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Adenylate Cyclase from *Brevibacterium liquefaciens*. III. *In Situ* Regulation of Adenylate Cyclase by Pyruvate

(cyclic AMP/DL-alanine/D-aminoacid oxidase)

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ABSTRACT **In the presence of DL-alanine intracellular cyclic AMP in nonproliferating cells of *Brevibacterium liquefaciens* increased rapidly to the maximum level of approximately 180 μM, and extracellular cyclic AMP increased to 100 μM within 4 hr at 25°. Adenylate cyclase (EC 4.6.1.1) induction was not observed during this incubation. The concentration of pyruvate in the total culture increased concomitantly with that of cyclic AMP and reached approximately 20 mM after 4 hr of incubation. Since the activity of cyclic nucleotide phosphodiesterase is extremely low in this bacterium, the accumulation of cyclic AMP with DL-alanine appeared to be due to the activation of adenylate cyclase by pyruvate. D-alanine was more effective than L-alanine in producing pyruvate, and a high activity of D-alanine oxidation was detected in the cell lysate of *B. liquefaciens*.**

Thus, adenylate cyclase in this bacterium appeared to be regulated *in vivo* by pyruvate which was formed, in this case, predominantly from D-alanine through the action of D-aminoacid oxidase (EC 1.4.3.3). Pyruvate, added extracellularly, also caused a rapid accumulation of intracellular cyclic AMP. Glucose did not change the level of cyclic AMP significantly. It also did not affect the intracellular accumulation of cyclic AMP with DL-alanine.

In 1963 Okabayashi *et al.* (1) reported the occurrence of cyclic AMP in the culture broth of *Brevibacterium liquefaciens*, a Gram-positive bacterium. They found that the bacterium accumulated as high as 3 mM of this nucleotide when cultured in the presence of glucose and DL-alanine (2–4). It was suggested that the level of cyclic AMP might be affected by the composition of the culture media, but the mechanism of regulation of adenylate cyclase has not been studied further.

Adenylate cyclase (EC 4.6.1.1) from *B. liquefaciens* was partially purified by Hirata and Hayaishi (5), and later purified to a crystalline form by Takai *et al.* (6). A unique characteristic of this enzyme is that it is strongly activated in the presence of pyruvate *in vitro*.

In this communication we investigated whether the increase of cyclic AMP with DL-alanine and glucose is due to the induction of adenylate cyclase or to the activation of the enzyme by pyruvate *in vivo*. Available evidence indicates that the total amount of enzyme protein remains essentially constant but the activity is enhanced by the intracellular accumulation of pyruvate.

MATERIALS AND METHODS

Cyclic [^{3}H]AMP (16.3 Ci/mmole) and [^{14}C]ATP were purchased from Schwarz BioResearch. Cyclic AMP and rabbit muscle lactate dehydrogenase were obtained from Boehringer Mannheim, Japan. Pyruvic acid was from Nakarai Chemicals (Japan).

Brevibacterium liquefaciens (American Type Culture Collection 14929) was grown in the presence of glucose with DL-alanine as the nitrogen source as described by Hirata and Hayaishi (5), unless otherwise indicated. Cells were harvested in their post-exponential phase by centrifugation, washed with saline, and resuspended in a salt medium (pH 7) containing 7 g of K_2HPO_4, 3 g of KH_2PO_4, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter (*Medium A*). The density of the cells in the suspension was between 3 and 5 g of wet-packed cells per 100 ml of medium. The incubation was carried out at 25° with or without supplements under continuous shaking in a water bath. The number of cells did not significantly increase under the experimental conditions employed. Cells were separated from the medium by filtration through a nitrocellulose filter (0.3 μm pore size, Sartorius Membranefilter, Germany).

For the determination of cyclic AMP, samples were acidified in 0.05 M HCl, heated at 100° for 3 min, and, after neutralization, assayed for cyclic AMP according to the method of Gilman (7). Pyruvate was determined by an enzymatic method (8), in which samples were treated by heating at 100° for 3 min. For the extraction of the adenylate cyclase activity to be determined, cells were centrifuged at 4°, washed with and then suspended in distilled water, and then dispersed in 5 volumes of 0.8 M sucrose containing 0.05 M Tris·HCl (pH 8) and 5 mM EDTA. After 20 min of preincubation at 25°, lysozyme was added to give a final concentration of 0.4 mg/ml. The lysis of the cells was checked turbidimetrically at 650 nm upon 200-fold dilution with water and it usually reached completion within 30–40 min. An aliquot of the lysate was then assayed for adenylate cyclase activity as described (5) except that NaF was omitted and that bovine serum albumin was added to a final concentration of 100 μg/ml and dithiothreitol to 1 mM. D-aminoacid oxidase (EC 1.4.3.3) activity was assayed at 25° in 50 mM potassium phosphate buffer (pH 7.2) containing 0.03 M KCN with D-alanine (50 mM) as substrate and dichloroindophenol as hydrogen acceptor. The decrease in absorption at 590 nm was followed. The formation of pyruvate was followed over the same time course and under the same conditions, except that KCN was omitted.

RESULTS

In the presence of DL-alanine and glucose the intracellular concentration of cyclic AMP increased rapidly from the basal level, and reached a maximum level of approximately 180 μM after 4 hr (Fig. 1A). In the presence of DL-alanine alone the

Abbreviation: cyclic AMP, cyclic adenosine 3′:5′-monophosphate.

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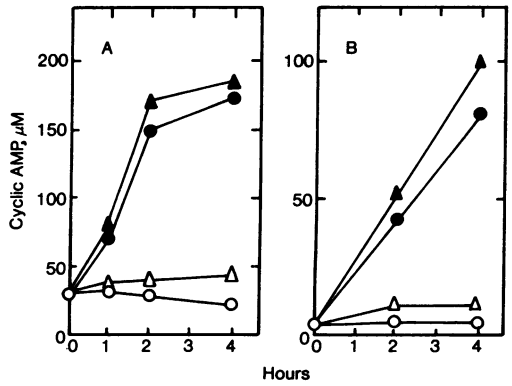

FIG. 1. Intra- and extracellular accumulation of cyclic AMP. Nonproliferating cells (5%, w/v) of *B. liquefaciens* were incubated in *Medium A* supplemented with nothing (○), 2% (w/v) glucose (△), 2% DL-alanine (●), or 2% glucose and 2% DL-alanine (▲). At time intervals aliquots were withdrawn and intracellular (A) and extracellular (B) concentrations of cyclic AMP were determined as described under *Materials and Methods*. For the concentration of intracellular cyclic AMP, 1 g of the wet packed cells is assumed to be 1 ml. The cell growth during incubation was negligible.

intracellular concentration of cyclic AMP increased in the same manner to the maximum level of 170 μM. The effect of glucose was very small. With glucose alone, the level of cyclic AMP increased only slightly.

The extracellular concentration of cyclic AMP increased with time and the concentration after 4 hr was 100 μM in the presence of DL-alanine and glucose (Fig. 1B). With DL-alanine alone the increase of extracellular cyclic AMP was similar but the level was slightly lower. Glucose alone did not affect the extracellular level of cyclic AMP significantly.

These results might be simply explained either by the elevated concentration of some activator of adenylate cyclase or by the induction of the enzyme, caused by the presence of DL-alanine. A high concentration (0.2 M) of either DL-, D-, or L-alanine activates this bacterial adenylate cyclase *in vitro* about 2-fold and 20-fold, at pH 7.3 and pH 9.0, respectively, but, taking account of the fact that the pH of the suspension remained constant around 7 in the present case, the direct activation of adenylate cyclase by alanine is not

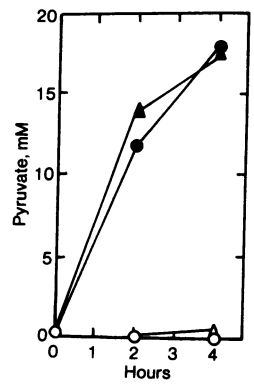

FIG. 2. Formation of pyruvate *in vivo*. Experimental conditions for incubation of the cells are the same as described in the legend to Fig. 1. At time intervals aliquots were withdawn and assayed for pyruvate in the total culture as described under *Materials and Methods*. (○), No supplement; (△), 2% glucose; (●), 2% DL-alanine; (▲), 2% glucose and 2% DL-alanine.

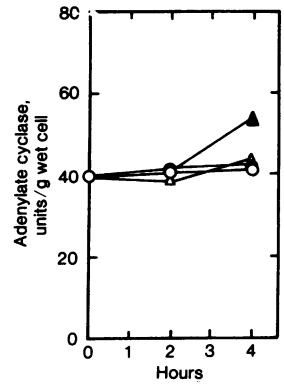

FIG. 3. Activity of adenylate cyclase. Experimental conditions for incubation of the cells are the same as described in the legend to Fig. 1. At time intervals aliquots of the suspension were withdrawn, lysed with lysozyme, and assayed for adenylate cyclase activity in the presence of 10 mM pyruvate as described under *Materials and Methods*. (○), No supplement; (△), 2% glucose; (●), 2% DL-alanine; (▲), 2% glucose and 2% DL-alanine.

sufficient to explain the rapid accumulation of cyclic AMP. When the cell suspension was assayed for pyruvate, a potent activator of the adenylate cyclase of *B. liquefaciens*, a large quantity of pyruvate was shown to be formed in the presence of DL-alanine or DL-alanine and glucose (Fig. 2).

The pyruvate concentration in the total culture reached 10–15 mM after 2 hr and nearly 20 mM after 4 hr. There was not a big difference between intra- and extracellular concentrations. No detectable amount of pyruvate was formed when *B. liquefaciens* was incubated with glucose alone.

To investigate the possibility of induction of enzyme, we assayed the amount of adenylate cyclase as a function of time of incubation. An aliquot of the cell suspension was withdrawn, lysed by lysozyme, and assayed for the full activity of the enzyme in the presence of pyruvate. During 4 hr of incubation the total activity of the enzyme did not change greatly in the presence of DL-alanine, glucose, or a combination of both in the incubation media (Fig. 3). With DL-alanine

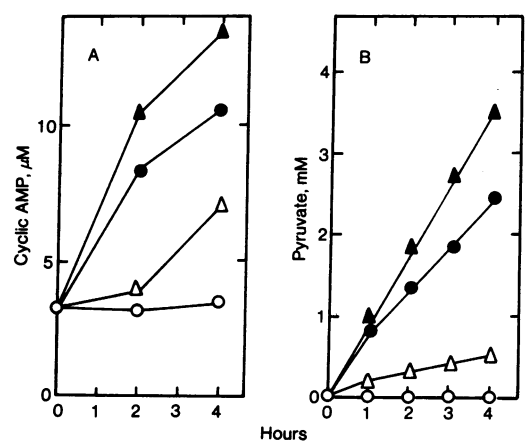

FIG. 4. Effect of DL-, D-, and L-alanine on the accumulation of cyclic AMP (A) and pyruvate (B). Nonproliferating cells (3%, w/v) of *B. liquefaciens* were incubated in *Medium A* supplemented with nothing (○), 2% (w/v) L-alanine (△), 2% D-alanine (●), or 2% DL-alanine (▲). At time intervals aliquots were withdrawn and the concentrations of cyclic AMP and pyruvate in the total culture were determined as described under *Materials and Methods*.

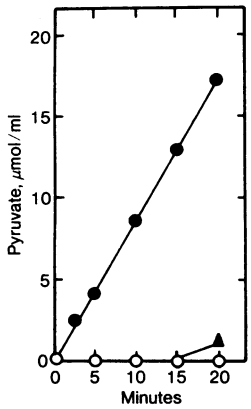

FIG. 5. Formation of pyruvate from D- or L-alanine *in vitro*. Cell lysate was prepared from DL-alanine-grown cells and assayed for the formation of pyruvate *in vitro* with D- or L-alanine (50 mM) as substrates as described under *Materials and Methods*. (○), No substrate; (●), D-alanine; (▲), L-alanine. Pyruvate formation was expressed as μmole/ml of lysate.

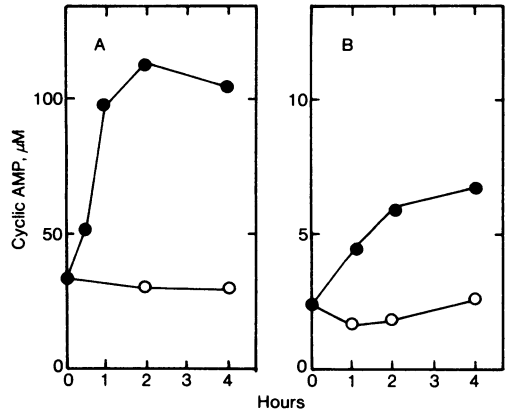

FIG. 6. Effect of pyruvate on the intracellular (A) and extracellular (B) concentrations of cyclic AMP. Nonproliferating cells (5% w/v) of *B. liquefaciens* were incubated in *Medium A* supplemented with no pyruvate (○), or 20 mM pyruvate (●). At intervals aliquots were processed and assayed for cyclic AMP as described under *Materials and Methods*.

and glucose together the enzyme activity increased slightly, but it was not enough to explan the remarkable increase of cyclic AMP.

The concentration of ATP in the total culture was investigated during the incubation by means of luciferase (9). It was found to be about 8 μM and did not change significantly during the incubation.

To determine which of the optical isomers of alanine is responsible for these phenomena, we investigated the effect of DL-, D-, or L-alanine on the accumulation of cyclic AMP and pyruvate. Cyclic AMP was most effectively accumulated in the total culture with DL-alanine; D-alanine increased the formation to a slightly lesser but comparable extent. L-alanine appeared to be much less effective in the formation of cyclic AMP (Fig. 4A). The effect of these additions on the formation of pyruvate in the total culture was similar to the effect on cyclic AMP formation (Fig. 4B). DL-alanine was the most effective and D-alanine was more effective than L-alanine.

The formation of pyruvate from alanine was investigated *in vitro* employing a cell lysate prepared with lysozyme. As shown in Fig. 5, a considerable amount of pyruvate was formed *in vitro* when D-alanine was employed as substrate. The rate of formation was calculated to be 4.8 μmoles/min per g of wet packed cells at 25°. The formation of pyruvate appears to be catalyzed by D-aminoacid oxidase. The enzyme activity was assayed by the reduction of dichloroindophenol with D-alanine or D-valine as substrate. The rate of reduction was nearly equal to that calculated from the formation of pyruvate. D-valine served as substrate as well as D-alanine but L-alanine, D-serine and D-leucine did not. D-alanine-dependent oxygen consumption was observed, and its rate corresponded to the value indicated above in rough estimate.

To confirm the effect of pyruvate on the accumulation of cyclic AMP, we added pyruvate to the culture medium and measured intra- and extracellular concentrations of cyclic AMP. In the presence of 20 mM pyruvate cyclic AMP was accumulated rapidly in the cell to 110 μM in 2 hr (Fig. 6A), while the level of extracellular cyclic AMP increased only slightly to 6 μM (Fig. 6B). Pyruvate added in the culture was metabolized by *B. liquefaciens*, and the remaining concentration after 2 hr was 3 mM. The total activity of adenylate cyclase was unchanged, and the cell growth was negligible.

DISCUSSION

The total amount of adenylate cyclase did not change significantly in the course of the experiment. Thus, the accumulation of cyclic AMP in the presence of alanine must be explained by the effect of pyruvate on the enzyme. Rapid accumulation of pyruvate was observed in the cell suspension when the bacteria were incubated with DL-alanine. The activation by pyruvate *in vivo* was supported by the observation that pyruvate added to the medium raised the level of cyclic AMP. Though glucose was reported to lower the level of cyclic AMP in *Escherichia coli* (10), it did not greatly change the accumulation or excretion of cyclic AMP in *B. liquefaciens*. The level of cyclic AMP was increased slightly in the presence of glucose. Pyruvate, which occupies a central position in the metabolism of carbohydrate, can arise directly from alanine. The formation of pyruvate from D-alanine by D-aminoacid oxidase was shown in *B. liquefaciens* that was grown with glucose and DL-alanine.

The possibility that cyclic nucleotide phosphodiesterase is involved in the regulation of the level of cyclic AMP in *B. liquefaciens* cannot be rigorously ruled out. However, the ratio of the activity of cyclic nucleotide phosphodiesterase to that of adenylate cyclase in *B. liquefaciens* is about two hundred times smaller than the value in *E. coli* (6, 11). Therefore, it seems unlikely that the rapid increase of cyclic AMP is caused by the decrease or inactivation of cyclic nucleotide phosphodiesterase.

It has been observed that *B. liquefaciens* grown in the presence of DL-alanine has a high activity of D-aminoacid oxidase but the cells grown in the presence of L-asparagine or NH_4Cl as a nitrogen source have only a negligible amount of activity†. Thus, it appears that in *B. liquefaciens* D-aminoacid oxidase is induced by the presence of DL-alanine. The oxidase acts on D-alanine to form pyruvate, which in turn activates adenylate cyclase to increase the level of cyclic AMP. However, the role of cyclic AMP here is still unknown.

† S. Tsuji, unpublished results.

The activation of adenylate cyclase by pyruvate seems not specific for *B. liquefaciens*. It has been reported that adenylate cyclase activity in crude extracts of certain other bacteria as *Micrococcus lysodeikticus*, *Micrococcus flavus*, *Arthrobacter globiformis*, and *Arthrobacter citreus* was effectively increased by pyruvate (12). Recently Kurashina has purified adenylate cyclase 2000-fold from *M. lysodeikticus*, using an affinity column coupled with an antibody against adenylate cyclase of *B. liquefaciens*. With the highly purified preparation it has been shown that pyruvate was essential for the enzyme activity‡. The physiological role of cyclic AMP and pyruvate-dependent adenylate cyclase in *B. liquefaciens* as well as in those micro-organisms is to be investigated.

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‡ Y. Kurashina, unpublished results.

1. Okabayashi, T., Ide, M. & Yoshimoto, A. (1963) *J. Bacteriol.* **86**, 930–936.
2. Okabayashi, T., Ide, M. & Yoshimoto, A. (1963) *Arch. Biochem. Biophys.* **100**, 158–159.
3. Okabayashi, T., Ide, M. & Yoshimoto, A. (1964) *Amino Acid Nucleic Acid (Tokyo)* **10**, 117–123.
4. Ide, M., Yoshimoto, A. & Okabayashi, T. (1967) *J. Bacteriol.* **94**, 317–322.
5. Hirata, M. & Hayaishi, O. (1967) *Biochim. Biophys. Acta* **149**, 1–11.
6. Takai, K., Kurashina, Y., Suzuki-Hori, C., Okamoto, H. & Hayaishi, O. (1974) *J. Biol. Chem.* **249**, 1965–1972.
7. Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 305–312.
8. Buecher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) in *Methods of Enzymatic Analysis*, ed. Bergmeyer, H. U. (Academic Press, New York), pp. 253–259.
9. Stanley, P. E. & Williams, S. G. (1969) *Anal. Biochem.* **29**, 381–392.
10. Makman, R. S. & Sutherland, E. W. (1965) *J. Biol. Chem.* **240**, 1309–1314.
11. Peterkofsky, A. & Gazdar, C. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2794–2798.
12. Ide, M. (1971) *Arch. Biochem. Biophys.* **144**, 262–268.