ON THE INVOLVEMENT OF P_i-BINDING PROTEINS IN P_i-UPTAKE IN THE YEAST *CANDIDA TROPICALIS*

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1. Introduction

Characterization and partial purification of P_i-binding proteins released by osmotic shock from *Candida tropicalis* have been reported [1]. Protein F_1 (characterized by a low affinity for P_i) was obtained in a highly purified state whereas protein F_2 (characterized by a high affinity for P_i) was slightly contaminated by F_1. To obtain data on the physiological significance of the phosphate binding proteins, antibodies F_1 and F_2 were raised in rabbits and the inhibitory effects of the antibodies on the P_i-binding activities of proteins F_1 and F_2 and on the P_i-uptake by whole cells are now reported.

2. Materials and methods

2.1 *Obtaining the proteins*

Candida tropicalis (strain 101) was grown as in [2]. Cells in exponential growth phase were incubated for 3 h in a medium lacking P_i [2], in order to stimulate the ability of the cells to take up P_i, and then subjected to a modification [3] of the cold osmotic shock procedure [4]. Cells (generally 30 g dry wt) were suspended (1 g dry wt/50 ml) in an hypertonic medium (20% sucrose, 1 mM EDTA, 30 mM Tris–HCl (pH 7.5) at 32°C for 15 min, centrifuged and resuspended in distilled water (1 g dry wt/50 ml) at 4°C for 15 min. After centrifugation, the shock fluid was recovered and clarified by filtration. Proteins present in the shock fluid were precipitated with $(NH_4)_2SO_4$ (65% saturation) in the presence of 50 mM Tris–HCl buffer (pH 7.5) containing 0.01% NaN_3 and collected by filtration through a millipore membrane filter (0.22 μm). The proteins were dissolved in 0.5 M Tris–HCl (pH 7.5) containing 0.1% NaN_3 and 0.25 M urea and dialysed overnight against buffer I (50 mM Tris–HCl (pH 7), 0.25 M urea, 1 mM KCl, 1 mM $MgCl_2$, 0.04% NaN_3). Proteins were concentrated on an Amicon membrane filter (minicon concentrator B15).

2.2. *Measurement of P_i-binding capacity*

The proteins were layered on a Sephadex G-150 column (85 ml gel) pre-equilibrated with buffer I. The column was eluted and 1.6 ml fractions collected. The fractions exhibiting P_i-binding capacity were pooled and concentrated by dialysis, using a minicon concentrator B15. P_i-binding capacity was measured as follows: an aliquot of proteins (0.200 ml) was incubated with ^{32}P for 15 h at 4°C or for 25 min at 32°C in buffer I, loaded on a Sephadex G-25 column (12 ml gel) and eluted. Fractions (0.5 ml) were collected and assayed for protein content (A_{280}) and radioactivity (Čerenkov method; Intertechnique SL 40 Scintillator).

2.3. *Purification of the P_i-binding proteins*

The fractions, obtained by filtration through Sephadex G-150 and showing P_i-binding capacity were loaded onto a DEAE-cellulose (Whatman, DE 52) or a DEAE-Sephacel (Pharmacia Fine Chemicals) column equilibrated with 20 mM Tris–HCl (pH 7.25) containing 0.1% NaN_3 and eluted with a 300 ml linear KCl gradient in the same buffer. 'Fraction 1' (F_1, eluted at 0.050 M KCl) and 'fraction 2' (F_2, eluted at 0.060 M KCl) were pooled and concentrated. They were either resuspended in buffer I, or in 50 mM Tricine buffer (pH 7). In the first case, fractions 1 and 2 were assayed for P_i-binding activity or layered on a Sephadex G-150 column to achieve purification.

In the second case, the proteins were used to stimulate P_i-uptake in osmotically shocked cells.

2.4. *Preparation of antisera and antibodies*

Proteins F_1 and F_2 after purification were injected into 2 rabbits at 3 time intervals (300 μg at each injection, in 50 mM tricine buffer, pH 7). After 3 months, the rabbit sera were tested for the presence of antibodies. Antisera used in the transport experiments were prepared as follows: the antisera were layered on a Sephadex G-25 column and eluted with buffer I, then concentrated, resuspended in 50 mM MES–KOH buffer (pH 6.5) and kept in a cold room for 2 or 3 h. Control rabbit serum was treated similarly. The antibodies were precipitated with $(NH_4)_2SO_4$ (40% saturation), dialyzed overnight, concentrated and resuspended in a buffer (ethylenediamine–Na 20 mM (pH 7), 0.04% NaN_3) and kept in a cold room.

2.5. *P_i-uptake by yeasts*

All experiments on P_i-uptake were done with cells pre-incubated for 2 or 3 h in a P_i-depleted medium. P_i-starved cells were centrifuged, rinsed and 2 kinds of experiments were then performed:

(i) Cells were subjected to the usual cold osmotic shock, then incubated for 15 min at 4°C in the presence of P_i-binding proteins eluted from DEAE-Sephacel (see section 2.3). P_i-binding proteins (1 mg) in 50 mM tricine buffer (pH 7), containing 4 mM $MgCl_2$, were added to cells (3 mg) dry wt) in the same tricine buffer (final vol. 1 ml). The suspension was diluted 10 times in the uptake medium (50 mM MES–KOH (pH 6.5), glucose 5 g/l). The control without P_i-binding proteins was also incubated for 15 min at 4°C, in the presence of 4 mM $MgCl_2$ in 50 mM tricine buffer (pH 7). After shaking for 5 min, radioactive P_i was added (final conc. 5×10^{-5} M).

(ii) Cells were suspended for 10 min at 4°C in MES–KOH buffer (50 mM, pH 6.5) in the presence of 0.2 ml of antisera or control serum (final vol. 0.4 ml). The cell suspension was diluted 10 times in the uptake medium (50 mM, MES–KOH or Hepes–KOH at different pH values, plus glucose 5 g/l). After shaking for 5 min, radioactive P_i was added (final conc. 5×10^{-5} M).

After the introduction of radioactive P_i, 0.5 ml cell suspension was withdrawn and filtered through a Sartorius membrane filter (3 μm). The radioactivity of the samples was measured (Čerenkov method).

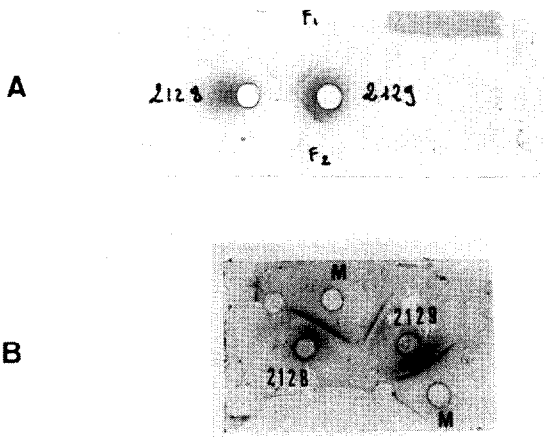

Fig.1. (A) Ouchterlony immunodiffusion test of P_i-binding proteins: 2128 (antiserum F_1); 2129 (antiserum F_2); F_1 and F_2 proteins purified as described in the text. (B) Ouchterlony immunodiffusion test of the protein mixture before purification (M).

3. Results and discussion

Fig. 1 shows the reaction of antisera against protein F_1 and the partially purified preparation F_2, and also against the unpurified proteins mixture. As expected, in Ouchterlony tests antiserum F_1 reacted with protein F_1, and antiserum F_2 with F_1 as well as F_2.

The effect of antibodies on the binding activities of proteins F_1 and F_2 was investigated. Fig.2 shows

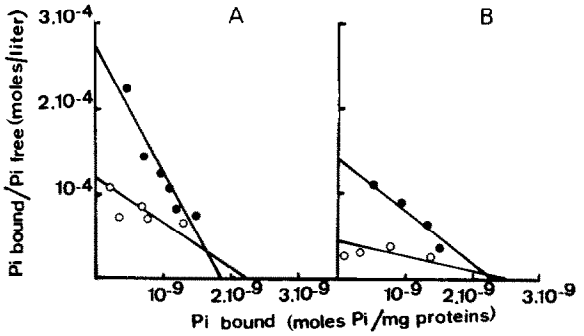

Fig.2. P_i-binding capacity of proteins eluted from a DEAE-Sephacel column (see text) as a function of P_i-concentration (expressed in mol/l), Eadie-Scatchard plot). (A) P_i-binding capacity of fraction 2 in the absence (•) or in the presence of antibody F_2 (○). (B) P_i-binding capacity of fraction 1 in the absence (•) or in the presence of antibody F_1 (○). In these experiments, the proteins released by osmotic shock after concentration were directly eluted from a DEAE-Sephacel column (see text). The dilution of antibodies used was 1/10.

that the antibodies inhibited the ability of proteins to bind P_i. The control serum did not affect the binding. Similar results have been obtained with *Escherichia coli* P_i-binding protein [5].

The effect of antisera F_1 and F_2 on P_i-uptake by whole, P_i-starved yeast cells at different external pH values are shown in fig.3. Antiserum F_1 decreased the P_i-uptake at neutral pH and exhibited only a slight inhibitory effect at acidic pH; the reverse was true for antiserum F_2. It must be remembered that this antiserum contained two antibodies, F_1 and F_2, as shown in fig.1.

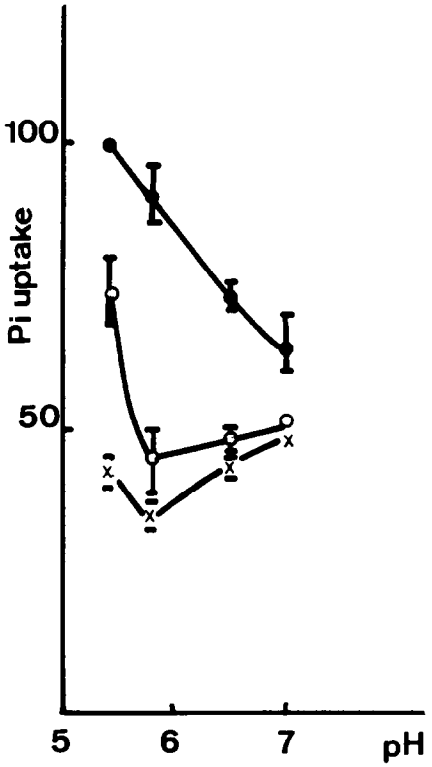

Fig.3. Effect of antisera F_1 (○) and F_2 (X) on P_i-uptake by P_i-starved yeast cells (initial velocities) at different external pH values. Control curve (●). The results are expressed in % of the control; P_i-uptake at pH 5.4 was taken as 100 (maximal P_i-uptake as a function of pH). The controls in all cases contained a control serum. Cells (0.2 mg dry wt) were incubated for 10 min at 4°C, in MES–KOH buffer (50 mM, pH 6.5), in the presence of 0.2 ml antisera or control serum. The cell suspension was diluted 10 times in the uptake medium (50 mM MES–KOH or Hepes–KOH at different pH-values, plus glucose 5 g/l). Averages of ≥4 expt are presented. Centrifugation after incubation with antisera did not change the results.

Proteins F_1 and F_2 added to shocked cells (see section 2) were found to stimulate P_i-uptake (control 100%, F_1; 145 ± 12%, F_2; 125 ± 12%, av. 10 expt). The order of magnitude of stimulation of P_i-uptake was similar to that observed in *E. coli* [5]. Several attempts to restore P_i-uptake in protoplasts were not successful and only a very short transient stimulation was observed (not shown). This is in contrast to the results obtained on P_i and ribose uptake by *E. coli* spheroplasts. However, P_i-binding proteins were released during protoplast formation, since the presence of P_i-binding proteins in the supernatant of protoplast preparations was demonstrated by immunological tests (not shown).

These results suggest that proteins F_1 and F_2, able to bind P_i, are involved in P_i-uptake in vivo. The P_i-binding proteins were found to stimulate P_i-uptake in shocked cells. Also, antisera F_1 and F_2 inhibited P_i-uptake by whole cells. Thus, it seems reasonable to conclude that the P_i-binding proteins are located near the cell surface. Furthermore, these findings, together with the evidence for a P_i-uptake system acting at neutral pH in *Saccharomyces* [8], lend support to a concept that protein F_1 plays a role in P_i-uptake at neutral pH whereas protein F_2 may be involved in P_i transport at acidic pH (pH 5.2–5.4, the physiological pH for growth) in the yeast *Candida tropicalis*.

References

[1] Jeanjean, R. and Fournier, N. (1979) FEBS Lett. 105, 163–166.
[2] Blasco, F., Ducet, G. and Azoulay, E. (1976) Biochimie 58, 351–357.
[3] Wiley, W. R. (1970) J. Bacteriol. 103, 656–662.
[4] Neu, H. C. and Heppel, L. A. (1965) J. Biol. Chem. 240, 3685–3692.
[5] Medveczky, N. and Rosenberg, H. (1970) Biochim. Biophys. Acta 211, 158–168.
[6] Gerdes, R. G., Strickland, K. P. and Rosenberg, H. (1977) J. Bacteriol. 131, 512–518.
[7] Galloway, D. R. and Furlong, C. E. (1979) Arch. Biochem. Biophys. 197, 158–162.
[9] Roomans, G. M., Blasco, F. and Borst-Pauwels, G. W. F. H. (1977) Biochim. Biophys. Acta 467, 65–71.