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REGULATORY ASPECTS OF THE CYCLIC AMP RECEPTOR PROTEIN IN *ESCHERICHIA COLI* K-12

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SUMMARY: A highly sensitive radioimmunological assay has been set up to determine the amount of the cyclic AMP receptor protein (CAP) in crude *Escherichia coli* extracts. This method enables to detect cross-reacting material in mutants carrying a deletion in the *crp* gene (coding for CAP) and to measure the amount of CAP under various growth conditions. We present results showing that the synthesis of CAP is affected neither by the growth conditions of bacteria nor by the carbon source or by cyclic AMP.

It is currently believed that adenosine 3'-5' cyclic monophosphate (cAMP) *via* its receptor protein (CAP) exerts a positive control on catabolite sensitive operons in *Escherichia coli*. However, it was recently shown that the cAMP-CAP complex might be involved in the regulation of polar expression of polycistronic operons (1). This suggested that CAP has a more general function in bacteria: it is not only involved in promoting transcription initiation of certain operons but it might exert a regulatory control on transcription termination. Moreover, nothing is known concerning the regulation of CAP synthesis. To search for possible regulatory effects on CAP synthesis, it was necessary to develop a method for measuring its actual amounts in crude bacteria extracts. This method could also be useful to study mutants isolated as exhibiting pleiotropic carbohydrate negative phenotype even in the presence of cAMP, and mapped close, or at the *crp* locus (structural gene of CAP). Using a sensitive radioimmunological method, we present results showing that growth conditions do not modulate the synthesis of CAP. Furthermore, mutants carrying a deletion in the *crp* gene express cross-reacting material to a varying extent.

MATERIALS AND METHODS:

1. Strains and Media : The following *E. coli* K-12 strains were used: wild-type 3000 ; CA8306 [cya_{Δ} ; isolated by E. Brickman *et al.* (2)] ; CA8404 [cya_{Δ} , crp^{4}; isolated by L. Soll *et al.* (3)] ; CA8496 , CA84110 ,

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CA84118 , CA8445 , CA84113 , CA8439 are strains deleted for adenylate cyclase gene (cya_{Δ}) and carrying different deletions in *crp* gene (crp_{Δ}). They were kindly provided by J. Beckwith.

Bacteria were grown at 37°C in rich medium ML or in minimal medium 63 (4) supplemented with thiamine (5 µg/ml) and glycerol (0.4%) , glucose (0.4%), or succinate (0.2%) as carbon source. When specified, cAMP was added at $5 . 10^{-3} M$ final concentration.

2. Preparation of crude extracts : Overnight cultures were centrifuged, the cells suspended in buffer A (Na_2HPO_4 0,1 *M* ; pH 7,3) and sonically disrupted. Generally 1 g (wet weight) of bacteria was suspended in 2.5 ml of buffer A. The sonicated extract was centrifuged at 15,000 rpm for 15 min and the pellet discarded.

3. Purification of CAP : CAP was purified from *E. coli* strain K-12 KLF41/ JC1553 (kindly provided by B. Bachmann) using a purification procedure to be described elsewhere (5). Samples used were about 99% pure as judged from SDS polyacrylamide gel electrophoresis and had a specific activity of 10,000 ± 1000 U/mg. (One unit is the amount of protein that binds one pmole of cAMP).

3. Cyclic AMP binding assay of CAP : The assay was performed as described by Anderson *et al.* (6). ^{3}H cAMP (360 Ci/mole) used was from the Radiochemical Center, Amersham (England). Binding assays were conducted for various concentrations of the ligand and Scatchard plots showed no difference in the binding properties of CAP, either pure or in crude extracts. This allowed to compute the total concentration of receptor from the number of units determined in standard assays at a given concentration of cAMP ($10^{-6} M$).

4. Immunization procedures : Rabbits were immunized by injections in footpads with 500 µg of purifed CAP in Freund adjuvant. Two months later 300 µg of antigen in Freund adjuvant were injected intramuscularly. At varying time intervals beginning one week after the booster, the rabbits were bled. The antiserum was kept at 4°C with 0.1% sodium azide.

5. Iodination : Iodinated CAP was prepared at room temperature by using the chloramine T method (7). To 5 µl of CAP (6.5 µg) , 1 µl Na[^{125}I] (Commissariat à l'Energie Atomique) and 4 µl of chloramine T (12 µg) were added successively. After 30 sec , the reaction was stopped by addition of 4 µl sodium metabisulfite (60 µg). The radioactive mixture was immediately passed through a Sephadex G-25 column (24 x 1 cm) equilibrated and eluted with PBS (buffer A containing 0.5% bovine serum albumin). The eluted iodinated protein solution was centrifuged at 35,000 rpm for 2 hr (Beckman, Ti-50) to eliminate aggregated material. The iodinated CAP was stored at 4°C and was stable for one month. The yield of CAP iodination was about 80% corresponding to a specific radioactivity of 1400 Ci/mmole.

6. Radioimmunoassay : All immunological reactions were performed in PBS. To 100 µl iodinated CAP (12500 dpm) diluted in 1% normal rabbit antiserum, 100 µl of diluted anti-CAP antiserum and 100 µl of unlabelled CAP or crude bacterial extract at different concentrations were added. After 12 hr at 4°C, 20 µl of goat antibodies against rabbit immunoglobulins (Calbiochem) were added to each tube, followed by the addition of 6% polyethylene glycol. The tubes were centrifuged in the cold at 2000 x g for 20 min, the supernatant discarded and the radioactivity contained in the pellet measured in a Gamma counter.

RESULTS : In a first set of experiments, the validity of the radioimmunoassay was checked using purified CAP. A typical dose-response curve is shown in Figure 1. The optimal sensitivity of the radioimmunoassay was obtained with an

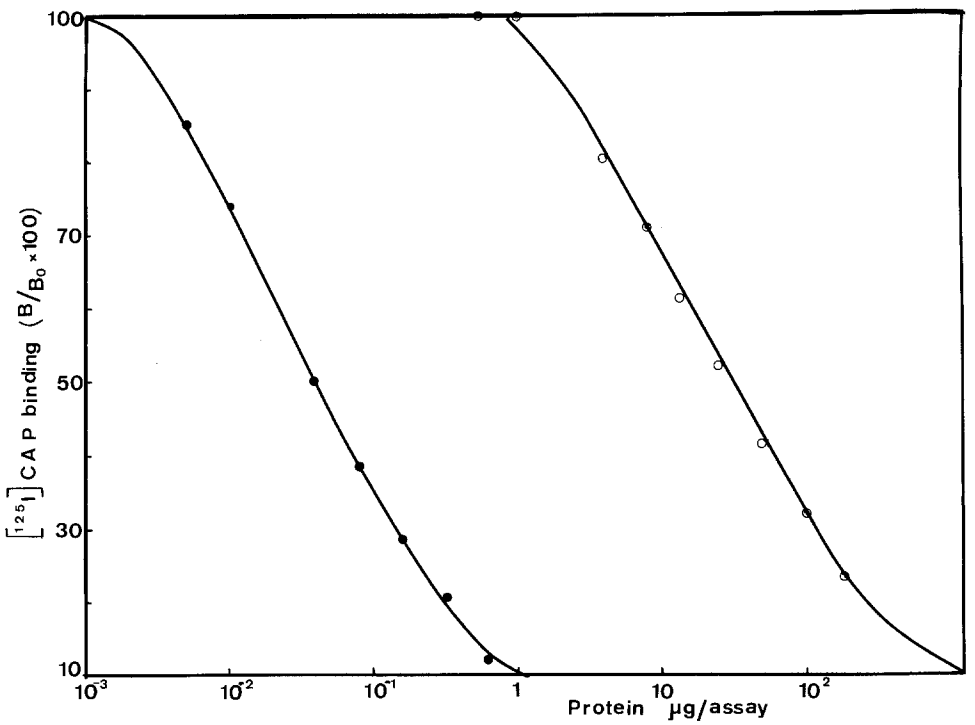

Figure 1. Radioimmunoassay of CAP.

Inhibition of [^{125}I]CAP binding to antiserum by increasing amounts of unlabelled CAP or of crude extracs from strain 3000 grown in 63 glucose minimal medium.
B_0 : initial binding corresponding to 45% of [^{125}I]CAP bound to the antiserum in the absence of unlabelled CAP.
B : per cent of residual [^{125}I]CAP bound in the presence of different amounts of unlabelled CAP (●—●) or of extract (O—O).

antiserum dilution of 1:10 000, (under these conditions, 45% of [^{125}I] CAP was bound). As it can be seen, the radioimmunoassay allows the measurement of low quantities such as 10 ng of CAP. The specificity was checked by determining the amount of CAP in bacterial extracts from wild-type strain. The inhibition curve obtained with this extract parallels the standard curve obtained with unlabelled purified CAP. To obtain 50% inhibition of [^{125}I] CAP binding to antiserum, 40 ng of purified CAP ($6.6 \cdot 10^{-13}$ moles) or 30 µg of total proteins of bacterial extract were necessary. This indicates that CAP represents 0.13% of the total bacterial protein and is in good agreement with the value obtained from purification yields.

Since the method was particularly sensitive and specific, it seemed appropriate to search for possible variations of CAP in bacteria under different growth conditions. To investigate whether CAP concentration depends upon the growth rate of bacteria or its expression is regulated by catabolite re-

TABLE I

Assay of CAP under various growth conditions by cAMP binding and radioimmunoassay

Strains	Media and Carbon Sources	Generation time (min)	Amount of extracts necessary to inhibit 50% of [^{125}I] CAP binding to antiserum (µg)	cAMP bound (pmoles cAMP/mg of protein)
3000	ML	25	34	7
3000	63+Succinate	120	22	7
3000	63+ Glycerol	90	30	6
3000	63+ Glucose	50	32	6
3000	63+ Glucose+ cAMP	55	34	5
CA8306	63+ Glucose	115	30	6
CA8306	63+ Glucose+ cAMP	80	36	5

pression: strains were grown in different media in the presence of carbon sources known to exert different catabolite repression effects. The results presented in Table I show that these different growth conditions do not result in a significant variation of CAP concentration, determined by both radioimmunoassay and cAMP binding. Moreover, addition of cAMP to growth media does not affect CAP synthesis. This latter point is strengthened by the experiment performed with a strain (CA8306) carrying a deletion of the adenylate cyclase gene (*cya*$_\Delta$) and thus unables to synthesize cAMP (second part of Table I). The amount of CAP found in extracts of *cya*$_\Delta$ strain (grown either in the absence or in the presence of cAMP) is the same as in the wild-type.

Several mutants affected in the *crp* gene were also studied for the presence of antigenically active CAP. One of these mutants, CA8404 (*cya*$_\Delta$, *crp*^{4}) was isolated as a pleiotropic carbohydrate-positive revertant in a *cya*$_\Delta$ background, being able to express catabolite-sensitive operons in the absence of cAMP (3). From Table II, it is apparent that this strain synthesizes CAP which is recognized to the same extent by the antiserum as wild-type CAP ;

TABLE 2

Assay of CAP in bacterial extracts of various mutants by cAMP binding and radioimmunoassay

Strains	Media	Amount of extract necessary to inhibit 50% of [^{125}I] CAP binding to antiserum (µg)	cAMP bound (pmoles cAMP/mg of protein
CA8404	63 + Glucose	30	2
CA8496	ML	35	0
CA84110	ML	40	0
CA84118	ML	35	0
CA8445	ML	> 3000	0
CA84113	ML	> 3000	0
CA8439	ML	> 3000	0

yet its cAMP binding activity is decreased by a factor of three, thus suggesting that *crp*[4] mutation affects the structure of CAP rather than its regulation. In bacterial extracts of mutants carrying deletions in the *crp* gene, no cyclic AMP binding activity could be detected. However, radioimmunoassay allowed to divide these mutants into two distincts classes. One group of mutants (CA8496 , CA84110 , CA84118) produce cross-reacting material recognized by the antiserum to the same extent as the wild-type protein. Most probably these mutants carry a small deletion in the *crp* gene resulting in a loss of the cAMP binding site without modifying its antigenic activity. In the second class of mutants (CA8445 , CA84113 , CA8439) no measurable amount of cross-reacting material was found indicating that they do not synthesize antigenically active protein.

DISCUSSION : Cyclic AMP receptor protein (CAP), encoded by the *crp* gene, plays an important role in the regulation of gene expression in *E. coli*. Its binding and chemical properties as well as its role in transcription stimulation have been extensively studied. Many *crp* mutants have been isolated and genetically characterized ; but CAP synthesis regulation has not yet been

studied. The investigation of the regulatory aspects of CAP synthesis requires, in the first place, a specific and sensitive assay of CAP, enabling to detect small variations of its steady-state level in bacterial extracts and to measure cross-reacting material from various *crp* mutants.

The main conclusion that can be drawn from our experiments is that the amount of CAP *(i)* does not depend on the generation time of bacteria since a four-fold variation in growth-rates does not affect CAP synthesis, *(ii)* does not depend on the nature of the carbon source. The different carbon sources were choosen for their known effects of repression (glucose) or derepression (succinate) of catabolite sensitive systems. No detectable variations of CAP level were observed indicating that CAP expression is not sensitive to catabolite repression , *(iii)* is not affected by cAMP: a cya_{Δ} strain contains the same amount of CAP as the wild-type (cya^{+}) strain. This latter result is noteworthy because it is known that in *crp* mutants very high levels of cAMP are found (8) suggesting that functional CAP might be involved in the regulation of adenylate cyclase.

So far, no specific regulation of CAP synthesis could be demonstrated. The possibility of a more complex mechanism of control, not as yet revealed, cannot be ruled out. We are confident that applying the combined immunological and binding assays, we might be able to detect mutants affected in specific regulation of CAP synthesis.

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