Characterization of *Escherichia coli* adenylate cyclase mutants with modified regulation

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In *Escherichia coli* there is a large increase of cAMP synthesis in crp strains, which are deficient in the catabolite gene activator protein. In this work it was shown that this increase in cAMP synthesis does not occur in crp crr strains, deficient in both the catabolite gene activator protein and enzymeIII-glucose, a component of the phosphotransferase system. It was also shown that the other components of the phosphotransferase system are required to obtain the increase of cAMP synthesis in a crp background. Adenylate cyclase mutants were obtained, by random mutagenesis, which had partial adenylate cyclase activity but which did not exhibit increased levels of cAMP in a crp background. For three mutants the mutation was identified as a single point mutation. This allowed the identification of residues arginine 188, aspartic acid 414 and glycine 463 which could be involved in the catabolite gene activator protein dependent activation process.

Introduction

Transcriptional regulation by cAMP in *Escherichia coli* is mediated by a cAMP receptor protein named catabolite gene activator protein (CAP) (Zubay et al., 1970). cAMP binds to CAP, converting it from an inactive form to one that can activate transcription. The concentration of cAMP required to obtain half-maximum binding of CAP-cAMP to a particular DNA-binding site varies considerably (Kolb et al., 1983; Berg & Von Hippel, 1988), suggesting a hierarchy at the transcriptional level (Alper & Ames, 1978). However, despite the established regulatory role of cAMP, the mechanism by which adenylate cyclase (AC) activity is regulated is not yet understood. In the presence of glucose, enzymeIII-glucose, a component of the phosphotransferase system (PTS), regulates uptake of non-PTS sugars (inducer exclusion: Magasanik, 1970) and AC activity. The model proposed for the regulation of AC was based upon genetic experiments which suggested that the phosphorylated form of enzymeIII-glucose was an activator of AC (Feucht & Saier, 1980; Postma et al., 1981). When glucose transport takes place, the intracellular concentration of phosphorylated enzymeIII-glucose decreases and correlates with a decrease in intracellular cAMP concentration. Several mutants of the structural gene for enzymeIII-glucose have been isolated which have lost inducer exclusion but which are still subject to glucose-mediated inhibition of AC (Feucht & Saier, 1980).

It has been known for nearly 20 years that the production of cAMP is increased 20- to 100-fold in crp mutants (Potter et al., 1974; Fraser & Yamazaki, 1978). This increase could not be accounted for by an elevated level of expression of AC (Mori & Aiba, 1985; Roy et al., 1988) and it was suggested that CAP could regulate the activity of AC (Joseph et al., 1982; Dobrogosz et al., 1983). However, the mechanism of regulation has not yet been elucidated. In the present work, we investigated the role of enzymeIII-glucose in the increase of cAMP synthesis in crp strains. We also describe a class of *E. coli* AC structural gene (cya) mutants which can still produce cAMP but which produce the same level of cAMP in crp and crp* strains.

Methods

**Bacterial strains.** The strains used in this work were *E. coli* K12 derivatives (Table 1). Growth media were either LB or minimal medium M63 (Miller, 1972) supplemented with the required amino acids (1 mM), thiamin (5 μg ml⁻¹) and different carbon sources (0.4%, w/v). Transductions using *Pluir* were done as described by Miller (1972).

**cAMP assays.** The secretion of cAMP by bacteria was assessed by using strain TP610A as an indicator bacterium. Strain TP610A is a spontaneous mutant of strain TP610 which produces red colonies on 1% (w/v) maltose MacConkey agar when supplemented with low levels of cAMP (15 μM instead of the 200-500 μM required by most *cya*
strains). A drop of an overnight culture of the strain to be analysed was plated on a lawn of strain TP610A and the plate was then incubated at 37 °C overnight. Strains secreting significant levels of cAMP produced a red halo around the culture drop due to the fermentation of maltose by strain TP610A.

cAMP production was quantified by a radioimmunological assay (Guidi-Rontani et al., 1981) with cultures grown in minimal medium M63. The amount of cAMP was expressed as pmol per mg dry weight heated for 5 min at 100 °C.

In vitro mutagenesis. In vitro mutagenesis with hydroxylamine was performed overnight as described by Manayan et al. (1988). After extensive dialysis, the mutagenized plasmid was used to transform the appropriate host cell.

Results

Increased cAMP production in a crp strain requires phosphorylated enzymeIII-glucose

Isogenic strains deficient in CAP (Δcrp-39), in enzyme-III-glucose (Δcrp) or in both CAP and enzymeIII-glucose were analysed for cAMP secretion on maltose MacConkey plates (see Methods and Fig. 1). Only the strain deficient in CAP alone secreted sufficient cAMP to produce red colonies of the indicator strain TP610A. Transformation of the strain deficient in both CAP and enzymeIII-glucose with pDIA4705, a derivative of pBR322 containing a functional crp gene, restored the red halo due to the excretion of cAMP (Fig. 1). This result indicated that the crp gene product is required for the production of high levels of cAMP in strains deficient in CAP.

The same experiment was carried out with strain TP9511, which is deficient in CAP and in enzymeI, Hpr and enzymeIII-glucose (the components of the PTS for

<table>
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<th>Table 1. E. coli strains and plasmids</th>
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<td>Strain or plasmid</td>
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<tr>
<td><strong>E. coli</strong></td>
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<td>TP2503</td>
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<td>TP2111</td>
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<td>TP2006</td>
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<td>TP610</td>
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<tr>
<td>TP2010</td>
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<td>TP9500*</td>
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<tr>
<td>TP2139</td>
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<td>TP2339</td>
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<td>TP2862</td>
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<tr>
<td>TP2811</td>
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<tr>
<td>TP9510*</td>
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<tr>
<td>TP9511*</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pSa206</td>
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<td>pDIA1900*</td>
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* Bacterial strains and plasmid derived during this work.
glucose transport). Even after transformation with pDIA4705 a red halo was not observed (data not shown). The phosphorylation of enzymeIII-glucose by the other components of the PTS is therefore necessary for the overproduction of cAMP in strains deficient in CAP.

In addition, total cAMP was determined after growth of strains in medium with pyruvate as sole carbon source. Total cAMP per mg dry weight of bacteria was constant during the exponential phase of growth. Mean values of three independent experiments were (pmol per mg dry weight of bacteria): 3000 for the wild-type strain TP2111, 50000 for the Acrp strain TP2139, 150 for the Acrr strain TP2862 and 200 for the Acrp Acrr strain TP9510. Thus in the Acrp strain, cAMP per mg dry weight of bacteria was increased about 20-fold as compared to the wild-type strain. These results were in agreement with the visual observations on MacConkey plates. The lower level of cAMP observed for the Acrr strain compared to the wild-type strain suggests that enzymeIII-glucose activates AC.

Screening and characterization of E. coli AC mutants

The 4.3 kb EcoRI-SalI fragment of pDIA100, a derivative of pBR322 containing the cya gene (Roy & Danchin, 1982), was inserted into EcoRI + SalI-digested pSa206, a low-copy-number plasmid (Close et al., 1984), to give pDIA1900 (Fig. 2). When the ΔcyA strain TP2010 was transformed with derivatives of pDIA1900 obtained by partial digestion with BclI, clones with an intermediate phenotype (CyA+/−, white colonies with a red centre) were obtained. Plasmids from such clones were invariably deleted from BclI(2) to BclI(3) or BclI(4) (see Fig. 2). This result was not surprising, as different truncated forms at the carboxy-terminal end of AC have been shown to retain AC activity (Roy et al., 1983a), but it showed that it was possible to screen AC mutants with a lower activity than the wild-type AC on MacConkey plates. The fact that the level of cAMP in a Δacrr strain was lower than that of a wild-type strain (see above) indicated that such mutants could be affected in the activation process mediated by enzymeIII-glucose.

pDIA1900 was mutagenized with hydroxylamine and then used to transform a ΔcyA strain (TP2010). CyA+/− clones appeared at a frequency of 24/1000. Four such clones (TP2010 containing pDIA1901, pDIA1902, pDIA1903 or pDIA1904) and a CyA− control (TP2010 containing pDIA1905) were isolated. TP2010 containing pDIA1901 was temperature-sensitive for the CyA phenotype, but TP2010 containing pDIA1902, pDIA1903 or pDIA1904 was not.

In order to compare the activities of the different proteins expressed from the mutagenized plasmids, strain TP9500 (ΔcyA) was transformed with pDIA1900, pDIA1901, pDIA1902, pDIA1903 or pDIA1904, and β-galactosidase activity produced with lactose or glucose 6-phosphate as sole carbon source was measured (Table 2). With TP9500 containing pDIA1901, pDIA1902 or pDIA1903, β-galactosidase activities were lower than that obtained with the strain containing the wild-type plasmid pDIA1900, but with TP9500 containing pDIA1904, β-galactosidase activity was very similar to that of TP9500 containing pDIA1900. With glucose 6-phosphate as carbon source, β-galactosidase activities were very low, as already reported by others for wild-type strains (Epstein et al., 1975). The data in Table 2 show that the levels of β-galactosidase measured in lactose compared to glucose 6-phosphate medium were higher in TP9500 containing pDIA1901, pDIA1902 or pDIA1903 (about 4-fold) than in TP9500 containing pDIA1900 or pDIA1904 (about 1.5-fold). In order to substantiate those results, the variations of cAMP per mg dry weight of bacteria in cells grown with lactose or glucose 6-phosphate were measured (Table 2); the results correlated with the variations in β-galactosidase levels.
Fig. 2. Partial restriction map of plasmid pDIA1900, containing the cya gene. The black box represents the coding region of the cya gene. Location of the promoters is represented by P1 and P2. The vertical arrows indicate the location of the mutations described in this work. cat' indicates a truncated chloramphenicol acetyltransferase gene.

Table 2. β-Galactosidase activity and cAMP levels in Δcya strains containing mutagenized plasmids

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<tr>
<th>Strain TP9500 (Δcya, lac+) containing:</th>
<th>β-Galactosidase activity [U (mg dry wt of bacteria)^{-1}] with:</th>
<th>Total cAMP* [pmol (mg dry wt of bacteria)^{-1}] with:</th>
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<tr>
<td></td>
<td>Lactose</td>
<td>Glc 6-P</td>
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<tr>
<td>pDIA1900 (WT)</td>
<td>2600</td>
<td>1800</td>
</tr>
<tr>
<td>pDIA1901</td>
<td>1210</td>
<td>360</td>
</tr>
<tr>
<td>pDIA1902</td>
<td>1900</td>
<td>490</td>
</tr>
<tr>
<td>pDIA1903</td>
<td>1570</td>
<td>340</td>
</tr>
<tr>
<td>pDIA1904</td>
<td>2440</td>
<td>1460</td>
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* Means ± SEM of three determinations.

Proteins expressed from mutagenized plasmids were characterized by minicell experiments in order to visualize possible truncated forms of the mutant proteins. The 4.3 kb EcoRI–SalI fragment of pDIA1901, pDIA1902, pDIA1903, pDIA1904 or pDIA1905 was inserted in pBR322 digested with EcoRI and SalI and the corresponding plasmids were used to transform the minicell-producing strain AR1062. Plasmid-encoded proteins, labelled with [35S]methionine, were analysed by polyacrylamide gel electrophoresis (Fig. 3). The mutant proteins expressed from pDIA1902, pDIA1903 and pDIA1904 (Fig. 3, lanes 3, 4 and 5) were similar in size to the wild-type AC (about 97 kDa, lane 6). One truncated form of about 52 kDa was expressed from pDIA1901 (lane 1). In the case of pDIA1905, the absence of protein on the gel (lane 2) was in agreement with the fact that a Δcya strain containing this plasmid remained Cya− (see above).

In order to locate the mutations, the BamHI(1)–BamHI(2) fragment (which contains the promoter region) or the EcoRI–HpaI fragment (which contains the catalytic site) or the HpaI–SalI fragment (see pDIA1900, Fig. 2) of each mutated plasmid was subcloned in pDIA1900, deleted for the corresponding fragment. The plasmids thus obtained were used to transform strain TP2339, which is deficient in both CAP and AC. The secretion of cAMP by the clones thus obtained was assessed using the indicator strain and compared to the secretion obtained with the wild-type plasmid. This allowed the determination of the fragment responsible for the mutated phenotype. For pDIA1902, pDIA1903 and pDIA1904, the mutations were located in the BamHI–HpaI fragment and thus the nucleotide sequences of the BamHI(2)–HpaI fragments of pDIA1902, pDIA1903 and pDIA1904 were determined. The mutations which were found corresponded to those usually obtained with hydroxylamine, which produces G.C→A.T transitions (Phillips & Brown, 1967). In each case, a single base change had occurred, leading to a single amino acid substitution. Amino acid residue 414 of the protein encoded by pDIA1902 was changed from aspartic acid to asparagine (Fig. 4a), amino acid residue 188 of the protein encoded by pDIA1903 was changed from arginine to histidine (Fig. 4b) and amino acid residue 463 of the protein encoded by pDIA1904 was changed from glycine to aspartic acid (Fig. 4c). The vertical arrows in Fig. 2 indicate the location of the different mutations.
Cyclases encoded by mutagenized plasmids do not produce high levels of cAMP in crp strains

The secretion of cAMP by strain TP2339, which is deficient in both CAP and AC, containing pDIA1900, pDIA1901, pDIA1902, pDIA1903 or pDIA1904 was assessed using strain TP610A as indicator as before. Strain TP2339 containing pDIA1900 produced a red halo on the plates but TP2339 containing pDIA1901, pDIA1902 or pDIA1903 did not. In the case of strain TP2339 containing pDIA1904, enough cAMP was secreted to allow the production of a small halo.

Total cAMP assays of the same strains grown on pyruvate were performed and the levels of cAMP obtained were compared to those obtained with strain TP9500, which is deficient in AC, containing the same plasmids (Fig. 5). When TP9500 was used as the host cell, the levels of cAMP obtained with the different mutated plasmids were lower than that obtained with pDIA1900 (about a 12-fold, 4-fold, 10-fold and 2-fold decrease with pDIA1901, pDIA1902, pDIA1903 and pDIA1904 respectively). The higher levels of cAMP observed for wild-type AC in a crp background were not obtained with AC encoded by pDIA1901, pDIA1902 or pDIA1903 (Fig. 5). In the case of pDIA1902, the crp strain had a lower level of cAMP than the crp+ strain; this was surprising and remains to be explained. In the case of pDIA1904, total cAMP was increased 5-fold (instead of 25-fold for wild-type AC), a result in agreement with the cAMP secretion observed on MacConkey plates.

![Polyacrylamide gel electrophoresis of plasmid-encoded proteins](image)

**Fig. 3.** Polyacrylamide gel electrophoresis of plasmid-encoded proteins expressed in minicells of strain AR1062. Lane 1, pDIA1901; lane 2, pDIA1905; lane 3, pDIA1902; lane 4, pDIA1903; lane 5, pDIA1904; lane 6, pDIA1900 (wild-type), lane 7, molecular mass markers.

![Location of the different mutations on the DNA](image)

**Fig. 4.** Location of the different mutations on the DNA and determination of the corresponding mutated amino acids. The numbering shown in this figure is similar to that used by Roy et al. (1983b). (a) pDIA1902, (b) pDIA1903, (c) pDIA1904.
Discussion

Strains deficient in CAP overproduce cAMP but the molecular mechanism underlying the increase in cAMP is not known. We have shown that it requires enzymeIII-glucose. The increase of cAMP in crp strains was also shown to be dependent on the presence of enzymeI and Hpr, the other components of the PTS. This result indicates that the phosphorylated form of enzymeIII-glucose is involved in the CAP-dependent activation process. To explain the decrease of cAMP synthesis in wild-type strains of Salmonella typhimurium grown on glucose, Postma (1982) proposed a model where phosphorylated enzymeIII-glucose was described as an activator of AC. By analogy with this model the simplest hypothesis to explain the high level of cAMP in wild-type strains of Salmonella typhimurium would be that CAP interacts with AC, preventing the activation of AC by phosphorylated enzymeIII-glucose. In the case of pDIA1903, the mutation was mapped to a BclI fragment which deletion studies showed to be essential for AC activity (see Fig. 2). This mutation was located in a region of the DNA which may code for the active site (or part of the active site) of AC, as shown by the fact that a 200 bp deletion in this region produces a Cya- phenotype (Brickman et al., 1973; Glaser et al., 1989). In this mutant, arginine residue 188 was changed to histidine. A change from arginine to histidine is a conservative change (Barker & Dayhoff, 1972) and the fact that such a change caused a mutant phenotype suggests that Arg-188 is an important residue.

The AC protein encoded by pDIA1904 showed a 2-fold decrease in cAMP synthesis as compared to wild-type AC in a crp+ background and a 10-fold decrease in a crp background (with pyruvate as sole carbon source). Therefore glycine residue 463 (which was changed to aspartic acid in this mutant) may not be directly related to the regulation mediated by CAP in a wild-type strain. Finally, it must be emphasized that Asp-414 and Arg-188 seem to be essential for the CAP-dependent activation process. Neither mutation completely abolished AC activity but both inhibited the increase in activity normally observed in crp strains. If this increase is indeed dependent upon a direct interaction between...
AC and enzyme III-glucose, mutants with higher AC activity than the wild-type should be obtained even in crr strains.

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References


