NMR study of the structural changes induced in the *E. coli* lac promoter by the specific binding of the CAP protein

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ABSTRACT

We have studied the binding of the CAP protein to an 18 base pair lac promotor sequence comprising the core of the CAP recognition sequence. Specific binding of this sequence was established by competition binding assays and comparison of the relative affinities of a number of lac promotor, lac operator, and unspecific sequences of different lengths. The effect of the binding of CAP to the 18 base pair promotor sequence and, for comparison, to an 18 base pair symmetric operator and an oligonucleotide of unrelated sequence have been studied by $^1$H NMR. Binding of CAP does not bring about any changes in the chemical shift values of the imino proton resonances of the DNA, but causes the selective line broadening of two of the resonances. The comparison of these data with results of gel retardation assays published previously (1) allows the identification and localization of a kink induced in the DNA by the CAP binding to its specific site on the lac promotor.

INTRODUCTION

The expression of numerous genes of *E. coli* is controlled by the cyclic AMP binding protein (CAP; also referred to as Crp) (2). CAP is a homodimeric protein with a molecular mass of 23 619 per subunit, the sequence of which is known (3,4). The cAMP-dependent binding of CAP to the specific binding sequence within the promotor region of the CAP-inducible genes results in a repression or, more often, in an enhancement of the rate of transcription (5). The classical example of CAP-dependent activation of transcription is the lactose operon (6).

Two basic models, which are not mutually exclusive, have been proposed for the mechanism of the transcription activation by CAP: CAP could promote the formation of an active RNA polymerase-promotor complex either by altering the structure of the promotor DNA (7) or by formation of protein-protein contacts to the RNA polymerase (8). A third possibility of transcription enhancement by CAP binding is simply the blocking of unproductive RNA polymerase binding sites on the promotor (9). Even though the structure of the CAP-cAMP complex is known at high resolution from X-ray crystallographic studies (10,11), and the specific promotor binding sites have been well characterized by genetic and biochemical methods (5,12–14), the mechanism of CAP action is still not well understood.

Physical methods have been applied to detect possible structural changes in the DNA structure induced by the specific binding of CAP-cAMP. Gel retardation assays (1,15,16) and electrooptical measurements (17) have provided evidence of a bending of the promotor DNA in the specific complex with CAP.

The oligonucleotide imino proton NMR resonances can be used as a probe for
Table 1. Sequences of the lac promoter and lac operator fragments. EcoRI restriction fragments (a–d) are numbered with respect to the start point of the E. coli P1 promoter, so that the −60 region represents the CAP binding site. The base pair numbering of the synthetic oligonucleotides (e–g) is arbitrary.

| a: pRC 50 / 31bp+ | 5' AATTCCGTGAGTTAGCTCATTAGGCGG3' |
| b: pRC 35 / 45bp+ | 5' AATTCCCGCAATTAATGTGAGTTAGCTCATTAGGCACCCGG3' |
| c: pRC 19 / 44bp L8 | 5' AATTCCGCAATTAATGTGAGTTAGCTCATTAGGCACCCGG3' |
| d: pRC 2 / 89bp L8 | 5' AATTCCGGGGTGCCTAATGAGTGAGCTAACTTACATTAATTGCGTGCGCAACGCAATTAATGTAAGTTAGCTCATTAGGCACCCGG3' |
| e: 18bp promotor | 5' A A T G T G A G T A G C T C A C A 3' |
| f: 14bp promotor | 5' G T G A G T A G C T C A C 3' |
| g: 18bp sym. operator | 5' A T T G T G A G C G C T C A C A A T 3' |

Conformational changes induced in the DNA structure by the binding of ligands (18–20). In the present paper we report the results of an NMR investigation of the effects of complex formation with CAP on the structure of an 18 bp oligodeoxynucleotide comprising the central sequence of the CAP binding site within the lac promoter of E. coli.

MATERIALS AND METHODS

Protein isolation
CAP was purified from an overproducing E. coli strain with the crp gene in pBR322 (21). This strain yielded up to 1 mg protein per g cells. The purification procedure was a combination of the methods of Boone & Wilcox (22) and Eilen et al. (23). This involved a PEI-precipitation in the first step and then chromatography on Bio Rex 70, hydroxylapatite
Table 2. $R_1$: CAP affinities relative to the sequence 45+ (see table 1). $R_2$: CAP affinities relative to the 40bp lac operator (27). $R_3$: CAP affinities of the synthetic fragments relative to the sequence 45+ (calculated from $R_2$ and the relative CAP affinity of the 45+ sequence and the 40bp lac operator).

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<td>1850</td>
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and DEAE-cellulose. The protein was stored in 50% glycerol at $-20^\circ$C. The CAP was more than 95% pure as monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis. From protein samples with reduced DNA binding activity highly active CAP could be recovered after precipitation by dialysis of the concentrated protein (>1mg per ml) against a tenfold volume of a 60% (NH$_4$)$_2$SO$_4$ solution (24) containing 380mg per ml (NH$_4$)$_2$SO$_4$ and 7mM $\beta$-mercaptoethanol adjusted to pH 7.9, overnight, 4°C.

DNA fragments

The E.coli strains HB101/pRC35 (45bp+), pRC50 (31bp+), pRC19 (44bp L8), and pRC41 (43bp) harboring short lac-promotor fragments inserted into the plasmids pBR325 and pJE13 (25) were constructed in the following way: wild type and L8-UV5 mutant lac promoter fragments, 203bp long, were isolated from the plasmids pOE326 and pOE214, respectively. The 203bp fragments were cut with the restriction enzymes BstNI and Hhal. The resulting 42bp fragments were trimmed with Nuclease S1 and DNA-polymerase, blunt-end-ligated to EcoRI linkers and, after cutting the excess linker, loaded on a 5% acrylamide gel. The EcoRI promoter fragments were eluted from the gel and ligated into the EcoRI site within the chloramphenicol gene of the plasmid pBR325 and later into the plasmid pJE13, which is essentially identical with the plasmid pARA1 (26). Wild-type and L8 mutant lac promotor fragments as short as 31bp (size of the EcoRI fragment) were obtained which comprise only the tight CAP binding site 1 of the lac promoter (Table 1).

The 40bp and 25bp lac operators were isolated as EcoRI restriction fragments from the stains HB101/pOE101 (27) and HB101/pOE425 (25), respectively. All restriction fragments used for the CAP binding studies were purified by preparative polyacrylamide gel electrophoresis.

The synthetic oligonucleotides used for the binding studies and NMR measurements, the 18bp wild-type lac promotor, and the 18bp symmetric lac operator were synthesized by the phosphoramidite procedure.

Binding assays

The reaction buffer contained 10mM Tris-HCl (pH 7.95 at 22°C), 10mM Mg(OAc)$_2$, 7mM $\beta$-mercaptoethanol, 0.1mM EDTA, 25µg BSA per ml, and 10% glycerol. The protein, $^{32}$P-labeled, and unlabeled DNA were diluted into the reaction buffer. The concentration of cAMP was 0.5mM. The total volume of an assay mixture was 50µl. Samples were incubated 10 min. at 22°C. 20µl each were applied to two nitrocellulose filters (type HA 45µm, Millipore). The washed filters were transferred into 10ml scintillation cocktail (Quickszint 212, Zinsser, Frankfurt, FRG) and measured for bound radioactivity.

In the competition filter binding experiments, the 45+ lac promotor or the 40bp lac operator sequence were labeled with $^{32}$P. In the presence of less than saturating amounts
of CAP increasing concentrations of unlabeled competitor DNA were added. The experiments were done at concentrations of the labeled oligonucleotides about tenfold higher than the dissociation constants of their CAP complexes. The concentrations of cold competitor oligonucleotides necessary to displace the labeled DNA in the CAP complex directly reflect under the experimental conditions the relative CAP affinities (R) of the cold and the labeled oligonucleotide; R is defined as the ratio of the dissociation constants. Competition curves were evaluated by least square fit using the program SYSTAT (Systat Inc., Evanston, Ill., USA).

**NMR measurements**

The sample buffer contained 75 mM NaCl, 7.5 mM potassium-phosphate (pH 8.0), 0.1 mM EDTA, and 10% 2H2O. The CAP protein was transferred into the ‘NMR’-buffer by chromatography on Sephadex G50 (Pharmacia) and concentrated by ultrafiltration (Centricon, Amicon) up to a concentration of 30 mg per ml. The CAP protein has the tendency to aggregate at high concentrations when mixed with DNA and cAMP. This problem was overcome by lyophilizing a dilute sample and dissolving it again in 300 µl H2O/2H2O. The complex was found to have a higher solubility than the protein itself. 1H NMR spectra were recorded at 500 MHz on a Bruker AM 500 NMR spectrometer. Chemical shifts were measured relative to the TSP (sodium 3-trimethylsilyl-(2,2,3,3-2H4)propionate) 1H-resonance. Chemical shift values were determined with an accuracy of ±0.01 ppm for the titration experiments and of ±0.03 ppm for the NOE measurements. The H2O solvent resonance was suppressed by means of the Redfield 2-1-4 pulse sequence (28). Prior to Fourier transformation and phase correction, spectra were resolution-enhanced by a Lorenzian to Gaussian transformation as provided by the Bruker DISNMR/P program.

**RESULTS**

**Competition binding assays**

In order to establish the relative affinities of short lac promotor fragments for the CAP protein, a number of oligonucleotides varying in size and sequence were assayed by competition filter binding. The more recently developed gel retardation assay was found to be difficult to apply to the assay of CAP binding to short oligonucleotides. Competition experiments were done with wild-type and L8 lac promotor fragments, lac operator fragments, and short unspecific oligonucleotides, the sequences of which were unrelated to the specific CAP binding site. The fragments tested and their CAP affinities relative to the 45 bp promotor fragment are listed in Tables 1 and 2.

**1H NMR measurements**

1. Assignment of the oligonucleotide imino proton resonances

The NH-N-(imino) proton resonances of the 18 bp synthetic wild-type lac promotor fragment were assigned from the NOE effects to the imino protons of their neighboring base pairs in a series of one-dimensional NOE experiments. The assignment of the 18 bp symmetric lac operator was taken from (20). In Fig. 1. the sequences of both 18 bp oligonucleotides and their assigned imino proton spectra are shown. The assignment of
Fig. 2. pH-dependence of the imino proton resonances of the 18 base pair promoter (A) and 18 base pair symmetric operator in complex with CAP protein at 298K.
the 18 bp promoter fragment is consistent with the assignment of a homologous 22 bp lac promoter fragment reported by Lee et al. (29).

2. Complex formation with CAP

The influence of CAP binding on the oligonucleotide imino proton spectra was studied at various degrees of saturation of the DNA with CAP. The spectra are shown in Fig. 1A, B. While an unspecific line broadening of the imino proton resonances was observed upon addition of CAP, no changes in the chemical shift values were detected. Instead, specifically the resonances of two base pairs (TA3, GC12) in the 18+ lac promoter sequence and of one base pair (TA3) in the 18 bp symmetric lac operator are unusually broadened (eventually beyond detection) as a result of the complex formation with CAP. Upon addition of cAMP no further changes in the spectra of the imino proton resonances were detected.

Gradual lowering the pH value causes the resonances of the TA 3 base pair to show up again in both oligonucleotides around pH 6 (Fig. 2). In contrast, the imino proton resonance of GC 12 in the promotor fragment is broadened even at low pH values (Fig. 2A).

The imino proton resonances of an unspecific 20 bp oligonucleotide showed the general increase in line width upon addition of CAP found also for the complex formation of CAP with the specific sequences, indicating binding, but no specific changes in the line widths or in the chemical shift values.

DISCUSSION

We describe in this paper the effects of CAP binding on the structure of an 18 bp oligonucleotide comprising the core of the CAP recognition sequence within the lac promotor of E.coli. By competition filter binding experiments, the relative binding constants and thus, the changes in the free enthalpy of complex formation of a series of oligonucleotides were determined relative to a 45 bp wild-type promotor fragment comprising the sequence from −78 to −42 in the lac promotor (Table 1). As expected, the affinity of CAP for the 18 bp oligonucleotide was drastically reduced, but the lac promotor fragments as well as the symmetric lac operator still showed significant specific binding as compared with a 20 bp and even with a 43 bp unspecific DNA sequence.

The 1H NMR resonances of the DNA imino protons can conveniently be used for the detection of local changes in the helix conformation induced by the binding of a protein, as these resonances do not generally overlap with protein signals. Being located in the interior of the helix, the imino protons provide a sensitive probe for changes in the DNA helix conformation. In two of the most extensively studied model systems, the phage lambda cro protein and the lac repressor headpiece, the complex formation with their respective specific DNA binding sites induces changes in the chemical shift values of the imino proton resonances, indicating small changes in the helix conformation, while the overall DNA structure is maintained (18–20). In contrast, the binding of CAP induces a specific line broadening in the resonances of two base pairs of its promotor recognition sequence (and in one base pair of the symmetric lac operator studied for comparison), while no changes in the chemical shift values of the imino protons could be detected.

Generally, two possible explanations for this line broadening can be proposed: 1. A selective increase in the relaxation rate owing to a decrease in the mobility of the particular proton as a result of the complex formation with the protein. This mechanism seems highly unlikely, in particular since the base pair GC 12 has so far not been shown to be involved
in a direct interaction with CAP. The line broadening can be due to an exchange process, either between two conformational states of the helix at the particular base pair in the free state and in the complex with CAP or between the imino proton and the protons of the solvent H$_2$O. At present, the data do not allow us to distinguish between these two mechanisms, i.e., conformational and chemical exchange. In fact, it is possible that a conformational change induced at a certain position leads also to an enhanced chemical exchange with the water.

By gel electrophoresis analysis it has been demonstrated that CAP induces a bending in its binding site at the lac promotor (1). The bending angle has been estimated and the bending site has been mapped. The comparison of these results with our data allows us to define the structural distortion of the DNA upon CAP binding in more detail. A model assuming a gradual bending of the DNA within a number of base pairs is incompatible with the data presented in this paper. Rather, the data demonstrate a sharp structural transition confined to single base pairs, i.e., a sharp kinking of the DNA rather than a gradual bending. The base pair GC 12 of the 18 base pair promotor sequence (corresponding to base pair -60 in the lac promotor) is located within the bending region determined by gel electrophoresis analysis (base pairs -58 to -60) (1). Thus, the kink site would be located slightly upstream from the axis of symmetry of the CAP binding site, which is between the base pairs -61 and -62. In addition to the GC 12, the imino proton resonance of the base pair TA 3 is broadened in the lac promotor as well as in the symmetric operator sequence. As this base pair is located near the fraying end of the oligonucleotide helix, the effect is most likely not specific but rather reflects the influence of the complex formation on the fraying of the terminal bases of the DNA. This is corroborated by the differential pH dependence of the line broadening of the GC 12 and TA 3 resonances. As a result of either the weakening of the CAP-DNA interaction at low pH values or the general decrease in the imino proton exchange rates, which reach a minimum at about pH 6 (30), the specific line broadening of TA 3 completely disappears at pH values lower than 6. In contrast no pH dependence of the the specific line broadening of the GC 12 resonance is detected as long as the CAP DNA binding is stable. Significantly longer oligonucleotides could not be used in the present study because of the increasing complexity of the spectra.

It may be surprising that no changes were detected between the spectra of the CAP-DNA complex in the presence and in the absence of cAMP. However, one should keep in mind that under the conditions of the NMR measurements (c ~ 0.1mM), binding of CAP to DNA is almost always achieved, even in the absence of cAMP, even at low pH and even for an unspecific sequence (see results). In other words, the spectra will not show differences in the affinities but in the structure of the complexes. Of course we cannot exclude structural differences between the CAP-DNA complex and the ternary cAMP-CAP-DNA complex, which may not be reflected by the imino proton resonances of the DNA, but as far as the DNA conformation is concerned, no such differences could be observed. This supports a model of the cAMP action which postulates a transition in the free CAP from a low-DNA-affinity to a high-affinity state rather than a difference in the structure of the specific CAP-DNA complex. A similar mechanism of induction has been found for other regulatory DNA-binding proteins.

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REFERENCES


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