The Effect of Anaerobiosis and Uncouplers on the Stimulation of β -Galactosidase Synthesis by Cyclic 3',5'-Adenosine Monophosphate in *Escherichia coli*

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(Accepted for publication 31 January 1972)

SUMMARY

Anaerobiosis, dinitrophenol and sodium azide decreased catabolite repression of β -galactosidase caused by glucose in non-growing *Escherichia coli* K-12. Anaerobiosis had no effect on the efficiency of exogenous cyclic AMP in stimulating enzyme synthesis. In contrast, dinitrophenol and sodium azide made the synthesis of β -galactosidase more responsive to exogenous cyclic AMP because the effect of cyclic AMP with dinitrophenol or with sodium azide was markedly greater than the sum of the separate effects of cyclic AMP and of each of the uncouplers. This effect of the uncouplers was unrelated to their action on oxidative phosphorylation because it still occurred under anaerobic conditions. It was also not due to increased accumulation of cyclic AMP by enhanced uptake or by inhibition of cyclic AMP-phosphodiesterase.

INTRODUCTION

Cyclic 3',5'-adenosine monophosphate (cyclic AMP) plays a role in the regulation of the synthesis of β -galactosidase and some other enzymes. Catabolite repression and transient repression involve depletion of the intracellular cyclic AMP (Makman & Sutherland, 1965; Perlman & Pastan, 1968; Chambers & Zubay, 1969; de Crombrughe, Perlman, Varmus & Pastan, 1969; Jacquet & Kepes, 1969). Metabolism of the sugar is not necessary for transient repression (Pastan & Perlman, 1969; Tyler & Magasanik, 1970), but the more permanent catabolite repression depends on the efficiency of the catabolism of the sugar and on the consumption of its products by biosynthetic processes. Interference with the sugar catabolism by inhibition of oxidative phosphorylation, or by other means, decreased catabolite repression (Mandelstam, 1961; Dobrogosz, 1965, 1966; Okinaka & Dobrogosz, 1967), whereas inhibition of anabolic processes did the reverse (Magasanik, 1961; Mandelstam, 1961).

In the present study we explored whether interference with the catabolism of glucose by inhibitors of oxidative phosphorylation, like anaerobiosis and uncouplers, decreased catabolite repression of β -galactosidase by diminishing the depletion of the intracellular cyclic AMP, or by participation in some other interaction which also affected the synthesis of β -galactosidase.

METHODS

Chemicals. The uncouplers used were 2,4-dinitrophenol (DNP) (Eastman Kodak Company, Rochester, New York, U.S.A.) and sodium azide (Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.). [^{14}C]Alanine (110 mCi/mmol) was from Schwarz Bio-Research, Inc., Orangeburg, New York, U.S.A. All other chemicals were as described by Aboud & Burger (1970).

Fig. 1. Effect of cyclic AMP and anaerobiosis on β -galactosidase synthesis. A suspension of bacteria in a nitrogen-deprived medium was divided into four equal portions. Those portions which were to be incubated anaerobically were extensively bubbled with N_2 before any addition and while sampling (each 20 min for assay of [14 C]leucine incorporation and β -galactosidase activity). Between samplings the flasks were tightly stoppered. At zero time each portion received IPTG (0.2 mM), glucose (0.5 % w/v), [14 C]leucine (0.05 μ Ci/ml) and non-radioactive leucine (10 μ g/ml) and were incubated under the following conditions: (●), aerobic without cyclic AMP; (▲), aerobic with 1.0 mM-cyclic AMP; (○), anaerobic without cyclic AMP; (△), anaerobic with 1.0 mM-cyclic AMP.

Organisms. *Escherichia coli* K-12 HfrH, requiring thiamine, was used throughout most of this study, except for one experiment in which *E. coli* B was used.

Media and growth conditions. Bacteria were subcultured at least once a week on Bacto red bile lactose agar (Difco Laboratories, Detroit, Michigan, U.S.A.). After incubation for about 20 h at 37 °C the agar plates were kept in the cold (4 °C) and were used for inoculation of liquid media.

An overnight culture, grown without shaking in a nitrogen-rich medium (Aboud & Burger, 1970) containing 0.2 % (w/v) glycerol at 37 °C, was diluted 1:100 in the same medium and incubated with shaking at 37 °C. In one experiment glycerol was replaced by 1 % (w/v) glucose. At the exponential phase, the bacteria were harvested by centrifuging at 4 °C, washed once with a nitrogen-deprived medium (the nitrogen-rich medium from which all nitrogen sources were omitted), and resuspended in the nitrogen-deprived medium to a density of about 60 μ g dry wt bacteria/ml.

Induction and assay of β -galactosidase. A suspension of washed organisms was induced to synthesize β -galactosidase by 0.2 mM-isopropyl-thio- β -D-galactopyranoside (IPTG) in the presence of 0.5 % (w/v) glucose, and assayed as described by Aboud & Burger (1970).

Incorporation of radioactive amino acids. Total protein synthesis was determined by the incorporation of [14 C]leucine (0.05 μ Ci/ml) added with non-radioactive leucine (10 μ g/ml), except in one experiment when the incorporation of [14 C]alanine (0.05 μ Ci/ml) added with non-radioactive alanine (10 μ g/ml) was measured. Incorporation of radioactive amino acids was assayed as described by Aboud & Burger (1971a).

Anaerobic conditions. Nitrogen was bubbled through the bacterial suspension before starting the experiment and while sampling; between the samplings the flasks were tightly stoppered.

Fig. 2. Effect of cyclic AMP and uncouplers on β -galactosidase synthesis. A suspension of bacteria in nitrogen-deprived medium received IPTG, glucose, [^{14}C]leucine and non-radioactive leucine (as for Fig. 1) and was immediately divided into four equal portions which were incubated with: (a) (●), no further additions; (▲), 0.125 mM-DNP; (○), 1.0 mM-cyclic AMP; (△), DNP+cyclic AMP. (b) (●), No further additions; (○), 20 mM- NaN_3 ; (▲), 1.0 mM-cyclic AMP; (△), NaN_3 +cyclic AMP. Samples were taken each 20 min for assay of β -galactosidase activity and [^{14}C]leucine incorporation.

RESULTS

Effect of cyclic AMP and anaerobiosis on the differential rate of enzyme synthesis. In none of the experiments was the effect of anaerobiosis and cyclic AMP higher than the sum of their separate effects; in some cases, as in Fig. 1, the combined effect was smaller than the sum.

Effect of cyclic AMP and uncouplers. The effect of cyclic AMP with 0.125 mM-DNP (Fig. 2a) or 20 mM- NaN_3 (Fig. 2b) was markedly higher than the sum of the separate effects of each. Measurements of [^{14}C]alanine incorporation gave similar results with DNP. The stimulatory effect of the uncouplers was therefore not due to a specific inhibition of leucine uptake. None of these uncouplers showed any effect on the activity of β -galactosidase. Their effect was therefore exerted on the synthesis of the enzyme.

Effect of cyclic AMP with uncouplers under anaerobic conditions. If the effect of the uncouplers in combination with exogenous cyclic AMP was connected with their interference with oxidative phosphorylation, there should be no such effect anaerobically when oxidative phosphorylation was already inactive. Dinitrophenol alone had no significant effect anaerobically, but had a similar effect as under aerobic conditions when cyclic AMP was present (Fig. 3). Similar experiments with NaN_3 were unsuccessful, because it inhibited more than 60% of the [^{14}C]leucine incorporation and β -galactosidase formation at 0.5 mM while lower concentrations were ineffective.

Effect of DNP in organisms containing a low level of cyclic AMP-phosphodiesterase. Theophylline, an inhibitor of cyclic AMP-phosphodiesterase, increased the stimulation of β -galactosidase synthesis by exogenous cyclic AMP in bacteria containing a high level of cyclic AMP-phosphodiesterase, such as glycerol-grown *Escherichia coli* K-12 (Aboud & Burger, 1971a), but had no effect on cyclic AMP action in organisms lacking cyclic AMP-phosphodiesterase, such as *E. coli* B, or having the enzyme only in low levels, such as glucose-grown *E. coli* K-12. Dinitrophenol also had no effect on *E. coli* B, whether or not exogenous cyclic AMP was present (Fig. 4a). However, unlike theophylline, DNP had some effect on

Fig. 3. Effect of cyclic AMP and DNP on β -galactosidase synthesis under anaerobic conditions. Suspension preparation and anaerobiosis production are described in Fig. 1. After receiving IPTG, glucose, $[^{14}\text{C}]$ leucine and non-radioactive leucine at zero time, each portion of the suspension was incubated under the following conditions: (●), aerobic with no further additions; (▲), anaerobic with no further additions; (■), anaerobic with 0.125 mM-DNP; (△), anaerobic with 0.125 mM-DNP and 1.0 mM-cyclic AMP; (○) anaerobic with cyclic AMP. The activity of β -galactosidase and the incorporation of $[^{14}\text{C}]$ leucine were assayed in samples taken each 20 min.

Fig. 4. Effect of cyclic AMP and DNP on β -galactosidase synthesis in organisms containing low levels of cyclic AMP-phosphodiesterase. The effect of DNP on cyclic AMP action was tested in (a) *Escherichia coli* B and (b) glucose grown *E. coli* K-12. Other experimental details were as described in Fig. 2: (●), no additions; (▲), DNP; (○), cyclic AMP; (△), DNP+cyclic AMP.

Fig. 5. Effect of various concentrations of cyclic AMP with and without DNP on β -galactosidase synthesis. A suspension of glycerol-grown *Escherichia coli* K-12 was divided into several equal portions. Each two portions were subjected to a different concentration of cyclic AMP, one in the presence of 0.125 mM-DNP (▲) and the other without DNP (●). After 45 min incubation, samples were taken from all portions for measuring β -galactosidase activity and [14 C]leucine incorporation.

the action of cyclic AMP in *E. coli* K-12 grown with 1% (w/v) glucose instead of glycerol (Fig. 4b).

Effect of DNP on the relation between β -galactosidase synthesis and cyclic AMP concentration. The relation between the differential rate of β -galactosidase synthesis and cyclic AMP concentration was estimated by incubating samples of an induced suspension of organisms with various concentrations of cyclic AMP for 45 min, after which β -galactosidase formation and [14 C]leucine incorporation were assayed. There was a linear correlation up to about 2 mM and a plateau at about 4 mM. With DNP the level of the plateau was higher and it was reached only at a higher concentration of cyclic AMP (Fig. 5).

DISCUSSION

Catabolite repression of β -galactosidase, caused by glucose in non-growing *Escherichia coli* K-12, was decreased by anaerobiosis or less effectively by the uncoupling compounds DNP and NaN_3 . Uncouplers, in contrast to anaerobiosis, interfere with the active transport

of β -galactosides (Pavlasova & Harold, 1969), and such an interference should result in a lower rate of β -galactosidase synthesis even if uncouplers release catabolite repression as efficiently as does anaerobiosis.

Since DNP had no significant effect anaerobically on the rate of β -galactosidase synthesis, when oxidative phosphorylation was already inactive (Fig. 3), the release of catabolite repression by anaerobiosis and the uncouplers must partly result from the inhibition of oxidative phosphorylation.

Catabolite repression is apparently a consequence of depletion of intracellular cyclic AMP (Makman & Sutherland, 1965; Perlman & Pastan, 1968; Chambers & Zubay, 1969; de Crombrughe *et al.* 1969; Jacquet & Kepes, 1969). The inhibitors of oxidative phosphorylation might have released catabolite repression by suppressing the depletion of intracellular cyclic AMP or by some other means. There was a linear correlation between the differential rate of β -galactosidase formation and the concentrations of cyclic AMP up to 2 mM, while higher concentrations caused little further synthesis (Fig. 5). Therefore, if the inhibitors released catabolite repression only by elevating the intracellular cyclic AMP level, the effect of a combination of any of them with 1 mM-cyclic AMP should be analogous to that of increasing amounts of cyclic AMP, i.e. equal to or lower than the sum of the separate effects of the inhibitors and the exogenous cyclic AMP. The effect of cyclic AMP under anaerobic conditions was indeed equal to the sum of its separate effect plus that of anaerobiosis, or in some cases it was lower. This does not prove that anaerobiosis increased the level of the intracellular cyclic AMP, nor does it exclude this possibility. Inhibition of oxidative phosphorylation by the uncouplers might also raise the intracellular level of cyclic AMP. However, even if this is the case, the uncouplers have some additional effect, not given by anaerobiosis, which makes the synthesis of β -galactosidase more responsive to exogenous cyclic AMP in their presence than in their absence (Fig. 2, 5). This effect was unrelated to an action on oxidative phosphorylation because, at least in the case of DNP, it occurred also anaerobically (Fig. 3). Other effects of uncouplers, which are not shown by anaerobiosis, have been reported (Galeotti, Kováč & Hess, 1968; Pavlasova & Harold, 1969).

The effect of the uncouplers also could not be due to inhibition of cyclic AMP-phosphodiesterase since DNP increased the effect of exogenous cyclic AMP in glucose-grown *Escherichia coli* K-12 which contained only small amounts of this enzyme (Fig. 4*b*). Further, if the effect of the uncouplers was due to inhibition of cyclic AMP-phosphodiesterase or if they increased the accumulation of the exogenous cyclic AMP inside the organisms by enhancing its uptake, then the uncouplers would not affect the level of the plateau in the curve of the relation between the differential rate of β -galactosidase formation and the concentration of cyclic AMP in the medium, and this plateau would be reached in the presence of the uncouplers with a lower concentration of cyclic AMP. However, with DNP the plateau level was considerably higher and it was reached with a concentration of cyclic AMP almost twice as high as that needed in the absence of DNP (Fig. 5).

It has been reported (Postgate & Hunter, 1963, 1964; Strange & Dark, 1965; Strange & Hunter, 1966; Calcott & Postgate, 1971) that carbon sources and some other substrates, which limit the growth rate of certain Gram-negative bacteria, accelerate their death when they are subsequently incubated in non-nutrient buffer. This phenomenon is known as 'substrate-accelerated death'. In most of our experiments glycerol was the limiting substrate during the growth of the bacteria. Such bacteria might, accordingly, be susceptible to glycerol-accelerated death. Both DNP and azide have been shown to protect from glycerol-accelerated death (Postgate & Hunter, 1964). It could, therefore, be argued that in the presence of the uncouplers there were more viable bacteria which could respond to cyclic AMP, thus

the stimulation of the differential rate of β -galactosidase synthesis in such a suspension is higher than in a suspension without uncouplers. However, it should be noted that during the incubation in the nitrogen-deprived medium, in our experiments, glucose replaced glycerol as a carbon source. Contradictory reports as to whether glucose induces substrate-accelerated death in glycerol-limited bacteria have been published (Postgate & Hunter, 1964; Strange & Dark, 1965). In an experiment not presented here, we assayed the colony-forming bacteria and after 2 h incubation we could not detect any significant effect on viability by glucose or by the uncouplers. This could, very possibly, be due to the presence of magnesium ions in the nitrogen deprived-medium, since Mg^{2+} had been found to prevent substrate-accelerated death (Postgate & Hunter, 1963, 1964).

The uncouplers make the synthesis of β -galactosidase more responsive to exogenous cyclic AMP by a mechanism that is not clear. However, some hypotheses can be suggested.

Unlike transient repression, the permanent catabolite repression depends on the balance between the catabolism of the carbon source and the anabolic pathways. Perhaps catabolite repression depends not only on the level of the intracellular cyclic AMP but also on the accumulation of a metabolite produced during the catabolism of the carbon source, which has a contradictory effect to that of cyclic AMP. The extent of catabolite repression might depend on the ratio between the levels of cyclic AMP and of that metabolite. Our experiments (Aboud & Burger, 1971*b*) strongly suggest that ATP plays such a role in the synthesis of β -galactosidase and that there is a contradictory relationship between this compound and cyclic AMP. Furthermore, some reports (Galeotti *et al.* 1968; Pavlasova & Harold, 1969) indicate that uncouplers, in contrast to anaerobiosis, prevent the utilization of ATP in some reactions at the substrate level. By analogy, the uncouplers might similarly prevent the action of ATP in catabolite repression so that cyclic AMP could act without the inhibitory effect of ATP. This hypothesis explains why the synthesis of β -galactosidase becomes more sensitive to cyclic AMP and responsive to higher concentrations of this nucleotide in the presence of the uncoupler.

Another possible explanation comes from evidence that β -galactosidase is formed in the cell membrane (Chefurka, Yapo & Nisman, 1970) and that uncouplers, unlike anaerobiosis, cause changes in the cell membrane (Pavlasova & Harold, 1969). Changes in the site of β -galactosidase synthesis may possibly alter the response of this process to cyclic AMP in a way described above.

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