ON THE SURFACE LOCALIZATION OF ENZYMES IN E. COLI

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Good evidence exists for the surface localization of certain enzymes in several organisms (1-6). Malamy and Horecker proposed that the alkaline phosphatase of Escherichia coli is at the cell surface, and reported that the enzyme is quantitatively released when EDTA-lysozyme spheroplasts are made. The same procedure releases the latent RNase (7) and an RNA-inhibited DNase (R. J. Hilmoe). We now find that three additional enzymes are set free: a Co^{++} stimulated 5'-nucleotidase, not previously described; an acid phosphatase; and a cyclic phosphodiesterase, recently discovered by Y. Anraku * (8). It is probably significant that all of them are degradative. We have also developed a new method for releasing these enzymes (except the nucleases) in high yield without greatly impairing cell viability.

EXPERIMENTAL

Published methods were used for growing cells (7) producing spheroplasts (1,7) and measuring RNase (7), DNase (9), β-galactosidase, and alkaline phosphatase (1,7). For cyclic phosphodiesterase the reaction mixture (0.05 ml) contained (in μmoles): A-cyclic P, 0.06; $MgCl_2$, 0.25; $CoCl_2$, 0.05, Na acetate buffer, pH 6, 2.5; and excess purified alkaline phosphatase. For 5'-nucleotidase, 0.1 ml contained (in μmoles): 5'-AMP, 0.44; $CoCl_2$, 0.1; $CaCl_2$, 1.0; Na acetate buffer, pH 5.8, 10. For acid phosphatase, 0.1 ml contained (in μmoles): glucose-6-phosphate, 0.5; Na acetate, pH 5.65, 9. After addition of test enzyme the mixtures were incubated for 20 minutes at 37° and P_i was measured. Activities were expressed as μmoles split per hour.

Viability counts were done on serial dilutions of cells in a minimal salts medium. Treated cells were compared with the original suspension.

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* He has purified this interesting new enzyme, which hydrolyzes nucleoside 2',3'-cyclic phosphates, 900-fold and pointed out its metabolic significance. We are grateful for permission to refer to this work prior to its publication.

RESULTS

All of the six enzymes mentioned in the introduction were released when EDTA-lysozyme spheroplasts (1,7) were made from E. coli strains B, K12S, K12λ, C_4F_1, and U7. Enzymes were also released in the following way: 1 g (wet weight) of cells was washed 3 times with 0.01 M Tris, pH 8, and suspended in 80 ml of 20% sucrose-0.03 M Tris-5 x 10^{-4} M EDTA. The mixture was gently agitated for 10 minutes at 23° in a 2-liter flask and centrifuged. The pellet was resuspended in 80 ml of cold water ("cold water wash"), and after 10 minutes of gentle agitation at 3° the mixture was centrifuged. The "cold water wash" contained 70 to 100% of the alkaline phosphatase, cyclic phosphodiesterase, 5'-nucleotidase, and acid phosphatase, but less than 10% of the RNase and DNase (Table I). The following "internal" enzymes were not released in significant amount into the medium surrounding spheroplasts or into the "cold water wash": β-galactosidase *, glucose-6-phosphate dehydrogenase *, glutamic dehydrogenase *, polynucleotide phosphorylase, RNA phosphodiesterase (10,

TABLE I. Release of enzymes on spheroplast formation and on treatment with sucrose-Tris followed by a "water wash"

Fraction	Alkaline phosphatase	Cyclic phosphodiesterase	RNase	DNase	DNA Polymerase
C4 F1-Low P Medium					
(a) Sonic extract, cells	4,080	600	5,100	19,000	11,280
(b) Spheroplast supernatant	3,680	673	3,640	9,760	0
(b) Spheroplast lysate	600	132	2,620	-----	11,200
(c) "Cold water wash"	3,600	625	80	300	60
			5'-Nucleotidase	Acid P'ase	Inorganic PPase
K12S-High P Medium					
(a) Sonic extract, cells	0.8	384	1,450	710	9,000
(b) Spheroplast supernatant	0	324	1,490	565	600
(b) Spheroplast lysate	trace	100	20	16	8,900
(c) "Cold water wash"	trace	340	1,200	675	50

Experimental procedures described in text. All activities are expressed as enzyme units derived from 1 g (wet weight) of cells. With K12S, the medium contained 0.06 M phosphate to suppress alkaline phosphatase. Leakage of β-galactosidase was less than 1%.

* Malamy and Horecker (1) first showed that these were retained within spheroplasts.

11), inorganic pyrophosphatase, DNA polymerase, leucine aminopeptidase, and the histidine activating enzyme.

This washing procedure, which does not involve lysozyme nor produce spheroplasts, is of considerable theoretical interest. It releases 5-10% of the cellular proteins; these are revealed as about 15 discrete bands on electrophoresis in starch gel agar **. In addition, much of the acid-soluble, 260 mμ absorbing material of the cell is lost into the medium. In spite of all this, a high degree of viability (60-90%) is maintained. From the practical point of view, the procedure provides an easy route for purification of surface enzymes and for subsequent preparation of cell extracts nearly free of these degradative activities. Enzymes in the "cold water wash" were separated by DEAE chromatography (Fig. 1), giving the following over-all purification (compared with sonic extracts), and yields: 5'-nucleotidase, 70-fold, 70% recovery; cyclic phosphodiesterase, 80-fold, 70% recovery, acid phosphatase, 120-fold, 70% recovery.

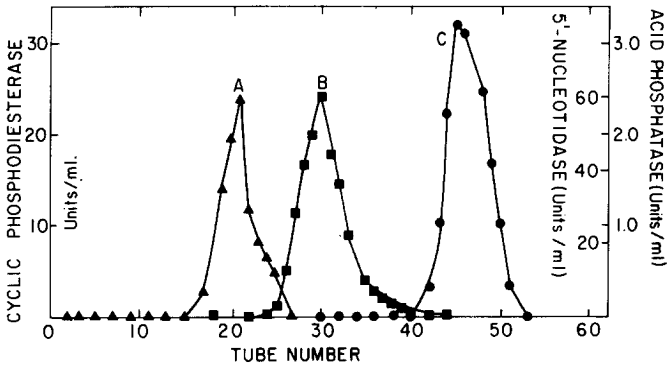

Fig. 1. Separation of cyclic phosphodiesterase (Peak A), 5'-nucleotidase (Peak B), and acid phosphatase (Peak C) by DEAE-cellulose chromatography. The column (2.5 x 12 cm) was equilibrated with 5×10^{-3} M Tris-HCl, pH 7.4-1×10^{-3} M $MgCl_2$, and 100 ml of "cold water wash" (see text) was applied. The "water wash" was derived from E. coli K12S and is described in Table I; it contained 0.067 mg protein per ml. The column was washed with 100 ml of the same buffer, after which a gradient was applied and fractions of 4.7 ml were collected. Mixing vessel: 150 ml starting buffer. Reservoir: 5×10^{-3} M Tris-HCl, pH 7.4-1×10^{-3} M $MgCl_2$-0.2 M NaCl. Flow rate, 2ml/min.

** We are grateful to Dr. Elliot Vesell for these determinations.

The 5'-nucleotidase was stimulated 5.5-fold by Co^{++}, 2.5-fold by Ca^{++} and 12-fold by the combination. Other ions, including Fe^{++} and Ni^{++} were ineffective (see (12)). It was highly specific for ribo- and deoxyribonucleoside 5'-phosphates. The acid phosphatase may be similar to one of the DEAE fractions described earlier (13); it had no metal requirement and was active in the presence of 0.01 M EDTA. Hexose phosphates were by far the best substrates and when phosphate was esterified to C-1 it had to be in α-linkage.

The fact that these enzymes are specifically removed from the cell suggests a surface localization. Further, there was close quantitative agreement between the activity measured with suspensions of intact cells and an equivalent extract. Intact cells displayed 70% of the alkaline phosphatase, 70% of the acid phosphatase, 100% of the cyclic phosphodiesterase and 100% of the 5'-nucleotidase activity of the sonic extract. As with invertase (6), the Michaelis-Menten constant, K_m, and pH optimum were similar whether the substrate was incubated with purified enzyme or whole cells (Table II). Properties such as metal

TABLE II. Comparison of intact cells and DEAE fractions

	5'-Nucleotidase		Acid phosphatase		Alkaline phosphatase	
	DEAE	Cells	DEAE	Cells	DEAE	Cells
K_m	$2x10^{-4}$	$1.2x10^{-4}$	$5.5x10^{-4}$	$5x10^{-4}$		
pH Optimum	5.5-6	5.5-6	5.6	5.5-6	8.5	8.5-9
Co^{++} Stimulation	5.5-fold	3.0-fold				

The 5'-nucleotidase and acid phosphatase of E. coli U7 cells was compared with DEAE fractions, using 5'-AMP and glucose-6-phosphate, respectively. For alkaline phosphatase, strain C_4F_1 and p-nitrophenyl phosphate were used. Buffers (acetate or Tris) were 0.05 M.

stimulation or RNA inhibition could also be demonstrated with intact cells. In addition, mutants lacking alkaline phosphatase were able to grow on 5'-AMP or A-cyclic-P as the source of carbon and phosphate, but 2'-AMP was ineffective. This is consistent with the specificity of the surface enzymes known to be present. Finally cells containing the non-specific alkaline phosphatase showed equally rapid uptake of the ^{14}C-labeled adenine moiety, whether provided as 3'-AMP or 2'-AMP. Cells lacking this enzyme but containing 3'-nucleotidase (8) showed much

poorer uptake of radioactivity with 2'-AMP. *

In several experiments spheroplasts were made of K12λ during the course of mitomycin induction. The new exonuclease (9) formed during induction was released while β-galactosidase was retained within spheroplasts. One may speculate whether nucleases located at or near the cell surface play a role in the degradation of foreign nucleic acids. The nucleases secreted into the medium by gram-positive organisms, such as B. subtilis and St. albus, may be similar, for we find that they lack the RNase that is associated with ribosomes of gram-negative bacteria.

This segregation of phosphatase, nuclease, and diesterase enzymes in a separate cellular compartment reminds one of similar phenomena in other forms, such as the occurrence of mammalian lysozymes and the surface localization of alkaline phosphatase in kidney tubule cells. Presumably it is of general biological signnificance to separate degradative from synthetic functions in this manner.

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* Current studies with electron microscopy by Drs. B. Wetzel and S. Spicer also indicate a surface localization of phosphatases in E. coli.

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