ADENYLATE CYCLASE OF *BREVIBACTERIUM LIQUEFACIENS*

Inactivation by Neuraminidase, Phospholipase A and Phospholipase C

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SUMMARY

A soluble adenylate cyclase of *Brevibacterium liquefaciens* was stimulated by DL-lactate as well as pyruvate. The purified enzyme stained positive with the periodate-Schiff reagent. Activation by the two metabolites was rapid and reversible, and was abolished by a preliminary incubation with neuraminidase, phospholipase C or phospholipase A. Neuraminidase and phospholipase C destroyed the response to pyruvate and lactate with similar kinetics, whereas phospholipase A affected the lactate response more than the pyruvate response. Loss of adenylate cyclase activity was not due to inhibition caused by products released by the three enzymes; neither was adenylate cyclase activity restored by the addition of sialic acid nor phosphoglycerides. These results suggest that adenylate cyclase activity is associated with a lipo-glycoprotein and that the enzyme may have been detached from the bacterial cell membrane.

INTRODUCTION

Studies of adenylate cyclase partially purified from *Escherichia coli* (1-3), *Streptococcus salivarius* (4), *Saccharomyces fragilis* (5) and *Brevibacterium liquefaciens* (6,7) have been reported. The enzyme from *B. liquefaciens* is of particular interest because it is soluble and highly active, and requires pyruvate in addition to Mg^{++} for maximum activity. In the absence of added pyruvate the purified enzyme is essentially inactive.

We report here that adenylate cyclase from *B. liquefaciens* is also activated by DL-lactate, that the activation by pyruvate or lactate is rapid and reversible, and that the response to these metabolites is destroyed by a preliminary incubation with neuraminidase, phospholipase A or phospholipase C.

MATERIALS AND METHODS

Purification and Assay of Adenylate Cyclase: Adenylate cyclase was purified to the step of A-50 anionic exchange column chromatography; the assay was essentially the same as described previously (8). Briefly, the reaction mixture of 0.1 ml contained 2 mM [^{14}C]-ATP (sp. act. 85 mμCi/μmole), 100 mM Tris-Cl, pH 9.0, 20 mM Mg^{++}, 20 mM pyruvate or 40 mM DL-lactate and an appropriate amount of enzyme. After 30 min at 30°, the reaction was

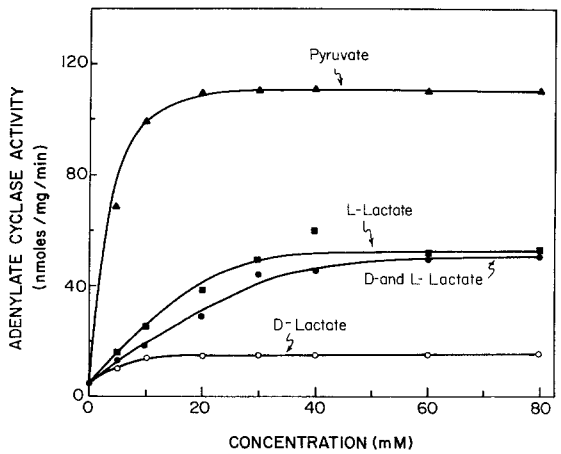

Figure 1. Dose response of adenylate cyclase to pyruvate, L-lactate, D-lactate and DL-lactate. The final concentrations of pyruvate and lactate are indicated along the abscissa. The amount of enzyme used in this experiment was 15 μg. Other components of the reaction mixture were identical to the conditions described in the text.

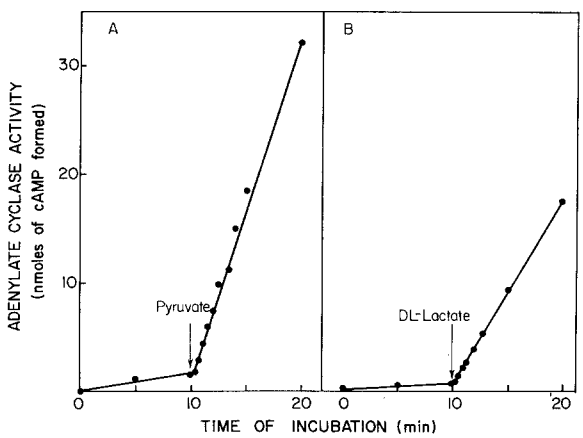

Figure 2 (A and B). Activation of adenylate cyclase by pyruvate and DL-lactate. The reaction mixture of 1.5 ml contained the usual components and 250 μg protein. A fraction was removed at various times. The arrow indicated the time of addition of pyruvate (A) or lactate (B).

terminated by boiling for 2 min. Cyclic AMP was separated from ATP by paper chromatography and the radioactivity determined in a liquid scintillation counter.

Protein Determination: Protein was determined in the homogenate according to Gornall *et al.* (9) with bovine serum albumin as a standard. Protein concentration in the purified samples was estimated by the spectrophotometric technique of Warburg and Christian (10).

Chemicals and Enzymes: [^{14}C]-ATP, [^{14}C]-pyruvate, ATP, cyclic AMP, and

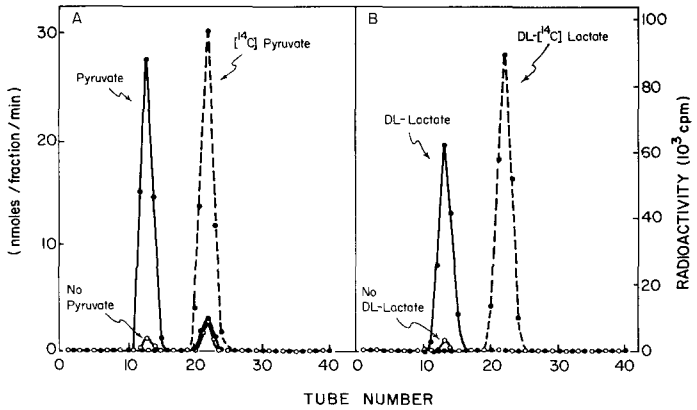

Figure 3 (A and B). Reversible removal of pyruvate or lactate from adenylate cyclase. Adenylate cyclase (1.5 mg) was incubated in 1 ml of a reaction mixture containing 20 mM DL-[^{14}C]-pyruvate (1 μCi); 100 mM Tris-Cl, pH 9.0, and 20 mM Mg^{++} at 30° for 30 min. The mixture was then transferred to a Sephadex G-25 column (0.9 X 60 cm) maintained at 4°. The column was eluted with 100 mM Tris-Cl, pH 8.3. Fractions of 1.4 ml were collected and assayed for adenylate cyclase activity in the presence or absence of exogenous pyruvate and for radioactivity. The experiment in Fig. 3B was done under identical conditions, except that 40 mM [^{14}C]-DL-lactate (1 μCi) was used instead of [^{14}C]-pyruvate. Adenylate cyclase from the column was assayed in the presence or absence of an exogenous DL-lactate.

neuraminidase were purchased from Schwartz BioResearch. DL-[^{14}C]-lactate was obtained from New England Nuclear, phospholipase A and phospholipase C from Calbiochem, and bovine serum albumin free of fatty acids from Sigma. Reagents were used without further purification.

RESULTS AND DISCUSSION

Hirata and Hayaishi (6) first reported that the activity of adenylate cyclase from *B. liquefaciens* depended greatly on pyruvate. Other *a*-ketoacids were stimulatory, but to a lesser extent. We now find that another closely related metabolite, L-lactate, also stimulates the bacterial enzyme. Figure 1 depicts the response of adenylate cyclase to different concentrations of pyruvate and lactate. Half-maximum activation was obtained with 3 mM pyruvate or 10 mM L-lactate. L-lactate produced about 50% as much stimulation as pyruvate did, and D-lactate about 10%. The combined effect of suboptimum concentrations of a mixture of D- and L-lactate was essentially additive. At optimum concentrations, the stimulation obtained by the DL-mixture was comparable to that by the L-isomer alone. In contrast, adenylate cyclase activity in the presence of a mixture of pyruvate and DL-lactate was invariably less than that of pyruvate alone (Table 1). This might mean that the two modulators compete for the same effector site.

Activation of adenylate cyclase by pyruvate or by DL-lactate was rapid; there was no

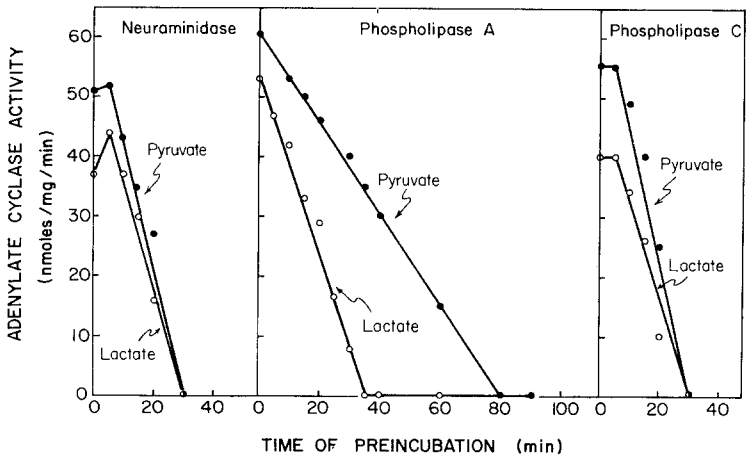

Figure 4. Effect of neuraminidase, phospholipase A and phospholipase C on the response of adenylate cyclase to pyruvate or DL-lactate. Adenylate cyclase (700 μg) was incubated at 30° in a reaction mixture of 1 ml containing 20 mM Tris-Cl, pH 7.0, 2 mM $CaCl_2$ and neuraminidase (1 μg), phospholipase A (100 μg) or phospholipase C (100 μg). A fraction was withdrawn at various times for assay of adenylate cyclase activity in the presence of 20 mM pyruvate or 40 mM DL-lactate. The activity of untreated adenylate cyclase was 130 and 105 nmoles/mg/min in the presence of 20 mM pyruvate and 40 mM of DL-lactate, respectively.

detectable time lag after the addition of pyruvate or lactate to the reaction mixture (Fig. 2; also see ref. 6).

To determine whether activation of adenylate cyclase by pyruvate was a reversible process, we incubated the enzyme with [^{14}C]-pyruvate at 30° for 30 min. The incubation mixture was then passed through a Sephadex G-25 column, which separated the enzyme from pyruvate (Fig. 3A). The enzyme, eluted from the column free of pyruvate, was essentially inactive unless exogenous pyruvate was added to the reaction mixture. This indicated that the effect of pyruvate was readily reversible. Lactate had a similar effect (Fig. 3B).

The response of the bacterial enzyme to pyruvate and lactate could be destroyed by prior incubation with neuraminidase, phospholipase A or phospholipase C. The kinetics of adenylate cyclase inactivation by neuraminidase or phospholipase C were similar with respect to the response of pyruvate and lactate (Fig. 4). Phospholipase A affected the response to lactate faster than that to pyruvate. At a time when the activity with lactate was abolished, about half the activity with pyruvate survived the treatment. The remaining activity was lost after prolonged incubation with the enzyme. After treatment with neuraminidase, phospholipase A or phospholipase C, the activity of adenylate cyclase could not be reconstituted with the addition of sialic acid, phosphatidyl serine, phosphatidyl ethanolamine, phosphatidic acid, cardiolipin, or phosphatidyl choline.

Neuraminidase catalyzes the hydrolysis of glycoproteins to yield sialic acids.

Table 1. The Responsiveness of Adenylate Cyclase to Pyruvate and DL-Lactate

Addition (mM)	Specific Activity (nmole/mg/min)
None	4.2
Pyruvate (10)	134
Pyruvate (20)	140
Pyruvate (40)	141
DL-Lactate (20)	41
DL-Lactate (40)	115
DL-Lactate (60)	116
Pyruvate (10) + DL-Lactate (20)	114
Pyruvate (20) + DL-Lactate (40)	123

The amount of adenylate cyclase used in this experiment was 13 μg. Other components of the reaction mixture are described in the text.

Phospholipase A catalyzes the release of fatty acids (such as oleic and palmitic acids) from carbon 2, whereas phospholipase C catalyzes the release of a phosphoryl derivative from carbon 3 of the glycerol moiety. Loss of adenylate cyclase activity due to inhibition by these products appeared unlikely. First, the activity of adenylate cyclase was not inhibited in the presence of 2 uM each of oleic acid, palmitic acid, or *o*-phosphorylethanolamine. Second, the inhibition by phospholipase A was not affected when bovine serum albumin was added to the reaction mixture. Bovine serum albumin would have complexed with free fatty acids released by phospholipase A and would have overcome the inhibition.

The concentrations of phospholipase A and phospholipase C used in the experiments were high relative to that of adenylate cyclase. Inactivation could have been caused by a trace contamination of proteolytic enzymes in these commercial preparations. However, using ^{125}I-labeled bovine serum albumin as a substrate, we did not detect any proteolytic activities in the reagents.

Adenylate cyclase of *B. liquefaciens* stained positive with the periodate-Schiff reagent (11), indicating it was a glycoprotein. The fact that adenylate cyclase activity was lost after treatment with phospholipases suggested that phospholipids were required for enzymic activity and that this activity was associated with a lipo-glycoprotein. Although the enzyme appears

soluble, it may have been dissociated from the cell membrane as a result of sonication, which was the initial step in the purification process.

The loss of activity of the bacterial enzyme upon treatment with phospholipases is reminiscent of the finding of Rubalcava and Rodbell (12) who observed that adenylate cyclase of rat liver plasma membranes lost its response to glucagon when treated with phospholipase C, and that the loss of response increased with progressive hydrolysis of phospholipids in the membrane preparation.

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