¹⁹F-n.m.r. studies of ligand binding to 5-fluorotryptophan- and 3-fluorotyrosine-containing cyclic AMP receptor protein from *Escherichia coli*

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Two fluorine-containing analogues of the cyclic AMP receptor protein (CRP) from *Escherichia coli* were prepared by biosynthetic incorporation of 5-fluorotryptophan (5-F-Trp) and 3-fluorotyrosine (3-F-Tyr). The ¹⁹F-n.m.r. spectrum of the [5-F-Trp]CRP showed two signals corresponding to the two tryptophan residues, and that of the [3-F-Tyr]CRP showed six signals (two overlapping) corresponding to the six tyrosine residues: these results are as expected for a symmetrical dimer. A comparison of the ¹⁹F-n.m.r. spectra of the CRP analogues in the presence and in the absence of cyclic AMP reveals that the chemical shifts of both tryptophan residues and of two of the six tyrosine residues show differences. Since none of these residues is in direct contact with the bound nucleotide (although Trp-85 is fairly close), these shift changes must arise from induced conformational effects. The ¹⁹F-n.m.r. spectra of complexes with cyclic GMP showed chemical-shift perturbations different from those caused by cyclic AMP, indicating that different conformational changes are induced by the binding of cyclic GMP. The ¹⁹F-n.m.r. spectrum of the complex of [3-F-Tyr]CRP with tubercidin 3',5'-(cyclic)monophosphate (which can activate transcription) showed essentially the same chemical-shift changes as seen for the cyclic AMP complex, indicating that similar conformational changes have been induced by the nucleotide binding. [3-F-Tyr]CRP in the presence of an equimolar amount of the 20 bp self-complementary DNA oligomer 5'-AATGTGAGTTAACTCACATT-3' and excess cyclic AMP gave an ¹⁹F-n.m.r. spectrum that was almost identical with that for the [3-F-Tyr]CRP–cyclic AMP complex, indicating that the binding of DNA does not induce significant conformational changes involving the tyrosine residues. Proteolysis of [3-F-Tyr]CRP with chymotrypsin produced a 31 kDa fragment that is a dimer containing the cyclic AMP-binding domain. This fragment contains five of the six tyrosine residues, and its ¹⁹F-n.m.r. chemical shifts were essentially the same as those of the intact protein except for one missing signal (signal F): this signal could be assigned to Tyr-206 and shown to be unperturbed by the binding of cyclic nucleotide to the intact [3-F-Tyr]CRP. The similarity of the ¹⁹F-n.m.r. chemical shifts in the α -fragment and the intact CRP indicates that the α -fragment retains the same structure as found in the intact protein. The binding of cyclic AMP to the α -fragment caused chemical-shift perturbations of signals from the same two residues (Tyr_A and Tyr_B) that are influenced in complexes with intact CRP, with similar but not identical perturbations being observed. The overall results provide evidence for specific conformational changes in the protein when different cyclic nucleotides bind to CRP, but give no indication of further conformational changes in the α -core of the protein when DNA binding takes place.

INTRODUCTION

In bacteria, the activation of catabolite-sensitive genes is controlled by the level of cyclic AMP (c-AMP) in the cell in a process mediated by the binding of c-AMP to a receptor protein (CRP). This protein regulates the expression of several genes by binding tightly to specific DNA sites in the presence of c-AMP [1,2]. The genes encoding the enzymes involved in the catabolism of lactose, arabinose, maltose and other sugars are typical examples of genes controlled in this way. In some cases CRP binding activates transcription (as for *lac* [3] and *ara* [4] operons), whereas in other cases it leads to repression of transcription (as for the *CRP* structural gene [5]) by influencing the RNA polymerase in a manner

as yet undefined. One model for the transcriptional regulation proposes that the CRP and RNA polymerase interact directly with each other when bound to the promoter [6], whereas another model postulates changes in the DNA conformation on binding CRP [7].

CRP is a dimer (total molecular mass approx. 47 kDa) of two identical subunits each having a c-AMP-binding site and a DNA-binding site [8,9]. Specific binding of CRP to DNA has been found for sequences in the DNA regulation site that comprise the sequence 5'-TGTGA-3' separated by a 6 bp block (with little sequence conservation) from an inverted repeat of the 5'-TGTGA-3', or a closely related structure. It is assumed that these two regions of the DNA sequence bind to the two subunits of the CRP dimer at binding sites similar to those found for

Abbreviations used: c-AMP, cyclic AMP; c-GMP, cyclic GMP; c-TuMP, tubercidin 3',5'-(cyclic)monophosphate; CRP, c-AMP-receptor protein; [5-F-Trp]CRP, 5-fluorotryptophan-containing CRP; [3-F-Tyr]CRP, 3-fluorotyrosine-containing CRP.

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other repressors with similar structural characteristics, such as the *cro*-repressor [10,11] and the lambda repressor [12]. In these cases the crystal-structure studies have indicated the presence of a helix-turn-helix motif implicated in DNA binding, and Steitz and co-workers [7,13] have identified a similar motif in the crystal structure of the CRP-c-AMP complex. As yet no crystal structure information is available on CRP-DNA complexes.

Steitz and co-workers [7,13] have discussed how the allosteric conformational changes might be induced by binding c-AMP in terms of either altering the relative orientation of the two subunits in the dimer or altering the relative orientation of the two domains in each subunit. Studies on mutant CRP proteins that can function without c-AMP have allowed Garges & Adhya [14] to implicate specific residues in α -helix D (in the DNA-binding domain) in the allosteric conformational changes. These residues are close to residues in α -helix C (in the α -core containing the c-AMP-binding site) and are thus located in the 'hinge region' connecting the two domains. Garges & Adhya [14] have postulated that c-AMP binding to residues in the α -helices C (as shown by Steitz and co-workers [7,13]) transmits conformational changes, via other residues in α -helix C, to interacting residues on the neighbouring α -helix D and ultimately on to α -helix F, which is thought to be directly involved in the DNA binding.

Tubercidin 3',5'-(cyclic)monophosphate (c-TuMP) has been found to be as effective as c-AMP in stimulating *gal* transcription in the presence of CRP; other cyclic nucleotide analogues, such as cyclic GMP (c-GMP), are inactive [9,15].

Fried & Crothers [16] have studied the binding of CRP to restriction fragments containing the *lac* promoter region and found that the tight specific binding ($8.4 \times 10^{10} \text{ M}^{-1}$) requires only a single equivalent of c-AMP to be bound to the dimer. They conclude that the c-AMP affinities for the two CRP sites are unequal. More recently Gaston *et al.* [17] have also reported on the tight binding of CRP for nucleotide sequences containing the consensus region. Takahashi *et al.* [18] have reported ionic-strength-dependent co-operative binding effects of c-AMP to CRP in the absence of DNA, and other workers [19] have measured the overall association constant in the range 10^5 – 10^6 M^{-1} . It seems likely that conformational changes will accompany ligand binding both for the CRP-c-AMP complex and for the CRP-c-AMP-DNA complex and that these conformational changes will be involved in the final understanding of the regulation of the transcription process. Several studies have already used physical techniques to demonstrate that CRP undergoes conformational changes on binding c-AMP [20–22]. Gronenborn & Clore [22] have used transferred nuclear-Overhauser-effect measurements to show that the glycosidic bond of c-AMP has a syn conformation when bound to CRP in solution, in contrast with the anti conformation found in the crystal structure [7,13]. They have also made some studies on the protein ^1H -n.m.r. spectrum at 270 MHz and characterized the pH-dependence of four of the five histidine residues by monitoring the relatively sharp imidazole C-2 proton signals [23]. The remainder of the spectrum was very complex and, even with access to higher magnetic-field values and two-dimensional n.m.r. techniques, it will probably be necessary to resort to selective deuteration techniques to

make the specific signal assignments required in order to obtain detailed conformational information. Another useful method of resolving resonances for individual amino acid residues in large proteins results from incorporating fluorine-labelled amino acids into the protein [24–29]. The ^{19}F nucleus has a high natural sensitivity for n.m.r. detection, and its shielding is very sensitive to its environment. This makes it a very useful probe for detecting conformational changes in molecules. There are no background ^{19}F -n.m.r. signals, the only signals observed being those from the selected fluorine-labelled amino acids incorporated into the protein. In the present paper we report on the preparation of two fluorine-labelled analogues of *Escherichia coli* CRP containing 5-fluorotryptophan (5-F-Trp) and 3-fluorotyrosine (3-F-Tyr) respectively and on ^{19}F -n.m.r. studies aimed at detecting conformational changes accompanying the binding of ligands.

MATERIALS AND METHODS

Ligands

c-AMP and c-GMP were purchased from Sigma Chemical Co. c-TuMP was prepared as follows. Tubercidin 5'-monophosphate (from Sigma Chemical Co.) (50 mg, 154 μmol) was converted into the 3',5'-cyclic derivative by following the procedure of Smith *et al.* [30], with the 4-morpholine-*NN'*-dicyclohexylcarboxamidinium salt of the nucleotide. The cyclized material was purified on a column of DEAE-cellulose (HCO_3^- form) by elution with a gradient of 0–0.4 M-triethylamine bicarbonate as described by Tanaka *et al.* [31], yielding 11.4 mg (35 μmol). Purity was established by h.p.l.c. analysis [Waters C_{18} $\mu\text{Bondapak}$ column eluted with 10 mM- KH_2PO_4 , pH 5.5, and 10% (v/v) methanol at 1.5 ml/min].

The self-complementary 20 bp DNA (I) 5'-AATGTG-AGTTAACTCACATT-3' was synthesized on a Beckman System 1 Plus DNA synthesizer by using the phosphoramidite method.

Preparation and purification of ^{19}F -labelled CRP proteins

E. coli GCSC strain 2849 (a gift from Dr. B. J. Bachmann, *E. coli* Genetic Stock Center, Yale University) is auxotrophic for phenylalanine, tyrosine and tryptophan. The lowest non-growth-limiting concentration of these amino acids in the M9/glucose minimal medium that we have used is shown in Table 1. The organism was transformed with plasmid pBS_{crp}2 (Cossart & Gicquel-Sanzey [32]), which incorporates the coding region for *E. coli* CRP. After selection and checking to ensure that auxotrophy and overproduction of the protein were retained, the tolerance of the organism to fluorinated amino acids in the minimal medium was determined. None of the amino acids could be replaced completely by the fluorinated analogue, but sufficient growth could be achieved in the presence of the mixtures of fluorinated and non-fluorinated analogues as shown in Table 1.

The transformed organism, renamed *E. coli* 2849t, was cultured at 37 °C in 40 litres of medium containing M9 salts, 0.2% glucose, 2 mM- MgSO_4 , 0.1 mM- CaCl_2 , 2 g of ampicillin, 1 μM -*p*-hydroxybenzoic acid, 1 μM -*p*-aminobenzoic acid, 1 μM -2,3-dihydroxybenzoic acid, plus tyrosine, tryptophan and phenylalanine each at 50 mg/litre

Table 1. Concentrations of amino acids used in conjunction with *E. coli* 2849t to produce fluorinated CRP in M9/glucose medium

Amino acid	Lowest non-growth-limiting concentration (mg/l)	Concentration used in large-scale growth (mg/l)	
		Non-fluorinated	Fluorinated
L-Tyrosine	20	7	18 (3-fluoro)
L-Tryptophan	10	7	15.5 (5-fluoro)
L-Phenylalanine	30	10	20 (3-fluoro)

or, alternatively, the mixture of fluorinated and non-fluorinated analogues at the concentrations indicated in Table 1. When the culture was about to enter stationary phase, the bacteria were rapidly chilled to 4 °C, then harvested by continuous centrifugation in a Sharples centrifuge. Extraction and purification were carried out exactly as described in Donoso-Pardo *et al.* [19]. Purity of the products was established by SDS/PAGE.

By using this method, samples of CRP containing 3-fluorotyrosine or 5-fluorotryptophan were obtained. The CRP containing 3-fluorophenylalanine proved to be highly unstable, being precipitated from solution as the purification proceeded. Consequently we have been unable to produce sufficient amounts of this material for n.m.r. experiments.

Pure fluorinated CRP samples were stored as (NH₄)₂SO₄ precipitates at 4 °C. Samples for ¹⁹F-n.m.r. experiments were made by dialysing the precipitates in small volume against the required (aqueous) buffer, determining the concentrations of the resulting solution by absorption spectroscopy and then dialysing again against buffer in ²H₂O/¹H₂O (1:4, v/v) containing the ligand at known concentration.

Typical samples contained 0.5–1.0 mM-protein in 2 ml of 500 mM-KCl/10 mM-phosphate in ²H₂O/¹H₂O (1:4, v/v). Most of the experiments were conducted at pH 8.0 and 293 K, but some studies over the pH range 6–8 and temperature range 280–313 K were also carried out. The pH measurements were made with a Radiometer model 26 pH-meter equipped with combination glass electrode; the pH values are meter readings uncorrected for any deuterium isotope effects.

Dissociation constants were measured by the precipitation method as described previously (Donoso-Pardo *et al.* [19]).

N.m.r. measurements

The ¹⁹F-n.m.r. measurements were carried out at 188.1 MHz with a Bruker WM200 spectrometer operating in the Fourier-transform mode. The spectra were typically obtained by using a spectral width of 10 kHz, a 90° (9.5 μs) pulse width and collecting between 10000 and 50000 transients with a repetition time 0.2 s. The ²H₂O in the solution was used as a field frequency lock. No proton decoupling was used. Trifluoroacetic acid, denatured [3-F-Tyr]CRP and free 5-fluorotryptophan

Fig. 1. ¹⁹F-n.m.r. spectra of [5-F-Trp]CRP (a) alone, (b) with excess c-AMP and (c) with excess c-GMP

Spectra were recorded at 188.1 MHz in 500 mM-KCl/10 mM-phosphate, pH 8.0 in ²H₂O/¹H₂O (1:4, v/v) at 293 K. The spectra are referenced to the ¹⁹F signal of free 5-fluorotryptophan (in 0.1 M-NaOH).

were used as external references for measuring the ¹⁹F chemical shifts.

RESULTS

[5-F-Trp]CRP

The ¹⁹F-n.m.r. spectrum of [5-F-Trp]CRP shows two resonances corresponding to the two tryptophan residues in each of the monomeric subunits of CRP (see Fig. 1). This is as would be expected if the two monomeric units were identical. Signal B, which appears 0.2 p.p.m. upfield of the position of free 5-fluorotryptophan, is broader than the downfield signal A at 2.7 p.p.m. [5-F-Trp]CRP examined in the presence of excess c-GMP is seen to show negligible effects on the chemical shifts of the two signals (Table 2). In the complex with excess c-AMP, however, modest changes in the chemical shifts of both signals are detected, resonance B being shifted upfield by 0.6 p.p.m. and resonance A being shifted downfield by 0.6 p.p.m.

Signal A is broader in the spectrum of the

Table 2. ^{19}F chemical shifts of [3-F-Tyr]CRP, [3-F-Tyr] α -CRP and [5-F-Trp]CRP, examined alone and in complexes with excess ligands

Errors are ± 0.05 p.p.m. except where stated otherwise. ^{19}F chemical shifts are referenced to denatured [3-F-Tyr]CRP for [3-F-Tyr]CRP and [3-F-Tyr] α -CRP and to free 5-fluorotryptophan (in 0.1 M-NaOH solution) for [5-F-Trp]CRP. The ^{19}F chemical shifts of denatured [3-F-Tyr]CRP and free 5-fluorotryptophan are 61.2 and 46.6 p.p.m. respectively from trifluoroacetic acid.

	^{19}F chemical shift (p.p.m.)						
	Tyr _A	Tyr _B	Tyr _C	Tyr _D	Tyr _E	Tyr _F	Trp _A Trp _B
[3-F-Tyr]CRP (alone)	3.07 (± 0.1)	1.30	0.6 (± 0.2)	0.6 (± 0.2)	-0.12	-0.74	
+c-GMP	1.95 (± 0.1)	1.22	0.3 (± 0.2)	0.3 (± 0.2)	-0.20	-0.78	
+c-AMP	3.41 (± 0.1)	0.7 (± 0.2)	0.7 (± 0.2)	0.7 (± 0.2)	0.04	-0.75	
+c-TuMP	3.6 (± 0.1)	0.6 (± 0.2)	0.6 (± 0.2)	0.6 (± 0.2)	0.09	-0.75	
+c-AMP-DNA(I)	3.2 (± 0.1)	0.9 (± 0.2)	0.4 (± 0.2)	0.4 (± 0.2)	-0.16	-0.76	
[3-F-Tyr] α -CRP (alone)	2.87 (± 0.1)	1.37	0.6 (± 0.2)	0.6 (± 0.2)	-0.16		
+c-AMP	3.26 (± 0.1)	0.88	0.60	0.15	-0.11		
[5-F-Trp]-CRP (alone)							2.7 -0.2
+c-GMP							2.7 -0.3
+c-AMP							3.3 -0.8

[5-F-Trp]CRP-c-GMP complex (85 Hz) than in the spectra of [5-F-Trp]CRP alone and [5-F-Trp]CRP-c-AMP (60–65 Hz). The corresponding value for signal B is 75–85 Hz for all three samples.

[3-F-Tyr]CRP

CRP contains six tyrosine residues per monomer. In the ^{19}F -n.m.r. spectrum of [3-F-Tyr]CRP at 293 K five resonances were resolved (see Fig. 2), the chemical shifts of signals C and D being identical with a value of 0.6 p.p.m. downfield of the 3-fluorotyrosine signal from the denatured protein (used as an external reference). The linewidths of most of the signals are of the order of 50–60 Hz, but peak A (3.07 p.p.m.) is much broader, with a linewidth of approx. 120 Hz at 293 K; the signal became narrower when the temperature was raised to 303 K, behaviour suggestive of some exchange process.

In order to assist in signal assignment in the spectrum of [3-F-Tyr]CRP a proteolysis fragment was examined. Proteolysis with chymotrypsin is known to occur at Phe-136 to produce a fragment (molecular mass 31 kDa) that is a dimer containing the c-AMP-binding N-terminal domain (' α -CRP') [33]. This α -CRP fragment contains only five of the six tyrosine residues (Tyr-23, Tyr-40, Tyr-41, Tyr-63 and Tyr-99). The n.m.r. spectrum of [3-F-Tyr] α -CRP is seen to be very similar to the spectrum of the intact protein except that signal F (at -0.74 p.p.m. in [3-F-Tyr]CRP) is missing. This signal must therefore be due to Tyr-206. Such an assignment can be made with confidence because of the close agreement between the spectrum of α -CRP and the corresponding signals in the spectrum of the intact protein. Differences in the chemical shifts of corresponding resonances are smaller than 0.2 p.p.m. Most of the linewidths are, as expected, narrower in the spectrum of the α -fragment, being of the order of 45–50 Hz. Resonance A, however, was broadened in the spectrum of α -CRP, having a linewidth of 140 Hz.

Fluorination of a tyrosine in the 3-position of the aromatic ring is known to decrease the pK value for the

dissociation of the phenolic hydroxy group by approx. 2 pH units; the pK value of free 3-fluorotyrosine is 8.2, and an upfield change in ^{19}F chemical shift of 0.6 p.p.m. accompanies dissociation of the hydroxy group [25]. The pK values of the 3-fluorotyrosine residues in CRP will, of course, depend on their particular environments in the protein. A pH titration was carried out on [3-F-Tyr]CRP to see whether any of the ^{19}F chemical shifts have contributions from partial dissociation of the hydroxy group. Variation of the pH in the range 6.1–8.0 (meter readings) showed that none of the signals was sensitive to pH within the experimental uncertainty of approx. ± 0.05 p.p.m. It is therefore concluded that all the chemical shifts reported here are representative of 3-fluorotyrosine residues in an undissociated state.

Binding of cyclic nucleotides

Measurement of the c-AMP dissociation constant for the binding to the fluorinated proteins showed no significant difference from that for the native protein. Under the conditions of the experiment more than 99% of the protein was complexed with c-AMP.

The ^{19}F -n.m.r. spectra of [3-F-Tyr]CRP complexed with excess c-GMP or c-AMP were recorded and are shown in Fig. 3. Five peaks were resolved in the spectrum of the [3-F-Tyr]CRP-c-GMP complex, one of them (at 0.30 p.p.m.) representing two superimposed resonances. The most significant change was an upfield shift of signal A from 3.07 p.p.m. to 1.95 p.p.m. and a concomitant decrease in its linewidth (by approx. 30 Hz). Only modest changes were observed for the chemical shifts of the remaining resonances (see Table 2), but their linewidths were increased by approx. 15–20 Hz. This result is consistent with the broadening of one peak in the spectrum of the [5-F-Trp]CRP-c-GMP complex.

The effects of binding of c-AMP to [3-F-Tyr]CRP are markedly different from those observed for c-GMP. The ^{19}F -n.m.r. spectrum of the [3-F-Tyr]CRP-c-AMP complex shows four resolved peaks. The chemical shift of the broad signal A is shifted downfield by approx.

Fig. 2. ^{19}F -n.m.r. spectra of (a) [3-F-Tyr]CRP and (b) α -fragment of [3-F-Tyr]CRP

Spectra were recorded at 188.1 MHz in 500 mM-KCl/10 mM-phosphate, pH 8.0, in $^2\text{H}_2\text{O}/^1\text{H}_2\text{O}$ (1:4, v/v) at 293 K. The spectra are referenced to the ^{19}F signal of denatured [3-F-Tyr]CRP.

0.34 p.p.m. to 3.41 p.p.m. with no apparent change in its linewidth. Signal B is shifted upfield by approx. 0.6–0.7 p.p.m. but changes in the chemical shifts of the remaining signals C, D, E and F are less than 0.1 p.p.m.

The effects of c-TuMP binding on [3-F-Tyr]CRP have also been studied: the ^{19}F -n.m.r. spectrum of this complex (see Fig. 3d) is very similar to that for the complex formed with c-AMP.

Binding of c-AMP to [3-F-Tyr] α -CRP

α -CRP, produced by proteolysis with chymotrypsin, retains its capability to bind c-AMP. The n.m.r. spectrum of [3-F-Tyr] α -CRP in the presence of excess c-AMP is shown in Fig. 3(e). Similarly to the results obtained for free [3-F-Tyr]CRP and [3-F-Tyr] α -CRP, there are no large differences between the spectra of the [3-F-Tyr]CRP-c-AMP and [3-F-Tyr] α -CRP-c-AMP complexes, apart, of course, from the missing signal of Tyr-206 and a sharpening of the resonances. Signals C and D, which, at 293 K, overlap in the [3-F-Tyr]CRP-c-AMP spectrum, become clearly resolved in the spectrum of the [3-F-Tyr] α -CRP-c-AMP complex, having chemical shifts of 0.15 and 0.60 p.p.m. (Fig. 3e). Signal A from [3-F-Tyr] α -

Fig. 3. ^{19}F -n.m.r. spectra of (a) [3-F-Tyr]CRP, (b) [3-F-Tyr]CRP in the presence of excess c-GMP, (c) [3-F-Tyr]CRP in the presence of excess c-AMP, (d) [3-F-Tyr]CRP in the presence of excess c-TuMP and (e) α -fragment of [3-F-Tyr]CRP in the presence of excess c-AMP

Spectra were recorded at 188.1 MHz in 500 mM-KCl/10 mM-phosphate, pH 8.0, in $^2\text{H}_2\text{O}/^1\text{H}_2\text{O}$ (1:4, v/v) at 293 K. The spectra are referenced to the ^{19}F signal of denatured [3-F-Tyr]CRP.

CRP shifts downfield by 0.39 p.p.m. on binding to c-AMP; this is very similar to the downfield shift seen when c-AMP binds to the intact CRP analogue (0.34 p.p.m.). Signal B shifts upfield by 0.5 p.p.m. for α -CRP and 0.7 p.p.m. for intact CRP. These results indicate that the c-AMP is binding to the fragment and the intact CRP in a similar though not identical manner.

DNA binding to [3-F-Tyr]CRP-c-AMP

Addition of the 20 bp oligomer (I) to the [3-F-Tyr]CRP-c-AMP solution used for the earlier ^{19}F -n.m.r.

studies caused precipitation of the complex. By changing the ionic-strength conditions (to 200 mM-KCl/10 mM-MgCl₂/10 mM-phosphate) and pH (to pH 7.0) it was possible to prepare a solution containing approx. 0.5 mM-protein in the presence of 1 molar equivalent of the DNA 20 bp oligomer (I) and excess c-AMP. The ¹⁹F-n.m.r. spectrum was essentially the same as that for the [3-F-Tyr]CRP-c-AMP complex recorded under the same conditions (see Table 2).

DISCUSSION

¹⁹F chemical shifts

A large contribution to the shielding of ¹⁹F nuclei arises from electric-field effects associated with Van der Waal's interactions between the fluorine and neighbouring atoms (separated by distance r_i) [24–26,34]. This interaction depends directly on Σr_i^{-6} and may be regarded as an approximate measure of the degree to which a fluorinated amino acid residue is 'buried' within the protein-ligand complex. Other contributions to the shielding can result from ring-current shift effects, from inductive effects (for example from changes in ionization state) or from interactions with solvent. If the fluorine-labelled CRP is assumed to have a similar structure to the non-labelled CRP, then from the structural data of Steitz and co-workers [7,13] one can show that the predicted ring-current shift contributions [35] are small for the [5-F-Trp]CRP and [3-F-Tyr]CRP complexes studied here. Changes in inductive effects are also unimportant, since it has already been noted that for [3-F-Tyr]CRP the ¹⁹F chemical shifts were insensitive to changes in pH over the pH range 6–8, indicating that all the 3-fluorotyrosine residues remain in an undissociated state under the pH conditions used in this study. The measured ¹⁹F chemical shifts can thus be taken as representative of the polarity of the environment (solvent accessibility) and of the conformation of the protein in the neighbourhood of the fluorine-labelled amino acid residues.

Although only one of the ¹⁹F signals has been assigned at the present time, the fact that the remaining signals can be assigned unambiguously to a specific residue type (either tryptophan or tyrosine) allows several useful observations to be made.

Symmetry of the CRP dimer

In the crystal-structure studies of the CRP-c-AMP complex Steitz and co-workers [7,13] found two different conformations for the subunits of the CRP dimer, the differences being mainly in the so-called 'hinge' region between the c-AMP-binding domain and the DNA-binding domain. The ¹⁹F-n.m.r. studies on the c-AMP complexes of [5-F-Trp]CRP and [3-F-Tyr]CRP do not provide any evidence for such conformations in solution. For example the spectrum of the [5-F-Trp]CRP-c-AMP complex shows only two signals, corresponding to the two non-equivalent residues Trp-13 and Trp-85. Likewise the ¹⁹F-n.m.r. spectrum of the [3-F-Tyr]CRP-c-AMP complex can be explained in terms of six signals from the six tyrosine residues (Tyr-23, Tyr-40, Tyr-41, Tyr-63, Tyr-99 and Tyr-206). Similar behaviour is also seen for the uncomplexed protein. It is possible of course that the conformational differences are present but not detected by the limited number of resonances considered here. We

also cannot exclude the possibility that the n.m.r. spectra are reflecting a time-averaged structure of two forms undergoing rapid interconversion. Indeed signal A in the spectrum of [3-F-Tyr]CRP (either uncomplexed or with c-AMP) shows some characteristics of a species involved in exchange: at 293 K signal A is much broader than the other signals but becomes narrower when the temperature is raised to 303 K. Intermediate exchange effects can selectively broaden one of the signals if there is an interconversion between two non-equivalent conformations where only one of the fluorotyrosine residues experiences a large difference in ¹⁹F shielding between the two forms.

Structural integrity of the isolated c-AMP-binding domain

Five of the fluorine-labelled tyrosine residues are in the c-AMP-binding domain (α -CRP), and the fact that the ¹⁹F chemical shifts of these are very similar to those in the intact CRP (see Table 2) indicates that the α -CRP has retained the same secondary structure as the c-AMP-binding domain in intact CRP in the regions probed by the 3-fluorotyrosine residues. The similarity in spectral features also extends to the broadening seen on signal A in both the α -fragment and intact CRP. These data suggest that the interactions between the DNA-binding domain and the c-AMP-binding domain are localized to regions devoid of tyrosine residues and that the interactions are not important for controlling the overall integrity of the structure of the α -CRP fragment. This finding is consistent with a model in which the CRP monomer exists as two isolated binding domains interconnected by a 'hinge' region between α -helix C of the c-AMP-binding domain and the α -helix D of the DNA-binding domain [14].

Binding of cyclic nucleotide perturbs CRP conformation

The results presented in Figs. 1 and 3 and Table 2 for [5-F-Trp]CRP and [3-F-Tyr]CRP complexed with c-AMP and c-GMP indicate clearly that there must be conformational changes induced in the protein when the nucleotides bind. It is sometimes difficult to decide whether a change in ¹⁹F chemical shift reflects a structural re-arrangement of the protein or whether it arises from effects resulting from direct interactions with the bound ligand. However, in this case, the crystal-structure data of Steitz and co-workers [7,13] indicate that none of the tryptophan and tyrosine residues is expected to make direct interactions with the bound c-AMP (although Trp-85 is fairly close to the binding site). For this reason, the substantial differences seen in the ¹⁹F-n.m.r. spectra of the complexes and the free CRP analogues must result from induced conformational effects (see Figs. 1 and 3). Because of the high sensitivity of ¹⁹F shielding to the environment of the fluorine nucleus it is possible that large effects seen in the spectra could arise from comparatively small differences between the structures. The crystal structure of CRP-c-AMP [7,13] shows that one of the two tryptophan residues (Trp-85) in each subunit is very close to the c-AMP-binding site, whereas the other, Trp-13, is located in α -helix A near the N-terminal end of the protein. The aromatic ring of Trp-13 is much more accessible to the solvent than is Trp-85, and the ¹⁹F chemical shift of Trp-13 should be closer to the value of free 5-fluorotryptophan. Thus the most likely assignment would seem to be that signal A in the spectra shown in

Fig. 1 corresponds to Trp-85 and signal B to Trp-13. Steitz and co-workers [7,13] have suggested that a hydrogen bond exists between the 2'-hydroxy group of c-AMP and the carbonyl group of Ala-84. If this is so, then one might expect some, albeit attenuated, interaction with Trp-85. The difference of 0.6 p.p.m. between the chemical shifts of signal A in CRP and in CRP-c-AMP is quite consistent with this model. The observation that the ¹⁹F chemical shift of Trp-13 is also perturbed by c-AMP binding means that induced changes in the protein structure produce effects far beyond the immediate reaches of the c-AMP-binding site. These conformational changes are not reproduced by c-GMP binding, which leaves the ¹⁹F shifts of 5-fluorotryptophan essentially unperturbed.

The results with the [3-F-Tyr]CRP produce further evidence that the CRP-c-AMP and CRP-c-GMP complexes have different conformations. In this case two of the six tyrosine residues (Tyr_A and Tyr_B) are perturbed significantly in different ways by c-AMP and c-GMP. c-AMP binding causes the fluorine nucleus in 5-F-Tyr_A to be deshielded (0.4 p.p.m.) and that in 5-F-Tyr_B to be shielded (0.6 p.p.m.), whereas c-GMP binding results in the fluorine nucleus in 5-F-Tyr_A being shielded (1.1 p.p.m.) and that in 5-F-Tyr_B being almost unaffected (< 0.1 p.p.m.). c-TuMP, however, produces similar spectral changes to c-AMP, indicating similar changes in protein conformation. These results are not entirely unexpected, since both c-AMP and c-TuMP induce specific DNA-binding activity and c-GMP does not [9,15], and this difference cannot be due to solely local conformational changes but must also involve long-range conformational changes eventually influencing the remote DNA-binding domain. This long-range transmission of conformational information could be effected by small relative movements of domains of secondary structure that result in a larger cumulative effect at the hinge region. However, it is not possible to use the ¹⁹F chemical-shift data reported here to speculate on the transmission pathway for the conformational changes: for this one needs to assign the ¹⁹F signals to specific residues in the protein. These are not yet available, but the lowest-field signals A and B (from residues influenced by cyclic nucleotide binding) are most likely to arise from deeply buried solvent-inaccessible residues. In the case of the one assigned residue (Tyr-206 to signal F), the insensitivity of its ¹⁹F chemical shift to the addition of c-AMP and c-GMP indicates that there has been no local change in conformation around this residue, which is located in the DNA-binding domain of the protein. The ¹⁹F chemical shifts of the five 5-fluorotyrosine residues in the CRP core containing the c-AMP-binding domain similarly show very little perturbation when DNA binds, indicating that large conformational perturbations involving the tyrosine residues are not induced by the DNA binding. It would appear from these results that the structures of the DNA-binding headpiece on one hand and the CRP core on the other are rather independent of each other. An additional illustration of this mutual independence is provided by the ¹⁹F-n.m.r. spectra of [3-F-Tyr]CRP and [3-F-Tyr]α-CRP. The chemical shifts of nuclei in corresponding residues are very similar, indicating that the structure of the α-CRP core remains essentially the same even when the C-terminal headpiece is removed. If the inverse relation is also true and if the local conformation of the headpiece is largely in-

dependent of the state of the α-core, then the function of the core would be that of a variable spacer between the two headpieces. Binding of c-AMP would simply have the effect of adjusting the average separation between the helix-turn-helix motifs of the individual subunits in such a way that the headpieces could complement the structure of a DNA double helix without experiencing undue mechanical stress. In terms of the model proposed by Garges & Adhya [14], the binding of c-AMP would induce conformational changes in the α-core that lead to interactions between residues in α-helix C (in the α-core) and in α-helix D (in the DNA-binding domain) at the hinge region: this would result in an overall relocation of the two DNA-binding domains into an orientation suitable for specific DNA binding but with no gross changes in overall structure occurring within these domains.

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