19F NMR evidence for interactions between the c-AMP binding sites on the c-AMP receptor protein from *E. coli*

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The 19F NMR spectra of 3-fluorotyrosine containing c-AMP receptor protein (CRP) from *E. coli* have been recorded in the presence of increasing amounts of c-AMP. One of the signals (from Tyr B) shifts upfield by 0.6 ppm in the presence of excess c-AMP and shows both slow and fast exchange behaviour during the titration. This is evidence for interactions between the two c-AMP binding sites on the CRP dimer leading to different dissociation rate constants (≤ 75 s^{-1}; ≥ 350 s^{-1}) for complexes containing one and two c-AMP molecules.

c-AMP binding protein; 19F - NMR; Fast exchange; Slow exchange

1. INTRODUCTION

In *Escherichia coli*, a c-AMP receptor protein regulates the expression of several genes by binding specifically to particular DNA sites in the presence of c-AMP [1,2]. Typical examples are found for the genes encoding the enzymes involved in the catabolism of lactose, arabinose, maltose and other sugars. In some cases, the CRP binding activates transcription (as for lac operon [3]) while for others CRP binding leads to repression (as for the CRP structural gene [4]). The mechanism by which the RNA polymerase activity is influenced by CRP is still unknown. One proposed model of the regulation has suggested a direct interaction between CRP and RNA polymerase on the promoter [5], whereas another has postulated a change in DNA conformation on binding CRP [6]. CRP is a dimer (total molecular mass ca 47 kDa) of two identical subunits each having a c-AMP binding site and each having a DNA binding site [7,8]. Specific binding of CRP to DNA has been shown 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' sequence or a closely related structure. These two regions of the DNA sequence were postulated to bind to the two subunits of the CRP dimer at binding sites involving helix turn helix motifs as found for other transcription factors with similar characteristics [9-12]. Recently Steitz et al. [13] have reported crystal studies on a com-

plex of CRP with c-AMP and DNA where they observed a symmetrical dimer with the helix-turn-helix motifs binding to the DNA in the predicted manner. Their results also indicated the substantial bending of the DNA when bound to CRP, a feature which had been predicted from earlier theoretical studies (Warwicker et al. [14]) and also deduced from gel retardation studies [15-17]. Fried and Crothers [18] have shown that in the case of CRP binding to restriction fragments containing the lac promoter region, only a single equivalent of c-AMP is required to induce the tight binding of the CRP dimer to the DNA, they concluded that c-AMP binding to the two sites on the CRP dimer must be unequal. Takahashi et al. [19] had earlier reported ionic strength dependent cooperative binding effects of c-AMP on CRP in the absence of DNA; at a salt concentration of 200 mM negative cooperativity ($K_1 = 4.6 \times 10^4$ M^{-1}; $\alpha = 0.30$) was observed while at 500 nM the binding was almost non-cooperative. More recently other workers [20,21] have reported very similar findings. In this present work, we have examined 19F NMR spectra of 3-F-tyrosine [3-F-Tyr] containing CRP recorded in the presence of increasing amounts of c-AMP with the aim of detecting any differences in the two binding sites. Here we examine complexes of CRP formed with c-AMP under non-saturating and saturating conditions and provide evidence for the non-equivalence of the binding sites even under conditions where the binding is known to be non-cooperative.

2. MATERIALS AND METHODS

c-AMP was obtained from Sigma Chemical Co. The preparation and purification of [3-F-Tyr] CRP has been described previously [22].

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Two samples were used for the NMR studies; one contained 1.72 mM protein (monomer) in 2.5 ml of 500 mM KCl/10 mM phosphate in $^2H_2O/H_2O$ (1:9 v/v) and the other contained 1.36 mM protein (monomer) in 2.5 ml of 200 mM KCl/10 mM phosphate in $^2H_2O/H_2O$ (1:9 v/v). The experiments were carried out at pH 7.5 and 283 K (500 mM) and 293 K (200 mM). The c-AMP titrations were carried out by adding 10 μl aliquots of concentrated c-AMP solution (19.2 mM and 20.43 mM respectively), each addition corresponding to approximately 0.05 molar equivalents of the CRP monomer concentration. The CRP and c-AMP concentrations were determined from UV absorption spectroscopy measurements. The pH measurements were made with a Radiometer Model 26 pH meter equipped with a combination glass electrode and the reported values are meter readings without corrections for any deuterium isotope effects.

The ^{19}F NMR measurements were carried out at 188.1 MHz with a Bruker WM200 spectrometer operating in the Fourier transform mode. The spectra were obtained by using a spectral width of the 10 000 Hz, a 90° pulse width and collecting 5000 transients with a repetition time of 0.41 s. The ^2H_2O in the samples was used as a field frequency lock. No proton decoupling was used. The ^{19}F chemical shifts were referenced externally to the ^{19}F signal of denatured [3-F-Tyr] CRP.

3. RESULTS AND DISCUSSION

Previous studies on [3-F-Tyr] CRP [21] have shown that the ^{19}F NMR spectrum has 6 signals, two of which overlap. These signals account for the 6 tyrosines per monomer and indicate that the tyrosines are symmetrically related within the dimer. In the presence of excess c-AMP two (A and B) of the 6 (three overlapping) signals observed in the spectrum show appreciable shift differences from those of free protein. Observation of only 6 signals is consistent with inherent symmetry in the structure of fully liganded CRP. As discussed above, others [19-21] have observed that the two binding sites have different affinities for c-AMP and it is possible to demonstrate directly from NMR measurements that the first and second binding sites of CRP are non-equivalent.

Fig. 1 shows the ^{19}F NMR spectra recorded for [3-F-Tyr]CRP (500 mM KCl/10 mM phosphate) in the presence of progressively increasing amounts of c-AMP. The 6 signals designated A-F are indicated in Fig. 1 which shows the spectrum of the [3-F-Tyr]CRP in the absence of c-AMP. Fig. 1 (top) shows the spectrum of the protein in the presence of excess c-AMP where it is seen that the signal corresponding to 3-F-Tyr$_B$ has been substantially shifted (0.6 ± 0.03 ppm upfield). When 0.18 molar equivalents of c-AMP (with respect to CRP monomer) have been added (Fig. 1) signal B has decreased in intensity but has not changed its chemical shift. This is characteristic of slow exchange between bound and free species were signal B corresponds to free protein. For the sample with 0.31 molar equivalents of c-AMP (with respect to CRP monomer) (Fig. 1), the signal at the position of B in the free protein has been replaced by a very broad signal with an overall upfield chemical shift. As increasing amounts of c-AMP are added to the protein sample, this signal continues to shift progressively towards the chemical shift
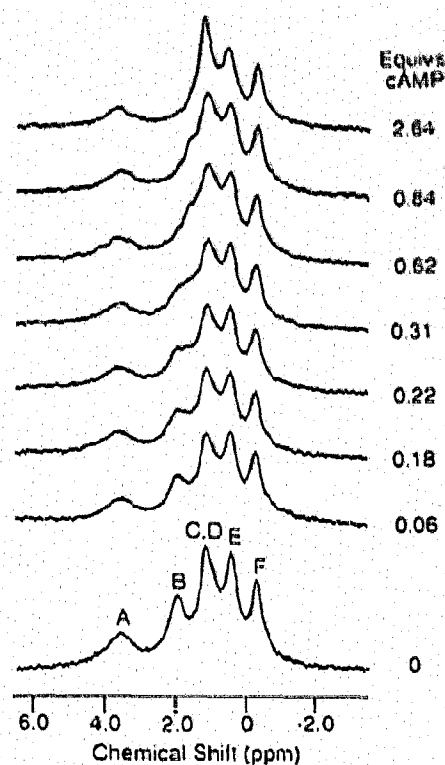
position of the fully bound species (Fig. 1). This latter behaviour is characteristic of fast or intermediate exchange on the chemical shift timescale. The titration was repeated using the CRP sample in 200 mM KCl/10 mM phosphate and the same pattern of fast and slow exchange behaviour was again seen in the initial stages of the titration. The limits on the dissociation rate constants can be estimated from the spectra: by simulating the spectra for a two site exchange process[23,24] between species with a chemical shift difference of 120 Hz and with signal linewidths of 90 Hz, the rate for the slow exchange process can be estimated to be ≤ 75 s^{-1} and the rate for the fast (or intermediate) exchange process ≥ 350 s^{-1}. The data cannot be explained in terms of a single rate process with intermediate exchange: such simulations indicated that a substantial shift in signal B would be seen in the presence of 0.2 equivalents of c-AMP and no such changes were observed. Clearly the observed complex behaviour involving both slow and fast exchange processes between bound and free species could not arise if the first and second binding sites for c-AMP on the CRP dimer are independent and equivalent. If both sites had been able to bind c-AMP independently and with equal binding affinity then, under conditions of fast exchange, one would have ex-

pected signal B to shift progressively towards the resonance frequency position of Tyr B in the titration. For the case of independent and equal binding affinity under conditions of slow exchange one would have expected signal B from the free species to remain unshifted and to decrease in intensity throughout the titration and a new signal with constant chemical shift to appear corresponding to Tyr$_B$ in the bound species. Since neither pattern of behaviour is seen one must consider the possibility of cooperative interactions between the two c-AMP binding sites.

If the binding of the two c-AMP molecules (C) to the CRP dimer (P) is regarded as a two step process where

$$P + C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PC_1 \qquad \text{Association constant, } K_1$$

$$PC_1 + C \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PC_2 \qquad \text{Association constant, } K_2$$

The different exchange behaviours seen in Fig. 1 indicated that the dissociation rate constant for the first step of the binding, $PC_1 \rightarrow P + C$, is smaller than that for the second step, $PC_2 \rightarrow PC_1 + C$. If the former dissociation rate constant is sufficiently low to result in slow exchange between bound and free species and the latter sufficiently high to give fast or intermediate exchange conditions then the observed behaviour shown in Fig. 1 can be explained. In the initial stages of the titration against c-AMP the predominant species would be P and PC_1 and these would coexist under conditions of slow exchange; thus a signal would remain at the position of signal B in the free protein as long as there is free protein P present in the equilibrium mixture. Increasing amounts of PC_1 and PC_2 will be formed as the titration progresses and if these are in fast or intermediate exchange then they will give rise to averaged signals which will shift progressively from the shift value in PC_1 to the shift value in PC_2 as the titration with c-AMP progresses. This is the behaviour seen in Fig. 1. The averaged signal is very broad indicating that PC_1 and PC_2 are in intermediate exchange.

Using the binding data of Ren et al. [21] measured at 400 mM KCl/40 mM Tris HCl ($K_1 = 1.9 \times 10^4$ M^{-1}; cooperativity parameter $\alpha = 0.84$) the concentrations of P, PC_1 and PC_2 were calculated as a function of total ligand concentration and then used to calculate simulated spectra for a system with slow exchange between P and PC_1 and fast exchange between PC_1 and PC_2 (Fig. 2). A comparison of the behaviour of signal B in the observed and calculated spectra confirms that the proposed model gives the general spectral changes observed (a better fit of the observed and simulated data could have been obtained by assuming that PC_1 and PC_2 are in intermediate exchange). Thus while the
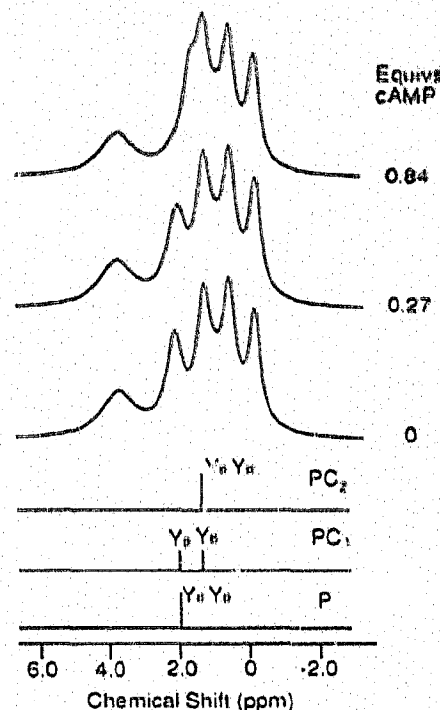

Fig. 2. Calculated ^{19}F NMR spectra for the Tyr$_B$ signal in [3-F-Tyr]CRP as a function of c-AMP concentration. The spectra were simulated using the PANIC program (Bruker) to calculate spectra of P, PC$_1$ and PC$_2$ (with PC$_1$ and PC$_2$ in fast exchange) and then summing these with the Bruker Aspect software. The chemical shifts of the two Tyr$_B$ residues in P, PC$_1$ and PC$_2$ are indicated schematically on the figure.

equilibrium binding data suggest that there is little cooperativity between the two c-AMP binding sites under high ionic strength conditions the sites do interact to influence the kinetics of the dissociation and association rate constants. Combining the values of the dissociation rate constants ($k_{-1} \leq 75$ s^{-1}; $k_{-2} \geq 350$ s^{-1}) with the equilibrium binding constant data gives estimates for the association rate constants of ($k_1 \leq 10^6$ mol^{-1}s^{-1}; $k_2 \geq 5 \times 10^6$ mol^{-1}s^{-1}) (these are in accord with the rates previously determined by Wu and Wu [25] from stopped-flow fluorescence measurements although their experiments did not resolve the second binding site). Thus the presence of c-AMP in the first binding site of the CRP dimer increases both the association rate constant and the dissociation rate constant of c-AMP in the second site. Furthermore, the data at 200 and 500 mM salt indicate that there is a substantial difference between the dissociation rate constants at the two sites regardless of ionic strength: thus the ionic strength dependent changes in binding constant cooperativity could be controlled by ionic strength dependent association rate constants.

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