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Reversible inactivation and binding to mitochondria of nitrate reductase by heat shock in the yeast *Hansenula anomala*

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Heat shock from 25°C to 40°C of *Hansenula anomala* cells resulted in a rapid and reversible inactivation of the NADPH-nitrate reductase (NR) activity. The inactive enzyme retained partial activity with the non-physiological co-substrates, reduced methyl viologen and reduced flavin mononucleotide. The inactive NR pelleted after centrifugation at 12,000 × *g* for 30 min and was associated with mitochondria. In untreated cells around 10% of the total NR is inactive and associated with mitochondria, while the active enzyme is soluble. In vitro, inactive NR could be partially dissociated from the mitochondria by incubating them at pH 11.5 or in the presence of 15 mM CHAPS.

Yeast; Nitrate reductase; Heat shock; Mitochondria

1. INTRODUCTION

In *Hansenula anomala* and other nitrate assimilatory yeasts [1], nitrate is transformed into ammonium by the nitrate assimilatory pathway. First, nitrate is reduced to nitrite by nitrate reductase (NR) and then, nitrite is reduced to ammonium by nitrite reductase. A variety of regulatory mechanisms have evolved in different organisms to control NR [2]. In *H. anomala* induction by nitrate and partial repression by reduced nitrogen sources (nitrogen metabolite repression) are the only mechanisms of regulation presently known [1,3].

Conflicting results exist about the cellular localization of NR in *H. anomala*. While some authors reported association of the enzyme with mitochondria [4,5], others found the enzyme in the soluble fraction [6,7].

Since NR appears to be the main point of regulation in the nitrate assimilation pathway, we have tried to find out if *H. anomala* presents mechanisms involved in the modulation of NR activity. Here we report the reversible inactivation of NADPH-NR activity by heat shock and a correlation between the degree of inactivation of the enzyme and its association with mitochondria.

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Abbreviations: NR, nitrate reductase; MV_r, reduced methyl viologen; FH_2, reduced flavin mononucleotide; CHAPS, 3-{(3-cholamidopropyl)dimethylammonio}-1-propanosulfonate; PMSF, phenylmethylsulphonyl fluoride.

2. MATERIAL AND METHODS

2.1. *Yeast strain and growth conditions*

Hansenula anomala (CECT 1112) was from the CECT (Valencia, Spain). Cells were grown at 30°C with shaking in liquid medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulphate (Difco), 2% glucose and 20 mM sodium nitrate as the sole nitrogen source.

2.2. *Heat-shock treatment*

Cells harvested in the mid-exponential phase of growth were resuspended at 10 mg (wet weight)/ml in fresh medium, incubated sequentially for 30 min at 30°C and 30 min at 25°C and tranferred to a prewarmed flask at 40°C for the time indicated.

2.3. *Preparation of cell-free extracts and NR assay*

Preparation of extracts and NADPH-NR and reduced methyl viologen (MV_r)-NR assays were carried out as in [3]. Reduced flavin mononucleotide (FH_2)-NR was determined using 0.2 mM flavin mononucleotide, reduced with sodium dithionite.

2.4. *Cellular fractionation*

A crude membrane fraction was obtained as in [8] in 100 mM Tris-HCl, pH 7.4, 20 μM FAD, 0.6 M sorbitol, 4 mM EDTA, 2 mM dithiothreitol, 0.1% aprotinin, 10 mM benzamidine and 2 mM PMSF. The membrane fraction was resuspended in buffer A (50 mM Tris-ClH pH 7.4, 250 mM NaCl, 0.25 M sucrose, 20 μM FAD, 2 mM EDTA, 2 mM dithiothreitol, 0.1% aprotinin, 10 mM benzamidine and 2 mM PMSF) at 0.7–1.5 mg of protein/ml. The suspension (2–3 mg of protein) was centrifuged in a continuous metrizamide gradient (15–45% w/v) for 2 h at 80,000 × *g*. The following activities were used as markers: cytochrome *c* oxidase for mitochondria [9], vanadate-sensitive ATPase for plasma membrane [9], NADPH-cytochrome *c* reductase for endoplasmic reticulum [10] and α-mannosidase for vacuoles [10].

2.5. *Isolation of mitochondria*

Mitochondria were prepared as in cellular fractionation. The brown band corresponding to mitochondria was carefully removed from the gradient, diluted with 4 vols. of buffer A and sedimented by centrifugation at 12,000 × *g* for 20 min.

2.6. *NR solubilization*

Isolated mitochondria from heat-shocked cells were resuspended at 0.7–1.5 mg of protein/ml in the following solutions: buffer A, (control); solution B (buffer A brought to pH 11.5 with NaOH); buffer C (buffer A containing 20% (v/v) glycerol and 15 mM CHAPS). The suspensions were incubated 30 min at 4°C with shaking and centrifuged as in cellular fractionation.

2.7. *PAGE and Western blot*

12.5% PAGE and Western blot were performed as in [11,12]. Anti-NR antiserum [3] and anti-rabbit IgG conjugated to peroxidase were used.

Protein concentration was measured according to Bradford [13]

3. RESULTS AND DISCUSSION

3.1. *Reversible inactivation of NR by heat shock*

When *H. anomala* was transferred from 25°C to 40°C a rapid loss of its NADPH-NR activity was observed. MV_r-NR and FH_2-NR were significantly less inactivated (Fig. 1). When the cells were transferred back to 30°C the NADPH-NR activity was recovered, even in the presence of 40 μg/ml of cycloheximide (Fig. 1).

When the crude extracts were electrophoresed in non-denaturing conditions and NR determined by Western blot it was seen that the levels of NR protein changed in parallel with NADPH-NR activity (data not shown).

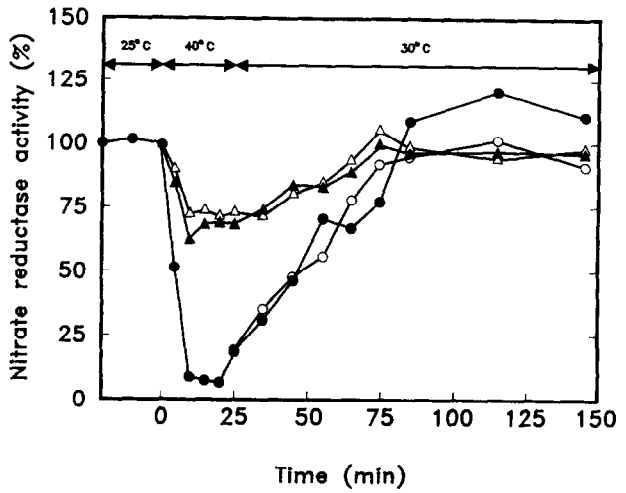

Fig. 1. Reversible inactivation of NR by heat shock. *H. anomala* cells were heat shocked for 20 min at 40°C. After this treatment the cells were transferred to 30°C and divided into two aliquots: 40 μg/ml of cycloheximide were added to one of them. The following activities were measured with time: NADPH-NR (●), NADPH-NR in cells with cycloheximide (○), MV_r-NR (△), FH_2-NR (▲).

3.2. *Inactive NR is associated with mitochondria*

In contrast with NR from untreated cells, NR from

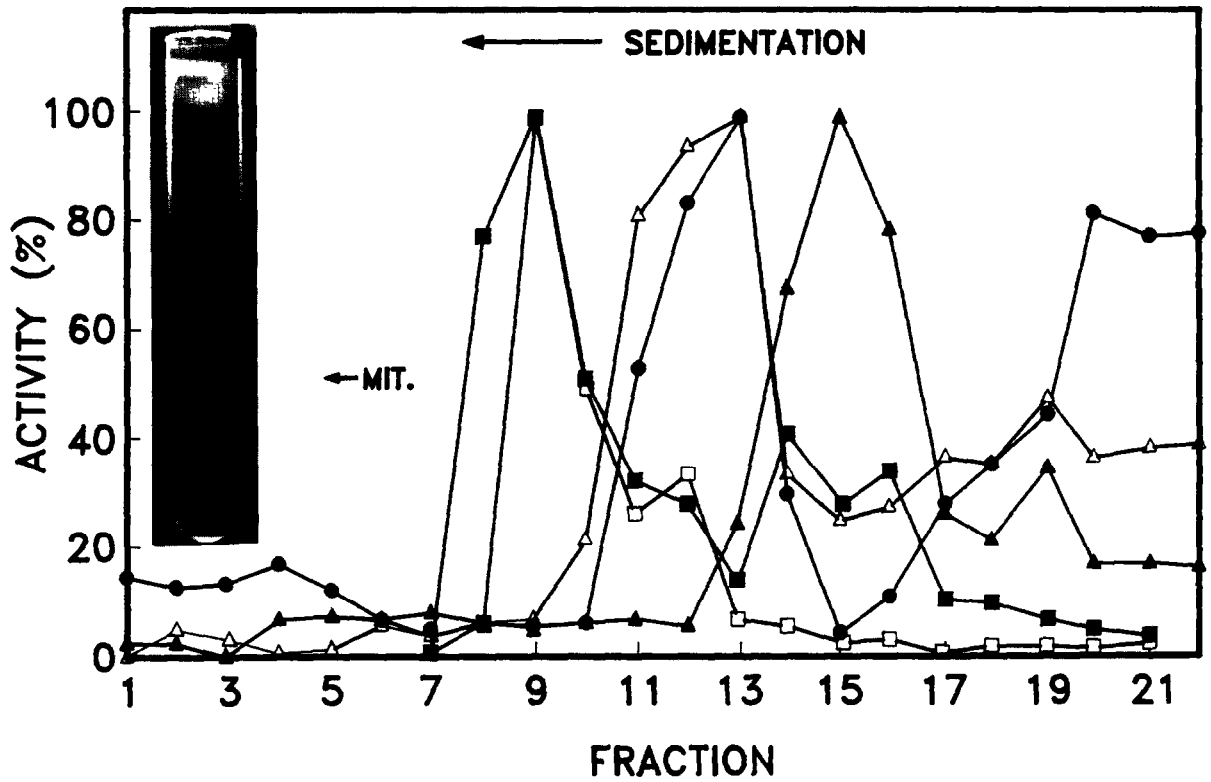

Fig. 2. Localization of NR in heat-shocked cells. The membrane fraction of heat-shocked cells (2–3 mg of protein) was centrifuged at 80,000 × *g* for 2 h in a continuous metrizamide gradient (15–45% w/v). The gradient was fractioned in aliquots of 0.5 ml and cytochrome *c* oxidase (□), MV_r-NR (■), plasma membrane ATPase (△), NADPH-cytochrome *c* reductase (●) and α-mannosidase (▲) were determined in each aliquot. (Inset) Band distribution along the gradient.

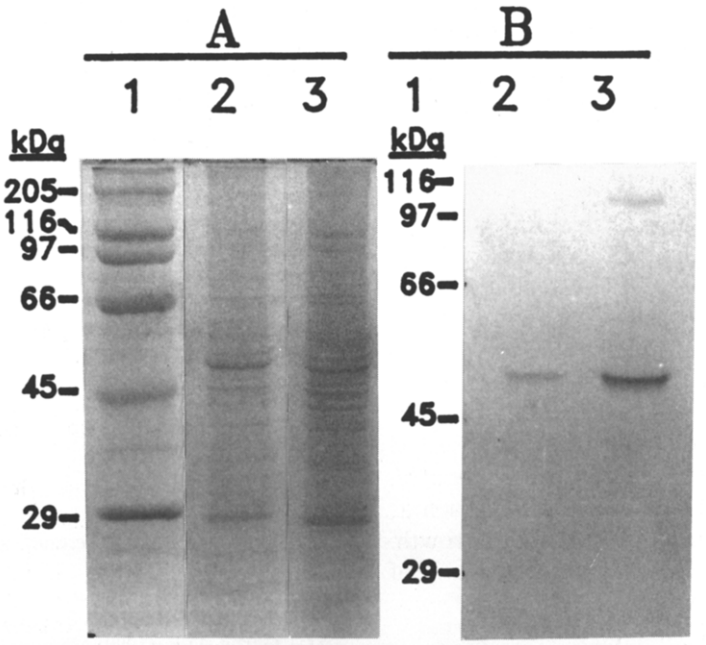

Fig. 3. Levels of NR protein on isolated mitochondria from untreated and heat-shocked cells. (A) SDS-PAGE Coomassie stained. (B) Western blot analysis. Lane 1, molecular weight markers; lane 2, isolated mitochondria from untreated cells (40 μg protein); lane 3, isolated mitochondria from heat-shocked cells (40 μg protein). The immunodetected band of 52 kDa corresponds to NR subunits.

heat-shocked cells did not enter in 5% non-denaturing PAGE nor in 0.5% agarose gels, and eluted in the exclusion volume on Sepharose CL-4B gel filtration (data not shown). These results suggest that during the process of inactivation NR has changed its mass either by the formation of aggregates or by binding to some structure.

When extracts from heat-shocked cells were centrifuged at 12,000 × g for 30 min NR was present in the pellet, while in the same conditions NR from untreated cells remained in the supernatant (data not shown). Fractionation of the pellet (membrane fraction) by centrifugation in a continuous metrizamide gradient, showed that MV_r-NR activity co-sedimented with a brown band containing cytochrome c oxidase, indicating that NR is associated with mitochondria (Fig. 2). The localization of plasma membrane, endoplasmic reticulum and vacuoles in the gradient ruled out the non-specific association of NR with other organelles.

Table I

Levels of NR activity on isolated mitochondria from untreated and heat-shocked cells

	Activity[a] (nmol NO_2^- $min^{-1}mg^{-1}$)	
	Control	*H. anomala*
NADPH-NR	< 1	<1
MV_r-NR	30 ± 4	343 ± 10
FH_2-NR	1 ± 0.2	10 ± 2

[a]Values represent the mean of two independent experiments with standard deviation. However, the levels of NR associated with mitochondria depend a lot on the inactivation reached: in the results shown here the inactivation was around 95%.

The levels of NR protein associated with mitochondria from untreated and heat-shocked cells are shown in Fig. 3. The corresponding activities are shown in Table I.

While heat shock of *H. anomala* cells produces an association of NR with mitochondria, in untreated cells around 10% of total NR was found associated with mitochondria. These results are consistent with those obtained by Pichitony and Metenier [4,5] on the localization of NR in *H.anomala*. The fact that NR associated with mitochondria does not show NADPH-NR activity (Table I) could explain the results of Zauner and Dellweg [6] who did not find insoluble NR. Also a careful examination of the results of Minagawa and Yoshimoto [8] show that about 8% of NR was associated with mitochondria in their experiments, which is in good agreement with our findings.

3.3. *NR solubilization*

Incubation of isolated mitochondria of heat-shocked cells with 15 mM CHAPS or at pH 11.5 dissociated around 45% NR. After centrifugation of the suspension in the metrizamide gradient the solubilized NR was found at the same density range as the soluble active NR. NR solubilized with CHAPS presented MV_r-NR activity but not NADPH-NR. The enzyme solubilized

by treatment at pH 11.5 was completely inactive (data not shown)

Partial solubilization of inactive NR with CHAPS suggests that the enzyme is bound to mitochondria but not aggregated, since protein aggregates produced by heat shock are not easily solubilized even by detergents [14].

The mechanism of inactivation by heat shock has not been elucidated but the fact that MV_r-NR and FH_2-NR were much less affected that NADPH-NR suggests that the inactivation takes place in the FAD domain. Association of the enzyme with mitochondria could be responsible for the inactivation or conversely the inactivated enzyme could be more easily associated with mitochondria. The process of inactivation–reactivation observed upon temperature shifts could also take place as a response to changes in enviromental conditions, such as nitrogen source, pH of the medium or phase of growth. It could have been selected to avoid the production of toxic nitrite under stress conditions.

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