CONFORMATIONAL SELECTION OF *syn*-cAMP UPON BINDING TO THE cAMP RECEPTOR PROTEIN

A ^{1}H NMR study

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Received 4 November 1981

1. Introduction

3′,5′-Cyclic AMP (cAMP) receptor protein (CRP) is a dimer of apparently identical subunits, each of M_r = 22 500 [1,2], which mediates control of catabolite-sensitive operons in *Escherichia coli* [3,4], cAMP acting as an effector [5]. The cAMP · CRP complex interacts with specific DNA sites at or near promotors [6,7], stimulating the initiation of mRNA synthesis [8,9]. The mechanism of the activation of transcription, however, is still poorly understood. Elucidation of the various possible underlying interactions in the proposed models [10] at the molecular and atomic levels will require extensive structural studies of all the complexes involved. Recently, the X-ray crystal structure of the cAMP · CRP complex has been determined at 2.9 Å resolution [11], but the conformation of bound cAMP was not resolved. We present here the first study on the solution structure of CRP in the presence and absence of cAMP by ^{1}H NMR spectroscopy, and determine the conformation about the glycosidic bond of bound cAMP by measurements of transferred nuclear Overhauser enhancements (NOE). We show that, in contrast to free cAMP which exists as a *syn/anti* equilibrium mixture in solution with a predominance of the anti conformer [12,13], cAMP bound to CRP is solely in the *syn* conformation.

2. Experimental

CRP was purified by the method in [14] and was >99% pure as judged by SDS–polyacrylamide gel electrophoresis. cAMP was obtained from PL Biochemicals and used without further purification. d8-cAMP was prepared by heating a solution of cAMP in D_2O at 80°C for 7 h. All other chemicals used were of the highest purity commercially available.

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

^{1}H NMR spectra were recorded at 270 MHz on a Bruker WH 270 spectrometer operating in Fourier transform mode. 2000 transients for standard spectra and 200 transients for NOE measurements, obtained by quadrature detection with an acquisition time of 0.975 s, using 8192 data points for a spectral width of 4.2 kHz, were averaged. Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 1 Hz for standard spectra and 2 Hz for NOE difference spectra. NOEs were measured by irradiation at a selected position for a duration of 1.5 s prior to the observation pulse. Systematic NOE measurements, irradiating at 20 Hz intervals, were carried out over the entire aromatic and sugar proton regions of cAMP in the presence and absence of CRP. Chemical shifts are expressed relative to internal (1 mM) dioxan (3.71 ppm downfield from dimethylsilapentane-5-sulphonate). All spectra were recorded at 20°C.

3. Results and discussion

In fig.1 the aromatic portion of the ^{1}H NMR spectra of both CRP and the cAMP · CRP complex is

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

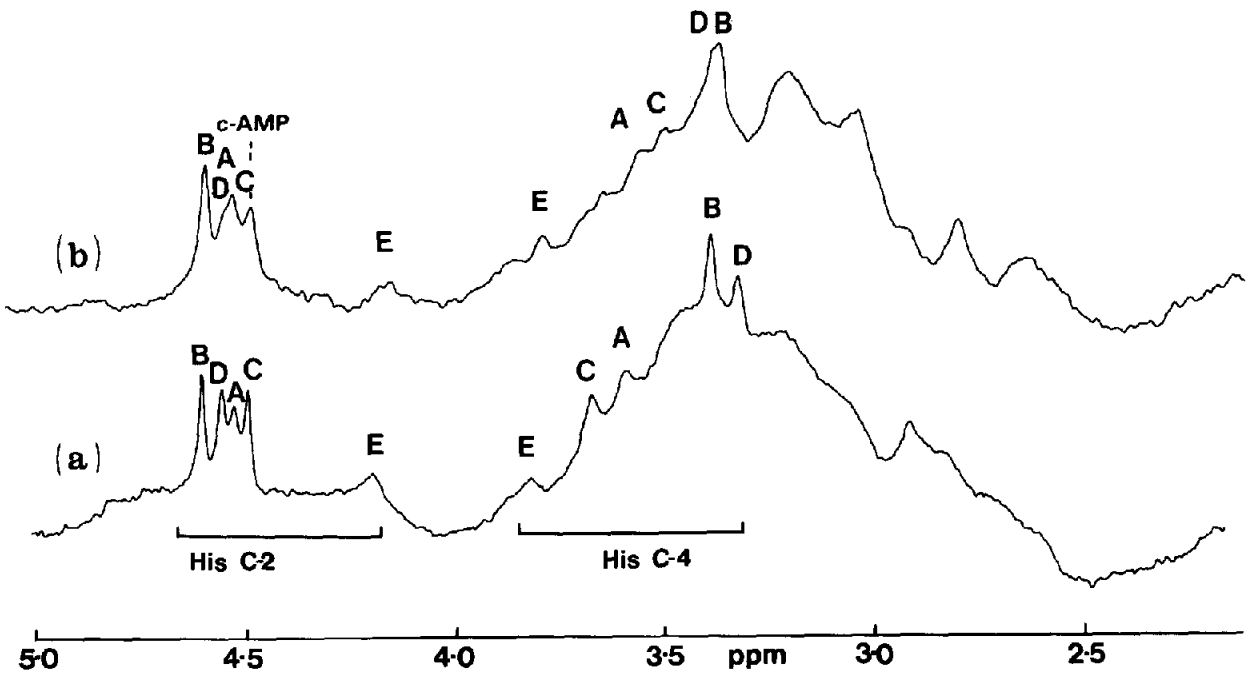

Fig.1. Aromatic portion of the 270 MHz ^{1}H NMR spectra of (a) CRP and (b) the cAMP · CRP complex. The 5 sets of His C(2)- and C(4)-proton resonances are labelled A–E. The C(2)- and C(4)-proton resonances for each His residue were connected by virtue of their pK-values (obtained by pH titration) and by intraresidue nuclear Overhauser enhancement. The samples contained 0.28 mM CRP; cAMP was 0.72 mM; from the K_a-values (3.5×10^4 M^{-1}) and cooperativity parameter ($\alpha = 1.7$) obtained by extrapolation of the data in [14] to the experimental conditions of ionic strength (0.55 M), the saturation of both binding sites on the protein with cAMP was calculated as ~95%. All other experimental conditions are in section 2.

shown. The most striking features are the extremely sharp lines for 4 of the 5 histidine (His) residues (labelled A–E). The C(2)- and C(4)-proton resonances of residues A–D have a natural linewidth of 3–5 Hz, suggesting significant internal mobility. The signals of the C(2)-protons of residues A–D exhibit a similar chemical shift in the range 4.5–4.6 ppm, while that of residue E is shifted to higher field (4.18 ppm) due to its low pK (~5). All the other His residues are titratable with pK-values in the region of 6.5–7.0. The markedly increased linewidth of both the C(2)- and C(4)-proton resonances of His E (~15 Hz) suggests that this amino acid lies in a rigid structure inside the protein, and has therefore lost most of its internal mobility. This is consistent with the finding that its imidazole ring only starts to become protonated at very low pH-values (<6), indicating that it is buried in the deprotonated state at physiological pH-values.

Addition of cAMP to CRP results in an overall broadening of the lines of the spectrum under conditions where both binding sites on the protein are ~95% saturated with cAMP. This implies that the conformational change of CRP upon binding cAMP consists in part of a tightening up of the flexible protein into a more rigid structure. This is consistent with the results obtained by small angle X-ray scattering which show a decrease in the radius of gyration upon cAMP binding due to an overall contraction of the CRP molecule [15].

In the spectrum of the cAMP · CRP complex no signals of the bound ligand can readily be seen, and no bound frequencies could be detected by the transfer of saturation technique for the protons of the adenine ring and the H1′ proton of the sugar ring. We were, however, able to demonstrate a transferred nuclear Overhauser effect.

If bound and free cAMP were in slow exchange, positions for bound signals differing from the free positions by >40 Hz should be detectable by transfer of saturation, as one of the components involved in the transferred NOE is the basic mechanism of transfer of saturation, namely magnetic exchange between

free and bound states. If, on the other hand, bound and free cAMP were in fast exchange, a shift of the free signal on binding should be observed if the chemical shift difference were >10 Hz; such a shift was not detected. We therefore conclude that the positions of the bound H1′ and H2/H8 resonances of cAMP coincide with the positions of the corresponding signals in free cAMP, and that bound and free cAMP are in fast exchange on the NMR time scale.

Systematic measurements of NOEs on cAMP and cAMP deuterated in the 8 position (d8-cAMP) in the presence and absence of CRP were used to obtain specific information on the local conformation of the bound cyclic nucleotide since the magnitude of a NOE is proportional to the inverse sixth power of the distance between the observed and irradiated nucleus. For the experiments in the presence of CRP an ~10-fold molar excess of free over bound cAMP was employed. Table 1 summarizes the observed NOEs and their magnitudes. Fig.2 shows NOE difference spectra obtained by subtracting a spectrum with irradiation of the H2/H8 peak, the H1′ peak and the H5′ peak from spectra where the irradiation frequency is offset by 150 Hz from the respective signal as a control. In free cAMP (i.e., in the absence of CRP) one observes a positive NOE on the H8 resonance on irradiating the H1′ and H2′ signals; no NOE from the H5′ signal to the H2 signal could be detected. In the presence of CRP, however, we observe large negative transferred NOEs from the positions of the free H1′ and H5′ protons to the position of the H2/H8 proton (under the conditions used, the H8 and H2 resonances of cAMP are superimposed at 4.50 ppm). Since these NOEs are negative they must arise from dipolar relaxation between protons characterized by long correlation times (in contrast to the very short correlation times of small molecules such as cAMP where positive NOEs are observed) and, therefore have to involve the bound protons of the cAMP · CRP complex via exchange of magnetization between the free and bound states. Using d8-cAMP as the ligand abolishes the negative transferred NOE on irradiating the position of the H1′ resonance, which clearly shows that it involves only the H8 and not the H2 proton, but reveals a negative transferred NOE from the H5′ to the H2 resonance.

The observation of substantial negative transferred NOEs in the range −20% to −25% between the H1′ resonance of the sugar ring and the H8 resonance of the adenine ring and between the H5′ sugar resonance and the H2 adenine resonance via the protein bound cAMP, implies that these atoms have to be very close to each other (≪4 Å [16]), and oriented towards each other. Since we were not able to observe a negative transferred NOE from the H2′ to the H8 proton in the presence of CRP, in contrast to the positive NOE observed between these 2 protons in the absence of CRP, we conclude that the conformation about the glycosidic bond of cAMP bound to CRP has to be *syn*, since in the *anti* conformation the H8 proton is close enough to the H2′ proton to yield a NOE. The fractional population of the *anti* form [with χ (O4′–C1′–N9–C4) ~ 240°] and the *syn* form [with $\chi \sim 60°$] of ~70% and ~30%, respectively, in free cAMP [12], is thus altered upon binding to CRP

Table 1
Observed transferred NOEs involving the adenine ring and sugar ring protons of cAMP in the presence of CRP and the corresponding NOEs seen for free cAMP (i.e., in the absence of CRP)

	Irradiated resonance (δ ppm from dioxan)	Observed resonance	% NOE
cAMP + CRP	H8 (4.50 ppm)	H1′[a]	−20
	H1′ (2.38 ppm)	H8[a]	−25
	H2′ (1.00 ppm)	no NOE detectable	
	H5′ (0.68 ppm)	H2[b]	−20
cAMP − CRP	H8 (4.50 ppm)	H1′[a]	+10
	H1′ (2.38 ppm)	H8[a]	+11
	H2′ (1.00 ppm)	H8[a]	+13
	H5′ (0.68 ppm)[c]	no NOE detectable	

[a] Not observed when d8-cAMP is used
[b] Observed both with cAMP and d8-cAMP
[c] The resonance of the H5′ proton is ~0.02 ppm downfield from that of the H4′ proton, and ~0.2 ppm upfield from that of the H5″ proton. However, NOEs from the H4′ and H5″ resonances to either the H2 or H8 resonance are very unlikely as the distances between these protons are too large for all conformations about the glycosidic bond, as judged on the basis of a space-filling model of cAMP. (Note the H5′ proton is axial to the H4′ proton whereas the H5″ proton is equatorial to the H4′ proton)

Since the H8 and H2 resonances of the adenine ring have virtually identical chemical shifts (~4.50 ppm) under the conditions used, NOEs involving the H8 and H2 resonances were distinguished using d8-cAMP: cAMP and d8-cAMP were 3.3 mM; CRP was 0.14 mM, yielding ~10-fold molar excess of free over bound cAMP. All other experimental conditions are given in section 2

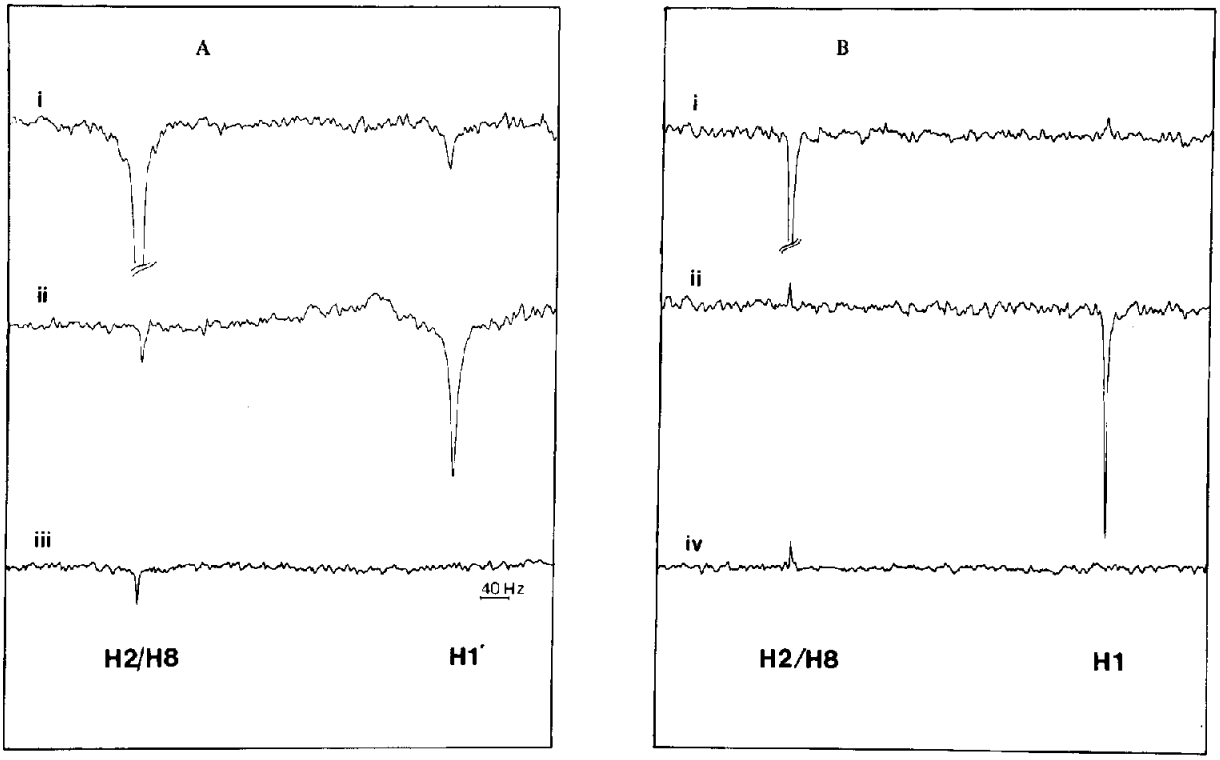

Fig.2. NOE difference spectra in the aromatic region of the 270 MHz ^{1}H NMR spectrum of cAMP in (A) the presence and (B) the absence of CRP: (i) spectrum with irradiation at 4.50 ppm (H2/H8 resonance) minus control spectrum with irradiation at 3.94 ppm; (ii) spectrum with irradiation at 2.38 ppm (H1′ resonance) minus control spectrum with irradiation at 2.94 ppm; (iii) spectrum with irradiation at 0.69 ppm (H5′ resonance) minus control spectrum with irradiation at 0.13 ppm using d8-cAMP; (iv) spectrum with irradiation at 1.00 ppm (H2′ resonance) minus control spectrum with irradiation at 1.56 ppm. Cyclic AMP and d8-cAMP were 3.3 mM; CRP was 0.14 mM yielding ~10-fold molar excess of free over bound cAMP. All other experimental conditions are in section 2.

in such a way that cAMP is bound entirely in the *syn* conformation. Furthermore, it seems likely that the torsion angle χ about the glycosidic bond of cAMP to CRP is $<60^\circ$ since irradiating the H5′ resonance showed no positive NOE on the H2 resonance in the absence of CRP (i.e., for free cAMP), but yielded a substantial negative transferred NOE (−20%) in the presence of CRP (i.e., for bound cAMP). Using space filling models it can be shown that the distance between the H5′ and H2 protons is at a minimum for $\chi \sim 45^\circ$.

These results demonstrate that the binding of cAMP to CRP involves conformational selection of the *syn* conformer of cAMP which is the minor form in free solution. In free nucleotides the *syn* conformer is promoted by substitution of the purine ring in the 8 position with bulky substituents (8Br-cAMP, 8oxo-cAMP) or intramolecular hydrogen bonding between N(3)–OH(5′) as found for *N*(6)-dimethyl-2′,3′-*O*-isopropylidene–adenosine [17]. One possible way of achieving this conformational selection in cAMP upon binding to the protein in favour of the *syn* form might be the formation of a hydrogen bond of a Watson–Crick type between a protein residue and the N1 of the adenine ring. The changes in the protein, as judged from the differences in the ^{1}H NMR spectra, however, seem to be of a rather subtle nature.

From the observation of only 5 His resonances for CRP alone and the cAMP · CRP complex, it seems very likely that the conformation of the 2 subunits are identical in solution, given the amino acid analysis finding of 5 His residues/subunit [1]. The narrow linewidths of the C(2)- and C(4)-proton resonances of 4 of the 5 His residues can either be attributed to an

overall mobility within the CRP molecule or to their location in a flexible portion of the protein. One such flexible region could be the smaller C-terminal domain which contains an area that is not well defined in the electron density map [11], possibly due to its internal mobility.

Acknowledgements

G. M. C. and A. M. G. thank Sir Arnold Burgen for continual encouragement and support. G. M. C. and A. M. G. also acknowledge the receipts of short term FEBS and EMBO travelling fellowships to purify CRP in Toulouse. We also thank Mr Colin Young for carrying out a 400 ℓ fermentation and Mr Michael Bardet for technical assistance in the purification of CRP.

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