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

Manda E. Gent, Silvia Gärtner, Angela M. Gronenborn, Rodica Sandulache and G. Marius Clore

Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, FRG

1Present address Department of Biochemistry and Applied Molecular Biology, University of Manchester Institute of Technology, Manchester M60 1QD, UK

2To whom reprint requests should be sent

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<sup>180</sup> abolish specific DNA binding, whereas those at the position Arg<sup>185</sup> have very little effect. Both positions have previously been implicated as crucial for the specific interaction between CRP and DNA. 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<sup>181</sup>, 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 TGGTGA 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<sup>180</sup> abolish CRP-mediated transcription enhancement; substitutions of Arg<sup>185</sup>, 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<sup>180</sup> — Lys, Arg<sup>180</sup> — Leu, Arg<sup>185</sup> — Lys, Arg<sup>185</sup> — Leu and Ser<sup>128</sup> — Ala variants. The first two positions of amino acid substitutions (Arg<sup>180</sup>, Arg<sup>185</sup>) have been proposed to be crucial for DNA binding (Weber and Steitz, 1984; Ebright et al., 1984b), whereas Ser<sup>128</sup> 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<sup>−</sup>) 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<sub>4</sub>)SO<sub>4</sub> precipitation, ion-exchange chromatography and gel filtration. They were > 95% pure as judged by SDS—polyacrylamide electrophoresis and their concentrations were determined spectrophotometrically using ε<sub>278</sub> = 4.1 × 10<sup>4</sup>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<sub>2</sub>, 5% glycerol, 0.1 mM dithiothreitol, 25 mM NaCl and 25 mM CaCl<sub>2</sub> 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 Hinfl/HaeIII fragment carrying the lac wild-type region. It was radioactively end-labelled using Klenow Polymerase and [α<sup>−32</sup>P]ATP. The approximate concentration of the DNA fragment employed in the binding assays was 10<sup>−10</sup> 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<sub>2</sub>, 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 boric acid, 2.5 mM EDTA and 0.2 mM cAMP. The gels were autoradiographed at −70°C with intensifying screens for 12 h.
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<sup>180</sup>— Leu and Arg<sup>185</sup>— Lys) showed the same pattern of proteolysis as a control Arg<sup>185</sup>—Leu mutant. There is, however, a reproducible difference in the behaviour of the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— 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<sup>180</sup>— Lys mutant. Here too, specific DNA binding is apparent, albeit with a slightly reduced affinity (K<sub>app</sub>~8 x 10<sup>-7</sup> M<sup>-1</sup>) 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<sup>180</sup> is crucial for DNA binding as has been proposed by the various models (Webber and Steitz, 1984; Ebright et al., 1985b). The difference found for the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— Lys 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<sup>185</sup> is not necessary for specific DNA binding by CRP. Thus the present models need to be amended in this respect. The Arg<sup>185</sup>— 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<sup>185</sup>— 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<sup>185</sup>— 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<sup>185</sup>— 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<sup>185</sup>—

ed out with these mutant proteins and neither protein is able to form 1:1 complexes, even at concentrations up to 5 x 10<sup>-7</sup> M protein. There is, however, a reproducible difference in the behaviour of the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— 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<sup>180</sup>— Lys mutant. Here too, specific DNA binding is apparent, albeit with a slightly reduced affinity (K<sub>app</sub>~8 x 10<sup>-7</sup> M<sup>-1</sup>) 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<sup>180</sup> is crucial for DNA binding as has been proposed by the various models (Webber and Steitz, 1984; Ebright et al., 1985b). The difference found for the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— Lys 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<sup>185</sup> is not necessary for specific DNA binding by CRP. Thus the present models need to be amended in this respect. The Arg<sup>185</sup>— 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<sup>185</sup>—

ed out with these mutant proteins and neither protein is able to form 1:1 complexes, even at concentrations up to 5 x 10<sup>-7</sup> M protein. There is, however, a reproducible difference in the behaviour of the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— 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<sup>180</sup>— Lys mutant. Here too, specific DNA binding is apparent, albeit with a slightly reduced affinity (K<sub>app</sub>~8 x 10<sup>-7</sup> M<sup>-1</sup>) 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<sup>180</sup> is crucial for DNA binding as has been proposed by the various models (Webber and Steitz, 1984; Ebright et al., 1985b). The difference found for the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— Lys 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<sup>185</sup> is not necessary for specific DNA binding by CRP. Thus the present models need to be amended in this respect. The Arg<sup>185</sup>— 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<sup>185</sup>—

ed out with these mutant proteins and neither protein is able to form 1:1 complexes, even at concentrations up to 5 x 10<sup>-7</sup> M protein. There is, however, a reproducible difference in the behaviour of the Arg<sup>180</sup>— Lys versus the Arg<sup>185</sup>— 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<sup>180</sup>— Lys mutant. Here too, specific DNA binding is apparent, albeit with a slightly reduced affinity (K<sub>app</sub>~8 x 10<sup>-7</sup> M<sup>-1</sup>) relative to wild-type CRP.
Lys change has no effect. The results on the Arg$^{185}$ mutants, however, do not exclude a possible role of Arg$^{385}$ in hydrophobic interactions at the protein–DNA interface, for example involving the C$^\alpha$ 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 ($K_{\text{eq}}$ = $3 \times 10^5$ 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.

Acknowledgements

This work was supported by the Max-Planck Gesellschaft and Grant no GR 658/3-1 of the Deutsche Forschungsgemeinschaft (G M C and A M G).

References


Received on February 11, 1987, revised on March 26, 1987.