

Mutations in the cyclic AMP binding site of the cyclic AMP receptor protein of *Escherichia coli*

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Mutants in the cyclic AMP binding site of the cyclic AMP receptor protein (CRP) of *Escherichia coli* have been constructed by oligonucleotide-directed mutagenesis. They have been phenotypically characterized and their ability to enhance the expression of catabolite-repressible operons has been tested. In addition, the binding of cyclic nucleotides to the mutants has been investigated. It is shown that the six mutants made fall into one of three classes: (i) those that bind cyclic AMP better than the wild type protein (Ser-62 → Ala) and result in greater transcription enhancement; (ii) those that bind cyclic AMP similarly to wild type (Ser-83 → Ala, Ser-83 → Lys, Thr-127 → Ala, Ser-129 → Ala); and (iii) those that do not bind cyclic AMP at all (Arg-82 → Leu). Implications of these findings with respect to present models of the cyclic nucleotide binding pocket of CRP are discussed.

INTRODUCTION

The expression of catabolite-repressible operons in *Escherichia coli* is cyclic AMP-dependent. It is mediated via the cyclic AMP receptor protein (CRP) which, when complexed with cyclic AMP, binds to specific sequences in the promoter region of the respective genes (for a recent review see Busby, 1986). CRP is composed of two identical subunits of 209 amino acids (Aiba *et al.*, 1982; Cossart & Gicquel-Sanzey, 1982), each of which is divided into a structural and a functional domain: the larger *N*-terminal domain carries the cyclic AMP binding site and is involved in subunit–subunit interaction, while the smaller *C*-terminal domain is responsible for sequence-specific DNA binding (Eilen *et al.*, 1978; McKay & Steitz, 1981). On binding cyclic AMP the protein undergoes a defined conformational change such that the complex is capable of activating transcription (Krakow & Pastan, 1973; Wu *et al.*, 1974). Although a large number of cyclic nucleotide analogues can bind CRP, only a few are able to induce the correct conformational change required to activate transcription. These are 1-, 3- or 7-deaza-cyclic AMP, 2- and 8-aza-cyclic AMP, as well as the two cyclic AMP-S diastereomers (Ebright *et al.*, 1985). From the crystal structure of the cyclic AMP–CRP complex contacts between CRP residues and the bound nucleotide have been identified (McKay *et al.*, 1982). These contacts, however, had to be revised (Ebright, 1986) as the incorrect enantiomorph of cyclic AMP was erroneously fitted in the structure. Further, whereas cyclic AMP is found in the anti-conformation in the crystal structure (McKay & Steitz, 1981), the cyclic nucleotide conformation of the complex in solution is syn (Gronenborn & Clore, 1982).

In order to understand the allosteric change brought about by cyclic AMP, several laboratories have investigated CRP mutants which function *in vivo* in the absence of cyclic AMP. These mutations have been called a

variety of names, most commonly *crp** (Sanders & McGeoch, 1973; Botsford & Drexler, 1978; Melton *et al.*, 1981). They have been categorized into two classes: class I mutants can be stimulated by either cyclic AMP or cyclic GMP, while class II mutants do not respond to cyclic nucleotides at all (Aiba *et al.*, 1985). Determination of the sequences of a number of these mutants has shown that most of the *crp** mutants are located at the end of helix C or the beginning of helix D, thus in a region which may be crucial for the reorientation of the *C*-terminal domain with respect to the *N*-terminal part of the protein (Garges & Adhya, 1985; Aiba *et al.*, 1985; Harman *et al.*, 1986). It has therefore been argued that these mutant proteins have already achieved the 'active' conformation in the uncomplexed state (Garges & Adhya, 1985; Anderson, 1986).

We have used oligonucleotide directed mutagenesis selectively to change amino acids in CRP (Gent *et al.*, 1987*a,b*) and determine which amino acids are important for its function. In this paper we have extended our previous studies to the cyclic AMP binding site of CRP and focus on amino acids which have been proposed as important contacts between the protein and the cyclic nucleotide (McKay *et al.*, 1982; Ebright, 1986). Five residues were investigated, four of which were thought to interact specifically with the cyclic nucleotide, either with the cyclic phosphate moiety (Arg-82, Ser-83) or the adenine ring (Thr-127, Ser-128). The fifth residue (Ser-62) was chosen, because, although not directly interacting with the cyclic nucleotide, it is located on a stretch of polypeptide chain comprising residues 61 to 64 on β -strand 5 which lines one side of the binding pocket. The amino acid changes carried out consisted of removing the functional group, i.e. Ser → Ala, or Arg → Leu. In the case of Ser-83, two amino acid substitutions were made, one which removed the supposedly hydrogen bonding OH (Ala), and a second in which the polar, small Ser side-chain was replaced by a bulky positively charged one (Lys) to probe for steric interaction. Strains carrying the

Abbreviations used: CRP, cyclic AMP receptor protein.

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mutant genes were tested for sugar fermentation patterns, which reflects transcription activity and cyclic nucleotide binding. Three types of mutants were found: (i) those that do not bind cyclic AMP and are therefore not capable of transcription activation (Arg-82 → Leu); (ii) those that bind cyclic AMP with similar, but somewhat reduced affinity with respect to wild type CRP, and, thus, can only activate transcription at elevated levels of cyclic AMP (Ser-83 → Ala, Ser-83 → Lys, Thr-127 → Ala, Ser-128 → Ala); and (iii) those that bind cyclic AMP better than wild-type CRP resulting in transcription activation at significantly lower cyclic AMP levels as compared to wild-type (Ser-62 → Ala). The results obtained with these mutants are discussed with respect to the present models of the cyclic AMP binding pocket.

MATERIALS AND METHODS

Chemicals

Cyclic AMP, cyclic GMP, 8-Br-cyclic AMP and the cyclic AMP-S (S_p) isomer were purchased from Boehringer Mannheim; the cyclic AMP-S (R_p) isomer was a gift from F. Eckstein. [^3H]Cyclic AMP, [^{32}P]ATP and [^{35}S]ATP were obtained from Amersham.

Bacterial strains

The *E. coli* strains used were, pp47 (*crp*⁻) kindly provided by H. Aiba and TP2339 (F^- , *lac* ΔX 74, *cya*Δ, *crp*Δ 39, *arg*H1, *xyl*) obtained from the Institute Pasteur (Paris, France) (courtesy of Dr. H. Buc). The plasmid pUC9-CRP is a derivative of pUC9 (Viera & Messing, 1982) which carries the *crp* structural gene on a 750 bp *Hind*III/*Eco*RV fragment inserted into the multiple cloning site.

Site-directed mutagenesis

CRP mutants were constructed by the gapped duplex method (Kramer *et al.*, 1984) using single-stranded M13mp9 carrying the *crp* gene on a 750 bp fragment as a template. Approx. 20 base long oligonucleotides were used as mutagenic primers, carrying the desired mismatches approximately in the middle of their sequence (but slightly shifted towards the 3' end). Phage harbouring the mutations were initially identified by dot blot hybridization using the ^{32}P -labelled oligonucleotide as a probe. The frequency of mutation was between 10% and 30%. After plaque purification the single strand of a mutant clone was sequenced by the dideoxy method (Sanger *et al.*, 1977) to establish that the desired mutations were the sole ones within the entire coding region. The mutated *crp* gene was subsequently cloned into the plasmid pUC9 and the recombinant plasmids were used for transformation of the CRP-deficient strains pp47 or TP2339. The sequence of the mutant gene in the plasmid was again checked by sequencing (Chen & Seeburg, 1985).

Sugar utilization test

Sugar utilization was tested on EMB agar containing 1% of the respective sugar, 50 μg of ampicillin/ml and cyclic nucleotides or other added supplements as indicated.

β-Galactosidase assay

Cells were grown in EMB medium containing lactose. When the optical density at 600 nm reached 0.5–1.0, 0.5

Table 1. Summary of plasmids carrying mutations in the cyclic AMP binding site

Plasmid	Type of mutation
pUC9	No <i>crp</i> gene
pUC9- <i>crp</i>	wild type
pUC9- <i>ag1</i>	Ser-83 → Ala
pUC9- <i>ag2</i>	Ser-83 → Lys
pUC9- <i>ag3</i>	Arg-82 → Leu
pUC9- <i>ag4</i>	Ser-62 → Ala
pUC9- <i>ag5</i>	Thr-127 → Ala ₁₀₀
pUC9-CAP4	Ser-128 → Ala

absorbance units were removed, centrifuged and the cells resuspended. β-Galactosidase activity was determined according to Miller (1972).

Cyclic AMP binding assay

Cyclic AMP binding was determined using the $(\text{NH}_4)_2\text{SO}_4$ precipitation procedure as described by Anderson *et al.* (1971) either in crude extracts or for purified protein. The equilibrium binding curves were fitted to a rectangular hyperbola:

$$\text{counts in pellet} = \frac{S \cdot K_{\text{app}} \cdot [\text{cAMP}]}{(1 + K_{\text{app}} \cdot [\text{cAMP]})}$$

where K_{app} is the apparent equilibrium association constant, [cAMP] the concentration of added cyclic AMP (note that [cAMP] ≫ [CRP]), and S a scale factor. Fitting was performed using the FACSIMILE program (Chance *et al.*, 1977) which employs Powell's method of non-linear optimization (Powell, 1972).

RESULTS

Expression of mutant protein

Table 1 lists the plasmids constructed and used in our investigation as well as the corresponding amino acid changes. All of these result in the production of CRP protein when introduced into a *crp*⁻ host strain, with the obvious exception of the parent plasmid pUC9. This is easily appreciated from the gel shown in Fig. 1, on which the total soluble proteins of overnight cultures of pp47 harbouring the various plasmids are separated. Comparable amounts of CRP are found in all cases. The protein is expressed from the *lac* promoter using its own ribosome binding site. A possible interference by CRP autoregulation (Aiba, 1983) was circumvented by omitting the CRP binding sites located at the 5' end of the structural gene in our constructs.

Phenotypic characterization of the mutant *crp* genes

The influence of the various CRP mutations on sugar utilization was investigated to characterize the variants and, in particular, to compare them with known *crp** mutations. The requirements of wild-type and mutant CRPs to promote the utilization of arabinose, lactose, galactose and maltose were tested in *crp*⁻ host cells transformed with the respective plasmids. The resulting fermentation patterns are shown in Table 2. All mutants are capable of fermenting the different sugars to varying degrees, with the exception of the plasmid pUC9-*ag3*

Table 2. Fermentation characteristics of strain pp47 carrying the vector alone, a *crp* wild-type plasmid and various mutant plasmids on various carbon sources

Colour of CRP mutants, + + + +, green; + + +, dark purple with a green shine; + +, dark purple; +, purple; ±, pink; -, white.

Plasmid in pp47	Colour when grown with:			
	Lactose	Galactose	Maltose	Arabinose
pUC9	-	-	-	-
pUC9- <i>crp</i>	+ + + +	+ + + +	+ + + +	+ + + +
pUC9- <i>ag1</i>	+ + + +	+ + + +	+ +	+
pUC9- <i>ag2</i>	+ + + +	+ + + +	+	+
pUC9- <i>ag3</i>	±	-	±	+
pUC9- <i>ag4</i>	+ + + +	+ + + +	+ + + +	+ + + +
pUC9- <i>ag5</i>	+ + + +	+ + + +	+ +	+ +
pUC9-CAP4	+ + + +	+ +	+	+ +

(Arg-82 → Leu) which exhibits the same negative response as the parent plasmid pUC9. It should be noted that the plasmid pUC9-*ag4* (Ser-62 → Ala) shows a stronger positive response than the wild-type-carrying plasmid for all sugars. In order to test for cyclic nucleotide dependence of CRP activity, all the plasmids were also introduced into the strain TP2339 which carries deletions of the *cya* and *crp* genes. Sugar utilization was subsequently tested as a function of exogenously added cyclic nucleotides. The results for galactose are listed in Table 3. A similar pattern, though not as pronounced, was obtained for maltose. Lactose utilization could not be tested as this strain also carries a deletion of the *lac* gene. As can be seen from the results all plasmids which were able to compensate for the lack of CRP in the *crp*⁻ strain showed positive fermentation patterns in the presence of exogenously supplied cyclic AMP.

We also tested other cyclic nucleotides for their ability to activate galactose expression. Cyclic GMP gave negative responses for all plasmids tested, as did 8-Br-

cyclic AMP. The two isomers of cyclic AMP-S, however, were able to activate the sugar operons with the wild-type plasmid already showing a positive response at a concentration of 0.02 mM for the *R_p* isomer; the *S_p* isomer, on the other hand required a higher concentration (> 2 mM) than cyclic AMP for activation.

Under normal circumstances CRP-dependent operon expression is repressed by glucose (Monod, 1947; for a recent review see Postma, 1986), a phenomenon known as catabolite repression which is thought to be caused by lowering of cyclic AMP levels leading to the dissociation of the active CRP-cyclic AMP complex. To test for catabolite repression we examined sugar utilization (lactose, galactose and maltose) as a function of added α-methyl-glucoside (10–100 mM), a non-hydrolysable PTS carbohydrate. At 10 mM some lowering of *lac* expression is observed, while for *gal* the effect is visible only from 20 mM upwards. In general, however, these effects are small, i.e. the strong positive *lac* and *gal* expression, as manifested by a green shine on EMB plates, is reduced to either dark purple (*gal*) or to a weak green shine (*mal*).

β-Galactosidase activity

Lac promoter activity for the various mutants was also measured by the β-galactosidase assay. The average of three independent measurements is summarized in Table 4. Once again *ag4* (Ser-62 → Ala) shows higher activity than wild type CRP, *ag5* (Thr-127 → Ala) exhibits approximately equal activity, and *ag1* (Ser-83 → Ala) and *ag2* (Ser-83 → Lys) display somewhat reduced activity. CAP4 (Ser-128 → Ala) shows markedly lower activity than the wild type, and in the case of *ag3* (Arg-82 → Leu) no appreciable amount of β-galactosidase activity could be detected.

Cyclic AMP binding

All of the mutants constructed were envisaged to have some effect on cyclic AMP binding. Binding affinities for cyclic AMP were measured either on purified protein (CRP wild-type, CAP4) or in crude extracts (CRP wild-type and all constructed mutants) by the (NH₄)₂SO₄ precipitation assay (Anderson *et al.*, 1971). Approximate

Table 3. Galactose utilization of strain TP2339 transformed with the vector alone, or plasmids carrying either the *crp* wild-type gene or mutants thereof, as monitored on EMB/galactose indicator plates

Colour of CRP mutants: + + + +, green; + + +, dark purple with a green shine; + +, dark purple; +, light purple; ±, pink; -, white. N.D., not determined; G.I., growth inhibition.

Plasmid in TP2339	Measure of galactose utilization							
	[Cyclic AMP]			[Cyclic AMP-S]			[Cyclic GMP] 0.1–5 mM	[8-Br-cyclic AMP] 0.1–5 mM
	0 mM	0.5 mM	2.5 mM	<i>R_p</i> 0.02 mM	<i>R_p</i> 0.2 mM	<i>S_p</i> 2 mM		
pUC9	-	-	-	-	-	-	-	-
pUC9-CRP	-	+ + + +	+ + + +	+ + + +	G.I.	±	-	-
pUC9- <i>ag1</i>	-	+	++	-	+ + + +	±	-	-
pUC9- <i>ag2</i>	-	±	++	-	+ + + +	+	-	-
pUC9- <i>ag3</i>	-	-	-	N.D.	N.D.	N.D.	-	-
pUC9- <i>ag4</i>	-	+ + + +	G.I.	N.D.	N.D.	N.D.	-	-
pUC9- <i>ag5</i>	-	+	++	N.D.	N.D.	N.D.	-	-
pUC9-CAP4	-	-	±	N.D.	N.D.	N.D.	-	-

Table 4. Equilibrium constants for the binding of cyclic AMP to wild type and mutant CRPs determined by the $(\text{NH}_4)_2\text{SO}_4$ precipitation assay, and β -galactosidase activity in strain pp47 containing plasmids which carry either no, wild-type or mutant *crp* genes

Plasmid in pp47	$10^{-5} \times K_A$ (M^{-1})	β -Galactosidase activity*
pUC9‡	No binding detectable	0.04
CRP†	2.3 (± 0.6)	
pUC9-CRP‡	2.5 (± 0.7)	1.00
pUC9-ag1 (Ser-83 \rightarrow Ala)‡	2.0 (± 0.5)	0.70
pUC9-ag2 (Ser-83 \rightarrow Lys)‡	1.8 (± 0.6)	0.73
pUC9-ag3 (Arg-82 \rightarrow Leu)‡	No binding detectable	0.06
pUC9-ag4 (Ser-62 \rightarrow Ala)-‡	6.0 (± 1.1)	1.49
pUC9-ag5 (Thr-127 \rightarrow Ala)‡	2.5 (± 1.0)	1.13
CAP4 (Ser-128 \rightarrow Ala)†	2.3 (± 0.9)	0.45

* The β -galactosidase binding data are presented as the averages of three independent measurements, normalized to wild type (set to 1). The relative errors in the values are approx. $\pm 20\%$.

† Purified protein.

‡ Cell extract.

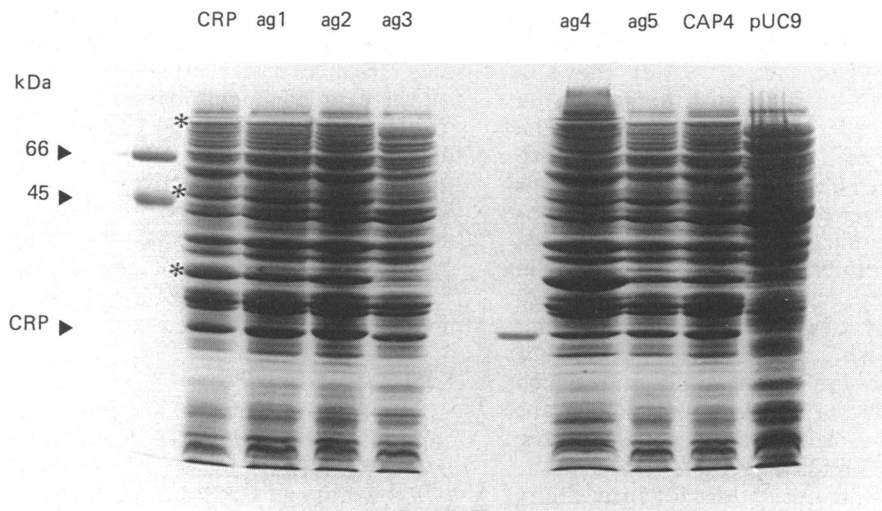


Fig. 1. CRP expression in strain pp47 containing pUC9 or recombinant plasmids carrying the wild-type or mutated *crp* genes

Overnight cultures of pp47 containing the indicated plasmid were lysed in a French press; 50 μl of extract was separated on a 15% polyacrylamide/SDS gel. The left lane contains two marker proteins (bovine albumin, egg albumin), in the middle purified CRP is run as a standard. Bands marked with an * represent proteins overproduced in the presence of high amounts of active CRP.

equilibrium constants were obtained by fitting the equilibrium binding data as described in the Materials and methods section. They are summarized in Table 4. Ag4 (Ser-62 \rightarrow Ala) exhibited the strongest binding, followed by wild-type CRP. All other mutants showed similar or slightly reduced binding as compared with wild type CRP, with ag3 (Arg-82 \rightarrow Leu) exhibiting no binding.

DISCUSSION

Activity of mutant proteins

We have constructed and characterized *crp* mutations in the cyclic AMP binding site. In contrast to the so-called *crp** mutants, our mutants are not able to express CRP-dependent operons in cells that lack cyclic AMP.

Most are, however, capable of inducing *lac* or *gal* expression in a strain lacking a functional *crp* gene, when introduced on a plasmid. Only the Arg-82 \rightarrow Leu mutant is not able to activate transcription. It is worth noting that the mutant carrying the Ser-62 \rightarrow Ala change is a better transcription activator than wild-type CRP. This is most likely due to the fact that the binding of cyclic AMP to this mutant protein is increased, thus inducing the allosteric transition to its active conformation at a lower cyclic AMP concentration relative to the wild-type situation.

In addition to a high expression level of CRP as seen from the gel shown in Fig. 1, active CRP is also responsible for the overexpression of other proteins. For wild-type CRP and all mutants, apart from ag3 (Arg-82 \rightarrow Leu), thus for all active CRP variants, three

additional bands are seen at approx. 32, 50 and 100 kDa. They probably originate from cellular proteins whose expression is under CRP control such as the various enzymes involved in sugar metabolism.

Cyclic nucleotide binding

The binding constants for cyclic AMP as determined by the $(\text{NH}_4)_2\text{SO}_4$ precipitation test are more or less identical for all mutants apart from ag3, which does not bind at all, and ag4, which binds better than wild-type. This correlates well with the results of the β -galactosidase assay with the exception of CAP4 (Ser-128 \rightarrow Ala). Thus β -galactosidase levels seem to reflect the cyclic AMP affinity for CRP with CAP4 being somehow different. As the cyclic AMP binding constant for CAP4 and wild type CRP are the same, this result suggests that the mutant protein cannot efficiently carry out the conformational change required for transcription activation.

From the results of the cyclic nucleotide dependence of fermentation patterns summarized in Table 3, it is clear that in those cases where the mutant CRP is able to bind cyclic AMP, exogenous addition of it will lead to a positive response. Further, the relative binding strength of the different cyclic AMP-S analogues can also be demonstrated. It has been established that cyclic AMP-S (R_p isomer) binds much better to wild-type CRP than cyclic AMP, which in turn binds better than cyclic AMP-S (S_p isomer) (Ebright, 1986). This is very clearly reflected on the EMB/galactose plates supplemented with the respective cyclic nucleotide. While *gal* activation is already seen at 0.02 mM-cyclic AMP-S (R_p isomer), the same activation level requires 0.5 mM-cyclic AMP or > 2 mM-cyclic AMP-S (S_p isomer). Results obtained with some of the mutants, e.g. ag1 (Ser-83 \rightarrow Ala), showed an identical pattern; however, all concentrations were up by a factor of 10. It is interesting to note that very similar ratios were found for cyclic nucleotide-induced β -galactosidase stimulation, namely cyclic AMP-S (R_p) \gg cAMP > cAMP-S (S_p) (Scholübbbers *et al.*, 1984). We also tested for cyclic AMP and 8-Br-cyclic AMP dependence of *gal* activation and found the same results for all mutants as was known for wild-type CRP, namely that these cyclic nucleotide analogues are not able to promote the allosteric transition required for activity (Ebright *et al.*, 1985). Thus all changes introduced into the protein by mutagenesis did not alter the known cyclic nucleotide selectivity of the wild-type protein.

Functional amino acids in the cyclic AMP binding pocket

How can the observed changes in activity be interpreted in terms of structure of the cyclic AMP binding site? The latest model for the interactions between CRP and cyclic AMP was presented by Weber *et al.* (1987), and a modification of their model is schematically illustrated in Fig. 2.

The Arg-82 side chain was thought to be involved in an ionic interaction with the equatorial phosphate oxygen. That indeed the interaction between Arg-82 and cyclic AMP is very important for binding has been substantiated by our Arg-82 \rightarrow Leu mutant which no longer binds cyclic AMP and is therefore not able to activate transcription. The better binding of cyclic AMP-S (R_p isomer) to wild-type CRP, however, casts some doubt upon the selection of the equatorial phosphate oxygen for this interaction.

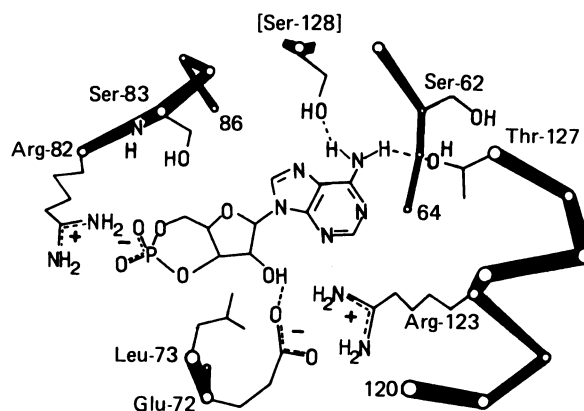


Fig. 2. Schematic representation of the cyclic AMP binding site in CRP

This drawing represents a modified version of the cyclic AMP binding pocket as shown by Weber *et al.* (1987), taking into account the results of the present study. In particular the Ser-83 hydroxyl is no longer hydrogen bonded to the axial phosphate oxygen. Instead the bidentate Arg-82 guanidinium group forms an ion pair with the negative phosphate comprising both the axial and equatorial oxygens. Hydrogen bonds between Thr-127 and Ser-128 on the one hand, and the 6-amino group of the adenine ring on the other, are still indicated although they may not necessarily be present.

The proposed interaction of the Ser-83 hydroxyl with the axial phosphate oxygen cannot be very important since both substitutions of Ser-83 by Lys or Leu do not abolish binding but only lower the binding constant, possibly by interfering with the correct positioning of cyclic AMP due to steric hindrance. If the Ser hydroxyl group really would be responsible for the better binding of cyclic AMP-S (R_p) to CRP, both mutants in which this hydroxyl is no longer present, namely ag1 (Ser-83 \rightarrow Ala) and ag2 (Ser-83 \rightarrow Lys), should have lost this selectivity. This is clearly not the case, as can be judged from the data presented in Table 3. Cyclic AMP-S (R_p) activates *gal* transcription in the $\Delta crp\Delta cya$ strain carrying ag1 or ag2 already to the fullest level of 0.2 mM, whereas 20 mM exogenously added cyclic AMP are necessary for the same level of activation.

From the crystallographic model it is not easily appreciated why ag4 (Ser-62 \rightarrow Ala) is a better activator than the wild-type protein. The Ser hydroxyl is pointing away from the cyclic nucleotide binding site being in rather close contact with Val-49. Thus, the improvement in the binding of cyclic AMP may be solely due to the removal of a bad steric contact between amino acids lining the binding site rather than a direct positive interaction between Ala-62 and the cyclic nucleotide. It is interesting to note that there already exists a mutant at position 62. This is one of the *crp** mutants isolated by Aiba *et al.* (1985) and consists of a Ser \rightarrow Phe change. In contrast to our mutant, this mutant can only be activated at higher cyclic AMP concentrations (Aiba *et al.*, 1985). It seems unlikely that the bulky Phe side-chain could occupy the same position as the Ser or the Ala. The aromatic phenyl ring may actually slot into the cyclic AMP binding site, which in turn could result in a similar conformational change to that produced in the wild-type

case by cyclic AMP, thereby rendering this protein independent of the cyclic nucleotide.

Mutants ag5 (Thr-127 → Ala) and CAP4 (Ser-128 → Ala) were constructed in order to test the proposed contacts with the adenine base. Both Thr-127 and Ser-128, with the latter from the adjacent subunit, supposedly hydrogen bond to the 6-amino group on the adenine ring; the threonine hydroxyl interacts with the amino hydrogen pointing towards N-1 and the serine hydroxyl interacts with the amino hydrogen on the N-7 side as well as with the N-7 on the adenine ring (McKay *et al.*, 1982). As evidenced by the binding data for cyclic AMP as well as the activity *in vivo* of both mutants, both amino acids are not crucial for the binding of cyclic AMP. Nevertheless, they have some effect on transcription since *gal* activation in both cases requires higher cyclic AMP levels than was found for wild-type CRP. We believe that the actual binding constant of cyclic AMP binding to the mutant proteins could be reduced, although they appear to be approximately similar to wild-type in the $(\text{NH}_4)_2\text{SO}_4$ precipitation assay. On the other hand the effects seen could also be due to conformational differences between the wild type and mutant proteins. As can be seen from the data in Table 3, the Ser-128 → Ala change shows a lower activity than the Thr-127 → Ala substitution. Both, however, can be activated by high levels of cyclic AMP and in turn activate the respective sugar operons. Ag5 (Thr-127 → Ala) needs a 10 times higher cyclic AMP concentration than that required to activate the wild-type protein, while CAP4 (Ser-128 → Ala) seems to require even more. This factor of 10, as discussed before, represents a gross overestimation of the difference in binding constant, which probably is only small and may have its origin in differences leading to the conformational changes required for transcription activation. From the fact that in the *crp*⁻ strain both mutants can activate transcription of sugar operons, it is obvious that cyclic AMP does indeed bind to CRP mutant proteins and induces the active conformation. Furthermore, DNA binding experiments *in vivo* with CAP4 confirmed that this mutant binds to the *lac* site with comparable affinity ($K_{\text{app}} \sim 8 \times 10^7 \text{ M}^{-1}$) as compared to wild-type CRP ($K_{\text{app}} \sim 10^8 \text{ M}^{-1}$) (Gent *et al.*, 1987b) and is able to induce transcription from the correct *lac* promoter (unpublished results). It is therefore our belief that neither ag5 (Thr-127 → Ala) nor CAP4 (Ser-128 → Ala) exhibit differences in cyclic AMP binding constants large enough to reflect the loss of a hydrogen bond. The small changes observed may solely be due to minor conformational differences in the two mutant proteins which in turn will have effects on cyclic AMP and/or DNA binding.

In view of the results obtained from extensive studies with cyclic nucleotide analogues (Ebright *et al.*, 1985), it seems clear that analogues modified at N-6 can bind to CRP, can induce the correct allosteric conformational change but fail to activate transcription. Since this modification comprises the N-1 directed hydrogen atom of the 6-amino group, it seems likely that the N-7 directed hydrogen is the one responsible for any interaction. This is thought to be in contact with Ser-128, and it is the Ser-128 → Ala mutation which requires the largest amount of cyclic AMP for activation. The proposed interaction between Ser-128 and the N-7 position has already been disproved since 7-deaza-cyclic AMP is as, or more, potent than cyclic AMP in binding

and activity (Ebright *et al.*, 1985). Thus, although not as crucial for binding as Arg-82, which presumably provides most of the binding energy, Ser-128 may play a minor role in producing the right conformation for optimal binding.

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