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Site-directed mutants of the cAMP receptor protein — DNA binding of five mutant proteins

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Oligonucleotide-directed mutagenesis was employed to generate mutants of the cAMP receptor protein (CRP) of *Escherichia coli*. The mutant proteins were purified to homogeneity and tested for stability and DNA binding. It is shown that mutations at the position of Arg^{180} abolish specific DNA binding, whereas those at the position Arg^{185} have very little effect. Both positions have previously been implicated as crucial for the specific interaction between CRP and DNA. The Ser^{128} → Ala mutant shows a slight reduction in DNA binding affinity relative to wild-type. All mutants investigated show similar stability profiles to wild-type CRP with respect to thermolysin proteolysis as a function of temperature.

Key words: cAMP receptor protein/oligonucleotide directed mutagenesis/DNA binding

Introduction

The cAMP receptor protein (CRP) is a specific DNA-binding protein, acting as an activator of transcription for catabolite repressible operons in *Escherichia coli* (for a recent review see Busby, 1986). It contains a helix–turn–helix motif which is common to a large number of repressor-like DNA-binding proteins and plays a major role in protein–DNA interactions (Anderson *et al.*, 1982, 1985; Sauer *et al.*, 1982; Steitz *et al.*, 1982).

It has previously been shown that Glu^{181}, located in helix F, is crucial for the interaction with the CRP binding site in the *lac* operon (Ebright *et al.*, 1984a), contacting the CG base pair at position 4 of the TGTGA consensus sequence (Ebright *et al.*, 1984b). Further evidence for the involvement of particular amino acids comes from studies on the transcriptional activity of various mutant proteins (Gent *et al.*, 1987): deletion of helix-F as well as mutations of Arg^{180} abolish CRP-mediated transcription enhancement; substitutions of Arg^{185}, which has also been implicated in specific DNA recognition (Ebright *et al.*, 1984b; Weber and Steitz, 1984), however, behave essentially like wild-type CRP (Gent *et al.*, 1987).

In order to elucidate which amino acids are important for either DNA binding or the allosteric change induced by cAMP binding, we have constructed a large set of site-directed mutants in the carboxy-terminal domain and cAMP binding pocket of CRP. In this paper we report on the DNA-binding properties of five purified mutant proteins: namely, the Arg^{180} → Lys, Arg^{180} → Leu, Arg^{185} → Lys, Arg^{185} → Leu and Ser^{128} → Ala variants. The first two positions of amino acid substitutions (Arg^{180}, Arg^{185}) have been proposed to be crucial for DNA binding (Weber and Steitz, 1984; Ebright *et al.*, 1984b), whereas Ser^{128} is supposedly critical for cAMP binding (McKay *et al.*, 1982). To rule out any influence of possible differential stability of the various mutant proteins on the DNA binding experiments, purified proteins were tested for resistance towards proteolysis by thermolysin as a function of temperature.

Materials and methods

Bacterial strains and plasmids

The *E. coli* strain used for the production of mutant protein was pp47 (crp^-) kindly provided by H.Aiba. Mutant *crp* genes were either located on the expression vector PINIII A1 (Masui *et al.*, 1983) or pUC8 (Vieira and Messing, 1982).

Site-directed mutagenesis

CRP mutants were either constructed in M13mp8 or M13mp9 by the two-primer method (Zoller and Smith, 1984) as described previously (Gent *et al.*, 1987) or by the gapped duplex method (Kramer *et al.*, 1984). The sequence of the mutant genes was checked by dideoxy sequencing (Sanger *et al.*, 1977).

CRP purification

Wild-type and mutant CRPs were purified from plasmid harbouring *E. coli* pp47 by fractionated $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel filtration. They were >95% pure as judged by SDS–polyacrylamide electrophoresis and their concentrations were determined spectrophotometrically using $\epsilon_{278} = 4.1 \times 10^4$/M/cm for the dimer (Takahashi *et al.*, 1982).

Thermolysin digestion

Purified wild-type and mutant CRPs were digested with thermolysin (Sigma) at an enzyme substrate ratio of 1:50 (w/w) in a buffer containing 15 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 5% glycerol, 0.1 mM dithiothreitol, 25 mM NaCl and 25 mM $CaCl_2$ as described by Matsumura *et al.* (1986). Reactions containing 3–5 μg of protein were performed in a total volume of 30 μl for 15 min at temperatures ranging from 0 to 60°C as indicated in the legend to Figure 1, and were terminated by the addition of EDTA to a final concentration of 10 mM. Digestion products were separated by electrophoresis on 12% SDS–polyacrylamide gels (Laemmli, 1970).

Polyacrylamide gel electrophoresis on DNA–CRP complexes

This was essentially carried out as described previously (Garner and Revzin, 1981; Kolb *et al.*, 1983). The DNA fragment used was a 200-bp *Hin*fI/*Hae*III fragment carrying the *lac* wild-type region. It was radioactively end-labelled using Klenow Polymerase and [α-^{32}P]ATP. The approximate concentration of the DNA fragment employed in the binding assays was 10^{-10} M.

Samples containing varying amounts of CRP were incubated with the DNA fragment for 30 min at room temperature in 40 mM Tris pH 8.0, 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and 0.2 mM cAMP. Electrophoresis was performed on 12% polyacrylamide gels for 2–3 h in 30 mM Tris, 90 mM borate, 2.5 mM EDTA and 0.2 mM cAMP. The gels were autoradiographed at −70°C with intensifying screens for 12 h.

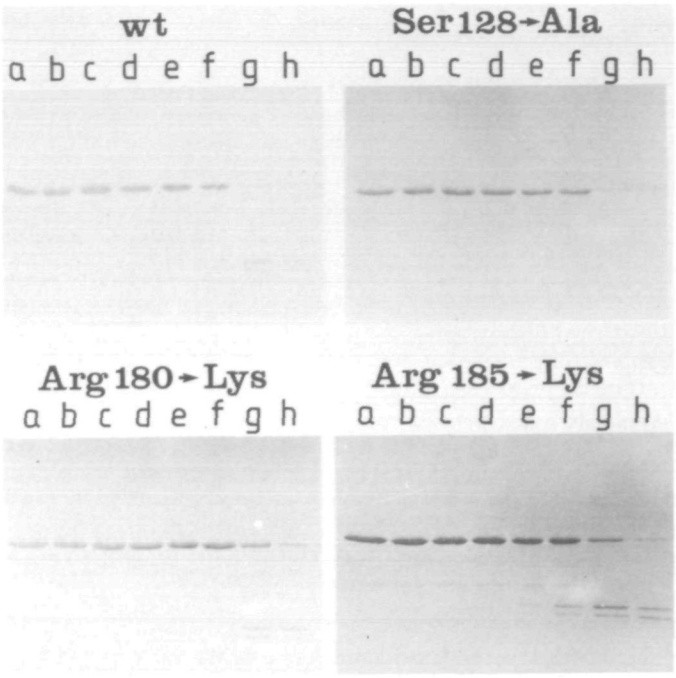

Fig. 1. Electrophoretic analysis of thermal stability of wild-type and mutant CRPs. Each panel shows the results of digestion with thermolysin at eight different temperatures **Lanes a, b, c, d, e, f, g** and **h**· 0, 25, 30, 37, 45, 50, 55 and 60°C respectively

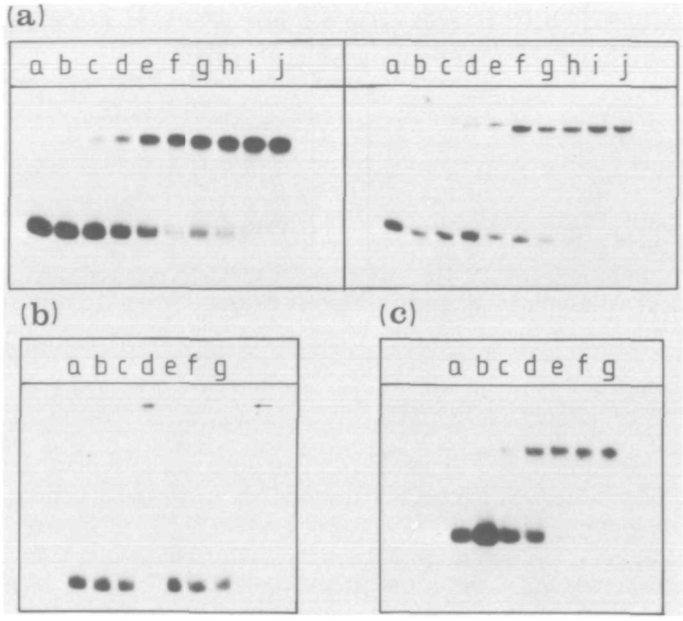

Fig. 2. Gel retardation assays for the binding of various mutant CRPs to a 200-bp wild-type *lac* DNA fragment (Note that each panel represents a different gel) **(A)** Arg^{185} → Leu mutant (left-hand panel **lanes a, b, c, d, e, f, g, h, i** and **j** 0, 1.5×10^{-9}, 3.1×10^{-9}, 6.2×10^{-9}, 1.2×10^{-8}, 1.9×10^{-8}, 2.4×10^{-8}, 3.1×10^{-8}, 3.8×10^{-8} and 4.4×10^{-8} M protein respectively), and Arg^{185} → Lys mutant (right-hand panel **lanes a, b, c, d, e, f, g, h, i** and **j** 0, 7.8×10^{-10}, 1.6×10^{-9}, 3.1×10^{-9}, 6.2×10^{-9}, 9.4×10^{-9}, 1.25×10^{-8}, 1.6×10^{-8}, 1.9×10^{-8} and 2.2×10^{-8} M protein respectively) **(b)** Arg^{180} → Lys mutant **(lanes b, c, d)** and Arg^{180} → Leu mutant **(lanes e, f, g)** **(Lanes a, b** and **e, c** and **f, d** and **g** 0, 5×10^{-8}, 2.5×10^{-7}, 5×10^{-7} M protein respectively.) The arrow indicates the origin **(C)** Ser^{128} → Ala mutant **(Lanes a, b, c, d, e, f** and **g** 0, 3.7×10^{-9}, 7.4×10^{-9}, 1.4×10^{-8}, 2.9×10^{-8}, 4.4×10^{-8} and 5.3×10^{-8} M protein respectively)

Results and Discussion

Resistance to proteolysis is a measure of the stability of a protein, since denatured or unfolded proteins are more sensitive to proteolysis than native ones (Heinrickson, 1977). In order to ascertain that the mutations introduced into CRP do not alter the stability of the proteins which, in turn, may affect their DNA binding affinities, we compared the resistance to digestion by thermolysin (Matsumura *et al.*, 1986) of wild-type and mutant CRPs. The results of this comparison for three mutants are shown in Figure 1. The single band corresponding to the wild-type and mutant CRPs begins to diminish between 45 and 50°C and products of proteolysis begin to appear. This suggests that the native conformation of both the wild-type and mutant proteins is partially denatured at these temperatures and hence becomes more digestible by thermolysin. At 60°C proteolysis is almost complete and only very faint bands of native protein and digestion products are visible. Digestions of the two other mutants (Arg^{180} → Leu and Arg^{185} → Leu) showed the same pattern of proteolysis as a function of temperature (data not shown). Thus the thermostability profiles of the mutant CRPs are very similar to that of the wild-type protein, and we conclude that the mutations introduced do not affect the stability of the mutant proteins to any substantial degree.

Figure 2a shows gel retardation assays (Garner and Revzin, 1986) carried out using a 200-bp fragment carrying the *lac* binding site and the two Arg^{185} mutants. Both mutants clearly show formation of 1:1 protein–DNA complexes, with the Arg^{185} → Lys mutant having the same binding affinity as the wild-type protein (K_{app} ~10^8 M^{-1}; Gent *et al.*, 1987). The Arg^{185} → Leu mutant shows marginally reduced specific DNA binding with 1:1 protein–DNA complexes being apparent at concentrations $>3 \times 10^{-9}$ M (K_{app} ~8×10^7 M^{-1}).

In contrast to the Arg^{185} mutants, the Arg^{180} mutants exhibit no specific DNA binding at all. Figure 2b shows a gel assay carried out with these mutant proteins and neither protein is able to form 1:1 complexes, even at concentrations up to 5×10^{-7} M protein. There is, however, a reproducible difference in the behaviour of the Arg^{180} → Leu versus the Arg^{180} → Lys mutant. In particular, the latter is obviously still capable of binding DNA non-specifically, as evidenced by the radioactivity retained in the slot (lane d) due to the formation of large non-specific protein–DNA aggregates. This is not the case for the Arg^{180} → Leu protein, where no evidence for any DNA binding was found, at least not up to concentrations of 5×10^{-7} M protein.

Figure 2c shows a binding assay carried out using the Ser^{128} → Ala mutant. Here too, specific DNA binding is apparent, albeit with a slightly reduced affinity (K_{app} ~8×10^7 M^{-1}) relative to wild-type CRP.

The *in vitro* DNA binding data presented in this paper agree well with our previous *in vivo* functional studies (Gent *et al.*, 1987), and clearly demonstrate the following facts. (i) Arg^{180} is crucial for DNA binding as has been proposed by the various models (Weber and Steitz, 1984; Ebright *et al.*, 1984b). The difference found for the Arg^{180} → Lys versus the Arg^{180} → Leu mutant further indicates that not only is specific binding abolished in the latter but non-specific binding also, at least up to the concentrations of protein employed. (ii) The guanidinium functional group of Arg^{185} is not necessary for specific DNA binding by CRP. Thus the present models need to be amended in this respect. The Arg^{185} → Leu change affects the formation of 1.1 CRP–DNA complexes only marginally, resulting in a very slight reduction (≤25%) in DNA binding affinity, whereas the Arg^{185} →

Lys change has no effect. The results on the Arg^{185} mutants, however, do not exclude a possible role of Arg^{185} in hydrophobic interactions at the protein–DNA interface, for example involving the C^{β} atom of the side-chain.

The cAMP binding site mutant Ser^{128} → Ala, which *in vivo* is still able to mediate transcription enhancement in the presence of cAMP (i.e. it is not a CRP^{*} mutant), albeit with a somewhat reduced activity (Gent *et al.*, 1987), is also still capable of binding DNA specifically. The concentration of protein necessary for complex formation, however, is slightly higher than that for wild-type CRP. This minor reduction in specific DNA binding affinity is not due to an alteration in cAMP binding affinity, since the equilibrium binding constant for the binding of cAMP to this mutant is identical to that for wild-type CRP within experimental error ($K_{ass} \sim 3 \times 10^5\ M^{-1}$; A.M.Gronenborn, unpublished results), as measured by the cAMP binding assay carried out as described by Pastan *et al.* (1974). Thus it may well be that the Ser^{128} → Ala change leads to a small structural alteration in the protein, which is transmitted to the DNA binding site, thereby slightly affecting the local conformation necessary for optimal DNA binding. What is clear, however, is that Ser^{128} is neither critical for cAMP binding nor for the cAMP-induced allosteric conformational change which is required for sequence-specific DNA binding. This is in contrast to the proposed critical role of Ser^{128} as deduced from the X-ray structure of the cAMP–CRP complex (McKay *et al.*, 1982). Careful examination of this structure, however, reveals that it contains the cyclic nucleotide in the wrong hand, i.e. the chirality at C1 is incorrect (cf. Figure 7 of McKay *et al.*, 1982; Ebright, 1986). Consequently, many of the proposed contacts between cAMP and CRP will have to be revised in the light of this incorrect structure.

Finally, no changes in the relative mobility of mutant CRP–DNA complexes with respect to the wild-type complex could be detected when run on a single gel (data not shown), indicating that the degree of CRP-induced DNA 'bending' (Wu and Crothers, 1984) is the same in all complexes.

Extending the approach outlined in this paper, namely the combination of site-directed mutagenesis with the fast and efficient gel retardation assay to probe the DNA binding properties of individual mutant proteins, will enable us to determine which other amino acids of CRP are important for specific DNA binding.

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