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Leucine transport in plasma membrane vesicles of *Saccharomyces cerevisiae*

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Yeast plasma membrane vesicles were obtained by the fusion of liposomes with purified yeast membranes by means of the freeze thaw-sonication technique. Beef heart mitochondria cytochrome-*c* oxidase was incorporated into the vesicles. Addition of substrate (ascorbate/TMPD/cytochrome *c*) generated a membrane potential negative inside, and an alkaline pH gradient inside the vesicle, that served as the driving force for leucine transport. Both ΔpH and $\Delta\psi$ could drive leucine transport. When ΔpH was increased in the presence of valinomycin and potassium, at the expense of $\Delta\psi$, leucine uptake increased by 10%.

Amino acid transport; Leucine transport; Electrochemical potential; Proteoliposome; Membrane vesicle; (Yeast)

1. INTRODUCTION

It is generally accepted that the transport of amino acids into yeast is driven by protonmotive force. Most of the evidence for this hypothesis comes from studies with intact cells (for review see [1]). Studies with whole cells, however, are often complicated by cellular metabolism and/or the difficulties encountered during the estimation of the components of the protonmotive force in the cells. The interference of metabolism and cell compartmentation can be overcome by employing plasma membrane vesicles. Since Kaback developed a procedure for the isolation of closed bacterial cytoplasmic membrane vesicles, this approach has been widely used in studies of solute transport in bacteria (for review see [2]). Reasonably sealed plasma membrane vesicles from *Saccharomyces cerevisiae* were prepared only recently using the technique of fusion of membranes with liposomes. Transport studies performed in such a system demonstrated a specific transport of D-glucose [3,4], transient protonmotive-force-driven leucine uptake [5] and membrane potential-driven ion transport [6]. To obtain more information on the role of protonmotive force on the transport of amino acids in yeast, a system is necessary which would generate and maintain for a reasonable long time period a membrane electrochemical potential difference of the same polarity as in whole cells. Such a system has been developed by Matsushita et al. [7] and by Driessen et al. [8,9], who demonstrated an accumulation of sugar and a large number of amino acids in bacterial membrane vesicles that have been fused with cytochrome-*c* oxidase proteoliposomes. The protonmotive force was generated by cytochrome oxidase with ascorbate/TMPD/cytochrome *c* as the electron donating system. In this study we

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; $DiSC_3(3)$, dipropylthiacarbocyanine; PC, phosphatidylcholine; PS, phosphatidylserine; TEA, triethanolamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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demonstrate that a similar system can be used in amino acid transport studies in yeast.

2. MATERIALS AND METHODS

2.1. *Yeast and its cultivation*

Saccharomyces cerevisiae DC XII was grown for 20 h at 30°C in a yeast semisynthetic medium [10] in which NH_4^+ was replaced by proline (2 mg/ml). After harvesting, the cells were washed twice with distilled water and kept overnight at 4°C.

2.2. *Preparation of protoplasts*

Protoplasts were prepared essentially according to Kovác et al. [11]. After 1 h incubation with snail gut enzyme or 40 min with lyticase (Sigma Chemical Co., St. Louis, MO), 95–98% of the cells were converted to protoplasts. They were washed twice with 50 mM Tris-HCl, pH 7.4, containing 0.9 M sorbitol, 10 mM $MgSO_4$, and preincubated for 1 h in the same medium with 0.8% glucose at 30°C.

2.3. *Preparation of plasma membrane*

Plasma membranes were prepared from the protoplasts by the concanavalin A method used by Opekarová et al. [5].

2.4. *Liposome preparation*

A mixture of acetone washed phosphatidylcholine (Sigma) (60 $mg \cdot ml^{-1}$) and phosphatidylserine, 1 mg/ml (Avanti Chem), in 50 mM H_3PO_4/TEA, pH 7, was sonicated to clarity using a probe sonifier (Branson Sonic B-12).

2.5. *Purification of cytochrome-c oxidase*

Cytochrome-*c* oxidase was purified from beef heart mitochondria using the method described by Ramirez et al. [12].

2.6. *Fusion of plasma membranes with liposomes and cytochrome oxidase incorporation*

Cytochrome-*c* oxidase was incorporated into the liposomes as previously described by Ramírez et al. [12]. The resulting proteoliposomes (0.8 ml) were mixed with 0.5 ml yeast plasma membranes (1.8 mg protein). The mixture was rapidly frozen in liquid nitrogen and then thawed slowly at room temperature. The turbid mixture was briefly sonicated at 4°C.

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

The membrane potential (interior negative) was determined by the fluorescence quenching of dipropylthiacarbocyanine ($DiSC_3(3)$) as described by Calahorra et al. [6]. The pH gradient was determined by the fluorescence changes of 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine). Liposomes in which 2 mM of dye was enclosed [13] were fused with plasma membranes as described above and the hybrid vesicles were passed through a Sephadex G 25 column to remove the external fluorescent probe. 10 μl of the vesicles were added to a cuvette with 2 ml fiinal volume of 50 mM H_3PO_4/TEA, pH 7.0, with or without 2.5 μM valinomycin and 5 mM KCl. The ΔpH formation was started by the addition of 1 mM ascorbate/TEA, pH 7.0, 0.1 mM TMPD and 150 μg cytochrome *c*. Fluorescence was measured at 467–510 nm.

2.8. *Transport assay*

The vesicles were diluted with 2.0 ml of 50 mM H_3PO_4/TEA, pH 7.0, 5 mM $MgSO_4$ to a final concentration of 0.8 mg protein per ml. At zero time, 50 μM [^{3}H]leucine (spec. act. 0.2 Ci/mmol; 7.4×10^6 Bq/mol), was added. 1 min later, 50 mM ascorbate/TEA, pH 7.0, 1 mM TMPD and 290 μg cytochrome *c* were added. Aliquots of 100 μl were withdrawn at intervals and diluted with 2.0 ml of ice-cold 100 mM LiCl. The suspension was filtered through 0.30 μm cellulose nitrate filters (Millipore) and washed once with 2.0 ml of LiCl. Filters were dried and transferred to scintillation vials with 5 ml of a scintillation cocktail and measured with a Packard liquid scintillation counter.

2.9. *Protein determination*

Protein was assayed according to Lowry et al. [14], using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Cytochrome-*c* oxidase incorporated into plasma membrane vesicles proved to be a powerful tool for generating a protonmotive force (alkaline and negative inside), which was able to drive the transport of a number of amino acids in bacterial vesicles [15]. Until now, however, only one report appeared on cytochrome oxidase functionally incorporated into yeast plasma membrane vesicles. Using a simple and efficient method for reconstitution [12], Calahorra et al. [6] demonstrated $^{86}Rb^+$ uptake into yeast plasma membrane vesicles with this respiratory enzyme incorporated. In this work we used the same method for reconstructing a system in which transport of amino acids in yeast can be studied.

We found that, as compared with vesicles containing only phosphatidylcholine (data not shown), cytochrome-*c* oxidase embedded liposomes prepared from the mixture of phosphatidylcholine and phosphatidylserine in a ratio 60:1 form more tightly sealed vesicles after fusion with the yeast membranes. This was demonstrated by higher values of respiratory control [12] and membrane potential formed in these vesicles.

On addition of the electron donating system to the suspension of yeast plasma membrane vesicles with cytochrome oxidase, a considerable membrane potential was formed, as demonstrated by the fluorescence quenching of the cyanine $DiSC_3(3)$ (fig.1A). This membrane potential could be abolished by CCCP. Considerably lower fluorescence quenching was observed (negligible $\Delta\psi$ formation) in the presence of valinomycin and

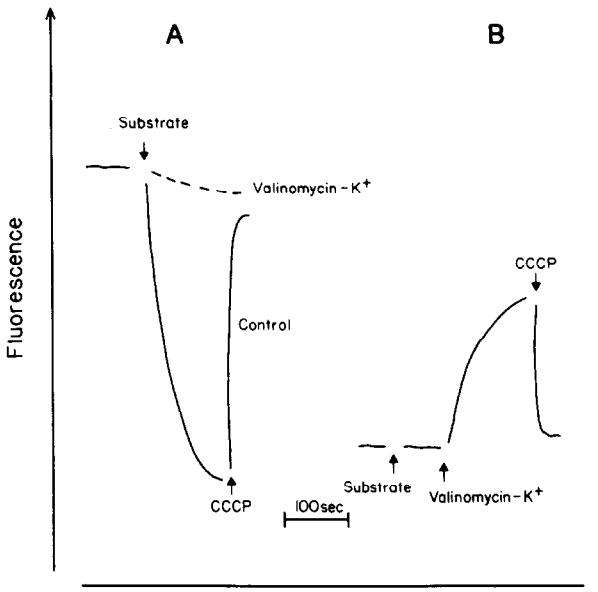

Fig.1. (A) Detection of a membrane potential in yeast plasma membrane vesicles with cytochrome oxidase incorporated. Effect of K^+ and valinomycin. Vesicles (50 μg protein) were added to 50 mM H_3PO_4/TEA, pH 7.0, and 0.5 M cyanine in a final volume of 2 ml. The reaction was started by the addition of substrate (1 mM ascorbate/TEA, pH 7, 0.1 mM TMPD, 150 μg cytochrome *c*) when indicated by the arrow; and collapsed with 2.5 μM CCCP. Where indicated, the experiment was performed in the presence of 5 mM KCl and 2.5 M valinomycin. (B) Detection of a pH gradient. Vesicles (50 μg protein) loaded with 2.0 mM pyranine were added in a cuvette with 2.0 ml final volume 50 mM H_3PO_4/TEA, pH 7.0. The reaction was started by the addition of substrate as in A. The addition of 5 mM KCl and 2.5 μM valinomycin generated an alkaline pH gradient inside that collapsed with 2 μM CCCP as indicated by the arrow.

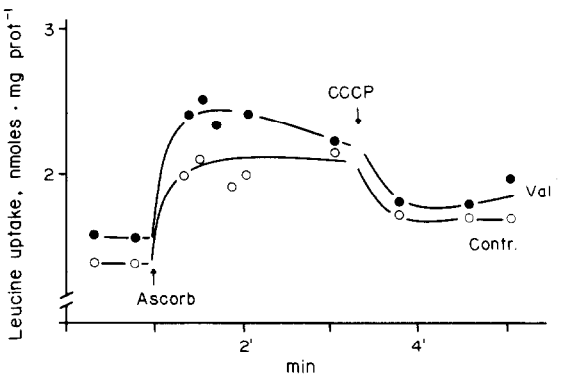

Fig.2. Leucine uptake by yeast plasma membrane vesicles with cytochrome-*c* oxidase incorporated. Vesicles were prepared as described in section 2 and added in the following medium: 5 mM $MgSO_4$, 50 M [^{3}H]leucine, 50 mM H_3PO_4/TEA, pH 7.0, in the presence (●) or absence (○) of 50 mM KCl and 2.5 μM valinomycin. The substrate (50 mM ascorbate/TEA, pH 7.0, 0.5 mM TMPD and 290 μg cytochrome *c*) was added as indicated. 6 μM CCCP was added as indicated.

potassium ions. The generation of the other component of the protonmotive force (ΔpH), monitored by pyranine (fig.1B), showed no pH gradient formation, unless valinomycin and potassium were present. This pH gradient, again, could be abolished by CCCP.

In our vesicle preparation, it is thus possible to create two sets of conditions for energizing amino acid transport, one with the protonmotive force consisting predominantly of $\Delta\psi$ in the absence of valinomycin and potassium, and another with it consisting essentially of the pH gradient, in the presence of the ionophore and K^+.

The leucine transport energized by the protonmotive force generated by cytochrome-*c* oxidase was assayed under both sets of conditions. On addition of a suitable electron donor system, a rapid leucine accumulation was observed (fig.2). In the presence of K^+ and valinomycin ('ΔpH' conditions), uptake was approximately 10% higher than in their absence ('$\Delta\psi$' conditions). No leucine uptake occurred in the presence of CCCP. Further, addition of the uncoupler to the vesicles in which leucine had already accumulated caused its efflux.

Protonmotive force formed on plasma membrane is the driving force for amino acid uptake in yeast, as shown for whole cells (review [1]), and demonstrated in yeast plasma membrane vesicles energized with valinomycin mediated diffusion potential [5]. The system we report in this work confirms these findings, and similarly, as in bacteria [15], opens the possibility of studying the role of particular components of the protonmotive force in energization of transport processes in yeast.

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