Determination of the conformations of cyclic nucleotides bound to the N-terminal core of the cyclic AMP receptor protein of *Escherichia coli* by ^{1}H-NMR

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1. INTRODUCTION

The cyclic AMP receptor protein (CRP) is a dimer of identical subunits (each of $M_r \sim 23\,000$) which mediates the regulation of a number of catabolite sensitive operons in *Escherichia coli*, cAMP acting as an effector [1–5]. We have shown by ^{1}H-NMR measurements of proton–proton *transferred* nuclear Overhauser enhancements (TRNOE [6]) that CRP exerts conformational selection on cyclic nucleotides, such that cAMP and its analogues which have a 6-NH_2 group and a deprotonated N(1) atom are bound in the *syn* conformation, whereas cGMP and its analogues which have a 6-keto group and a protonated N(1) atom are bound in the *anti* conformation [7,8]. This contrast with the situation in free solution where cyclic nucleotides exist as a *syn/anti* equilibrium mixture, cAMP being predominantly ($\sim$70%) *anti* and cGMP predominantly ($\geq$90%) *syn* [8–11].

Subtilisin digestion of the cAMP • CRP complex results in the formation of a stable N-terminal core, αCRP (composed of two subunits, each of $M_r \sim 12\,500$) which retains cAMP binding activity [12,13]. In the present paper we have determined the conformations of cAMP, cTuMP and cGMP bound to αCRP by proton–proton TRNOE measurements, and show that αCRP, in contrast to CRP, does *not* exert conformational selection on cyclic nucleotides, the conformation in the bound state being the predominant form in free solution.

Abbreviations: CRP, cAMP receptor protein of *Escherichia coli*; αCRP, N-terminal core of CRP produced by subtilisin digestion of the cAMP-CRP complex; cAMP, adenosine 3′,5′-cyclic phosphate; cTuMP, tubericidin 3′,5′-cyclic phosphate; cGMP, guanosine 3,5′-cyclic phosphate; NOE, nuclear Overhauser effect; TRNOE, *transferred* nuclear Overhauser effect

2. EXPERIMENTAL

CRP was prepared by the method of B. Blazy, M. Takahashi and A. Baudras (unpublished) and previously used in [7,8,14]. αCRP was prepared by subtilisin digestion of the cAMP • CRP complex by a modification of the procedure in [13]. cAMP and cGMP were obtained from PL Biochemicals and cTuMP from the UpJohn Co. Ltd., lyophilized from D_2O and used without further purification. All other chemicals used were of the highest purity commercially available.

Samples for ^{1}H-NMR were prepared by extensive dialysis of CRP and αCRP 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. The final concentrations in the samples used in the TRNOE measurements were: 0.1 mM CRP or αCRP (corresponding to 0.2 mM in cyclic nucleotide binding sites), between 1.4 and 2.2 mM cyclic nucleotide, 500 mM KCl, 50 mM potassium phosphate (pH* 6.5), 1 mM EDTA and 1 mM dioxan.

Proton–proton TRNOE measurements were

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carried out at 270 MHz using a Bruker WH270 spectrometer operating in Fourier transform mode with the pulse sequence $(t_1-t_2-\pi/2-AT)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (1 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). 750 transients, obtained by quadrature detection with 4096 data points for a spectral width of 4.2 kHz, were averaged for each spectrum. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. TRNOE measurements were carried out systematically at 20 Hz intervals throughout the entire aromatic and sugar proton regions of the spectrum. Chemical shifts are expressed relative to internal (1 mM) dioxan (3.71 ppm downfield from 2,2-dimethyl silapentane-5-sulphonate). All experiments were carried out at 20°C.

3. RESULTS

The proton–proton nuclear Overhauser effect (NOE) has been widely used in conformational analysis to demonstrate the proximity of two protons in space and determine their separation [15–19]. However, direct NOE measurements are usually not suitable to study the conformations of ligands bound to large proteins (M_r >20 000) as it is usually difficult to observe the resonances of the bound ligand directly. This problem can be overcome by the use of the *transferred* NOE (TRNOE) technique [6,7,8,20,21], the theory of which has recently been dealt with in detail [6]. The basis of the TRNOE involves the transfer of magnetic information concerning cross-relaxation between bound ligand protons from the bound state to the free state of the ligand by chemical exchange. By this means easily detectable *negative* TRNOE's arising from cross-relaxation between bound ligand protons characterized by long correlation times (as opposed to cross-relaxation between free ligand protons characterized by very short correlation times which give rise to *positive* NOE's) can be detected on free or averaged ligand resonances following irradiation of other ligand resonances (free, bound or averaged), thus conveying conformational information on the bound ligand [6].

The results of systematic TRNOE measurements

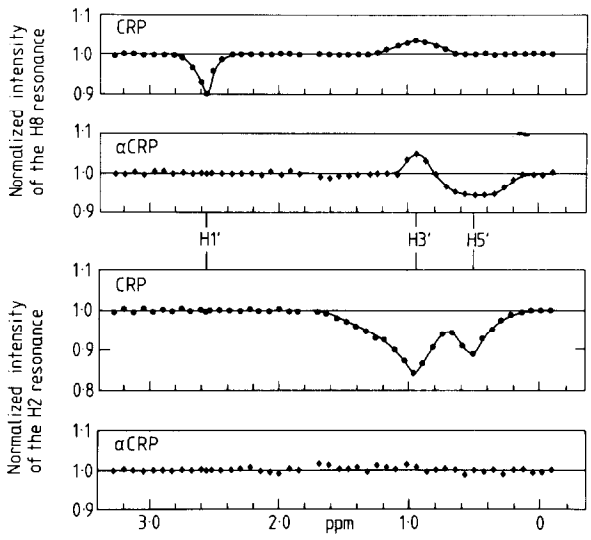

Fig.1. Normalized intensity of the averaged H8 and H2 resonances of the purine ring of cTuMP in the presence of CRP (•) and αCRP (◆) as a function of irradiation frequency. The molar ratio of free to bound cTuMP was 10 in the case of CRP and 6 in the case of αCRP, and the concentration of protein used in both cases was 0.1 mM (corresponding to 0.2 mM in cyclic nucleotide binding sites). Other experimental conditions are given in Section 2. Chemical shifts are expressed relative to dioxan (3.71 ppm downfield from 2,2 dimethyl silapentane-5-sulphonate).

on cAMP, cTuMP and cGMP in the presence of αCRP and on cTuMP in the presence of CRP, obtained by systematic irradiation at 20 Hz (≡ 0.074 ppm) intervals throughout the aromatic and sugar proton regions of the spectrum, are summarized in table 1. These experiments were carried out with a six to ten fold molar excess of free over bound cyclic nucleotide.

For cAMP and cTuMP in the presence of αCRP only a single *negative* TRNOE, characteristic of the *anti* conformation, is observed, namely on the H8 resonance of the purine ring following irradiation of the H5′ sugar resonance (table 1). In contrast, the only *negative* TRNOE's observed for cAMP [7,8] and cTuMP (table 1) in the presence of CRP are characteristic of the *syn* conformation, namely on the H8 resonance of the purine ring following irradiation of the H1′ sugar resonance and vice

Table 1

TRNOE's observed for cAMP, cTuMP and cGMP in the presence of αCRP and for cTuMP in the presence of CRP

Irradiated resonance[a]	Observed resonance[a]	% TRNOE			
		CRP	αCRP		
		cTuMP[b]	cAMP[c]	cTuMP[c]	cGMP[b]
H1′	H8	−10	–	–	−12
H8	H1′	−15	–	–	−14
H3′	H8	+4[d]	–	+5[d]	–
H5′	H8	–	−6[e]	−5	–
H3′	H2	−16	–	–	–
H5′	H2	−11	–	–	–

[a] Chemical exchange between the free and bound states of the cyclic nucleotides is fast on the chemical shift scale, and under the experimental conditions employed, the positions of the averaged ligand resonances are approximately at the positions of the corresponding resonances in free cyclic nucleotides

[b] The molar ratio of free to bound cyclic nucleotide was 10

[c] The molar ratio of free to bound cyclic nucleotide was 6

[d] The *positive* NOE on the H8 resonance following irradiation of the H3′ resonance arises directly from the free cTuMP. This *positive* NOE is the only one observed for free cTuMP in the absence of protein where it has a value of ∽ +5%; the observation of this single *positive* NOE for free cTuMP indicates that the predominant conformation about the glycosidic bond of cTuMP in free solution is *anti*

[e] In the case of cAMP the H8 and H2 resonances are superimposed so that the effects on these two resonances can only be distinguished using cAMP deuterated in the 8 position (d8-cAMP). The TRNOE observed on the H8/H2 resonance following irradiation of the H5′ resonance is not observed with d8-cAMP indicating that this TRNOE only involves the H8 proton and not the H2 proton

The TRNOE measurements were carried out by systematic irradiation at 20 Hz (≡ 0.074 ppm) intervals throughout the aromatic and sugar proton regions of the spectrum. The experimental conditions are given in section 2

versa, and on the H2 resonance of the purine ring following irradiation of the H5′ and H3′ sugar resonances. This is clearly illustrated in fig.1 where the normalized intensities of the H8 and H2 resonances of the purine ring of cTuMP in the presence of CRP and αCRP are plotted as a function of irradiation frequency. (It will be noted that in the presence of both CRP and αCRP a small *positive* NOE of ≲ +5% is seen on the H8 resonance of cTuMP following irradiation of the H3′ sugar resonance; this arises directly from free cTuMP).

For cGMP in the presence of αCRP the only *negative* TRNOE's observed, namely on the H8 resonance of the purine ring following irradiation of the H1′ sugar resonance and vice versa (see table 1), are characteristic of the *syn* conformation. This too contrasts with the situation observed for cGMP in the presence of CRP where the only *negative* TRNOE's observed, namely on the H8 resonance of the purine ring following irradiation of the H5′ and H3′ sugar resonances, are characteristic of the *anti* conformation [8].

Table 2

Conformations about the glycosidic bond and the glycosidic bond torsion angles of cAMP, cTuMP and cGMP bound to CRP and αCRP, determined from TRNOE measurements

Cyclic nucleotide	Conformation about the glycosidic bond (χ[a])	
	CRP	αCRP
cAMP	*syn* (χ = 45° ± 5°)[b]	*anti* (χ ~ 225°[d])
cTuMP	*syn* (χ = 60° ± 5°[c])	*anti* (χ ~ 225°[d])
cGMP	*anti* (χ = 225° ± 10°)[b]	*syn* (χ ~ 75°[d])

[a] The convention used in defining χ is the standard one given by Davies [11]: χ = 04′-C1′-N9-C4. χ is related to the angle χ' defined by Arnott and Hukins [22] and adopted by most crystallographers by the relation $\chi = 360° - \chi'$

[b] From Gronenborn and Clore [8]

[c] χ was determined by model building on the basis of the distance ratio

$$r_{H2-H5'}/r_{H2-H3'} = [N_{H2}(H3')/N_{H2}(H5')]^{1/6} = 1.06\ ^{+0.09}_{-0.07}$$

calculated using the values of the TRNOE's given in table 1. (The notation $N_i(j)$ denotes the TRNOE observed on the resonance of proton *i* following irradiation of the resonance of proton *j*.) The use of this equation is justified providing no *positive* NOE's on the H2 resonance following irradiation of the H3′ and H5′ resonances are observed for free cTuMP in the absence of protein [6], which is indeed the case. The error in the distance ratio and, hence, in the value of χ is based on an estimated error of ±3% in the observed TRNOE's

[d] As a TRNOE between only a single pair of protons was observed, the value of χ given is only approximate and is that for which the distance between the two protons involved in the TRNOE is at a minimum, namely the distance $r_{H8-H5'}$ in the case of cAMP and cTuMP and the distance $r_{H8-H1'}$ in the case of cGMP

The conformations about the glycosidic bond and the values of the glycosidic bond torsion angle χ (04′-C1′-N9′-C4) for cAMP, cTuMP and cGMP bound to CRP and αCRP, determined from the TRNOE measurements, are given in table 2.

4. DISCUSSION

The results of the TRNOE measurements presented here demonstrate that αCRP does *not* exert conformational selection on cyclic nucleotides, such that the conformations about the glycosidic bond of cAMP, cTuMP and cGMP bound to αCRP (see table 2) are the same as the predominant form in free solution, namely *anti* in the case of cAMP [8–10] and cTuMP (see footnote d to table 1), and *syn* in the case of cGMP [8–10]. This contrasts with the situation for CRP where conformational selection is exerted, cAMP and cTuMP being bound in the *syn* conformation and cGMP in the *anti* conformation (see table 2 and [7,8]).

It should be noted that although the TRNOE's observed for a given cyclic nucleotide in the presence of both CRP and αCRP arise from only a single conformation, the data do *not* allow one to conclude that a given cyclic nucleotide is bound exclusively in a single conformation. However, an upper limit for the proportion of a second minor conformation can be estimated on the basis of the dependence of the magnitude of the TRNOE's on the molar ratio of free to bound ligand, $[L_F]/[L_B]$ [6]. For a TRNOE, $N_i(j)$, observed on the resonance of proton *i* following irradiation of the resonance of proton *j*, for which no corresponding *positive* NOE is observed for the free ligand in the absence of protein, one can calculate that for values of $N_i(j)$ of −20%, −15%, −10% and −5% at $[L_F]/[L_B]$ = 10, the upper limits for the proportion of a second minor conformation are approximately 5%, 10%, 15% and 30% respectively, bearing in mind that under our experimental conditions the lower limit of detectability of a TRNOE lies in the range −1% to −2% [6,8]. Thus, the upper limit for the proportion of a second minor conformation of bound cAMP, cTuMP and cGMP lies in the range 5–15% in the case of CRP and 15–30% in the case of αCRP (see table 1 and [8]).

Although the available 2.9 Å resolution crystal structure of the cAMP · CRP complex [23] does not resolve the conformation of bound cAMP, it clearly shows that cAMP is buried in the interior of the β roll of the N-terminal domain of each subunit, with its phosphate group bound in the loop connecting strands 6 and 7 in the β roll, and the adenine ring located in the proximity of helix C of one or both subunits. This suggests that the conformational selection exerted on cyclic nucleotides by CRP and revealed by ^{1}H-NMR [7,8], arises from the formation of specific hydrogen bonds be-

tween groups of the purine ring (namely the 6-NH_2 group and the N(1) atom in the case of cAMP and its analogues, and the 6-keto and N(1)H groups in the case of cGMP and its analogues) and groups of one or more amino acid residues of helix C. This model is supported by the results presented here on αCRP. Comparison of the amino acid sequence of the C-terminal end of αCRP obtained by amino acid sequencing [24] with the amino acid sequence of CRP deduced from the nucleotide sequence of its structural gene [25,26] has shown that the site of subtilisin cleavage is located between Leu 116 and Ser 117. Inspection of the crystal structure of the cAMP • CRP complex [23] indicates that this cleavage site is located close to the N-terminal end of helix C (which extends from residue 111 to residue 134). Thus, in the case of αCRP where approximately three quarters of helix C are no longer present, conformational selection on cyclic nucleotides can no longer be exerted, the base of the bound cyclic nucleotide is free to rotate about its glycosidic bond, and the predominant conformation adopted by the bound cyclic nucleotide is the same as that of the free cyclic nucleotide. Moreover, the finding that the equilibrium association constants for the binding of cAMP and cGMP to both CRP and αCRP are comparable, lying in the range 10^4–5×10^4 M^{-1} ([14] and M. Takahashi, B. Blazy and A. Baudras, personal commun.), indicates: (i) that in the case of both CRP and αCRP the predominant contribution to the binding free energy arises from the interaction of the negatively charged phosphate group with a basic amino acid residue(s); (ii) that the base of the bound cyclic nucleotide is accessible to the solvent in the case of αCRP in contrast to the situation with CRP where it is buried within the protein [23]; and (iii) that the main driving force governing the conformational selection exerted by CRP over cyclic nucleotides arises because the selection of the minor free solution conformer (viz. *syn* in the case of cAMP and cTuMP, and *anti* in the case of cGMP) is energetically more favourable than the preservation of the major free solution conformer [viz. *anti* for cAMP ($\geq$70%), *anti* for cTuMP ($\geq$90%) and *syn* for cGMP ($\geq$90%)] as in the latter case the base of the cyclic nucleotide can no longer hydrogen bond with either solvent molecules or a residue(s) of the protein.

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