THE ENZYMIC DEGRADATION OF 3',5' CYCLIC AMP IN STRAINS OF E. COLI SENSITIVE AND RESISTANT TO CATABOLITE REPRESSION

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Summary: Two mutants of E. coli, resistant to catabolite repression, differ from a wild type strain, sensitive to catabolite repression, in having a defective 3',5' cyclic AMP degradative system.

Evidence that 3',5' cyclic AMP (cAMP) partially overcomes catabolite repression (CR) in Escherichia coli has been presented (Perlman and Pastan, 1968; Ullmann and Monod, 1968). We made similar observations and found that, when glucose served as source of carbon, cAMP decreased the intensity of both the severe "transient" and of the less severe "permanent" inhibition of the synthesis of β-galactosidase.

Efforts by a number of workers including ourselves (Hsie et al., 1969) to identify any one metabolite of glucose as the "effector" of CR have been unsuccessful. The failure to detect a metabolite directly responsible for CR, on the one hand, and the finding that cAMP partially overcame CR, on the other hand, suggests that cAMP may play a central role in the mediation of CR. One prediction of this hypothesis is that there may exist a class of mutants simultaneously resistant to CR and abnormal with respect to the metabolism of cAMP. In this communication we describe an enzyme system that catalyzes the formation of 5'AMP from cAMP in E. coli. Furthermore we report that certain mutants resistant to CR (CR^-) are also abnormal with respect to the degradation of cAMP.

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MATERIALS AND METHODS

Organisms and conditions of growth: Strains of E. coli, conditions of growth, and measurement of protein have been described (Hsie and Rickenberg, 1967; Rickenberg et al., 1968). AB 257 (CR^+) is sensitive to CR; AB 257^{pc-1} (CR^-) is resistant to both transient and permanent CR; LA12G (CR^-) is resistant to permanent, but still sensitive to transient CR (Tyler et al., 1967).

Preparation of bacterial extracts: Extracts were prepared from bacteria which had been washed twice and resuspended in 4×10^{-2} M Tris.HCl, pH 8.45, 4×10^{-4} M Mg^{++}. The bacteria were treated in a Raytheon 10 kc magnetostrictive oscillator for 20 min followed by centrifugation for 30 min at 45,000 x g. The supernates from the 45,000 x g centrifugation are referred to as "Crude Extracts". They were submitted to a second centrifugation at 160,000 x g for 17 hrs. The pellets from this centrifugation were resuspended in buffer and dialyzed against several changes of Tris buffer (as above) and are referred to as COMPONENT I. The supernates from the 160,000 x g centrifugation were also dialyzed against Tris buffer. The non-dialyzable material is referred to as COMPONENT II, the dialyzate as COMPONENT III.

Assay of cAMP degradation: We employ the terms "cAMP degradative activity" and "enzyme system" rather than cAMP phosphodiesterase because our current findings do not exclude rigorously the possibility that cAMP may be converted to 5'AMP by a series of reactions rather than by simple hydrolytic cleavage. The occurrence of a cAMP phosphodiesterase in E. coli was reported by Brana and Chytil (1965); the observations of these authors, however, also do not exclude a more complex mechanism of cAMP degradation. Our assay was based on the same principle as that described recently (Brooker et al., 1968) for the measurement of cAMP. Anion exchange resin binds and quenches the radioactivity of ^3H labeled cAMP but not of adenosine; this fact was utilized in the assay in which the 5'AMP formed in the degradative reaction was hydrolyzed by an excess of 5'-nucleotidase. The assay mixture, in a total volume of 0.5 ml, consisted of the enzyme preparation, 0.05 mg of nucleotidase (Sigma), and ^3H-cAMP (Schwarz BioResearch, Inc.) to a final concentration of

5×10^{-4} M and approximately 35,000 dpm. The buffer was the same as the one described above. The reaction, carried out with gentle shaking at 37°C in scintillation vials, was started by the addition of cAMP and terminated by the addition of 2 ml of absolute ethanol containing 300 mg of Dowex AG 2-X8, 200-400 mesh (Calbiochem), chloride form. After 10 min more absolute ethanol was added to solubilize the aqueous reaction mixture in 5 ml of the toluene scintillation cocktail. The radioactivity, representing adenosine derived from 5'AMP, was determined in a liquid scintillation counter. Duplicate samples were incubated for different periods of time and the radioactivity of reaction mixtures devoid of enzyme served as a measure of non-quenched cAMP. Specific cAMP degradative activity is expressed as millimicromoles of cAMP disappearing/min/mg of protein. Preliminary experiments showed that identical results were obtained when the Pi released in the reaction catalyzed by the added nucleotidase from 5'AMP was determined by the micromethod of Chen et al. (1956).

RESULTS AND DISCUSSION

Fig. 1 shows the degradation of cAMP by crude extracts of strains AB 257, AB 257^{pc-1}, and LA12G. The wild type and one CR-resistant mutant had identical activities; the second mutant had only low activity. Ultracentrifugation of a crude extract of AB 257 followed by dialysis of both pellet and supernate showed that at least three components, defined functionally as Components I, II, and III (cf. Materials and Methods) were required for full cAMP degradative activity (Table I). Recombination of Component I and undialyzed supernate in appropriate proportions led to a complete regain of the activity found in the crude extract. Addition of dialyzed supernate (Component II) to Component I, however, did not lead to a regain of more than approximately 30% of the original activity.

Table II shows that the cAMP degradative activity of mutant AB 257^{pc-1} differed from that of the other two strains with respect to Component I. An experiment designed to assess the relative contributions to cAMP degradation by the non-dialyzable (Component II) and dialyzable (Component III) fractions of wild type and mutant supernates is described in Table III. An extract of AB 257 served as source of Component I. To it were added either dialyzed or non-dialyzed supernates ob-

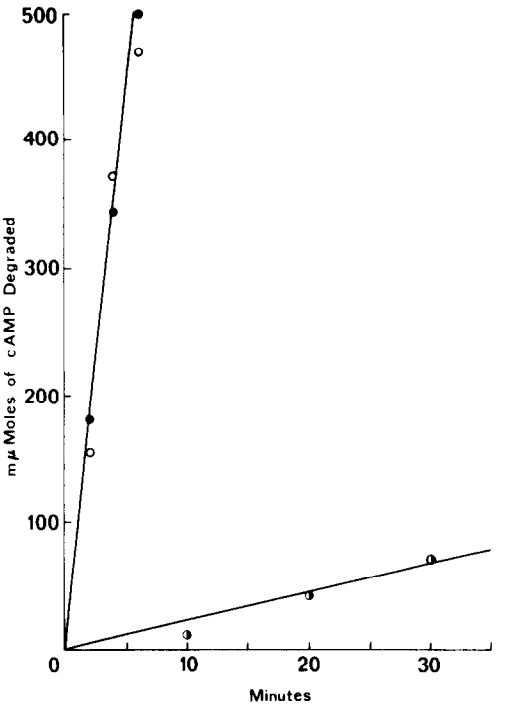

Figure 1. cAMP degradation by crude extracts of AB 257, AB 257^{pc-1}, and LA12G. The concentrations of protein employed in the assay were: AB 257, 3.5 mg; AB 257^{pc-1}, 3.5 mg; LA12G, 3.2 mg. The values in the figure are normalized for a concentration of 3 mg. The source of carbon in the medium was glucose.

●—●—● AB 257

◑—◑—◑ AB 257^{pc-1}

○—○—○ LA12G

tained from extracts of all three strains. It may be seen that all non-dialyzed supernates activated. Dialysis of the AB 257 and AB 257^{pc-1} supernates reduced activation whereas dialysis of the LA12G supernate abolished its ability to activate. Component II of strain LA12G was also ineffective in activating homologous, LA12G, Component I. These experiments showed that strain LA12G was abnormal with respect to the non-dialyzable supernatant fraction, i.e. Component II.

The experiments described so far showed that in addition to the sedimentable (Component I) and the non-dialyzable supernatant (Component II) fractions, a third, dialyzable fraction, (Component III), was required for maximal cAMP de-

TABLE I

Components of cAMP Degradative System

	Volume ml	Protein mg/ml	Total protein mg	Specific activity*	Total units
Crude extract	20.0	61.0	1,220	27.2	33,200
Supernate	17.0	10.9	187	0.9	17
Resuspended pellet	15.0	56.0	840	5.5	4,630
Resuspended pellet + supernate (1:1)				30.0†	25,200
Resuspended pellet + dialyzed supernate (1:1)				11.4†	9,580

*Specific activity: mμmoles of cAMP degraded/min/mg of protein.

†Specific activity based on protein of pellet only.

TABLE II

cAMP Degradative Activity of

CR^+ and CR^- Strains

Strain	Crude extract	Component I
AB 257 (CR^+)	27.2	5.5
AB 257^{pc-1} (CR^-)	0.7	0.2
LA12G (CR^-)	25.2	2.5

Numbers refer to specific activity. Cultures were grown with glucose as source of carbon.

A commercial preparation (Grain Processing Corp., Muscatine, Iowa) of a wild type (CR^+) K 12 strain, grown on a rich medium and harvested in the exponential phase of growth had a crude extract activity of 34 and Component I activity of 6.9.

TABLE III

Activation of Component I* by Supernatant Fractions

Derived from Centrifugates of CR^+ and CR^- Strains

Source of supernate	Activation by Non-dialyzed supernate	Activation by Dialyzed supernate (Component II)
AB 257 (CR^+)	18.1	4.5
AB 257pc-1 (CR^-)	14.0	3.8
LA12G (CR^-)	21.3	0

*Component I was derived from strain AB 257 and had a specific activity of 5.5. The values in the table represent the difference between the activity of unsupplemented and supplemented Component I. Numbers refer to specific activity.

gradative activity. The experiment presented in Table IV showed that the re-addition of the dialyzate to Components I and II restored cAMP degradative activity completely.

On theoretical grounds (paper in preparation) we anticipated that intermediates of carbohydrate metabolism might stimulate cAMP degradative activity and that Component III might be the source of these activators. Preliminary experiments, to be described in detail elsewhere, showed that in the absence of Component III a number of intermediates of carbohydrate metabolism stimulated cAMP degradation. Among the compounds tested and at a concentration of 4×10^{-4} M glucose-6-P, fructose-6-P, ribose-5-P, and 3-P-glyceraldehyde proved to be most effective; glucose and fructose stimulated only slightly; 2-P-glyceric acid and 3-P-glyceric acid inhibited. None of the compounds tested activated as effectively as Component III.

The cellular concentration of Component I found in AB 257 was the same irrespective of the source or concentration of carbohydrate on which the bacteria

TABLE IV

Activation of Components I* and II* by Dialyzate

Fractions	Specific Activity†
Component I	3.1
Components I + II	7.2
Components I + II + Component III (Dialyzate)	36.0
Component I + Crude Supernate	34.0
Crude Extract	29.2

Preparation of dialyzate (Component III): 50 ml of crude supernate were dialyzed against three changes of distilled H_2O (1000 ml total). The dialyzate was then concentrated to dryness *in vacuo* at 35°C, redissolved in 50 ml of Tris buffer, and appropriate volumes tested.

*All components were derived from strain AB 257. The extract was not the same one as that employed in the experiments described in the preceding tables. Component II and a mixture of Components II and III were devoid of cAMP degradative activity. The ratios of the combinations of individual components tested corresponded to their occurrence in the crude extract.

†Specific activity based on protein of Component I only.

were grown. Preliminary findings indicate that the cellular content of Component II varies with the concentration of sugar in the medium. The *in vitro* assay of cAMP degradative activity of crude extracts is probably not a true measure of the activity *in vivo*. It is likely that our conditions of assay are not physiological, particularly with respect to Component III. The fact that crude extracts of mutant IA12G, in spite of the abnormality in Component II, showed the same cAMP degradative activity as wild type AB 257 crude extracts (Fig. 1) also casts doubt on the physiological relevance of the assay.

The finding that several intermediates of carbohydrate metabolism stimulated the activity of the cAMP degradative system suggests that these, and undoubtedly other metabolites, have to be present in optimal proportions for maximal cAMP

degradative activity. We are now engaged in the purification of Components I and II. Preliminary findings indicate Component I to be a protein of a molecular weight of above 200,000 devoid of activity unless supplemented with Components II and III. Component II, also a protein, has a molecular weight of less than 100,000, is stable to heating at 70°C for 10 min, and has no cAMP degradative activity. There is no evidence that the mutations of strains AB 257^{pc-1} and LA12C lead to the formation of an inhibitor of the cAMP degradative system. Mixtures of crude extracts of AB 257 and mutants give additive activities.

Determinations of the steady state concentrations of cAMP in wild type and mutant strains grown on different sources of carbon showed consistently slightly higher levels in the mutants than in the parent strains (Janeček et al., in preparation).

Clearly an understanding of the role of cAMP in the regulation of energy metabolism requires the study of the behavior of both the adenyl cyclase and cAMP degradative systems. It is likely that the activities of both systems are affected by the cellular concentrations of intermediates of energy metabolism.

The present findings, showing a correlation between a defective cAMP degradative system and resistance to catabolite repression, are compatible with a central role of cAMP in the regulation of energy metabolism in E. coli. We are now looking for CR^- mutants normal in their metabolism of cAMP but defective with respect to other components of the hypothetical CR regulatory system.

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