THE RELEASE OF ENDONUCLEASE I FROM ESCHERICHIA COLI BY A NEW COLD SHOCK PROCEDURE

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Summary

A new cold shock procedure has been developed for releasing large quantities of endonuclease I from E.coli, which neither involves EDTA-lysozyme treatment nor osmotic shock. Treatment of cells with ice-cold 0.1M Tris-0.2M KCl buffer, pH 7.4 results in the release of endonuclease I into the medium. Although the loss of endonuclease I from the cells is a rapid process, its recovery in the shock fluid is gradual and approaches maximum in about 90 minutes. Certain divalent metal ions such as Mg^{++} and Mn^{++} strongly inhibit the release of endonuclease I. The cold shock procedure is rather selective and the mechanism of the release of endonuclease I is different from that of osmotic shock procedure.

Malamy and Horecker (1) first observed the quantitative release of alkaline phosphatase into the medium when E.coli cells were converted into spheroplasts by EDTA-lysozyme treatment. Since then, a number of other periplasmic enzymes have been reported to be selectively released into the medium by this method (2-5) and by osmotic shock (6-8).

The endonuclease I (EC 3.1.4.5) of E.coli discovered by Lehman et al (9) is an RNA-inhibitable, Mg^{++}-activated enzyme thought to be located in the periplasmic region (5). During the course of investigations on the turnover of endonuclease I in E.coli by inhibition of protein synthesis, an unusual phenomenon was noticed (see results) which led to the development of a new and simple method for releasing large amounts of endonuclease I into the medium. The unique features and kinetics of the release

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of endonuclease I by this new cold shock procedure are described in this communication.

Experimental

Organism. E.coli B was used in all the experiments.
Growth and Media. The medium contained per liter, 10g yeast extract (Difco), 11g K_2HPO_4, 8.5g KH_2PO_4 and 10g glucose (10). In some experiments, glucose was replaced by lactose to induce beta-galactosidase. The growth was monitored by measuring the absorbance of the culture at 650nm in a Gilford spectrophotometer.
Cold shock procedure. The culture was harvested at an A_{650} of approximately 0.8 by centrifuging 10ml samples in glass centrifuge tubes at room temperature. The tubes were wiped with tissue to remove as much of the medium as possible, and the cells were subjected to "cold shock" by rapidly resuspending in 10ml of ice-cold 0.1M Tris-0.2M KCl buffer, pH 7.4* and kept in an ice-bath at 0°C with occational shaking. At various time intervals, the tubes were centrifuged in cold and the supernatant, referred to as "shock fluid" was collected. The pellet was again resuspended in 10ml of 0.025M Tris-KCl buffer and sonicated in cold for 2 minutes (designated as "cellular"). For a control, the cells were centrifuged, resuspended in 0.025M Tris-KCl buffer at room temperature and sonicated in cold.
Enzyme assays. Endonuclease I was assayed in shock fluid, cellular and control cell extract after incubating at 37°C with 20 μg of RNase for 30 minutes to destroy the inhibitory RNA.

The reaction mixture (1ml) containing 350 μg calf thymus DNA, 75 μmoles Tris pH 7.4, 45 μmoles $MgCl_2$ and 50 μmoles NaCl was mixed with 0.5ml of enzyme (pretreated with RNase) and incubated at 37°C for 30 minutes. The reaction was stopped by adding 1ml of 1N perchloric acid. After 10 minutes in ice, the tubes were centrifuged and 1ml of the clear supernatant was withdrawn for the determination of acid-soluble deoxyribonucleotides by Burton's method (11). One unit of endonuclease I activity is defined as that amount of enzyme which causes the liberation of 0.1 μmole of acid-soluble DNA-nucleotides in 30 minutes at 37°C.

Ribonuclease (12), Co^{++}-stimulated 5'-nucleotidase (6), cyclic phosphodiesterase (6) and beta-galactosidase (2) activities were assayed by the published methods.

Protein was determined by the method of Lowry et al (13).

Results

Initial observations that led to the development of the new cold shock procedure. Chloramphenicol (CAP) inhibition of protein synthesis in rapidly growing cultures of E.coli results in loss of endonuclease I activity. However, it was noticed that the exponential loss of endonuclease I activity is preceded by an initial rise,

Abbreviations: CAP=Chloramphenicol, PI=Pentamidine isethionate.
* The pH of the buffer refers to a 0.1M solution at 37°C even-though the buffer may have been used at 0°C.

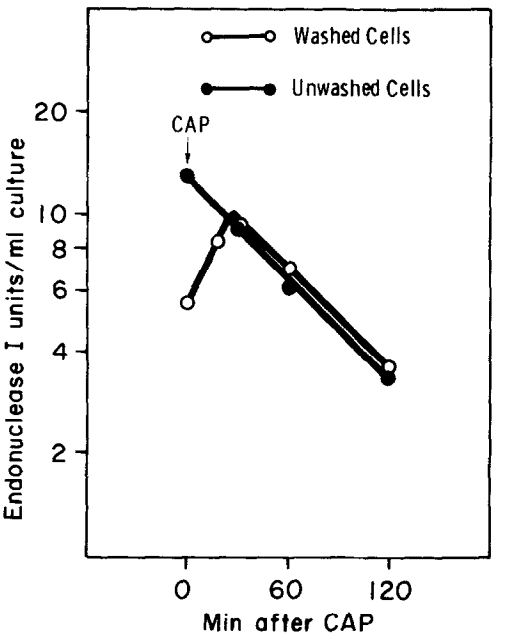
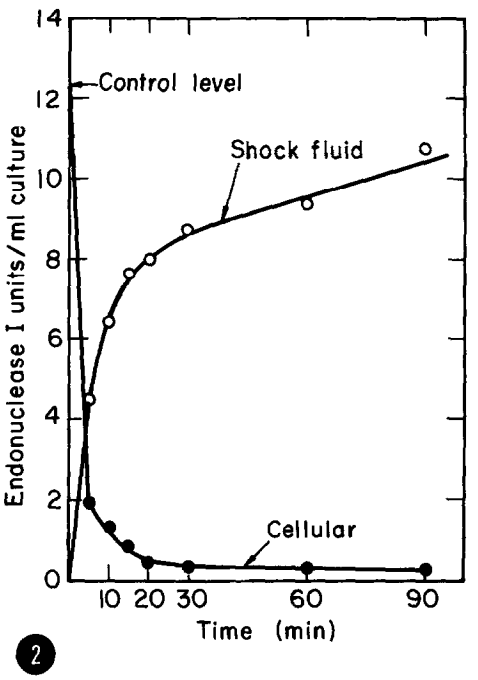


Figure 1. Effect of washing the cells on the CAP-induced turnover loss of endonuclease I. CAP (final conc.150 $\mu g/ml$) was added to log phase E.coli (A_{650}=1.2). Samples were removed at various time intervals, centrifuged in cold and the cells either unwashed or washed with ice-cold 0.1M Tris-0.1M NaCl buffer, pH 7.4 were sonicated in the same buffer.

as shown in Figure 1. This initial rise was later found to be an artifact. The usual cold wash of the cells with 0.1M Tris-NaCl buffer results in a partial release of endonuclease I activity into the washing fluid. This releasibility is blocked after CAP addition (details will be published elsewhere). Similar experiments with unwashed cells reveal no such initial rise and the loss of endonuclease I activity occurs immediately after CAP addition.

Following the observation that washing E.coli cells with cold Tris-NaCl buffer results in a partial release of endonuclease I, conditions were sought which would result in the most rapid and complete release of endonuclease I. It was found that the medium composed of 0.1M Tris-0.2M KCl pH 7.4 resulted in a maximum release of endonuclease I.



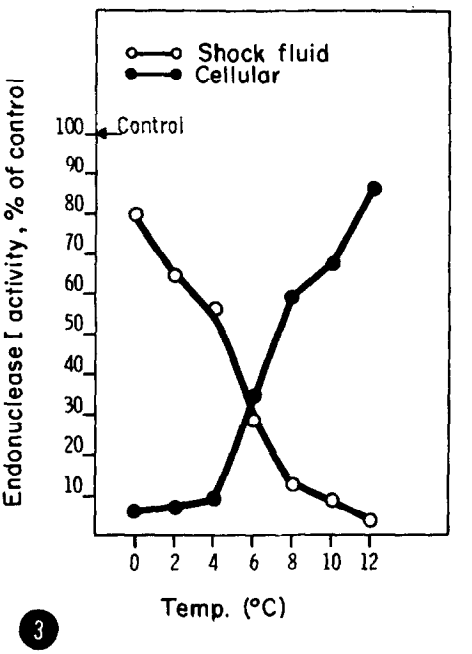


Figure 2. Kinetics of the release of endonuclease I by cold shock. Log phase E.coli cells were cold shocked as described in the text.

Figure 3. Release of endonuclease I from E.coli by Tris-KCl treatment at various temperatures. Cells were resuspended in Tris-KCl buffer previously equilibriated to their respective temperatures and held for 90 minutes.

Kinetics of the release of endonuclease I. The time course of the release of endonuclease I by cold shock is presented in Fig.2. Treatment of E.coli cells with ice-cold Tris-KCl buffer results in a rapid loss of endonuclease I activity from the cells (cellular), whereas the appearance or the recovery of the enzyme in the shock fluid is a gradual process requiring about 90min to approach a maximum. In most experiments, more than 90% of the total endonuclease I activity is lost from the cells in the first 5-10 min of cold shock. The recovery at the end of 90 min ranges from 80-90% of the total.

Temperature curve. The release of endonuclease I by Tris-KCl treatment at temperatures ranging from 0-12°C is shown in Fig.3. The

loss of endonuclease I from the cells remain more or less unaffected up to 4°C, whereas at higher temperatures progressively less enzyme is released and at 12°C virtually no endonuclease I is released into the medium.

In separate experiments it was observed that resuspending the cells with prewarmed buffer at 15°C, room temperature or at 37°C followed by chilling the tubes to 0°C did not release endonuclease I. For maximum release, it is essential to resuspend the cells quickly in ice-cold Tris-KCl buffer.

Effect of washing the cells on the cold shock release of endonuclease I. It is a common procedure to use washed cells to remove traces of the growth medium. However, washing the cells with various dilute salts and/or buffers at any temperature prior to cold shock resulted in a poor recovery of endonuclease I in the shock fluid (only 10-50% of total), although the loss from the cells remained unaffected (data not shown).

Effect of divalent ions. The release of endonuclease I by cold shock suggests a surface layer impairment, consistant with the known localization of this enzyme. Several divalent ions which are known to have a stabilizing effect on isolated protoplast membranes (14) and spheroplasts (15,16) were included in the cold shock medium to test whether the release of endonuclease I is in any way affected. Mn^{++}, Zn^{++} and Mg^{++} ions were found to block the release of endonuclease I much more effectively than Co^{++}, while Ca^{++} had little or no effect. The results of the effect of Mg^{++} on the cold shock release of endonuclease I presented in Table 1 indicate that Mg^{++} at 1×10^{-3}M and above concentrations completely blocks the release of endonuclease I.

Effect of polyamines. Polyamines such as spermine, spermidine and putrescine, which are also known to have a stabilizing effect

Table 1. Effect of Mg^{++} on the release of endonuclease I by cold shock.

	Endonuclease I activity(units/ml culture) Shock fluid	Cellular
Control, No cold shock	13.67 (100)	
Cold shock, $-Mg^{++}$	10.60 (77.5)	0.32 (2.3)
" $+10^{-4}M\ Mg^{++}$	4.20 (30.7)	4.85 (35.5)
" $+10^{-3}M\ Mg^{++}$	0.32 (2.3)	13.10 (95.8)
" $+10^{-2}M\ Mg^{++}$	0.29 (2.1)	13.60 (99.4)

The cells were cold shocked for 90 min in 0.1M Tris-0.2M KCl buffer containing various concentrations of $MgCl_2$. The values in the parentheses represent % of total endonuclease I activity.

on spheroplasts against osmotic damage (17,18) were found to be completely ineffective in preventing the release of endonuclease I by cold shock even at high concentrations (up to $10^{-2}M$).

Effect of various inhibitors. Pentamidine isethionate (PI), an inhibitor of protease activity (19) partially inhibits the release of endonuclease I when PI is present during cold shock (Table 2). This suggests a possible involvement of proteolytic activity in the release of endonuclease I.

However, cyanide ($2 \times 10^{-2}M$) and 2,4-dinitrophenol ($10^{-2}M$) had no effect on the release of endonuclease I when they were present during cold shock thus suggesting that the release of endonuclease I by cold shock is not an energy dependent process.

Release of other enzymes by cold shock. Besides endonuclease I, several other periplasmic enzymes are released in differential amounts by cold shock (Table 3). Endonuclease I is released preferentially compared to the other periplasmic enzymes studied with the exception of RNase. The activities of 5'-nucleotidase

Table 2. Effect of Pentamidine isethionate (PI) on the cold shock release of endonuclease I

	Endonuclease I activity (units/ml culture)	
	-PI	+PI
Shock fluid	10.00 (82.6)	4.85 (40.0)
Cellular	0.64 (5.2)	4.20 (34.6)
Control	12.13 (100)	12.00 (100)

The cells were cold shocked in Tris-KCl buffer for 90 min in the presence (0.2mM) or absence of PI. The values in the parentheses represent % of control.

Table 3. Release of enzymes from log and stationary phase E.coli by cold shock.

	Enzyme units/ml culture			
	Endonuclease I	5'-nucleotidase	Cyclic phospho diesterase	RNase
Log phase				
Control	13.4(100)	1.50(100)	2.04(100)	0.32(100)
Shock fluid	10.9(81.5)	0.62(41)	1.00(49)	0.15(47)
Cellular	0.5(3.7)	0.98(65)	1.26(61.5)	0.05(15.6)
Stationary				
Control	15.2(100)	21.20(100)	17.50(100)	1.98(100)
Shock fluid	7.6(50)	3.88(18.4)	1.42(8.1)	0.20(10.1)
Cellular	3.1(20.4)	22.20(100)	17.50(100)	0.24(12.1)

Log phase (A_{650}=0.8) and stationary phase (three hours after the culture entered the stationary phase) cells were cold shocked for 120 min. The values in the parentheses represent % of control.

and cyclic phosphodiesterase are never released in excess of 40-50% of their respective total. Interestingly, none of the other periplasmic enzymes are released from stationary phase cells, whereas endonuclease I and RNase are released (Table 3).

Beta-galactosidase, a representative of the internal enzymes is not released by cold shock. A constant fraction (about 10%) of the total protein is released into the shock fluid.

Discussion

The selective release of endonuclease I from E.coli by cold shock has certain unique features. Even though the loss of endonuclease I from the cells is rapid, its recovery in the shock fluid is a gradual process. The conditions for the release of endonuclease I by cold shock are entirely different from those of osmotic shock procedure. For instance, Nossal and Heppel(8) in their osmotic shock procedure used high concentrations of $MgCl_2$ (2×10^{-2}M) in stage II for releasing large amounts of endonuclease I. However, in the cold shock procedure, the presence of even low concentration of $MgCl_2$ (10^{-3}M) completely inhibits the release of endonuclease I.

The relative differences between endonuclease I and other periplasmic enzymes released by cold shock could be due to the differences in their intracellular localization. The fact that none of the other periplasmic enzymes except endonuclease I and RNase are released by cold shock from stationary phase culture is further indicative of the differences in their intracellular location. In this context, Obinata and Mizuno (20) observed that the releasability of endonuclease I not only differed with various treatments, but also varied with the growth phase suggesting that their intracellular location changed with the physiological state of the bacteria.

Several workers have demonstrated that treatment of E.coli with Tris-EDTA alters the permeability of cells (21-23). Neu et al (21) have shown that Tris-HCl itself changes the permeability of E.coli cells at 0°C, 23°C and at 37°C. However, the release of endonuclease I by Tris-KCl treatment is different in the sense that the release occurs only at 0°C but not at 23°C or 37°C,

whereas the release of endogenous constituents such as the free nucleotide pool occurs at all temperatures.

Lastly, in view of the observation that the periplasmic enzymes are released from E.coli by Tris-KCl or Tris-NaCl treatment, one should exercise caution in experiments dealing with periplasmic enzymes. Wherever possible, low concentrations of Mg^{++} should be included in the washing medium to prevent the leakage of periplasmic enzymes from the cells.

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