CHARACTERIZATION AND PARTIAL PURIFICATION OF PHOSPHATE-BINDING PROTEINS IN *CANDIDA TROPICALIS*

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1. Introduction

Energy-dependent phosphate uptake in the yeast *Candida tropicalis* is sensitive to osmotic shock [1]. This result suggests that phosphate-binding proteins are released by this procedure. The present paper confirms this observation and reports the characterization and partial purification of phosphate-binding proteins of *Candida tropicalis.*

2. Materials and methods

2.1. *Obtaining the proteins*

Candida tropicalis (strain 101) was grown as in [1]. *Candida* cells were incubated for 3 h in a medium lacking phosphate [1], in order to stimulate the ability of the cells to take up phosphate, and subjected to the modification [2] of the cold osmotic shock procedure [3]. 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) 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 0.01% NaN_3, 0.25 M urea and dialyzed overnight against buffer I (50 mM Tris–HCl (pH 7), 0.25 M urea, 1 mM KCl, 1 mM $MgCl_2$, 0.01% NaN_3). Proteins were concentrated on an Amicon membrane filter (minicon concentrator B_{15}).

2.2. *Measurement of phosphate-binding capacity*

Proteins (10 mg) determined spectrophotometrically at 280 nm, were equilibrated at 32°C with radioactive phosphate (10 nmol) in buffer I for 25 min, then 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 were assayed for protein content at 280 nm, for radioactivity (by using the Cerenkov method, scintillator Intertechnique SL 40), and for alkaline phosphatase activity (by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl phosphate in 0.5 M Tris–HCl (pH 8.5) containing 4 mM $MgCl_2$). The fractions labelled with radioactive phosphate, were pooled and concentrated by dialysis (minicon concentrator B_{15}). Phosphate binding capacity of these fractions was measured either as above or with the following modification: aliquots of proteins incubated with various amounts of ^{32}P for 15 h at 4°C in buffer I, layered on a Sephadex G-25 column (12 ml gel) and eluted with buffer I. Fractions (0.5 ml) were collected and assayed for protein content (A_{280}) and radioactivity (Cerenkov method).

2.3. *Purification of the phosphate-binding proteins*

The fractions containing phosphate-binding capacity obtained by filtration through Sephadex G-150 (fig.1, fractions 40–50) were pooled, concentrated and loaded on a DEAE-cellulose (Whatman, DE52) column pre-equilibrated with 20 mM Tris–HCl (pH 7.25), NaN_3 0.1% and eluted with a 300 ml linear gradient of KCl (0–0.5 M) in the same buffer.

3. Results and discussions

Figure 1 shows a typical distribution of phosphate-binding capacity and alkaline phosphatase activity

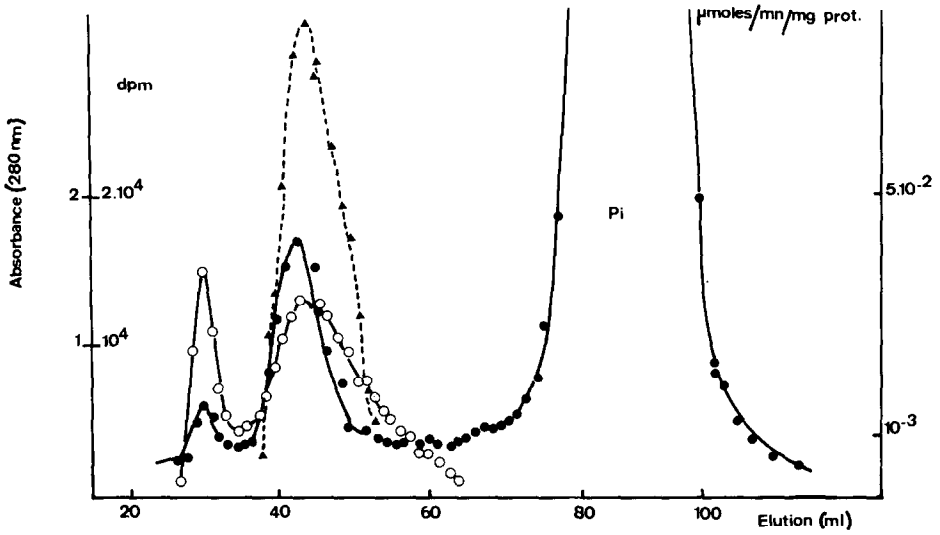


Fig.1. Elution profile from a Sephadex G-150 column of proteins released by osmotic shock from *Candida tropicalis*. (●——●) Radioactivity of the samples (in dpm); (○——○) A_{280}; (▲– – –▲) alkaline phosphatase activity (in mol *p*-nitrophenol liberated .min^{-1}.mg $protein^{-1}$).

in fractions collected by Sephadex G-150 filtration of proteins released by osmotic shock and obtained as in section 2. Radioactivity in peak I (excluded fractions) was attributed to protein aggregates and was discarded. Phosphate-binding and alkaline phosphatase activity were found in the second peak. These fractions (40–50, fig.1) were pooled, concentrated and their phosphate binding capacity was examined as indicated in section 2. The results of (P_i bound/mg protein) were plotted according to Eaddie Scatchard (fig.2). The curves of phosphate binding were biphasic, both a high affinity (3–5 μM) and a low affinity constant (17–22 μM) were found.

Arsenate (fig.3) competitively inhibited the binding of phosphate (the inhibition constant was the same order of magnitude as the high affinity constant,

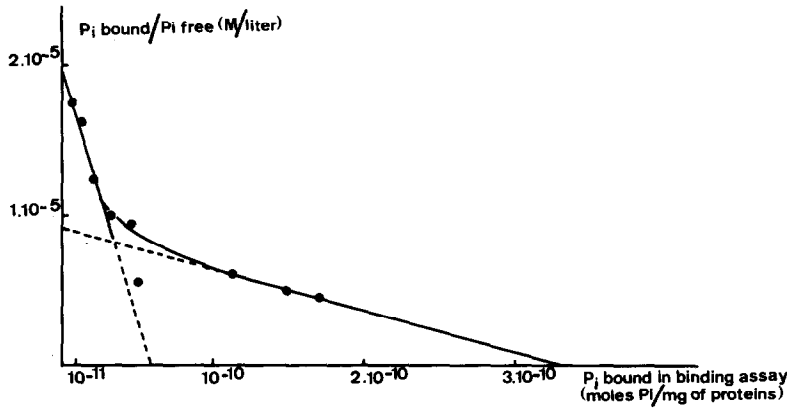


Fig.2. Phosphate-binding capacity of the proteins eluted from Sephadex G-150 column (see the text) as a function of phosphate concentration (Eaddie-Scatchard plot). Phosphate-binding capacity is in mol P_i/mg protein; phosphate concentration is in M.

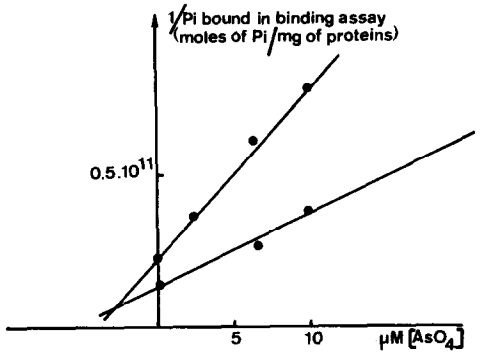


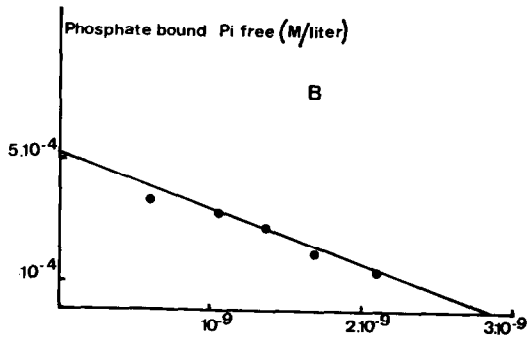
Fig.3. Competitive inhibition of the phosphate binding capacity in the presence of arsenate (Dixon plot). Phosphate binding capacity is in mol P_i/mg protein; arsenate concentration is in μM. Upper curve P_i concentration 2 μM; Lower curve P_i concentration 5 μM.

i.e., 3–5 μM); however, the binding was not affected by the presence of sulfate. The binding was also sensitive to mercaptoethanol (24 mM, 83% inhibition) and to *N*-ethylmaleimide (1 mM, 45% inhibition).

The proteins obtained by filtration through Sephadex G-150 column (40–50, fig.1) and exhibiting phosphate-binding capacity were loaded on a DEAE-cellulose column. The fraction (I) eluted by 0.050 M KCl was pooled, concentrated and its phosphate binding capacity was measured. This fraction binds phosphate and was characterized by a low affinity constant for P_i (17–22 μM) by different experiments (see fig.4A).

The molecular weight of the protein present in the fraction I able to bind phosphate determined by filtration through Sephadex G-150 column was ~100 000 (fig.5A). The samples characterized by higher radioactivity/protein ratios after filtration through Sephadex G-150 were found to contain one major component (designated as protein I) as shown by electrophoresis under non-denaturing conditions (data not given). The proteins, eluted by 0.060 M KCl (fraction II) were pooled, concentrated and their phosphate binding capacity was studied. The results (fig.4B) showed that this fraction bound phosphate and a high affinity constant (5–6 μM) was found. The molecular weight of the proteins of this fraction determined in the same conditions as fraction I, was ~70 000–75 000 (fig.5B). The samples (higher radioactivity:protein ratio) after filtration through Sephadex G-150 analyzed by electrophoresis under non-denaturing conditions was found to be constituted by two main proteins, one probably similar to protein I (the migration distance was identical to protein I), and a major component (protein II) which was responsible for 4 the high phosphate-binding capacity. The proteins able to bind phosphate did not contain alkaline phosphatase activity; this is an important observation since it is known that bacterial alkaline phosphatase released by osmotic shock does bind phosphate [4].

In conclusion, the results demonstrated that two proteins released by osmotic shock from *Candida*



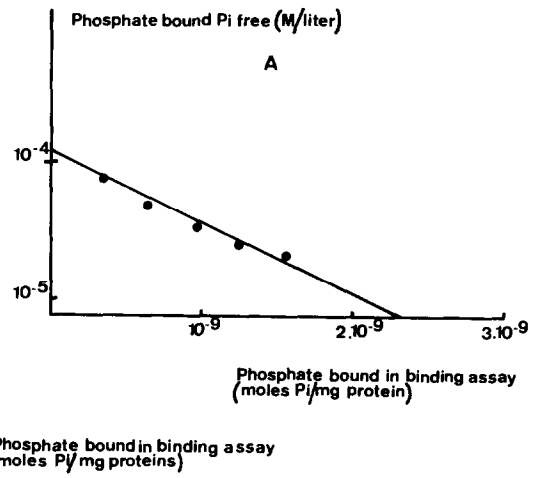


Fig.4. Phosphate-binding capacity of the proteins eluted from DEAE-cellulose column (see text) as a function of phosphate concentration (Eaddie-Scatchard plot). Phosphate-binding capacity is in mol P_i/mg protein; phosphate concentration is in M. (A) Phosphate-binding capacity of fraction I. (B) Phosphate-binding capacity of fraction II.

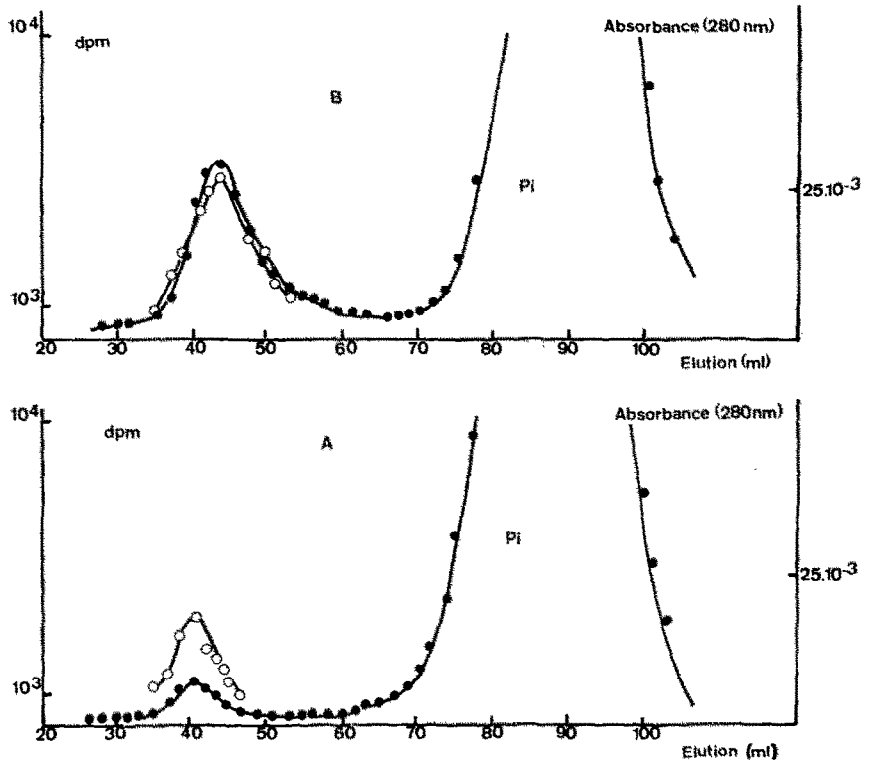


Fig.5. (A) Elution profile of fraction I (obtained from a DEAE-cellulose column) from a Sephadex G-150 column. (•——•) Radioactivity of the samples (in dpm); (○——○) A_{280} nm. (B) Elution profile of fraction II (obtained from a DEAE-cellulose column) from a Sephadex G-150 column. (•——•) Radioactivity of the samples (in dpm). (○——○) A_{280}.

tropicalis are able to bind phosphate. The first (protein I) has mol. wt ~100 000, and exhibits a low affinity constant; the other (protein II) has mol. wt ~70 000–75 000 and is characterized by a high affinity constant, approximately the same as the K_m for phosphate transport in phosphate-starved *Candida* cells. Phosphate-binding protein has already been isolated from *Escherichia coli* [5,6] and a number of proteins binding amino acids, sugars and sulfate, have been obtained from bacteria [7–11]. Generally, these bacterial binding proteins were mol. wt 37 000–45 000. There are very few data on the binding proteins in eucaryotic cells, except the characterization of the tryptophane-binding protein released by osmotic shock from *Neurospora* [2]. However, this protein has estimated mol. wt >200 000. The molecular weight of the phosphate-binding proteins described here is in the range of the molecular weight of other binding proteins already isolated.

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