Volume 213, number 1, 45–48 FEB 04454 March 1987

Protonmotive-force-driven leucine uptake in yeast plasma membrane vesicles

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Received 4 November 1986; revised version received 22 December 1986

Plasma membranes prepared from *Saccharomyces cerevisiae* by the concanavalin A method were fused with liposomes by a freeze-thaw sonication method. A $\Delta\psi$ of -109 mV was generated in the sealed vesicles by a valinomycin-mediated potassium diffusion potential. This component of protonmotive force was able to drive a transient uptake of leucine.

Membrane vesicle; Leucine; Amino acid transport; *(S. cerevisiae)*

1. INTRODUCTION

The evidence that at least some of the amino acid transporting systems in yeast are driven by protonmotive force comes from studies with intact cells (review [1]). Considerable insight could be gained by studying these phenomena in yeast plasma membrane vesicles in which the effects of cellular metabolism are eliminated.

The only protonmotive-force-generating system present in such membrane vesicles is the proton-translocating ATPase. The localization of the active site of this enzyme on the inner side of the membrane of right-side-out oriented vesicles excludes ATP hydrolysis as a mechanism for generating a protonmotive force. Hence, in order to study for instance protonmotive-force-driven amino acid transport, the protonmotive force has to be generated artificially. Procedures have been developed to generate artificially a membrane potential ($\Delta\psi$) [2] and/or a transmembrane pH gradient (ΔpH) [3] in bacterial membrane vesicles.

A method based on ionophore-mediated translocation of K^+ resulting in $\Delta\psi$ formation was used in this work to energize the uptake of leucine into yeast plasma membrane vesicles prepared by fusion of yeast plasma membranes with liposomes.

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2. MATERIALS AND METHODS

2.1. *Yeast strain and its cultivation*

Saccharomyces cerevisiae DC XII was grown for 26 h at 30°C in a medium described by Opekarovà et al. [4] in which NH_4^+ was replaced by proline (2 mg/ml). After harvesting the cells were washed twice with sterile distilled water and kept overnight at 4°C.

2.2. *Preparation of protoplasts*

Protoplasts were prepared essentially according to Kovàc et al. [5]. After 50 min incubation with the snail-gut enzyme, 95–98% of the cells were converted to protoplasts. These protoplasts were washed twice with 50 mM Tris-HCl (pH 7.4) containing 0.9 M sorbitol and 10 mM $MgSO_4$ and preincubated for 1 h in the same medium with 0.8% glucose.

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00145793/87/$3.50

2.3. *Preparation of plasma membranes*

Plasma membranes were prepared from the protoplasts by a slightly modified concanavalin A method described by Scarborough [6] and Stroobant and Scarborough [7].

2.4. *Liposome preparation*

Acetone-washed asolectin (50 mg) in 1 ml of 50 mM potassium phosphate (pH 7.0) was sonicated to clarity under a constant nitrogen stream at 4°C in a pulse mode (15 s sonication, 45 s intermission) using a probe sonifier Labsonic 2000 (B. Braun, Melsungen).

2.5. *Fusion of plasma membranes with liposomes*

A freeze-thaw sonication method described for fusion of proteoliposomes with bacterial vesicles was used [8]. The liposomes (0.8 ml) were vortex-mixed with yeast plasma membranes (0.5 ml, 8 mg protein). The mixture was rapidly frozen in liquid nitrogen and then thawed slowly at room temperature. The turbid mixture was sonicated on an ice bath for 5–8 s at half-maximal output of the sonifier.

2.6. *Preparation of K^+-loaded proteoliposomes*

A partially clarified suspension obtained after the last sonication step was transferred to a medium containing 10 mM potassium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM KCl (K^+-rich medium) with 4 μM valinomycin, and centrifuged at 30000 × g for 30 min.

2.7. *Determination of $\Delta\psi$*

Membrane potential (interior negative) was determined from the distribution of tetraphenylphosphonium (TPP^+) across the membrane using a TPP^+-selective electrode [9]. Reaction mixtures contained 10 mM sodium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM NaCl (K^+-free medium) and 8 μM TPP^+. At time zero, 10 μl K^+-loaded proteoliposomes was added to a total volume of 1 ml.

2.8. *Transport assay*

Concentrated K^+-loaded liposomes (6 μl, 0.08–0.1 mg yeast membrane protein) were rapidly diluted into 0.2 ml of either K^+-rich or K^+-free medium with 50 μM uniformly labeled [^{14}C]leucine (spec. act. 730 MBq/mmol). The transport assay was carried out at 30°C and terminated by the addition of 2 ml ice-cold 100 mM LiCl; the membrane vesicles were then filtered on 0.30-μm cellulose nitrate filters (Synpor, Czechoslovakia) and washed once with 2 ml ice-cold 100 mM LiCl. Dried filters were transferred to scintillation vials with 10 ml toluene-based scintillation cocktail and their radioactivity was measured with a Beckman LS 9000 liquid scintillation counter.

2.9. *Protein estimation*

Protein was assayed according to Lowry et al. [10].

3. RESULTS AND DISCUSSION

3.1. *Preparation of closed plasma vesicles*

The use of the concanavalin A method for isolating plasma membranes from yeast ensures a high purity of the plasma membrane fraction [6,7]. However, the procedure does not appear to yield closed vesicles since we were not able to demonstrate either $\Delta\psi$ formation or leucine uptake into this preparation (see below). The fusion of the yeast membranes with liposomes by a freeze-thaw sonication method, on the other hand, led to the formation of a system capable of generating artificially, under certain conditions, a membrane potential (interior negative) of a considerable magnitude.

3.2. *Generation of membrane potential in yeast plasma membranes*

The method for artificial generation of $\Delta\psi$ by a valinomycin-mediated K^+ diffusion potential, originally used in bacterial vesicles [2], proved to be also applicable to the proteoliposomes prepared by fusion of liposomes with yeast plasma membranes. Dilution of K^+-loaded proteoliposomes 100-fold into the K^+-free medium resulted in the formation of a $\Delta\psi$ as indicated by the decrease in TPP^+ concentration in the medium (fig.1, trace B). Dilution of the same proteoliposomes into the K^+-rich medium resulted only in a small decrease of the TPP^+ concentration due to non-specific binding of TPP^+ to membranes (fig.1, trace A). The maximum decrease in TPP^+ concentration corresponded to a membrane potential of -109 mV. An internal volume of the proteoliposomes of 4.3 μl/mg protein was used in the

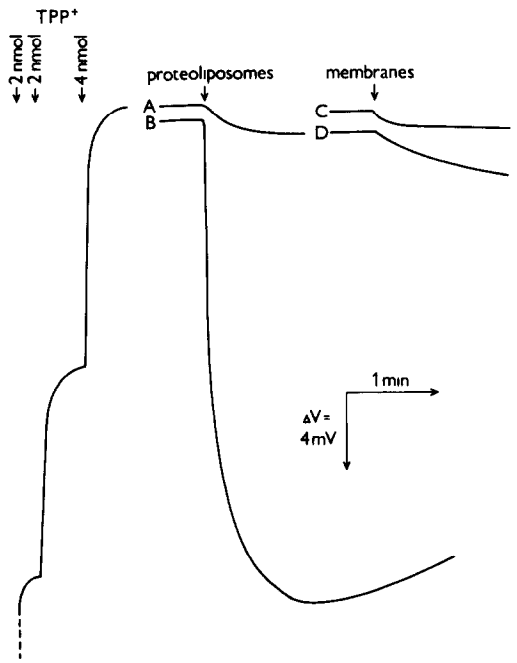

Fig.1. Tetraphenylphosphonium (TPP^+) uptake by proteoliposomes and yeast plasma membranes. Concentrated K^+-loaded proteoliposomes (10 μl, 0.16 mg protein) were added to a medium containing 10 mM potassium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM KCl and 8 μM TPP^+ (trace A) or 10 mM sodium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM NaCl and 8 μM TPP^+ (trace B). The same procedure was carried out with yeast plasma membranes (0.43 mg protein) which were subjected to the same K^+-loading treatment as the proteoliposomes (traces C,D). Calibration of the electrode was performed twice by a 2-fold increase of the TPP^+ concentration.

$\Delta\psi$ calculation (see below). The calculation included a correction for the concentration-dependent binding of TPP^+ to the membranes according to Lolkema et al. [11]. When the same set of experiments was performed in both K^+-rich and K^+-free media with a plasma membrane fraction not fused with liposomes, only the non-specific binding of TPP^+ was observed (fig.1, traces C,D). This is interpreted as reflecting the fact that no inner volume (i.e. closed vesicles) was present, the preparation consisting of membrane sheets or very leaky vesicles.

The formation of $\Delta\psi$ in fused membrane vesicles together with the transient leucine uptake (see below) in contrast to the absence of both $\Delta\psi$ formation and leucine uptake in non-fused membrane preparations provides strong evidence that the freeze-thaw sonication method resulted in the fusion of liposomes with yeast plasma membranes. Driessen et al. [8,12] proved by means of freeze-etch electron microscopy, sucrose density gradient centrifugation and labeling with non-exchangeable fluorescent phospholipid probes that the freeze-thaw sonication method results in an efficient fusion of bacterial vesicles with proteoliposomes. Using a similar method for fusion of yeast plasma membranes with liposomes, Franzusoff and Cirillo [13] obtained closed membrane vesicles exhibiting the same characteristics as intact cells for D-glucose specific transport activity (zero trans, equilibrium exchange and influx counterflow).

It is generally accepted that the uptake of amino acids into *S. cerevisiae* is energy-dependent and that at least some of the amino acid transport systems are driven by the proton-motive force. Up to now, however, only one report on amino acid transport by plasma membrane vesicles prepared from yeast has appeared [14]. The authors reported on amino acid transport into yeast plasma membranes vesiculated in an osmotic stabilization buffer. The amino acid uptake in these vesicles was affected by the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone. Apart from this finding the authors did not provide any evidence for the build-up of a protonmotive force in their system. Since the vesicle formation was done in the same buffer as the transport assays the formation of the protonmotive force is unlikely. In the present study an artificially formed protonmotive force (represented by one of its components, $\Delta\psi$) was used to energize the leucine transport.

3.3. *Protonmotive-force-driven leucine uptake*

The K^+-loaded proteoliposomes were diluted into either K^+-free (energized conditions) or K^+-rich medium (non-energized conditions). The time course of leucine uptake is shown in fig.2. A transient uptake of leucine was observed under energized conditions while no such overshoot occurred under non-energized conditions. Also, no leucine uptake was found in the presence of 50 μM carbonyl cyanide *m*-chlorophenylhydrazone and in a plasma membrane preparation not fused with liposomes (not shown).

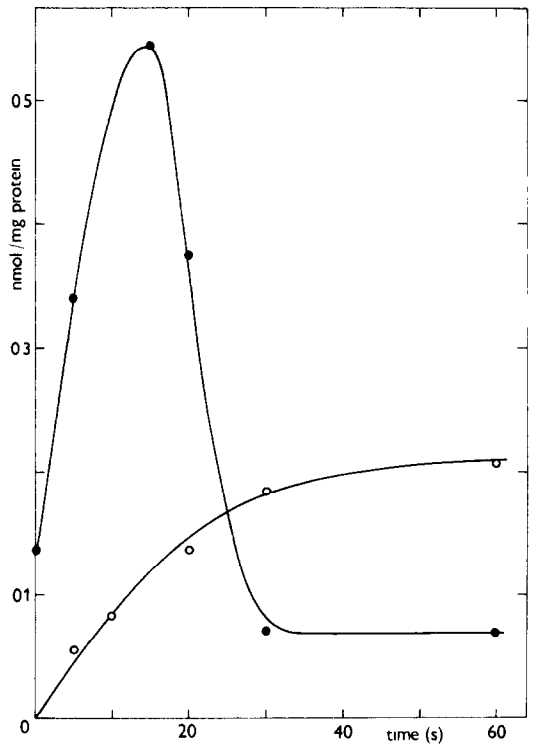

Fig.2. Leucine transport in yeast plasma membrane vesicles fused with liposomes by freeze-thaw sonication. K^+-loaded proteoliposomes (6 μl, 0.08–0.1 mg protein) were diluted into 0.2 ml medium containing 10 mM potassium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM KCl (○) or 10 mM sodium phosphate (pH 7.0), 5 mM $MgSO_4$, 100 mM NaCl (●). In both cases 50 μM [^{14}C]leucine (730 MBq/mmol) was present.

A fractional internal volume of 4.3 μl/mg protein was estimated for the proteoliposomes from the uptake of [^{14}C]leucine under non-energized conditions assuming that under these conditions the internal and external leucine equilibrated.

In bacterial membrane vesicles energized in the same way as those used in this study, Driessen (unpublished) observed the formation of inverted ΔpH (interior acid) generated as a result of a $\Delta\psi$-induced proton influx across the membrane. This inverted ΔpH compensates the $\Delta\psi$ and abolishes the overall protonmotive force after about 30 s while leaving the $\Delta\psi$ relatively stable. This explains the transient uptake of leucine in the energized proteoliposomes while a significant $\Delta\psi$ remains for a longer period. Also the lower level of [^{14}C]leucine at 60 s after the dilution of vesicles into K^+-free medium (fig.2) might reflect the inverted ΔpH formation resulting in a partial expelling of leucine from the vesicles. Further experiments are to be performed to check this possibility.

Since both the leucine transport systems [15] were present in the plasma membranes used for this study it is not clear which system mediates the observed protonmotive-force-driven leucine uptake.

ACKNOWLEDGEMENT

This work was partially supported by a grant to one of us (M.O.) from the Federation of European Biochemical Societies.

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