq +2\%$) on the H(8) resonance following irradiation of the H(2') resonance, indicating a large predominance of the syn conformer, with about 90% of the molecules in the syn conformation (Yathindra & Sundaralingam, 1974). 8-Bromo-cAMP is also predominantly in the syn conformation as the bulky bromine atom cannot be accommodated easily about the sugar ring (Davies, 1978). In the case of dibutyryl-cAMP, we were unable to detect NOE's to either the H(8) or the H(2) proton on irradiation of the resonances of

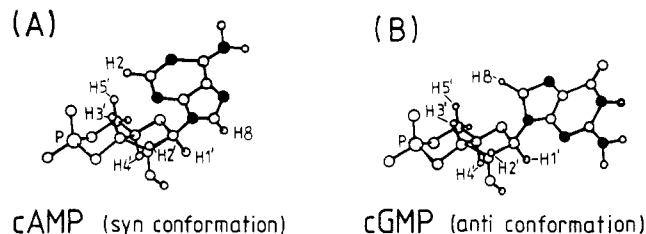


FIGURE 6: Conformations of (A) cAMP and (B) cGMP bound to CRP.

any of the sugar protons, possibly due to a steric effect of the dibutyryl side chains on the sugar base linkage, so that we can only assume that it exists in solution as a syn/anti equilibrium mixture similar to that of cAMP.

In the presence of CRP, the high selectivity of the negative TRNOE's observed for the cyclic nucleotides clearly demonstrates that a conformational selection of either the syn or anti form occurs upon binding to CRP. cAMP and its three analogues, cAMPS, 8-bromo-cAMP, and dibutyryl-cAMP, are bound in the syn conformation characterized by negative TRNOE's on the H(2) purine resonance but not on the H(8) purine resonance following irradiation of the H(5') and H(3') sugar resonances, and by a negative TRNOE on the H(8) resonance but not on the H(2) resonance following irradiation of the H(1') sugar resonance (Figure 6A). In contrast, cGMP and its analogue, cIMP, are bound in the anti conformation characterized by negative TRNOE's on the H(8) resonance but not on the H(2) resonance following irradiation of the H(5') and H(3') resonances, and by no TRNOE's on either the H(2) or the H(8) resonance following irradiation of the H(1') resonance (Figure 6B).

It is clear that the results of these TRNOE measurements do not prove that cAMP and its analogues are bound *entirely* in the syn conformation and that cGMP and cIMP are bound *entirely* in the anti conformation, although this may seem likely on heuristic grounds. We can, however, calculate an upper limit for the proportion of a second minor conformation (i.e., anti in the case of cAMP and its analogues and syn in the case of cGMP and cIMP) on the basis of the following observations: (1) under our experimental conditions, the sensitivity limit for the detection of a NOE is about 1–2%; (2) the magnitude of the largest negative TRNOE for which no corresponding positive NOE is seen in the free cyclic nucleotides, namely, that on the H(2) resonance following irradiation of the H(5') resonance in the case of cAMP and its analogues and that on the H(8) resonance following irradiation of the H(5') resonance in the case of cGMP and cIMP, has values lying in the range –10 to –20% at a molar ratio of free to bound cyclic nucleotide, $[L_F]/[L_B]$, of about 10 (see Table II); (3) it can be seen from Figure 5A that, for such TRNOE's, a negative TRNOE will no longer be detectable when $[L_F]/[L_B] > 200$ for the TRNOE's with a value of –20% at $[L_F]/[L_B] = 10$, and when $[L_F]/[L_B] > 60$ for the TRNOE's with a value of –10% at $[L_F]/[L_B] = 10$. From these observations, we conclude that the upper limit for the proportion of a second minor conformation lies in the range 5–15%.

To obtain quantitative conformational information on the glycosidic bond torsion angle χ [O(4')–C(1')–N(9)–C(4)] of the cyclic nucleotides bound to CRP, we have calculated the values of the distance ratio $r_{H(2)-H(5')}/r_{H(2)-H(3')}$ for cAMP and its analogues and $r_{H(8)-H(5')}/r_{H(8)-H(3')}$ for cGMP and cIMP by using the equation $r_{iB}/r_{jB} = (\sigma_{iB}/\sigma_{jB})^{1/6} = \{N_i(k)/N_j(i)\}^{1/6}$ (Noggle & Schirmer, 1971) where r_{iB} is the distance between the bound ligand protons i and x (and x is used to represent protons j and k), σ_{iB} the cross-relaxation rate be-

tween these two bound ligand protons, and $N_i(x)$ the normalized magnitude of the TRNOE observed on the resonance of proton i following irradiation of the resonance of proton x . The use of this equation to calculate distance ratios is justified for TRNOE's provided that $|a\sigma_{i_F x_F}| \ll |\lambda(1-a)\sigma_{i_B x_B}|$ where $\sigma_{i_F x_F}$ is the corresponding cross-relaxation rate between the free ligand protons i_F and x_F , a is the mole fraction of free ligand, and λ is the fraction of bound ligand present in the relevant conformation (Clore & Gronenborn, 1982a). This relation is satisfied for all the cases considered here as, in the absence of CRP, no positive NOE's could be detected on the H(2) resonance of cAMP and its analogues and on the H(8) resonance of cGMP and cIMP following irradiation of the H(3') and H(5') sugar resonances. Consequently, the presence of a small fraction of cyclic nucleotide bound in a second minor conformation will have no effect on the calculation of the distance ratios as, in all the cases considered here, $N_i(x)$ is proportional to $\lambda(1-a)\sigma_{i_B x_B}$ (Clore & Gronenborn, 1982a). From the distance ratios, the values of χ can be evaluated by simple model building, and these are given in Table II.

It is interesting to note that for the two naturally occurring cyclic nucleotides, cAMP and cGMP, both of which have very similar binding constants and bind to the same two sites (one per subunit) on CRP (Takahashi et al., 1980), the conformation about the glycosidic bond in the bound state, syn for cAMP and anti for cGMP, represents the minor conformer of the free cyclic nucleotide.

At present, the mechanism of conformational selection is unknown. However, it is clear that specific interactions between the protein and the cyclic nucleotides in a particular conformation must be involved. One possible mechanism could involve hydrogen bonding to the purine base. In this respect, one can note that the major difference between cAMP and its analogues on the one hand, and cGMP and its analogue cIMP on the other, is that in the former the N(1) atom is deprotonated and a hydrogen bond acceptor and the 6-NH₂ group is a hydrogen bond donor, whereas in the latter, the N(1) atom is protonated and a hydrogen bond donor and the 6-keto group is a hydrogen bond acceptor. Moreover, it is known that the syn conformation in free nucleotides is promoted by intramolecular hydrogen bonding (Rao & Sundaralingam, 1970; Plochocka et al., 1977). Thus, it may not be unreasonable to postulate that hydrogen bonding between a -NH-C(=O) fragment of the protein backbone and the N(1) atom and 6-NH₂ group of the adenine ring in a Watson-Crick base-pair-like structure, the adenine N(1) atom hydrogen bonding with the peptide NH group and the adenine 6-NH₂ group with the peptide C=O group, could be responsible for the selection of the syn conformation of cAMP and its analogues when bound to CRP. In the case of cGMP and cIMP, hydrogen bonding could occur between the purine N(1)H group and a peptide C=O group and the purine 6-keto group and a peptide NH group, resulting in the selection of the anti conformation.

The selection of the syn conformation could be one of the underlying forces for the unique action of cAMP on CRP, namely, enhancement of transcription of catabolite-sensitive operons. However, the selection of the syn conformer per se cannot be the sole feature required to produce the necessary conformational changes in CRP as dibutyl-cAMP and 8-bromo-cAMP, both of which are bound to CRP in the syn conformation, show little activity in promoting CRP-stimulated transcription (Pastan & Perlman, 1970; Anderson et al., 1972). This is probably due to the presence of large bulky groups (viz., the dibutyl chains and the bromine atom) preventing concomitant conformational changes in CRP occurring upon cyclic

nucleotide binding. In this respect, we note that of the six cyclic nucleotides examined, cAMP produced the most marked changes in the ¹H NMR spectrum of CRP.

Acknowledgments

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Proton Nuclear Magnetic Resonance Study of the Histidine Residues of the *Escherichia coli* Adenosine Cyclic 3',5'-Phosphate Receptor Protein. pH Titration Behavior, Deuterium Exchange, and Partial Assignments[†]

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ABSTRACT: The properties of the histidine residues of the cyclic AMP receptor protein (CRP) of *Escherichia coli* and its N-terminal core (α CRP) have been investigated by ¹H NMR. Comparison of the spectra of CRP and α CRP shows that of the five histidine residues per subunit present in CRP, three, histidines A, C, and D, lie in the N-terminal domain, and two, histidines B and E, lie in the smaller carboxy-terminal domain. The C(2) protons of histidines A, B, C, and D undergo rapid deuterium exchange at 37 °C with a $t_{1/2}$ of about 2 days, in contrast to that of histidine E, which remains unexchanged after 50 days. With the exception of histidine E, complete pH titration curves were obtained for the histidine residues. The pH titration curves for the imidazole proton resonances of the three histidine residues, A, C, and D, present in the N-terminal core are identical in CRP and α CRP. The pH titration curves

of the imidazole proton resonances of histidines A and B exhibit classical Henderson-Hasselbach behavior, titrating with a single pK. In contrast, the pH titration curves for the imidazole proton resonances of histidines C and D deviate markedly from Henderson-Hasselbach behavior and can be described quantitatively by a model in which their imidazole rings mutually interact. Partial assignments of the histidine residues are discussed on the basis of the data presented in this paper and data in the literature on the X-ray crystal structure of CRP [McKay, D. B., & Steitz, T. A. (1981) *Nature (London)* 290, 745-749] and on the nucleotide sequence of its structural gene [Aiba, H., Fujimoto, S., & Ozaki, N. (1982) *Nucleic Acids Res.* 10, 1345-1362; Cossart, P., & Gicquel-Sanzey, B. (1982) *Nucleic Acids Res.* 10, 1363-1378].

In the preceding paper (Gronenborn & Clore, 1982), we presented a ¹H nuclear magnetic resonance (NMR)¹ study on the binding of cyclic nucleotides to the cyclic AMP receptor protein (CRP) of *Escherichia coli*. CRP is a dimer of apparently identical subunits, each of molecular weight 22 500 (Anderson et al., 1971; Riggs et al., 1971). X-ray crystallographic studies (McKay & Steitz, 1981) have shown that each subunit is composed of two domains, a larger N-terminal domain which contains the cyclic nucleotide binding site and a smaller carboxy-terminal domain which was proposed to contain the DNA binding site. Subtilisin digestion of the cAMP-CRP complex separates these two domains and results in the formation of a stable N-terminal core, α CRP, in which each subunit has a molecular weight of 12 500 (Krakow & Pastan, 1973; Eileen et al., 1978). α CRP retains the ability

to bind cAMP (Krakow & Pastan, 1973) and more recently has been shown to bind DNA nonspecifically, stabilizing its double-helical structure (Takahashi et al., 1981). In the present paper, we present a detailed study by ¹H NMR of the histidine residues of CRP and α CRP. The possible assignments of the histidine residues are discussed in relation to the amino acid sequence of CRP deduced from the nucleoside sequence of its structural gene (Aiba et al., 1982; Cossart & Gicquel-Sanzey, 1982) and X-ray crystallographic data (McKay & Steitz, 1981).

Experimental Procedures

Materials. CRP was purified from *E. coli* KLF 41 by the method of Takahashi et al. (1980). α CRP was prepared by

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¹ Abbreviations: CRP, cyclic AMP receptor protein; α CRP, N-terminal core of CRP; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; SD_{ln}, standard deviation of the natural logarithm of an optimized parameter; NaDodSO₄, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.