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The isolation and reconstitution of the ADP/ATP carrier from wild-type *Saccharomyces cerevisiae*

Identification of primarily one type (AAC-2)

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Methods for isolation of the ADP/ATP carrier (AAC) from yeast (*Saccharomyces cerevisiae*) are described which allow separation of the carrier from the initially copurified porin which poses a specific problem in yeast. The procedure varies according to whether one wishes to obtain a stable CAT-AAC complex, the free and active AAC for reconstitution, or the SDS-denatured pure AAC peptide. CNBr cleavage of AAC enabled us to differentiate clearly between isogenes AAC-1 and AAC-2 recently found in yeast, due to the exclusive occurrence of a methionine (M-115) residue at the end of the first domain in AAC-2. Thus the AAC isolated from wild-type yeast is primarily or exclusively AAC-2. The isolated AAC is active in ADP/ATP exchange in reconsitituted liposomes with a V_{max} of 1100 μmol/min per g protein and $K_m = 15$ μM for ADP, and a V_{max} of 900 μmol/min per g protein and $K_m = 9$ μM for ATP.

ADP/ATP carrier; Cyanogen bromide cleavage; Reconstitution; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Among eukaryotic cells, yeast is at present the most suitable target for molecular biological and genetic manipulations. Therefore, for applying molecular biological methods, the characterization and isolation of the ADP/ATP carrier (AAC) from *Saccharomyces* are desirable. Originally, in isolated mitochondria from *Saccharomyces*, functional properties of the AAC were studied [1], such as the exchange kinetics and inhibition by atractylate. With procedures similar to those developed for bovine heart AAC [2], the isolation from yeast of two proteins of 37 and 34 kDa was reported [3]. The putative AAC of 37 kDa was separated by affinity chromatography. This molecular mass was considerably higher than found previously for the AAC from bovine heart and *Neurospora*. Also, the 37 kDa component was photoaffinity-labelled by arylazido-ADP [4]. The structural and functional properties of this protein were, however, not further characterized. Later, by genetic complementation of the yeast mutant op_1, an AAC was found which migrated only with an apparent molecular mass of 30–32 kDa [5]. cDNA sequence analysis of this AAC gene then revealed a value of about 33 kDa [6]. However, it was recently shown that *Saccharomyces* contains another nuclear gene for the AAC, called AAC-2, which codes for a protein of 33.6 kDa [7].

In view of the fact that the isolation of the AAC from yeast and its molecular and functional characterization remained unsatisfactory, and in view of the vast potential for molecular, biological and genetic manipulation of the AAC in yeast, it was important to develop a highly efficient isolation procedure. Furthermore, functional charac-

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Abbreviations: DTE, dithioerythritol; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; HTS, hydroxyapatite; CAT, carboxyatractylate; BKA, bongrekic acid

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terization of the isolated AAC after reincorporation into liposomes was required. It should also be decided as to what extent the two different gene products, AAC-2 and AAC-1, are actually expressed in the wild-type yeast.

2. MATERIALS AND METHODS

2.1. *Cultivation of yeast cells and preparation of mitochondria*

The haploid wild-type *S. cerevisiae* strain D 273-10B α was used. Yeast cells were grown in the usual synthetic culture medium with lactate as carbon source [8]. Mitochondria were isolated according to [8] with minor modifications. For isolation of intact AAC, 2 μmol CAT/mg protein and 50 μM ATP were added to the fresh mitochondria. For maximizing the yield of mitochondria, the cytochrome content of yeast cells was determined. On average, the cells contain 0.05 μmol/g (w/w) of cytochrome a/a_3. The purity of mitochondria was assayed by measuring the content of cytochrome a/a_3, usually amounting to 0.16 μmol/g protein.

2.2. *Isolation of AAC*

2.2.1. Isolation of intact AAC

100 mg yeast CAT-preloaded mitochondria were incubated in 5 ml homogenizer buffer [150 mM Na_2SO_4, 0.5 mM EDTA, 20 mM Tricine, 1 mM PMSF (pH 8.0); 1% TX-100 as detergent (detergent/protein, 1:1)]. PMSF was added to inhibit the high proteolytic activity in the detergent extracts. After 15 min incubation and ultracentrifugation at 100000 × *g* for 30 min the supernatant was applied to a hydroxyapatite column (6 × 2 cm) with an HTS-protein ratio of 200 μl/mg and eluted with a buffer containing 0.1 M NaCl, 10 mM Mops, 0.1 mM EDTA, 0.2% Triton X-100 (pH 7.2). The AAC-containing fractions (80–170% of the column volume) were collected (protein concentration 0.2 mg/ml) and concentrated by pressure dialysis to 2 mg/ml. This concentrate was then applied to an S-300 column (80 × 0.8 cm) and eluted in 1.8-ml fractions per 8 min with the same buffer (flow rate 225 μl/min).

2.2.2. High yield isolation of denatured AAC

The first purification step is the same as that in section 2.2.1, except that a detergent/protein ratio of 2.5:1 was used. The combined AAC-containing fractions of the HTS column at 0.7 mg protein/ml were concentrated by pressure dialysis to 6 mg/ml. The S-300 column (130 × 0.6 cm) was run with the buffer used for intact AAC, with the addition of 1% SDS, and 0.5-ml fractions were collected per 50 min (flow rate 33 μl/min).

2.3. *CNBr cleavage*

The purified AAC was incubated for 8 h in a CNBr/formic acid (80%) solution at 10 μg CNBr per μg protein (500-fold excess with respect to methionines). The cleavage products were separated by a gel-electrophoresis system [9], most suitable for separation of small peptides.

2.4. *Reconstitution*

Transport activity of the yeast AAC was determined with minor modifications using the inhibitor stop method according to [10]. Purification has to be rapid in view of the instability of the unliganded yeast AAC and is carried out in batch procedure [11]. Mitochondria were solubilized in 2% Triton X-100, 150 mM Na_2SO_4, 0.5 mM EDTA, 20 mM Tricine-NaOH (pH 8.0) (10 mg protein/ml). The HTS/protein ratio was 280 μg/mg. Proteoliposomes were prepared by sonication of phospholipids (8% phosphatidylcholine, 2% phosphatidylethanolamine purified from egg yolk, 1% cholesterol) under addition of AAC protein (100 μg/38 mg phospholipids) as in [10]. Liposomal suspensions contained 70 mg phospholipids/ml and 180 μg protein/ml. External nucleotides were removed by Sephadex G-75 gel filtration.

2.5. *Exchange*

For transport measurements, liposomes were incubated with internal substrate (20 mM) in a buffer comprising 20 mM Tricine-NaOH (pH 7.5) at 25°C. The exchange was started by adding [^{14}C]ADP or [^{14}C]ATP at 3.1, 9.5, 32.5 and 100 μM at $t = 0$ and stopped at $t = 5$, 10, 20, 30 s by addition of 3 mM CAT and 6 μM BKA. The samples were passed through small Dowex anion-exchanger columns and the ratio of ^{14}C-labelled ADP/ATP taken up was determined in the pass-through fraction.

2.6. *Preparation of antisera*

Polyvalent antibodies were produced in 3-month-old New Zealand rabbits by standard immunization procedures. 200 μg purified AAC in 1% SDS and 2 vols complete Freund's adjuvant were injected subcutaneously three times over a period of 8 weeks. Antisera were tested by routine ELISA. For immunoblotting proteins were run on 12.5% Laemmli gels [12].

3. RESULTS

3.1. *Isolation and purification of the AAC*

For isolation of intact AAC, carboxyatractylate (CAT)-AAC or atractylate (ATR)-AAC complexes are usually the most suitable forms because of their stability. For this purpose, fresh yeast mitochondria were loaded with CAT or ATR. In preliminary purification experiments with Triton X-100 extracts from yeast mitochondria, after passage through hydroxyapatite, two major bands in the range 29–31 kDa were obtained. In view of the fact that two genes for the AAC have been found [7], it seemed at first feasible that these two bands represent the products of these two genes. For further identification, immunoreactivity against antiserum for the AAC from *Neurospora crassa* was tested in Western blots, which showed that only the band of 31 kDa was reactive. This suggested that the 29 kDa band might be mitochondrial porin which has been previously shown to have a molecular mass of 29 kDa in yeast [13]. With antiserum against porin from

Neurospora this assignment was confirmed. In extracts from bovine heart, mitochondrial porin migrated more slowly than the AAC with an apparently greater molecular mass [14] and occurs in much lower amounts than in yeast and therefore caused no difficulty in the isolation of the AAC.

The segregation of porin from the AAC in yeast required special measures. One way was to change the extraction conditions, in order to segregate porin from the AAC at an early stage. On varying the ratio of Triton X-100 to mitochondrial protein, it was noted that a major portion of the AAC could be extracted within a narrow range of detergent/protein ratios of 0.8–1.2 before significant solubilization of the lower molecular mass component (fig.1A). Also, after passing these extracts through HTS at a Triton X-100/protein ratio = 1.4, the AAC could be obtained largely separated from the porin (fig.1B).

For identification of the 31 kDa component with the AAC, the isolation procedure was performed with mitochondria loaded prior to isolation with [^{3}H]CAT. Specific [^{3}H]CAT binding increased 10-fold from the raw extract after the HTS pass-through, when the content of the 29 kDa component was low at detergent/protein ratio = 1.2, and only 8-fold at a detergent/protein ratio = 3 with a greater content of this component.

Further purification, particularly from higher molecular mass proteins, was achieved by gel filtration on S-300. More important is the removal during this step of the bulk of Triton X-100 and of phospholipids. The AAC-containing fractions were collected and concentrated by pressure dialysis. The final protein concentration was usually 2–3 mg protein/ml containing Triton X-100/protein at a ratio of about 10. With this method medium yields of pure CAT-AAC or ATR-AAC complexes can be obtained, suitable for structural and some functional studies.

In order to prepare denatured AAC peptide in greater amounts, exhaustive extraction of the AAC using higher Triton X-100/protein ratios of 2–2.5:1 is required. In this case, the hydroxyapatite pass-through is significantly contaminated by porin. Separation of the AAC from porin can then be achieved by denaturing the HTS-protein with SDS and gel chromatography on S-300 (fig.2). Porin is eluted with an apparently higher molecular mass than the AAC. Presumably, porin

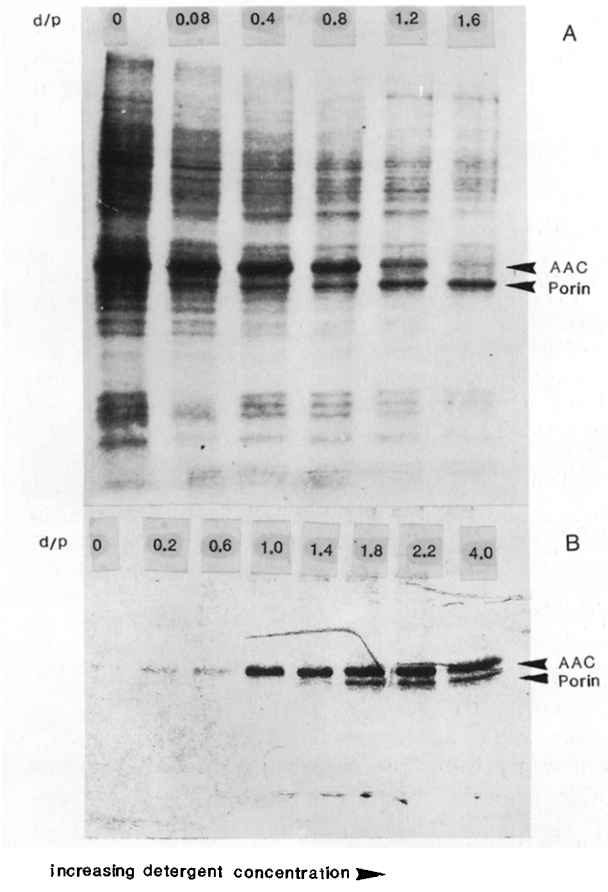

Fig.1. Extraction of the ADP/ATP carrier from yeast with increasing amounts of detergent. Partial segregation from the co-extracted porin. Gel electrophoresis of sediments of the corresponding extracts (A) and of the subsequent hydroxyapatite pass-through fractions (B). Carboxyatractylate-loaded yeast mitochondria were extracted in batches of 6 mg in 0.8 ml buffer containing 0.5 mM NaCl, 0.1 mM EDTA and 10 mM Mops (pH 7.2) with increasing amounts of Triton X-100 (detergent/protein wt ratio 0–3) as indicated. Incubation for 30 min at 4°C by shaking and subsequent centrifugation for 40 min at 80000 × *g*. In separate experiments the corresponding extracts were applied to small hydroxyapatite columns (3.1 × 0.35 cm) with a buffer of 110 mM NaCl, 0.2% Triton X-100, 0.1 mM EDTA, 10 mM Mops (pH 7.2). The major protein pass-through fractions were concentrated and applied to SDS gel electrophoresis in a 12.5% acrylamide gel (B).

forms trimers in SDS [15], whereas AAC exists as monomers [16].

3.2. *CNBr-cleavage pattern identifies the AAC species*

One of the questions to be solved with the

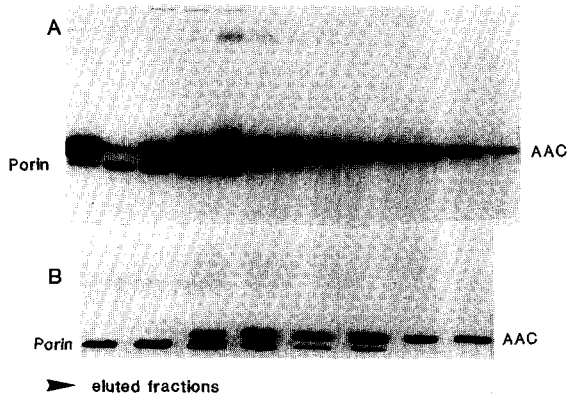

Fig.2. Gel chromatography of the HTS pass-through fractions. (A) Chromatography on S-300 of CAT-AAC complex extracted using a limited Triton/protein ratio (d/p = 1). Higher molecular mass proteins and porin were separated. The elution profile indicates the impurities in the first AAC-containing fractions. Column was run with the buffer used in fig.1. (B) Chromatography of SDS-denatured extracts obtained at higher Triton X-100/protein ratio (d/p = 2.5). Large amounts of essentially pure AAC and porin peptide were obtained by using the elution buffer in fig.1 supplemented with 1% SDS. Flow rate was decreased 10-fold compared with A.

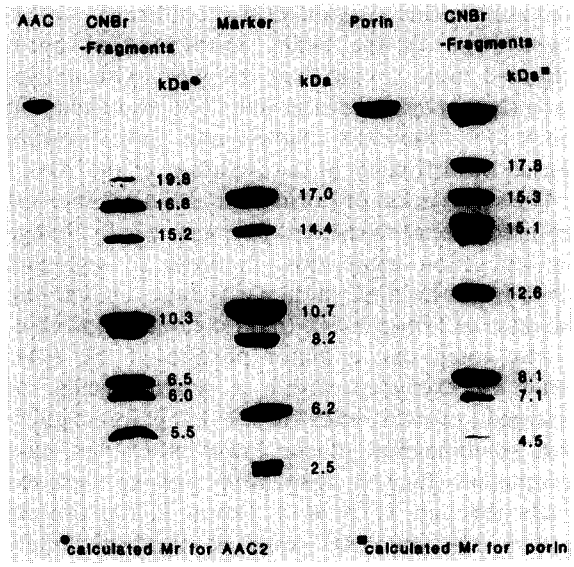

Fig.3. CNBr-cleavage pattern of purified AAC and, for comparison, of the porin from yeast. The two proteins were obtained and separated by gel chromatography on S-300 in SDS-containing buffer. The lower resolution of the low molecular mass AAC2 CNBr fragments is due to the higher amount of phospholipids in the AAC fractions. CNBr fragments of horse heart myoglobin were used as molecular mass standards. The AAC cleavage pattern is compared with the predicted CNBr fragments of the AAC-2 gene product [7] and of porin [17].

purified AAC is to what extent the two genes of AAC found in yeast are expressed in the wild-type *S. cerevisiae*. The amino acid sequence decoded from the cDNA shows that AAC-2 is distinguished by a methionine occurring after about the first third of the sequence [7]. In this respect, AAC-2 differs not only from AAC-1 but also from AAC in all other species currently known, where a methionine occurs only after two thirds of the sequence. The CNBr-cleavage pattern of AAC-2 should therefore clearly differ from that of AAC-1. Fortunately, the degree of CNBr cleavage for the yeast AAC can reach a very high value and therefore the CNBr-cleavage pattern is rather simple. This is compared with peptides of the AAC on the basis of the calculated molecular mass (fig.3). In order to ensure that the identity of the AAC fragments was different from that of porin, we also cleaved purified porin. Most prominent is a peptide of about 10 kDa which cannot be identified with any cleavage product of the hitherto known AAC-1. It can only be identified with the fifth CNBr fragment as predicted from the recently discovered AAC-2 gene product. In fact, scrutiny of the cleavage pattern shows that virtually all peptides can be explained as being cleavage products of the AAC-2, as calculated from the AAC-2 sequence.

3.3. *Reconstitution of transport activity with isolated AAC in liposomes*

A major goal in isolating the AAC is also studying its function. For this purpose, however, the AAC cannot be isolated in the stable CAT-AAC complex, but only in the free form which is much more labile. The rapid procedure developed for this purpose for the AAC from beef heart [10] was with corresponding modifications also applied to the AAC from yeast. Since the yield of AAC is too low at a Triton X-100/protein ratio = 1, this ratio was increased to 2 for this batch procedure by allowing for a less pure preparation. The supernatant from these centrifuged HTS mixtures could be incorporated into liposomes according to the method published for bovine heart AAC [10].

For studying the kinetics of ADP/ATP exchange, ^{14}C-labelled ATP or ADP was added to

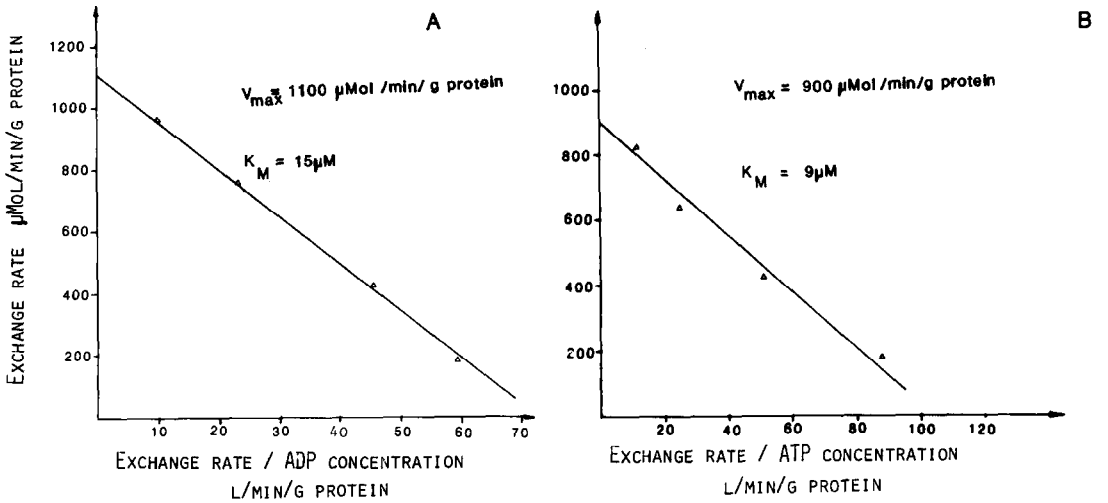

Fig.4. Concentration dependence of ADP (A) or ATP (B) exchange in proteoliposomes obtained by reincorporation of purified AAC into phospholipid vesicles. Determination of V_{max} and K_m of adenine nucleotide uptake from plots according to Eadie Hofstee. Initial exchange velocities were obtained from at least 4 inhibitor-stopped samples, at time intervals of 5, 10, 20 and 30 s.

ADP- or ATP-loaded AAC-containing proteoliposomes. The rate of uptake of radioactivity corresponds to exchange against endogenous unlabeled nucleotides. In fig.4 the results of the exchange rates as a function of the concentration of ADP and ATP are plotted according to Eadie Hofstee for evaluation of the maximum exchange rates and K_m values. From the approximately linear relation, maximum rates (μmol/min per g protein) of 1100 for ADP or 900 for ATP were extrapolated. The K_m value is about 15 μM for ADP and 9 μM for ATP. The rates are somewhat greater than those determined for reconstituted bovine AAC under optimal conditions. We can conclude that the isolated AAC is active and competent in ADP/ATP exchange.

3.4. *Antisera*

Antisera against the ADP/ATP carrier were raised in rabbits. For immunization, the isolated yeast AAC was prepared by gel chromatography on an S-300 gel column, using SDS as detergent. The antibodies were obtained by the footpad immunisation boosting procedure after the first complete adjuvant-mediated injection of SDS-AAC. The antiserum has a surprisingly high specificity for yeast AAC and cross-reacts only very poorly with mammalian AAC and even *Neurospora* AAC (fig.5). This is surprising in view of the fact that the antiserum against *Neurospora* AAC cross-reacts quite well with yeast AAC. The titer of the serum was determined by ELISA and shown to decrease to 10% of the maximum only at a dilution of more than 1:6000. Thus, for immunoblot applications the serum was applied in a dilution of 1:4000.

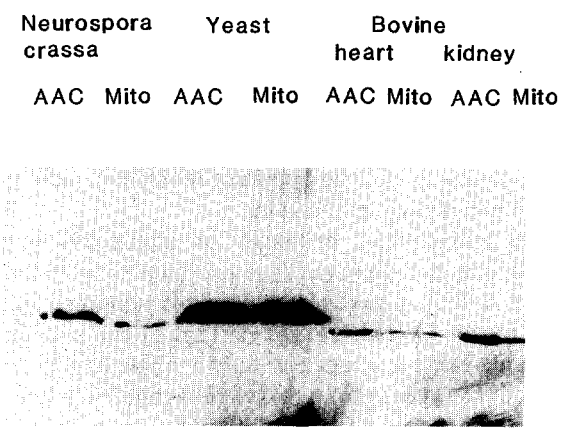

Fig.5. Immunoblots with antiserum raised against the purified AAC from yeast tested against AAC from various sources. For immuno-detection polyvalent antisera to AAC from yeast were diluted 1:4000. Each lane of the blotted gel contained equivalent amounts of mitochondrial protein (30 μg) or purified AAC-protein (5 μg).

4. DISCUSSION

The now classical procedures for the isolation of AAC from mitochondria can also be applied to the isolation of AAC from yeast when properly modified. The major problem is obviously removal of the porin which tends to accompany the AAC during the hydroxyapatite step. Previously, separation of the '37 kDa' species from '34 kDa' component was achieved only by affinity chromatography on an ATR-liganded column [3]. However, in most cases, affinity chromatography has the disadvantages of giving low yields of AAC and of denaturation by the adsorption procedure. The present isolation procedure therefore provides a means for future work on isolating the AAC from AAC mutants as well, whether generated by selection, gene disruption or site-directed mutagenesis. This is also particularly important for reconstitution of the unloaded AAC in order to study the functional differences of the AAC wild or mutant forms.

The higher molecular masses, as reported previously [3] of 37 and 34 kDa for the two components partially purified from yeast mitochondria are not easily explained. The 37 kDa component was identified by affinity chromatography of the AAC, photoaffinity-labelled with arylazido-ADP [3] and arylazido-ATR [4]. Comparison of the reported amino acid composition of the 37 kDa component with the now decoded sequence from the AAC2 cDNA reveals some significant differences (especially His, Met). With hindsight, there was probably only a calibration error of the AAC, since, as first shown by genetic complementation, followed by cDNA analysis and now with the isolated AAC, the apparent molecular mass of the AAC is only at 31 kDa.

The isolation of primarily or exclusively only one of the two possible gene products from yeast, of the AAC-2, could be convincingly demonstrated here by the CNBr-cleavage pattern of the purified protein. The occurrence of only a single band confirmed by the immunoblots also agrees with a single type of AAC. This is in accordance with results indicating that only the AAC-2 gene can complement the op_1 mutation with a single copy whereas complementation with the AAC-1 gene requires multiple copies.

The existence of two AAC genes in yeast and the present finding that primarily or exclusively the AAC-2 protein is functionally active in aerobically grown yeast cells, raises the question of whether the two isoforms are adapted to different requirements of yeast metabolism and are differently expressed during the life cycle. It remains to be ascertained in further investigations whether there is a dynamic change of isoform pattern.

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