SELECTIVE RELEASE OF RIBONUCLEASE-INHIBITOR FROM BACILLUS SUBTILIS CELLS BY COLD SHOCK TREATMENT

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B. subtilis cells secrete a ribonuclease into the external medium during the stationary phase of growth (Nishimura and Nomura, 1959). Smeaton, Elliott and Coleman (1965) have shown that in homogenates of ribonuclease secreting B. subtilis cells there is an inhibitor which apparently combines tightly with the enzyme. The inhibitor, which is completely specific for the B. subtilis ribonuclease and is found only in cells which have reached the stage of ribonuclease secretion, has the properties of a protein of low molecular weight (approx. 12,000). A procedure has now been found for altering the permeability of the cells by 'cold shock' treatment which permits release of the inhibitor without general cell lysis. Features of the cold shock phenomenon are also described.

EXPERIMENTAL

B. subtilis (Takamine strain)** cultures grown for 25 hr (Coleman and Elliott, 1965) were harvested and the cells washed in 0.05 M tris-HCl buffer, pH 8.0 at 30°.

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The cold shock treatment consisted of squirting thick suspensions of washed cells from a syringe into 20 volumes of stirred buffer at 0^{o} (or other selected temperature).

Fluorescence measurements were carried out with the fluorescen attachment on an Eppendorf spectrophotometer (Netheler and Hinz, GmbH, Hamburg). A 366 mu primary filter was used and fluorescence measured from 420 - 3,000 mu. The samples upon which fluorescence measurements were made contained 1.5 mg dry weight of cells, and N-tolyl- α-naphthylamine-8-sulphonic acid (2.5×10^{-4} M) in 2 ml of 1% ($\frac{w}{v}$) NaCl adjusted to pH 7 with Na HCO_3 (Newton, 1954). Under these conditions dye concentration was not limiting in fluorescence production.

Bacterial homogenates were prepared using a French pressure cell. Ribonuclease was prepared and assayed as described previously (Smeaton, Elliott and Coleman, 1965). In all assays for inhibitor, the preparation and enzyme were mixed in buffer and the reaction started by addition of RNA.

RESULTS

When extracellular ribonuclease is mixed with normal B. subtili cells the enzyme is not affected; this is as expected since such cells produce the enzyme. However, if the cells are first cold shocked, such suspensions progressively inactivate ribonuclease, the process reaching a maximum in 30 min at 25^{o} (Fig. 1). The amoun of enzyme inactivated corresponds to the total amount of inhibitor present in the cells (as measured in disrupted preparations) and suggests that all of the inhibitor becomes available for reaction with externally added ribonuclease. In addition to exposing the inhibitor to external RNAse, cold shock treatment allows release int

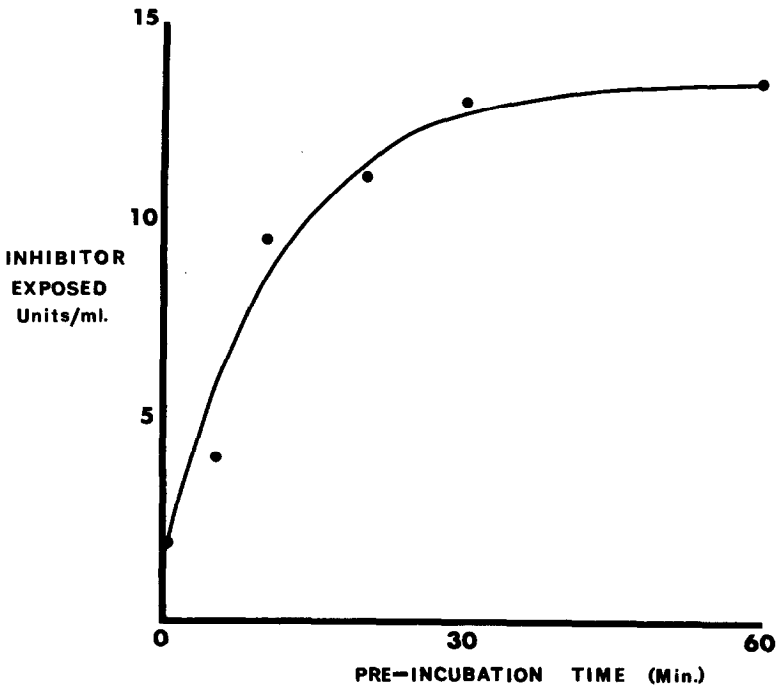

Fig. 1. Effect of cold shock on interaction of intact B. subtilis cells with external RNAse. Cold shocked cells were incubated at 25° with B. subtilis ribonuclease. Assays were started, at varying intervals, by the addition of substrate to this mixture and the amount of enzymic activity remaining was determined.

solution of up to 90% of the inhibitor when the cells are subsequently incubated in buffer either at 0° or 25°. This process is slower than the exposure to external enzyme and requires 4 hr for completion (Fig. 2). The release of inhibitor is relatively selective, being accommpanied by a loss from the cell of less than 4% of the total protein.

Somewhat surprisingly, the requirements for the cold shock effect proved to be extremely precise. The critical temperature zone through which the cells must be cooled to obtain the cold shock effect is remarkably well-defined at 16-14° (Fig. 3). 'Instantaneous' cooling is essential; when suspensions were cooled by swirling

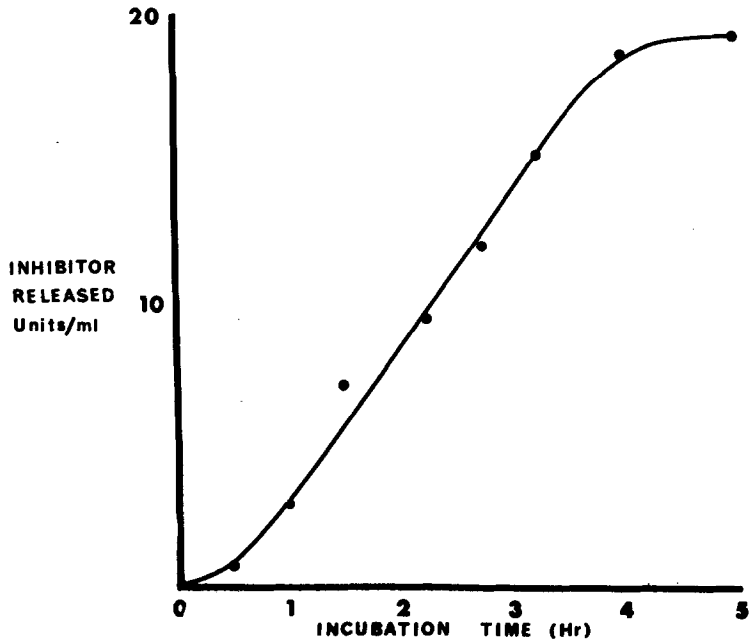

Fig. 2. Release of inhibitor from cells following cold shock. The shocked cell suspension was left at 0° with occasional stirring. Samples were taken at intervals, and following removal of the cells by centrifugation the supernatants assayed for released inhibitor. The total inhibitor present in a cell homogenate was 21 units/ml.

a flask in ice-water (under which conditions a temperature of 11° was reached in 1 min and 1° in 4 min) the cells were unaffected in the sense that no exposure or release of inhibitor occurred on subsequent incubation.

The effect of the treatment is to increase the permeability of the cell. This is indicated by studies with N-tolyl- α -naphthy lamine-8-sulphonic acid, a dye which fluoresces when combined with protein (Newton, 1954). Cold shocked cells induce as much fluorescence as completely disrupted preparations (Table I) showing that the dye can completely penetrate the cell, in contrast to normal cells which yield very little fluorescence when exposed to the dye.

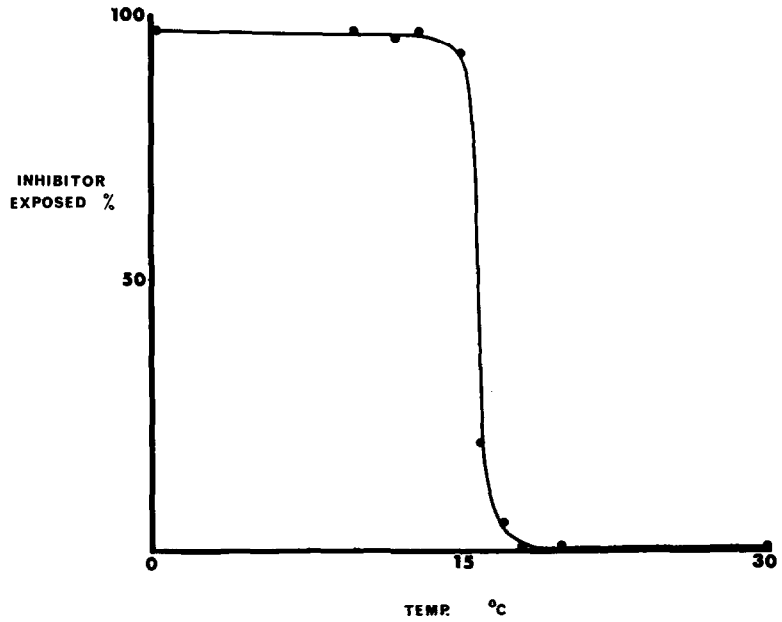

Fig. 3. The temperature change requirement for the cold shock effect. Suspensions of cells at 30^{0} were injected into 20 volumes of buffer held at the temperature indicated. The temperature rise during the addition was less than 0.5^{0}. Samples of the suspensions were then incubated with ribonuclease as described in Fig. 1 for 60 min to allow maximum inhibition of the enzyme. The enzyme remaining was then assayed.

It has also been found that cold shocked cells have almost completely lost the ability to incorporate C^{14}-valine into protein and to synthesise α-amylase. Log phase B. subtilis cells showed the same loss of protein synthesising ability on cold shocking. Viability studies were not possible because of clumping of the cells. However, it has been shown previously that sudden chilling can cause loss of viability in E. coli and aerobacter aerogenes (Meynell, 1958; Strange and Dark, 1962). In the latter case, viability loss was increased if the chilling medium contained added RNAse (Strange and Postgate, 1964).

TABLE I

Sample	Emitted Light 420 - 3000 mu (Units)
Reference cell no. 6732	1
Control cells	1.1
Shocked cells	3.1
Disrupted cells	3.07

Table I. The fluorescence developed by cold shocked cells in the presence of N-tolyl- α-naphthylamine-8-sulphonic acid compared to that of comparable amounts of untreated and French pressure cell disrupted cells. The conditions of measurement were as described in the text and the values have been normalised to compare with the standard reference cell.

DISCUSSION

The cold shock treatment provides a simple method for obtaining the RNAse inhibitor in relatively pure form and has been used successfully as the first step in purifying this material (Smeaton and Elliott, unpublished). The method might well be extendable to the preparation of other low molecular weight components of bacterial cells. A considerable amount of 260 mu absorbing material was released along with the inhibitor; Strange and Dark (1962) first found that amino acids, peptides and ATP were released by cold shocked cells of Aerobacter aerogenes.

It seems likely from the fluorescence studies that the cold shock treatment of B. subtilis cells leads to release of the inhibitor due to a general increase in permeability of the cells. This is in contrast to the effect on E. coli of osmotic shock whic apparently specifically releases superficially located hydrolytic enzymes (Nossal and Heppel, 1966, Neu and Heppel, 1965).

Although the dependence of the cold shock effect on rapid cooling has been reported previously (Sherman and Cameron, 1934; Meynell, 1958) the existence of such a narrowly defined critical temperature zone has not apparently been previously observed. The fact that the critical temperature zone is so high (16-14°) is somewhat surprising and suggests that it would be extremely easy to cold shock B. subtilis cells inadvertently by procedures such as washing a cell pellet with chilled buffer. Such cold shock treatment although almost completely destroying the capacity for protein synthesis, nevertheless leaves the cells unchanged in physical appearance either by gram stain examination or electron microscopy of sections. Meynell (1958) also found no change in electron micrographs of cold shocked E. coli.

Strange and Postgate (1964) have reported that chilling of A. aerogenes renders the cells sensitive to irreversible destruction by RNAse. This might suggest that inhibitor formation by B. subtilis is a mechanism to protect the cell against extracellular RNAse diffusing back into the cell possibly due to transitory impairment of membrane permeability characteristics.

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