DNA-sequence recognition by CAP: role of the adenine N⁶ atom of base pair 6 of the DNA site

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Received September 18, 1990; Revised and Accepted November 6, 1990

ABSTRACT

Two similar, but not identical, models have been proposed for the amino acid-base pair contacts in the CAP-DNA complex ('Model I,' Weber, I. and Steitz, T., *Proc. Natl. Acad. Sci. USA*, 81, 3973–3977, 1984; 'Model II,' Ebright, *et al.*, *Proc. Natl. Acad. Sci. USA*, 81, 7274–7278, 1984). One difference between the two models involves Glu181 of CAP. Model I predicts that Glu181 of CAP makes two specificity determining contacts: one H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site, and one H-bond with the adenine N⁶ atom of T:A at base pair 6 of the DNA half site. In contrast, Model II predicts that Glu181 makes only one specificity determining contact: one H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site. In the present work, we show that replacement of T:A at base pair 6 of the DNA half site by T:N⁶-methyl-adenine has no, or almost no, effect on the binding of CAP. We conclude, contrary to Model I, that Glu181 of CAP makes no contact with the adenine N⁶ atom of base pair 6 of the DNA half site.

INTRODUCTION

The *Escherichia coli* catabolite gene activator protein (CAP; also referred to as the cAMP receptor protein, CRP) is a sequence-specific DNA binding protein involved in transcription regulation; CAP functions by binding, in the presence of the allosteric effector cAMP, to specific DNA sites located at or near promoters (1,2). The three-dimensional structure of CAP in complex with cAMP has been determined to 2.5 Å resolution by x-ray diffraction analysis (3). The protein is a dimer of two chemically identical subunits, each of which is 209 amino acids in length and contains a helix-turn-helix DNA binding motif (see refs. 4,5). A model has been proposed for the alignment of the structure of CAP to the structure of DNA in the CAP-DNA complex (6,7). In the model, the CAP-DNA complex is 2-fold symmetric: one subunit of the CAP dimer interacts with one half of the DNA site; the other subunit of the CAP dimer interacts in a 2-fold symmetry related fashion with the other half of the DNA site. Two contacts between amino acids of the helix-turn-helix motif of CAP and base pairs of the DNA half site have been identified

experimentally (8–11): i.e., Arg180 of CAP has been shown to contact base pair 5 of the DNA half site, and Glu181 of CAP has been shown to contact base pair 7 of the DNA half site.

Two different detailed models have been proposed, listing contacts between amino acids of the helix-turn-helix motif of CAP and base pairs of the DNA half site (9,12). We will refer to the model of Weber and Steitz (12; see also ref. 13) as 'Model I'; and to the model of Ebright *et al.* (9; see also refs. 11,14) as 'Model II.' Figure 1 compares the two models. One difference between the two models involves Glu181 of CAP. Model I predicts that Glu181 of CAP makes two specificity determining contacts: one H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site, and one H-bond with the adenine N⁶ atom of T:A at base pair 6 of the DNA half site (12). In contrast, Model II predicts that Glu181 of CAP makes only one specificity determining contact: one H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site (9). The different predicted roles for Glu181 in the two models are due to essentially arbitrary differences in the side-chain torsion angles used in the model building. Both models are stereochemically acceptable; it is not possible to choose between the two models by model building.

In this paper, we have tested experimentally the prediction of Model I that Glu181 of CAP makes an H-bond with the adenine N⁶ atom of T:A at base pair 6 of the DNA half site (in addition to the H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site). To do this, we have assessed the effect on binding of CAP of replacement of T:A at base pair 6 by T:N⁶-methyl-adenine ('T:N⁶-MeA'). Replacement of T:A by T:N⁶-MeA eliminates the ability of the adenine N⁶ atom to make an H-bond with an amino acid (15). In addition, replacement of T:A by T:N⁶-MeA introduces steric bulk between the adenine N⁶ atom and any amino acid close to it (15). Replacement of T:A by T:N⁶-MeA does not disrupt Watson-Crick base pairing (16–20) and has no large effect on DNA conformation (17–20).

For comparison, we also have assessed the effects on binding of CAP of replacement of T:A at base pair 6 by A:T, by C:G, and by G:C.

The results show that replacement of T:A at base pair 6 by T:N⁶-MeA has no, or almost no, effect on binding of CAP. In contrast, replacement of T:A at base pair 6 by A:T, by C:G,

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Figure 1. The two models for contacts between amino acids of the helix-turn-helix motif of CAP and base pairs of the DNA half site. Base pairs 4 through 8 of the DNA half site are illustrated. *A*, Model I (12; see also ref. 13). Model I predicts that Glu181 ('E181') makes two contacts: one H-bond with the cytosine N4 atom of G:C at base pair 7 of the DNA half site, and one H-bond with the adenine N⁶ atom of T:A at base pair 6 of the DNA half site. *B*, Model II (9; see also refs. 11,14).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
ICAP	A	A	-	T	G	T	G	A	-	-	-	-	-	T	C	A	C	A	-	T	T	
	T	T	-	A	C	A	C	T	-	-	-	-	-	A	G	T	G	T	-	A	A	
ICAP-6'NMA-SYM						T	MA									MA	T					
ICAP-6'NMA-LEFT						T	MA									A	T					
ICAP-6'NMA-RIGHT						T	MA									MA	T					

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
ICAP	A	A	-	T	G	T	G	A	-	-	-	-	-	T	C	A	C	A	-	T	T	
	T	T	-	A	C	A	C	T	-	-	-	-	-	A	G	T	G	T	-	A	A	
ICAP-6A-SYM						A	T									T	A					
ICAP-6C-SYM						C	G									G	C					
ICAP-6G-SYM						G	C									C	G					

Figure 2. DNA sites utilized in this study. *Upper Panel*, DNA sites used to assess the effect of replacement of T:A at base pair 6 by T:N⁶-MeA. DNA fragment ICAP carries the consensus DNA site for CAP (7,22). 'MA' denotes N⁶-methyl-adenine. *Lower Panel*, DNA sites used to assess the effects of replacement of T:A at base pair 6 by A:T, C:G, and G:C.

or by G:C has a significant effect on binding of CAP. We conclude, contrary to Model I (12), that CAP makes no contact with the adenine N⁶ atom of T:A at base pair 6.

MATERIALS AND METHODS

CAP and CAP Derivatives

CAP was the kind gift of Dr. J. Krakow (Hunter College of CUNY, New York), and had been purified as described in ref. 21. The fraction of molecules active in sequence-specific DNA binding (0.64) was determined by titration with 5.0 nM

[³²P]-ICAP (7); all data are reported in terms of molar concentrations of active CAP dimers.

Synthetic DNA Fragments

40 base pair double-stranded DNA fragments containing the consensus DNA site for CAP, or substituted derivatives of the consensus DNA site for CAP (sequences in Figure 2), were synthesized, purified, and radiolabelled as described in ref. 7. N⁶-MeA was introduced using N⁶-methyl-deoxyadenosine- β -cyanoethylphosphoramidite (Pharmacia).

The presence of N⁶-MeA in synthetic oligodeoxy-

ribonucleotides was confirmed by digestion of the oligodeoxy-ribonucleotide with snake venom phosphodiesterase and bacterial alkaline phosphatase (ref. 23), followed by analytical reversed-phase HPLC. Analytical reversed-phase HPLC showed 1.0 mole N⁶-methyl-deoxyadenosine per 1.0 mole of oligodeoxyribonucleotide.

In Vitro DNA Binding Experiments

In vitro DNA binding experiments were performed using the nitrocellulose filter binding technique as described in ref. 7. Experiments were performed using Schleicher & Schuell BA83 filter membranes (0.22 $\mu\text{m} \times 25 \text{ mm}$) and a Hoefer filter manifold. Experiments were performed in 500 μl buffer containing 10 mM MOPS-NaOH (pH 7.3), 200 mM NaCl, 0.1 mM dithiothreitol, 50 $\mu\text{g/ml}$ bovine serum albumin, and 0.2 mM cAMP. [³²P]-labelled DNA (3 to 10 pM) and CAP were equilibrated at room temperature for 60 min, and were then filtered in $\approx 10 \text{ s}$ to 15 s under suction through filters pre-soaked in assay buffer minus bovine serum albumin. Filters were dried and Cerenkov radiation was determined in a Beckman LS8000 scintillation counter. All data were corrected for background (i.e., radioactivity retained on the filter in the absence of CAP).

Values of K_{obs} were extracted using Scatchard analysis for single-site binding (24), utilizing data obtained at 15% to 85% saturation. ΔG_{obs} , the binding free energy for CAP-DNA complex formation, was calculated using the equation $\Delta G_{\text{obs}} = -RT \ln(K_{\text{obs}})$. $\Delta \Delta G_{\text{obs}}$, the difference between the binding free energy for CAP-DNA complex formation in the case of DNA site i , vs. the binding free energy for CAP-DNA complex formation in the case of the consensus DNA site, was calculated using the equation $\Delta \Delta G_{\text{obs}} = -RT \ln(K_{\text{obs},i}) + RT \ln(K_{\text{obs,consensus}})$. The technique yielded high-precision data for $K_{\text{obs}} = 1 \times 10^7 \text{ M}^{-1}$ to $K_{\text{obs}} = 1 \times 10^{11} \text{ M}^{-1}$. The precision of the estimates of values of K_{obs} is $\pm 10\%$.

RESULTS

Effects of Replacement of T:A by T:N⁶-MeA

We have performed equilibrium DNA binding experiments to investigate the binding of CAP to the consensus DNA site (DNA fragment ICAP; sequence in Figure 2) and to derivatives of the consensus DNA site having T:N⁶-MeA at base pair 6 in both DNA half sites (DNA fragment ICAP-6'NMA-SYM), in the only the left DNA half site (DNA fragment ICAP-6'NMA-LEFT), and in only the right DNA half site (DNA fragment ICAP-6'NMA-RIGHT). The data are presented in Table 1. The consensus DNA site exhibits a binding constant for interaction with CAP of $4.5 \times 10^{10} \text{ M}^{-1}$. Replacement of T:A at base pair 6 by T:N⁶-MeA in both DNA half sites results in less than a 1.7-fold reduction in binding constant for interaction with CAP. This effect may be within the margin of error of the assay method. Replacement of T:A at base pair 6 by T:N⁶-MeA in only one DNA half site results in a 1.1-fold to 1.2-fold reduction in binding constant for interaction with CAP. This effect is within the margin of error of the assay method.

Effects of Replacement of T:A by A:T, C:G, and G:C

We also have performed equilibrium DNA binding experiments to investigate the binding of CAP to derivatives of the consensus DNA site having A:T, C:G, or G:C at base pair 6 in both DNA half sites (DNA fragments ICAP-6A-SYM, ICAP-6C-SYM, and ICAP-6G-SYM; sequences in Figure 2). The data are presented

TABLE 1. Effects of replacement of T:A by T:N⁶-MEA

DNA SITE	K_{OBS} (M^{-1})	$-\Delta G_{\text{OBS}}$ (KCAL/MOL)	$\Delta \Delta G_{\text{OBS}}$ (KCAL/MOL)
ICAP	4.5×10^{10}	14.4	[0]
ICAP-6'NMA-SYM	2.7×10^{10}	14.1	0.3
ICAP-6'NMA-LEFT	4.1×10^{10}	14.4	0.0
ICAP-6'NMA-RIGHT	3.7×10^{10}	14.3	0.1

TABLE 2. Effects of replacement of T:A by A:T, C:G, and G:C

DNA SITE	K_{OBS} (M^{-1})	$-\Delta G_{\text{OBS}}$ (KCAL/MOL)	$\Delta \Delta G_{\text{OBS}}$ (KCAL/MOL)
ICAP	4.5×10^{10}	14.4	[0]
ICAP-6A-SYM	5.2×10^8	11.8	2.6
ICAP-6C-SYM	5.8×10^8	11.8	2.6
ICAP-6G-SYM	5.8×10^8	11.8	2.6

in Table 2. Replacement of T:A at base pair 6 by A:T, by C:G, or by G:C in both DNA half sites results in an approximately 80-fold reduction in binding constant for interaction with CAP.

DISCUSSION

Replacement of T:A at base pair 6 of the DNA half site by T:N⁶-MeA has no, or almost no, effect on binding of CAP ($\leq 0.1 \text{ kcal/mol}$ per DNA half site). This effect is within the margin of error of our assay method (see Materials and Methods). The magnitude of the effect is small relative to the magnitude of the effect expected for elimination of an H-bond and introduction of steric bulk between H-bond acceptor and ligand ($> 1 \text{ kcal/mol}$ per DNA half site; see refs. 10,25,26). The magnitude of the effect also is small relative to the magnitude of the effect of replacement of T:A at base pair 6 of the DNA half site by A:T, C:G, or G:C ($\approx 1.3 \text{ kcal/mol}$ per DNA half site).

We conclude, contrary to Model I (Figure 1A; ref. 12), that CAP makes no contact with the adenine N⁶ atom of T:A at base pair 6 of the DNA half site.

We propose that specificity for T:A at base pair 6 of the DNA half site results from either: (i) a contact between CAP and an atom of T:A at base pair 6 other than the adenine N⁶ atom, or (ii) sequence-dependent effects on DNA conformation (i.e., sequence-dependent effects on DNA bending or DNA twist). We favor possibility *ii*. In the model for the structure of the CAP-DNA complex, there is a $> 30^\circ$ DNA kink between base pair 6 and base pair 7 (6,7); we speculate that T:A at base pair 6 facilitates formation of this DNA kink.

ACKNOWLEDGEMENTS

We thank Dr. J. Krakow for purified CAP. This work was supported by National Institutes of Health grants GM41376 to R.H.E.

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