ACTION OF PROTEINASES ON THE ARGININE TRANSPORT SYSTEM OF PURIFIED VACUOLES FROM SACCHAROMYCES CEREVISIAE.

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Summary: Thermolysin, a commercial bacterial proteinase, greatly activated the arginine transport system of isolated yeast vacuoles. Pronase had the same effect at low concentrations, but rapidly inactivated the transport system at higher concentrations. Arginine specifically protected the transport system form the inactivation by pronase. The protective effect of other amino acids correlated well with their affinity for the transport system. It is concluded that both thermolysin and pronase attack a membrane protein which restrains the transport of arginine, whereas the protein which carries the specific binding site of this transport system can be destroyed only by pronase.

Vacuoles isolated from yeast spheroplasts and purified contain a highly active transport system for arginine (1). The question whether this transport system is mediated by a membrane protein can be examined by studying the effect of proteinases. We considered vacuoles suitable for such an experiment: Saccharide residues which, it is suspected, protect the membrane from the attack of proteases (2), have been shown to be absent from the outer membrane surface of the vacuoles (3).

Material and Methods:

Preparation of yeast spheroplasts and vacuoles

Spheroplasts were obtained from growing cultures of Saccharomyces cerevisiae (strain LBG H 1022, Institute for Microbiology, ETH, Zürich). The spheroplasts were disrupted under isotonic conditions (0.6 M sorbitol containing 5 mM Tris-Pipes, pH 6.8 [TP-buffer]) by polybase-induced lysis as described (4) using 100 µg DEAE-dextran per 10^8 spheroplasts. The vacuoles were separated from the homogenate as described (4) but the method was scaled up by using a zonal rotor (type Beckman Ti-14). The homogenate was layered on a gradient

consisting of two washing layers (0.5 M sorbitol with 0.1 M sucrose, 100 ml, and 0.45 M sorbitol with 0.15 M sucrose, 100 ml, respectively) and a reception layer (0.6 M sucrose, 100 ml) on top of which the vacuoles float after centrifugation. All solutions were buffered with TP-buffer. After 30 min centrifugation at 100'000 x g, the gradient was displaced by 30 % sucrose and the fractions containing the vacuoles were combined. They were mixed with 4 volumes of 0.6 M sorbitol and the vacuoles sedimentated by 30 min centrifugation at 2'000 x g. The purified vacuoles were resuspended in 0.6 M sorbitol with TP-buffer, at a final concentration of 5×10^7 vacuoles per ml.

Protease treatment of the vacuoles and assay for arginine transport

1 ml portions of purified vacuoles were preincubated for 5 min at 25° C and then mixed with 50 µl of protease solution prepared with 0.6 M sorbitol containing TP-buffer and 10 mM $CaCl_2$. After specified intervals the vacuoles were separated from the proteases by centrifugation through a discontinuous isotonic density gradient (1). The vacuoles were taken up in 0.6 M sorbitol with TP-buffer, and the initial rate of the transport of 100 µM L-[^{3}H]arginine was determined as described (1).

Chemicals and protease preparations

L-[^{3}H]arginine was obtained from New England Nuclear, Boston, USA. Pronase was purchased from Koch-Light, Colnbrook Bucks, England, and Thermolysin (*Protease type X) from Sigma, St.Louis, USA. One proteolytic unit** was* defined as the amount of protease which liberates 1 µmol acid soluble tyrosine per min from 1 % casein under the conditions used for the protease treatment of the vacuoles (0.6 M sorbitol; TP-buffer; 0.5 M $CaCl_2$; 25° C).

Results and Discussion

Activation of vacuolar arginine transport by thermolysin

The effect of incubation of vacuoles with thermolysin, a bacterial protease preparation consisting of a single Ca^{2+}-depending proteolytic enzyme, was first tested. The treatment of vacuoles with thermolysin activated their transport of arginine. The maximal activity was attained after 10 min with 1U thermolysin and ranged from 170 to 600 % of the control in different experiments. It was higher for the vacuoles with a low absolute rate of transport in the untreated controls.

Apparently a fraction of vacuoles was lysed upon treatment with thermoysin. As compared with the untreated controls, only about 70 % of the vacuolar amino acids were recovered in the sediment. The amount of vacuolar amino acids sedimentated has been found to be a reliable measure for the amount of intact

* Abbreviation: 1 proteolytic unit = 1 U

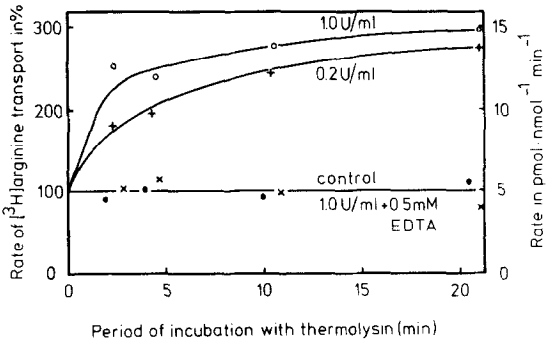

Fig. 1: Thermolysin activates the vacuolar transport system
5 x 10^7 vacuoles per ml were incubated with 50 µl of thermolysin (1.0 or 0.2 U) preincubated either with 10 mM $CaCl_2$ or 10 mM Tris-EDTA. At the time indicated the vacuoles were separated from the incubation mixture by centrifugation through discontinuous density gradients, resuspended in 0.6 M sorbitol with 5 mM Tris-Pipes, pH 6.8, and assayed for the initial transport rate of 100 µM L-[^{3}H]arginine.

vacuoles (1), and therefore served always as a basis of reference for the calculation of the transport rate.

Under the assay conditions 0.5 mM EDTA completely inhibited the proteolytic activity of thermolysin against casein. Correspondingly in the presence of 0.5 mM EDTA thermolysin had no effect on the arginine transport system (Fig. 1). This indicates that the effect of thermolysin is due to its proteolytic action. The increase in transport activity induced by thermolysin can be explained either as a proteolytic liberation of preexisting hidden carrier sites or as a dissection of a membrane protein which restrains the mobility of the carrier.

The dual effect of pronase on vacuolar arginine transport

Pronase, a protease preparation containing several proteolytic enzymes with a high potency to degrade native proteins, was next examined. A ratio of 3.3 mU pronase per 5 x 10^7 vacuoles activated arginine transport in the same way as thermolysin (Fig. 2). However, when higher pronase concentrations were applied (15 - 67 mU), a concomitant inactivation of the system became apparent. The decay of the transport activity followed first order kinetics

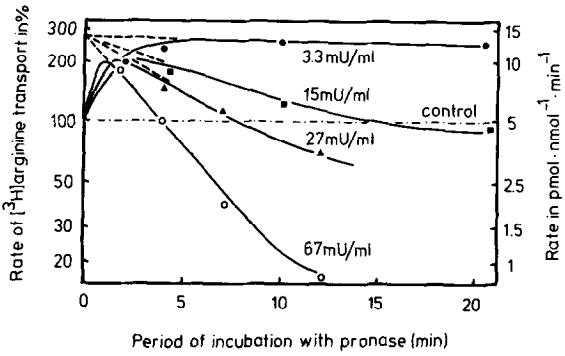

Fig. 2: Effect of various pronase concentrations on vacuolar arginine transport

As Fig. 1, but thermolysin replaced by 3.3, 15, 27, 67 mU of pronase.

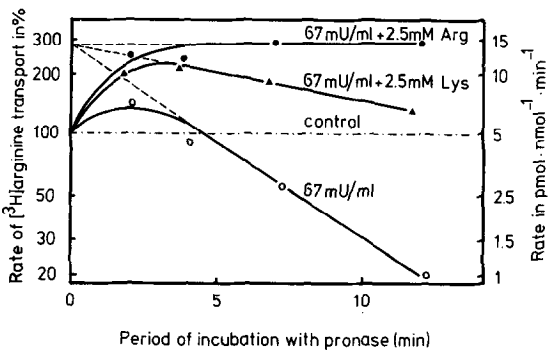

Fig. 3: Protection of the arginine transport system from proteolytic inactivation by its substrates, arginine and lysine

As Fig. 2, but with addition of 10 µl 0.2 M L-arginine or L-lysine 1 min before the addition of pronase.

over the first 7 min if the maximally activate d transport rate (260 %) was taken as the value for zero time. The decay constants were 0.14 min^{-1} and 0.7 min^{-1} for 15 mU and 67 mU pronase, respectively.

A possible explanation for the slowed decay after the first 7 min lies in the fact that some vacuoles were destroyed by the pronase treatment: After resuspension and transport assays, 30 % less vacuoles were sedimentated than

in the controls for all pronase incubations of more than 7 min. The internal arginine pool liberated from the destroyed vacuoles may protect the transport system of the remaining vacuoles, as demonstrated below.

Substrate amino acids protect the vacuolar arginine transport system from proteolytic inactivation

In the presence of 2.5 mM L-arginine, pronase still activated the arginine transport system (Fig. 3) but no longer displayed the subsequent inactivation. This is not due to an inhibition of pronase as its activity against casein was identical in the absence or presence of 2.5 mM arginine.

Tab. 1: Effect of different amino acids on the transport of L-arginine and on the proteolytic inactivation of the arginine transport system

The transport rate of vacuoles for 100 μM L-[^{3}H]arginine was determined in the presence of the amino acids listed. Vacuoles of the same batch were used for studying the effect of the amino acids on the kinetics of proteolytic inactivation of the arginine transport system. 5 x 10^7 vacuoles in 1 ml were incubated for 2, 5, 10 and 20 min with 50 µl pronase (67 mU) in the presence of 0.5 mM $CaCl_2$ and the amino acids listed. The vacuoles were then separated from the incubation mixture by centrifugation through discontinuous density gradients, resuspended, and their initial rate of arginine transport determined. From plots as shown in Fig. 3 for arginine and lysine, the rate constants of the proteolytic inactivation of the arginine transport system were estimated.

Amino acid	Conc.	Transport rate of 100 µM L-[^{3}H]arginine		Rate constant of the proteolytic inactivation of the arginine carrier	
	mM	pmoles per nmol amino acid per min	%	min^{-1}	%
-		7.7	100	0.56	100
L-leucine	2	6.7	87	0.56	100
L-lysine	2	3.4	44	0.26	46
L-histidine	2	2.2	29	0.19	34
D-arginine	2	1.0	13	0.075	13
L-arginine	0.5	1.3	17	0.12	21
L-arginine	2	0.6	8	0.00	0

The presence of 2.5 mM L-lysine, a basic amino acid with a poor affinity for the vacuolar arginine transport system reduced the rate of decay of the system only by ca. 70 % (Fig. 3).

In the experiment presented in Tab. 1, the relative affinities of a number of amino acids for the vacuolar arginine transport system were established by measuring their ability to inhibit arginine transport. The ability of the respective amino acids to inhibit proteolytic inactivation of the arginine transport system was tested with the same batch of vacuoles. For each of the amino acids tested, the ability to inhibit proteolytic inactivation of the arginine transport system corresponded closely to the respective affinity for the arginine transport system.

These findings strongly suggest that both effects have the same cause; we attribute this to the binding of the amino acid to the specific site of the arginine transport system and conclude that the protein carrying the specific site for arginine binding of the transport system is destroyed by pronase. However, this effect of the attack by pronase occurs only if the site is unoccupied by a substrate molecule.

Pronase has no effect on the arginine transport of spheroplasts

The arginine transport system of intact spheroplasts (1) was found to be resistant even to high concentrations of pronase (up to 1200 mU per 5×10^7 spheroplasts for 10 min). Possibly the saccharide residues covering the outer surface of the plasma membrane (3) protect the transport system of the spheroplasts from attack by pronase.

It is interesting in this connection to consider how saccharide residues able to bind Concanavalin A are distributed on the vacuolar membrane (3). Its outer surface, the susceptibility of which to pronase and thermolysin we examined in the present communication, is free of them. However the inner surface of the vacuolar membrane, exposed to several potent proteases located within the vacuole (6), was found to be covered with saccharide residues.

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