EFFECT OF TRYPSINIZATION ON THIAMINE TRANSPORT IN ESCHERICHIA COLI CROOKES

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Received September 10,1975

Summary

Trypsin treatment of intact Escherichia coli Crookes cells is a probe for analyzing the functioning of protein sites in thiamine transport. These sites are cryptic since cells with different levels of thiamine transport activity respond differently to trypsin treatment. Mild trypsin treatment of cells with normal transport activity enhanced the velocity of uptake and decreased the capacity; more rigorous treatment reduced both parameters. Cells with low activity showed greatly increased rates of uptake and capacities under all but the mildest treatment conditions. These observations are consistent with a trypsin unmasking of thiamine transport sites in low activity cells and a destruction of the sites in higher activity cells.

Neujahr (1, 2) has characterized an energy dependent thiamine transport system in Lactobacillus fermenti. This uptake is stimulated by Mg^{2+}, K^{+}, and ascorbate; shows pronounced temperature and pH optima; has saturation kinetics, is stereospecific with 260-fold accumulation; and is reversible. Kawasaki, et al. (3) found an energy- and temperature-dependent uptake of thiamine in Escherichia coli with a pH 6.5 optimum. The uptake follows Michaelis-Menten kinetics with a 175-fold concentration of thiamine pyrophosphate. Using thiamine monophosphokinase or thiamine kinase deficient mutants, Kawasaki and Yameda (4) showed that thiamine is the transported compound. Nishimune and Hayashi (5); Iwashima, Matsuura, and Nose (6); and Griffith and Leach (7) demonstrated the release of a thiamine binding protein upon osmotic shock and have reported its purification.

Treatment of mammalian cells with proteolytic enzymes functions as a probe of surface located proteins. The mechanism of passive ion permeability in red blood cells has been investigated by Passow (8) using pronase. Kahlenberg et al. (9) used proteases and phospholipases to determine relative surface accessibilities of glucose transport components of intact red blood

cells. This approach is less widely used with bacteria. Normura and Nakamura (10) used trypsin treatment to relieve colicin K inhibition. Tomasz (11) finds treatment of Pneumococci with trypsin destroys the cell-bound activator protein which is required for DNA-mediated transformation.

This paper describes the effects of trypsin treatment of E. coli cells under various conditions on the cells' ability to transport thiamine

Materials and Methods

Chemicals

^{35}S-Thiamine (specific activity 55 to 158 mCi/nmole) was obtained from Amersham-Searle. Bovine serum albumin, soybean trypsin inhibitor and phenylmethanesulfonyl fluoride (PMSF) were products of Sigma. Trypsin was obtained from Worthington Biochemical Corp. Chloramphenicol was purchased from Parke-Davis and Company. Gelman Metrical filters (pore size 0.45 μ) were from Fisher Scientific Company.

Growth of Cells

E. coli Crookes cells were grown in M-9 minimal medium (12) supplemented with 0.2% glucose on a rotary shaker at 37°.

Uptake Studies

Aliquots of a cell suspension were filtered on Gelman Metrical GA-6 filters and washed with buffer. The washed cells were suspended in 10 mM Tris buffer, pH 8.0.

To measure uptake, cells (a final A_{620} of approximately 0.1) were incubated at 37° with 10 mM glucose, 1 mM $MgSO_4$ and 0.1 mg/ml chloramphenicol for 30 min in a New Brunswick Scientific Co. Metabolyte Water Bath Shaker shaking at 100 rpm. ^{35}S-Thiamine was added to give a thiamine concentration of 1.0 μM. Aliquots were removed at appropriate intervals and filtered rapidly on Gelman Metrical GA-6 filters on a Bradley multi-cell filtration apparatus. The filters were washed with 1 ml of buffer, air-dried and counted in 10 ml of Bray's solution in a Packard Tri-Carb Liquid Scintillation Spectrometer to a 1% standard counting error.

Trypsin Treatment

Cells were grown, harvested and suspended as described above. Trypsin solution was added to cells in 10 mM Tris containing 0.1 mg/ml chloroamphenicol to give the desired trypsin concentration. The reaction mixtures were shaken for 1 hr, at which time a trypsin inhibitor was added, either 2 mM phenylmethanesulfonyl fluoride in water suspension or soybean trypsin inhibitor (1.5 times weight of trypsin). All additions were made from solutions or suspensions at 37°. The inhibitor/trypsin/cell suspension was incubated at 37° for 1 hr. The control cells were incubated at 37° for the duration of the treatment.

Results

During studies on thiamine uptake by E. coli Crookes strain variations were observed in the uptake capacity of cells at different times. When the

stock E. coli cells were streaked so that individual colonies could be obtained and their thiamine transport activity determined, three classes of response were demonstrated. High activity cells took up 1000-1500 pmoles, intermediate activity cells took up 500-1000 pmoles and low activity cells took up 20-100 pmoles per mg dry weight. These different types would remain fairly stable in liquid culture (about one week) but then upon plating would give rise to the three classes again. Although growth rates vary on M-9 medium (high activity cells faster than low activity cells) the variation in activity is not a function of time since inoculation or growth phase. The mechanism of this variation is not known, but the variation serves as an excellent source material for demonstrating structural correlations with changes in thiamine transport activity.

The effects of trypsin treatment (1 mg/ml trypsin) on normal cells (high activity) under standard conditions are shown in Fig. 1A. Under these conditions trypsin decreased the initial velocity of thiamine transport by 75%. Accumulation in the trypsin-treated cells saturated earlier than in untreated cells, which took up thiamine for over 2 hours. The level of thiamine

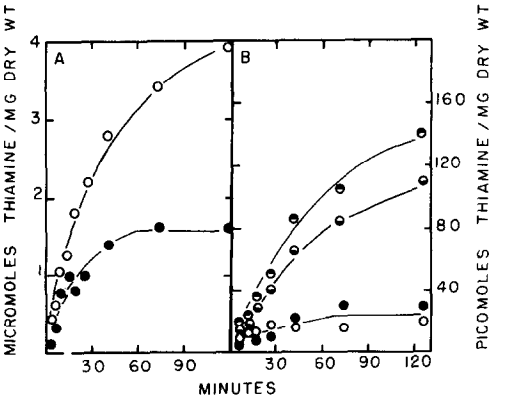

Figure 1. Effect of trypsin treatment on thiamine uptake.
A. Uptake by cells with high activity. Control cells O and cells treated with trypsin (1 mg/ml for 1 hr at 37°) ●.
B. Uptake by cells with low activity. Control cells O and cells treated with ●, 0.1 µg/ml; ◐, 10 µg/ml; and ◑, 1 mg/ml of trypsin for 1 hr at 37°. In both cases the action of trypsin was stopped by soybean trypsin inhibitor.

accumulated at 72 min in trypsin-treated cells was reduced 50%. However, milder trypsin treatment (at 30° for 45 min) and assay at 37° did not decrease the rate of uptake (325 pmoles/mg dry weight-min compared to treated, 289 pmoles/mg dry weight-min).

Trypsin treatment of cells with lower thiamine transport capacities produces a dramatically different effect from that seen with high-activity cells. Fig. 1B depicts the effect of trypsin at several concentrations. Instead of the reduced capacities seen in Fig. 1A, there was enhancement of uptake. At 70 min the treated cells accumulated almost 4 times as much thiamine as the untreated cells.

Since cells with high and low thiamine uptake capabilities clearly had different responses to trypsin treatment, the effect of trypsin treatment on intermediate capacity cells was studied. Variation in trypsin concentration yielded results shown in Table I. Increasing trypsin concentrations increased initial velocities and thiamine capacities up to a concentration of 10 μg/ml of trypsin. At higher trypsin concentrations the initial velocity drops sharply with a gradual decrease in capacity.

Part B of Table I shows a time study using trypsin at 1 mg/ml. A 5 min treatment reduces by approximately half the velocity of transport and the amount accumulated. Samples treated for longer times show a roughly linear increase in initial velocity. Treatment for 45 min or longer gave velocities greater than those of the control. Accumulation measured at 1 hr had the same pattern except that at the longest treatment times the accumulation reached a plateau.

Discussion

The effect of trypsin treatment of *E. coli* Crookes cells on thiamine uptake depends on 1) thiamine uptake capability of the cells and 2) the conditions used in proteolysis. In cells having a high transport capacity, stringent trypsin digestion (1 mg/ml, 37°) decreases both the initial velocities and maximal accumulation. Treatment under milder conditions had

Table I. Effect of Varying Trypsin Concentration on Uptake by Intermediate Activity Cells

A. Cells with intermediate thiamine uptake activity were treated with the indicated trypsin concentrations at 37° for 1 hr. The reaction was terminated with soybean trypsin inhibitor and uptake measured by the standard procedures.

Trypsin Concentration μg/ml	$v_{5\ min.}$[a] pmoles/min / mg dry weight	Thiamine Accumulated at 73 min pmoles / mg dry weight
None	23.3	560
0.1	27.9	630
1.0	30.9	1020
10	41.8	1190
100	33.1	1090
1000	28.5	1000

[a]Velocities at 5 min.

B. Cells with intermediate thiamine uptake activity were treated for the indicated times with 1 mg per ml trypsin.

Time of Trypsin Treatment, min	$v_{5-18\ min}$ pmoles/min / mg dry weight	Thiamine Accumulated at 60 min pmoles / mg dry weight
0	0.68	623
5	0.34	211
15	0.47	448
30	0.54	504
45	0.80	690
60	0.93	619

little effect. Trypsin treatment of low activity cells, even under the most stringent conditions of trypsin concentration, temperature and time of digestion, enhanced thiamine accumulation. These results can be interpreted as transport sites in the membrane which are accessible to trypsin (in high activity cells) and sites which are buried or "cryptic" (in low activity cells).

Kahlenberg, et al. (9) found an analogous situation for glucose transport sites in red blood cells. The concept of intermediate capacity cells having part of the thiamine transport sites exposed and part of them cryptic is substantiated by trypsin treatment for short time periods decreasing activity, whereas longer treatment periods stimulate the initial velocity of uptake.

The effects of the enzyme on transport behavior may be complex, since exposed sites can be destroyed at the same time as new sites are being exposed. There are two possible explanations for the phenomena observed in this work. One is that there is a single class of thiamine transport sites whose accessibility to trypsin and transport activity are determined by their location within the membrane (which varies with conditions such as temperature). The second is that there are two classes of sites with inherently different transport activities and susceptibilities to trypsin digestion.

The correlation between rate of growth and thiamine accumulation capacity suggests that in the slow-growing cells there are alterations of membrane components in addition to those directly responsible for the accumulation of thiamine. These might include ion transport or energy production components.

Acknowledgments

We thank Dr. Kermit Carraway for helpful comments in the preparation of this paper. This research was project number 1109 of the Oklahoma Agricultural Experiment Station and was supported in part by NSF grant #GB 8752, by American Cancer Society Institutional Grant #IN 91, and by NIH Research Career Program Award CA-K3-6487. Taken in part from C. A. C. Carraway's Ph.D. thesis. Journal Article J-2989 of the Oklahoma Agricultural Experiment Station.

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