

Affinity purification of molecular chaperones of the yeast *Hansenula polymorpha* using immobilized denatured alcohol oxidase

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We used peroxisomal alcohol oxidase (AO) for the affinity purification of molecular chaperones from yeasts. Methodical studies showed that up to 0.8 mg of purified bacterial GroEL was able to bind per ml of immobilized denatured AO column material. Using crude extracts of *Hansenula polymorpha* or *Saccharomyces cerevisiae*, several proteins were specifically eluted with Mg-ATP which were recognized by antibodies against hsp60 or hsp70. One *H. polymorpha* 70 kDa protein was strongly induced during growth at elevated temperatures, whereas a second 70 kDa protein as well as a 60 kDa protein showed strong protein sequence homology to mitochondrial SSC1 and hsp60, respectively, from *S. cerevisiae*.

Alcohol oxidase; Chaperone; HSP; Peroxisome; *Saccharomyces cerevisiae*; *Hansenula polymorpha*; Complex formation

1. INTRODUCTION

Molecular chaperones play an important role in protein translocation and modulation of folding and oligomerization of newly synthesized proteins [1]. Common feature of these proteins is their ability to interact with other proteins which assume a more or less unfolded structure [2,3]. The components of the 'chaperone system' generally do not act independently in guiding the folding proteins to their final conformation, but at some stages cooperate in a sequential reaction [4].

In the course of our studies on the biogenesis of yeast peroxisomes we are now investigating the possible role of molecular chaperones in peroxisomal protein translocation or assembly. In an initial series of experiments we studied the use of denatured peroxisomal alcohol oxidase (AO) from *Hansenula polymorpha* for the binding of a purified chaperone; we showed that bacterial GroEL can form complexes with denatured AO in vitro. Complex formation occurred in the absence of ATP; once-formed complexes were stable at low temperature even in the presence of Mg-ATP, whereas dissociation of the complex was dependent on ATP-hydrolysis at room temperature [5].

Based on these findings we explored the use of immobilized denatured AO for the affinity purification of

chaperones of the yeast *H. polymorpha*. The results of this study are presented in this paper.

2. MATERIALS AND METHODS

2.1. Organisms and growth conditions

Hansenula polymorpha de Moraes et Maya CBS 4732 was grown in continuous culture at 37°C on mineral medium [6] supplemented with 1% (w/v) methanol. In addition the organism was grown in batch culture in mineral medium using 0.5% (v/v) methanol as sole carbon source at 25°C or 43°C. For heat shock experiments, cells grown at 25°C were exposed to 43°C for 30 min. *Saccharomyces cerevisiae* 273-10B was grown on 0.1% (v/v) oleic acid at 30°C [7].

2.2. Preparation of crude cell extracts and organellar pellets

Crude extracts of cells were prepared as described previously [8]; organellar pellets from chemostat-grown *H. polymorpha* were obtained after differential centrifugation of homogenized protoplasts [9] obtained by Zymolyase treatment of intact cells. The 30,000 × g pellets were solubilized in buffer A, containing 25 mM Tris-HCl (pH 7.0), 50 mM KCl and 1 mM dithiothreitol (DTT) and used for further analysis.

2.3. Preparation of immobilized denatured alcohol oxidase

Purified peroxisomal alcohol oxidase (AO) of *H. polymorpha* was a gift from Unilever Research Laboratories. Native AO (10 mg/ml bed volume) was immobilized on CNBr-activated Sepharose 4B according to instructions of the manufacturer (Pharmacia, Sweden) and subsequently denatured with 8 M urea in 25 mM Tris-HCl, pH 7.0 for 24 h at 4°C. The column material was then extensively washed with buffer A.

2.4. Conditions of binding

For methodical studies, increasing amounts of purified GroEL were incubated with 100 µl aliquots of column material at 0°C for 15 min in buffer A. Samples of crude extracts prepared from yeast cells were

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incubated for 15 min with immobilized denatured AO in the presence of 1 mM Mg-ATP in buffer A at room temperature to dissociate possible existing complexes between chaperones and natural target proteins. After incubation, EDTA (pH 7.0; 50 mM final concentration) was added and the mixture was cooled on ice.

2.5. Conditions of elution

After binding of proteins to the column material, columns were poured and extensively washed in three subsequent steps, including 15 column volumes of (1) buffer A, (2) 0.2 M KCl in buffer A and (3) again buffer A. For methodical studies with GroEL and with chaperones present in crude extracts of yeasts the column was then eluted with 5 column volumes of 10 mM Mg-ATP in buffer A at room temperature and subsequently with 8 M urea.

For some studies an additional elution with 0.1 M acetic acid [10] was performed between the Mg-ATP and 8 M urea elution steps.

2.6. Analytical procedures

SDS-PAGE [11], protein sequencing [12] and Western blotting [13] was carried out according to published methods

3. RESULTS

Methodical studies, using purified bacterial GroEL as a model protein, were carried out to determine the capacity of the alcohol oxidase (AO) column for binding of molecular chaperones. These studies revealed that significant amounts of this purified bacterial protein were capable to bind to immobilized denatured AO, which could be quantitatively eluted by 8 M urea (Fig. 1). Elution did not occur by either KCl or Mg-ATP. The amount of bound GroEL increased with the amount of added protein until a plateau was reached at approximately 0.8 mg of protein/ml column material. A minor amount of the protein was detected in the flow through fraction prior to saturation of the column. This fraction most probably contains GroEL protein which has lost the ability to bind unfolded proteins.

Identical studies, performed on crude extracts of *H. polymorpha*, harvested from the mid-exponential growth phase on methanol, revealed that increasing amounts of proteins could be eluted with Mg-ATP

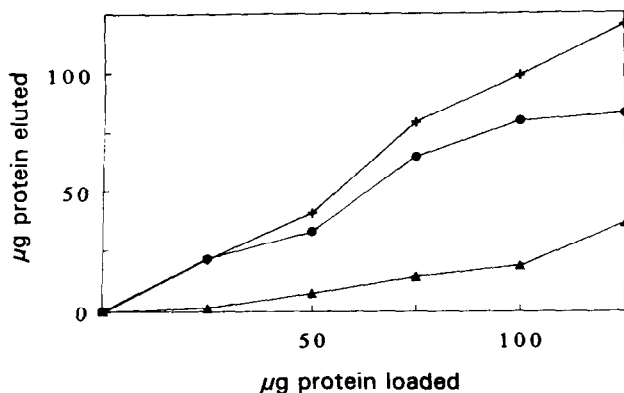


Fig. 1. Determination of the capacity of immobilized denatured AO column-material for purified bacterial GroEL. Binding was performed with 100 µl column material. Protein was only detected in the flow-through (▲—▲) and urea (●—●) elution. The sum of both elutions is indicated by (+—+) and equals the amount of protein loaded.

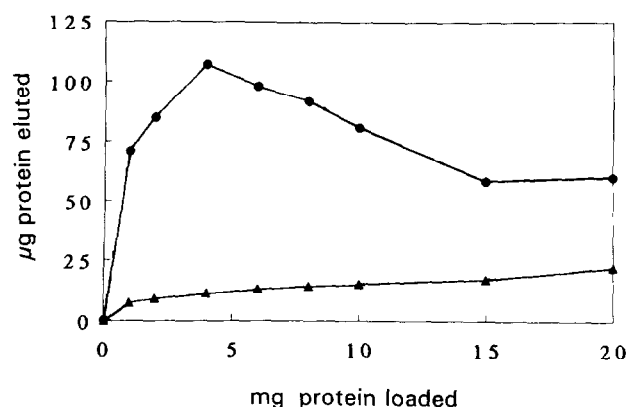


Fig. 2. Determination of amount of protein in Mg-ATP and urea elutions when increasing amounts of crude extracts of *H. polymorpha* are loaded on 100 µl columns. The amount of protein in the Mg-ATP elution (▲—▲) increases throughout the range tested whereas the urea elution (●—●) reaches a maximum of 120 µg when 4 mg of protein is loaded on the column.

throughout the range studied (up to 20 mg protein/100 µl column material; Fig. 2). In the urea elution, however, a maximum was observed when 4 mg of protein was loaded on the 100 µl column (Fig. 2). SDS-PAGE indicated that in Mg-ATP-elutes using crude extracts (Fig. 3, lane 2) of *H. polymorpha* grown in a methanol-limited chemostat at 37°C, three prominent protein bands of approximately 70 kDa were present together with one of 40 kDa and a minor protein band of approximately 60 kDa (Fig. 3, lane 3). All three 70 kDa protein bands were recognized by specific polyclonal antibodies raised against the conserved amino (N) terminal part of hsp70 of *Saccharomyces cerevisiae* (Fig. 3, lane 4). Data obtained from protein sequencing indicated that the upper 70 kDa protein was N-terminal blocked, the middle band appeared to consist of three yet unidentified proteins, whereas the sequence of the lower 70 kDa protein showed a 85% sequence identity in a 20 amino acids overlap to the N-terminus of the mitochondrial SSC1 protein of *S. cerevisiae* (Table I). The N-terminus of the 40 kDa protein showed 78% sequence identity to mitochondrial malate dehydrogenase of *S. cerevisiae* in a 23 amino acid overlap (Table I). Using extracts of heat shocked methanol-grown cells of *H. polymorpha*, an identical pattern of 60 and 70 kDa proteins could be detected in the Mg-ATP elution after Coomassie staining of SDS-PAA gels (Fig. 3, lane 5). However, specifically the 60 kDa protein appeared to be induced compared to untreated cells (Fig. 3, compare lanes 3 and 5). Several proteins present in this elution, including the above 60 kDa protein are stained more intensely by silver (Fig. 3, compare lanes 5 and 6). This 60 kDa protein, together with two additional proteins of approximately 60 kDa, but with a slightly higher electrophoretic mobility cross-reacted with antibodies against bacterial GroEL (Fig. 3, lane 7). The protein

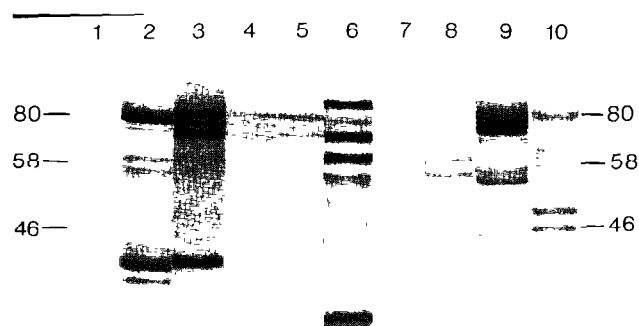


Fig. 3 7.5% SDS-PAA gels of elutions from immobilized denatured AO and Western blots of corresponding fractions after binding of proteins of crude extracts and isolated ($30,000 \times g$) organellar pellets of methanol-grown *H. polymorpha*. Lane 1 and 10: markers (46, 50, 58, 68, 80 and 94 kDa). Crude extracts (lane 2, Coomassie) of methanol-grown *H. polymorpha* were incubated with denatured immobilized AO and eluted with Mg-ATP (lane 3, Coomassie). Western blotting of the same fraction as shown in lane 3, decorated with antibodies against the N-terminal conserved part of hsp70 of *S. cerevisiae* revealed the presence of three 70 kDa bands cross-reacting with these antibodies (lane 4). Crude extracts of heat shocked methanol-grown *H. polymorpha* were used to study heat shock induction. Mg-ATP elutions were stained with Coomassie (lane 5), silver (lane 6) or after Western blotting decorated with antibodies against GroEL (lane 7). Proteins present in a $30,000 \times g$ organellar pellet (lane 9, Coomassie) were after binding eluted with 0.1 M acetic acid (lane 8, Coomassie).

with the highest apparent molecular weight, specifically enhanced after silver staining of the gel (Fig. 3, lane 6) was excised for N-terminal protein sequencing. The N-terminus of this protein showed 100% identity to mitochondrial hsp60 of *S. cerevisiae* in a 11 amino acid overlap (Table I) and most probably represents the *H. polymorpha* counterpart of mitochondrial hsp60, since it was also present in the $30,000 \times g$ organellar pellet (Fig. 3, lanes 8 and 9) obtained from methanol grown *H. polymorpha*. Subsequent studies on crude extracts of cells grown on methanol at 43°C showed that under these conditions specifically one of the 70 kDa protein bands was strongly enhanced (Fig. 4).

In an attempt to further exploit the potential of the method, control experiments were performed on crude extracts, prepared from oleate-grown *S. cerevisiae* (Fig. 5, lane 1). The Mg-ATP elution of such crude extracts, contained different prominent protein bands of approximately 60 and 70 kDa (Fig. 5, lane 2). Antibodies against the N-terminal conserved part of hsp70 of *S. cerevisiae* and mitochondrial hsp60 of *Neurospora crassa* showed cross-reactivity with bands of the corresponding molecular mass. The 70 kDa band appeared to consist of several proteins which were stained with different intensities, indicating differences in recognition by these antibodies of the individual proteins (Fig. 5, lanes 3 and 4). Following Mg-ATP elution, the column was subsequently eluted with 0.1 M acetic acid, resulting in elution of additional amounts of the 60 and 70 kDa proteins (Fig. 5, lanes 5–7) together with a range of proteins of lower molecular weight. This result sug-

gests that not all of the 60 and 70 kDa proteins were functionally eluted and furthermore, that other not yet identified proteins were able to bind to immobilized denatured AO. An example of this phenomenon is presented in Fig. 5, lane 5, showing the proteins present in the acetic acid elution.

4. DISCUSSION

In this paper a single-step affinity purification procedure for several molecular chaperones is presented. This method is based upon the biological activity of these proteins to bind to non-native protein, using denatured peroxisomal alcohol oxidase (AO) as a target protein and allows the analysis of chaperones present in crude protein extracts of vegetative yeast cells. Methodical studies, designed to determine the capacity of the AO column, using purified bacterial GroEL, showed that approximately 75% of the purified protein bound to immobilized denatured AO; this value is in agreement with earlier data obtained for free denatured AO [5], but strongly enhanced compared to binding studies based on the use of an immobilized abnormal fusion protein, in which 3–10% of molecular chaperone was bound [10]. By this method we have identified putative chaperones of approximately 60 and 70 kDa of the yeast *Hansenula polymorpha* together with a temperature-inducible 70 kDa protein. Protein sequence data of the N-termini of both a 60 and 70 kDa protein suggest that these represent homologues of the mitochondrial hsp60 and hsp70 of *Saccharomyces cerevisiae*, respectively. Additional evidence for the organellar location of the above 60 kDa protein was obtained from the fact that a 60 kDa protein with the same relative mobility was present in an

Table I

Amino (N) terminal protein sequence comparison of proteins which bound to denatured immobilized AO and could be specifically eluted with Mg-ATP

Hp70	:	STKVTGSGVIGIDLGTTNAA
	:
SSC1	:	STKVQGSVIGIDLGTTNSA
Hp60	:	SHKELKFGVEG
	:
hsp60	:	SHKELKFGVEG
Hp40	:	VKVTXVXGAAGGIGQPLSLMLKMN
	:
MMDH	:	YKVTVLGAGGGIGQPLSLLLKLN

Identical amino acids are indicated by :, homologous amino acids are indicated by .. X indicates an unidentified amino acid. Alignment of the N-termini of the *H. polymorpha* 70 kDa protein (Hp70) and the *S. cerevisiae* SSC1 protein (SSC1) showing 85% identity, the *H. polymorpha* 60 kDa protein (Hp60) and the *S. cerevisiae* mitochondrial hsp60 (Mhsp60) showing 100% identity and the *H. polymorpha* 40 kDa protein (Hp40) and *S. cerevisiae* mitochondrial malate dehydrogenase (MMDH) showing 78% identity.

30,000 $\times g$ organellar pellet, prepared from methanol-grown *H. polymorpha*. Neither purified GroEL nor putative chaperones present in crude extracts of *H. polymorpha* were quantitatively eluted with Mg-ATP. This effect was more strongly observed for hsp60 compared to hsp70 (Fig. 5, lane 5) and might be due to immediate rebinding of the chaperones to the denatured AO. This is in agreement with the proposed model for the functioning of hsp60, which predicts that protein release is coupled to folding and depends on the regulation by a GroES homologue. The hydrolysis of ATP, in the absence of GroES may lead to a partial release of the substrate after which rapid rebinding occurs [14]. It may be speculated that the addition of a competitive substrate during elution will enhance recovery of the bound 60 and 70 kDa proteins and will allow to functionally elute also other proteins, now found in the acetic acid and urea elutions. We do not yet know if binding of these proteins represents a biological function but it might be that these proteins bind to the column together with the identified proteins in multi-subunit complexes and have different requirements for dissociation.

When increasing amounts of crude extract are loaded on the column a maximum in the urea elution, but a steady increase in the Mg-ATP elution was observed. This might be explained in two ways. Firstly, all the binding places on the column material are occupied when 4 mg of proteins are loaded on the column (Fig. 2) and the affinity for denatured AO of the proteins that can be eluted by Mg-ATP is higher than that of those that can only be eluted by urea. Binding of these proteins is thus at the expense of binding of the only urea elutable proteins. Secondly, the ratio of natural target proteins in the crude extract versus denatured AO increases by addition of more concentrated crude extract, competing for the binding of putative chaperones.

We have no clearcut explanation for the presence of malate dehydrogenase in the Mg-ATP elution, but it is possible that some, perhaps partially denatured proteins interact unspecifically with the immobilized denatured AO.

The described method of AO affinity chromatogra-

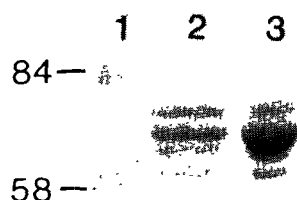


Fig. 4. Coomassie stained 7.5% SDS-PAA gel of Mg-ATP elutions from immobilized denatured AO after binding of crude extracts of *H. polymorpha* cells grown at 25°C (lane 2) and 43°C (lane 3) on methanol until the mid-exponential growth phase. Lane 1: markers (58 and 84 kDa).

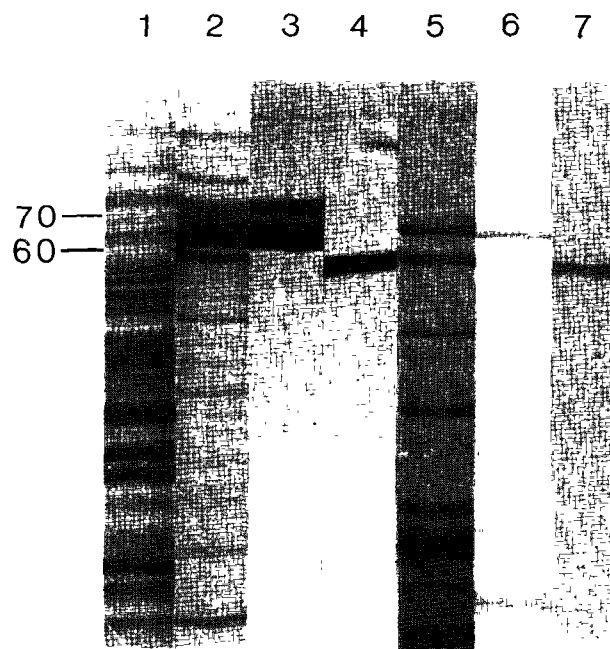


Fig. 5. 10% SDS-PAA gels of Mg-ATP and acetic acid elutions from immobilized denatured AO and Western blots of corresponding fractions after binding of crude extracts (lane 1) of *S. cerevisiae* grown on oleic acid. Mg-ATP elutions of immobilized denatured AO where stained with Coomassie (lane 2) or after Western blotting decorated with antibodies against the N-terminal conserved part of hsp70 of *S. cerevisiae* (lane 3) or mitochondrial hsp60 of *Neurospora crassa* (lane 4). Acetic acid elutions of immobilized denatured AO performed following the Mg-ATP elution were stained with Coomassie (lane 5) or after Western blotting decorated with antibodies against the N-terminal conserved part of hsp70 of *S. cerevisiae* (lane 6) or mitochondrial hsp60 of *Neurospora crassa* (lane 7).

phy will be useful as an efficient screening procedure to identify the chaperone components of different cellular fractions and organelles.

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