Nitrite causes reversible inactivation of nitrate reductase in the yeast *Hansenula anomala*

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The addition of nitrite, the product of the reaction catalysed by nitrate reductase, to cell suspensions of the yeast *Hansenula anomala* caused a reversible inactivation of NADPH-dependent nitrate reductase activity. The haem- and Mo-dependent and Mo-dependent activities of nitrate reductase, determined with the non-physiological electron donors FMNH₂ and reduced methyl viologen respectively, were less affected. A similar inactivation was found with the proton ionophores 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone. The inactive enzyme was found in the particulate fraction and cosedimented with the mitochondrial fraction. When the NADPH-dependent nitrate reductase activity was restored *in vivo* the enzyme was found in the soluble fraction. The inactivation of nitrate reductase by nitrite, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone was dependent on the external pH. The treatment of isolated mitochondria at alkaline pH with Triton X-100 solubilized about 30% of the inactive enzyme.

Keywords: *Hansenula anomala*, nitrate reductase, nitrite, mitochondria, yeast

INTRODUCTION

Hansenula anomala (currently called *Pichia anomala*) is a yeast able to assimilate nitrate as sole nitrogen source (Silver, 1956). Nitrate is reduced to nitrite by nitrate reductase (NR), and nitrite to ammonium by nitrite reductase. The assimilatory NRs so far described contain the prosthetic groups FAD, haem and Mo-pterin. The electrons flow from the electron donor NAD(P)H to nitrate through FAD, haem and Mo (Solomonson & Barber, 1990). Oxidation of the non-physiological electron donors FMNH₂ and MVr involves the prosthetic groups haem and Mo, and Mo, respectively. Regulation of nitrate assimilation takes place mainly at the level of NR, which has evolved a variety of regulatory mechanisms in different organisms. In general, nitrate is the inducer of NR synthesis while reduced nitrogen sources are repressors. For reviews on NR regulation in yeast, plants and filamentous fungi see Hipkin (1989), Solomonson & Barber (1990) and Marzluf (1993).

In *H. anomala*, NR is induced by nitrate and slightly repressed by reduced nitrogen sources (González & Siverio, 1992). Inactivation of NR in the early-stationary

phase, which is reversed *in vitro* by ferricyanide, has been found (Minagawa & Yoshimoto, 1984; C. González & J. M. Siverio, unpublished). Furthermore, a reversible inactivation as a response to heat shock has been reported (Siverio *et al.*, 1993).

It is conceivable that the reversible inactivation caused by heat shock could also be a response to other environmental conditions such as the nitrogen source, pH of medium, or growth phase, as well as to stress conditions. With this idea in mind we have studied the effect of nitrite on NR activity. Moreover, since nitrite is a weak acid (pK_a 3.2) it may act as a proton ionophore. Therefore, its effect on NR activity was compared with the proton ionophores DNP and CCCP.

METHODS

Yeast strain and growth conditions. *Hansenula anomala* (CECT 1112) was obtained from the Colección Española de Cultivos Tipo (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% (w/v) glucose; and 20 mM NaNO₃ as sole nitrogen source (YNBGN).

Effect of nitrite and proton ionophores on NR. Cells harvested in the mid-exponential phase of growth were resuspended at 10 mg (wet wt) ml⁻¹ in YNBGN, buffered with 25 mM MES/Tris at pH 4.5 when 10 mM nitrite was added and at pH 5.0 when 2 mM DNP was added. The cell suspension was

Abbreviations: NR, nitrate reductase; MVr, reduced methyl viologen; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chx, cycloheximide; YNBGN, medium containing yeast nitrogen base without amino acids or ammonium sulphate, plus glucose and nitrate.

incubated for 30 min at 30 °C with shaking and nitrite or DNP was added. At appropriate intervals, samples (50 mg wet wt) were added to 5 ml ice-cold water and centrifuged. The resulting pellet was washed again with 10 ml ice-cold water. The cells were kept at -20 °C until cell-free extracts were prepared.

When the effect of external pH on inactivation by nitrite or proton ionophore was studied, the cells were incubated at 20 mg (wet wt) ml⁻¹ for 30 min at 30 °C with shaking. The cell suspensions were then buffered with 1 vol. 50 mM MES adjusted with Tris to different pH values between 4.5 and 6.5. The cells were incubated with nitrite or DNP for 15 min.

Preparation of cell-free extracts and NR assay. Preparation of crude extract and NADPH-NR and MVr-NR assays were carried out as described by González & Siverio (1992). Cell-free extracts were centrifuged at 3000 *g* unless otherwise stated. Protein concentration was measured according to Bradford (1976).

Subcellular fractionation and isolation of mitochondria. Subcellular fractionation was carried out by centrifugation of a crude membrane fraction, obtained as described by Rickwood *et al.* (1987) in a continuous metrizamide gradient (15–45%, w/v) for 2 h at 80000 *g* (Siverio *et al.*, 1993). The following activities were used as markers: cytochrome *c* oxidase for mitochondria and vanadate-sensitive ATPase for plasma membrane (Navarrete & Serrano, 1983); NADPH-cytochrome *c* reductase for endoplasmic reticulum and α -mannosidase for vacuoles (Roberts *et al.*, 1991). Mitochondria were isolated after centrifugation by taking the brown band, visible in the gradient, and testing its purity using the marker enzymes listed above.

NR solubilization. Isolated mitochondria from cells treated with either nitrite or DNP were resuspended at 1 mg protein ml⁻¹ in the following solutions: solution A (control) 50 mM Tris/HCl pH 10.0, 150 mM NaCl, 2 mM EDTA, 20 μ M FAD, 2 mM NaNO₃ and 20% (v/v) glycerol; solution A containing 11.6 mM Triton X-100; solution B (control) 25 mM Tris/HCl pH 9.5, 2 mM EDTA, 20 μ M FAD, 2 mM NaNO₃ and solution B plus 500 mM Na₂SO₄. The suspension was mixed, incubated for 10 min at 4 °C and centrifuged at 200000 *g* for 30 min. The MVr-NR activity was determined in the supernatant.

RESULTS

Inactivation of NR by nitrite

H. anomala was able to grow in liquid medium with up to 20 mM nitrite as sole nitrogen source at the same growth

rate and to the same cell density as nitrate-grown cultures. The level of NR in cells grown with nitrite was 150 $\pm$ 25 nmol nitrite min⁻¹ (mg protein)⁻¹ (mean $\pm$ SD; *n* = 5), corresponding to about 50% of the activity measured in cells grown on nitrate. The addition of 10 mM nitrite to a cell suspension, even in the presence of nitrate, resulted in inactivation of NADPH-NR activity (Fig. 1). About 75% of the NADPH-NR activity was lost 25 min after nitrite addition. The partial activities of haem- and Mo-dependent NR, determined with electron donor FMNH₂, and Mo-dependent NR, determined with reduced methyl viologen (MVr) were less affected. The NADPH-NR activity recovered about 30 min after nitrite had been removed from the cell suspension and the cells

Fig. 1. Reversible inactivation of NR by nitrite. Nitrate-grown cells were resuspended in YNBGN (pH 4.5) and incubated for 30 min at 30 °C. At *t* = 0, 10 mM nitrite was added. After 30 min, cells free of nitrite were resuspended in YNBGN (pH 4.5) containing 40 μ g cycloheximide ml⁻¹. The levels of NADPH-NR (∇) and MVr-NR ($\blacksquare$) were determined; 100% activity of NADPH-NR and MVr-NR corresponds to 300 $\pm$ 30 and 450 $\pm$ 40 nmol nitrite min⁻¹ (mg protein)⁻¹, respectively (means $\pm$ SD; *n* = 5). Experiments were repeated five times without significant differences; results shown are from a single experiment.

Table 1. Levels of MVr-NR and NADPH-NR in the soluble and particulate fractions of cell extracts

Extracts were prepared from control cells, from cells incubated with 10 mM nitrite, and from cells incubated for 90 min in the presence of cycloheximide after nitrite removal. Values represent the mean of three independent experiments $\pm$ SD. Levels of NR in the particulate fraction depend on the levels of inactivation; here the inactivation was about 80%.

	Enzyme activity [nmol nitrite min ⁻¹ (mg protein) ⁻¹]					
	Control cells		After NO ₂ ⁻ incubation		After NO ₂ ⁻ removal	
	Soluble	Particulate	Soluble	Particulate	Soluble	Particulate
MVr-NR	410 $\pm$ 25	42 $\pm$ 5	86 $\pm$ 4	320 $\pm$ 15	385 $\pm$ 21	36 $\pm$ 5
NADPH-NR	295 $\pm$ 13	< 1	50 $\pm$ 5	< 1	223 $\pm$ 15	< 1

Fig. 2. Effect of external pH on NADPH-NR inactivation by nitrite or DNP. Nitrate-grown cells were resuspended in YNBGN buffered with 25 mM MES/Tris at different pH values and incubated for 15 min with nitrite or DNP. (a) NADPH-NR activities upon addition of nitrite to cell suspensions at pH 4.5 (■), pH 5.0 (□), pH 5.5 (▲) and pH 6.5 (△). (b) NADPH-NR activities upon addition of DNP to cell suspensions at pH 5.0 (■), pH 6.0 (□), pH 6.5 (▲) and pH 7.5 (△). Experiments were repeated three times without significant differences; results shown are from a single experiment. The NADPH-NR activity before the addition of nitrite or DNP was 300 ± 30 nmol nitrite min^{-1} (mg protein) $^{-1}$ and it was not affected significantly by the pH of the incubation.

further incubated in YNBGN. This recovery was also observed in the presence of the inhibitor of protein synthesis, cycloheximide at $40 \mu\text{g ml}^{-1}$, sufficient to inhibit the synthesis of NR (González & Siverio, 1992). These data show that the inactivation of NR is reversible.

The MVR-NR activity increased in the particulate fraction, obtained by centrifugation ($12000 g$ for 30 min) of a crude extract from cells incubated with nitrite, while the NADPH-NR activity was absent. After nitrite removal the NADPH-NR activity was restored and was found together with MVR-NR activity in the soluble fraction (Table 1).

The degree of inactivation of NADPH-NR by nitrite was dependent on external pH (Fig. 2a).

Fig. 3. Subcellular location of MVR-NR in cells incubated with nitrite. The membrane fraction of cells incubated with nitrite (2–3 mg protein) was centrifuged at $80000 g$ for 2 h in a continuous metrizamide gradient (15–45% w/v). The gradient was fractionated in aliquots of 0.5 ml beginning from the bottom of the tube. The distribution of (a) cytochrome c oxidase and MVR-NR (△), (b) NADPH-cytochrome c reductase, (c) plasma-membrane ATPase and (d) α -mannosidase is shown. The arrows in (b–d) indicate the MVR-NR peak fraction. Experiments were repeated three times without significant differences; results shown are from a single experiment.

Subcellular location of inactive NR

In order to localize the inactive MVR-NR present in the particulate fraction from cells incubated with nitrite, a crude membrane fraction prepared from cells incubated with 10 mM nitrite for 30 min was fractionated by ultracentrifugation in a density-gradient, resulting in cosedimentation of NR with the mitochondria (Fig. 3).

Table 2 shows the levels of NR cosedimented with mitochondria isolated from untreated cells, from cells incubated for 30 min with nitrite and from cells incubated in YNBGN plus $40 \mu\text{g cycloheximide ml}^{-1}$ for 120 min after nitrite removal. Purified mitochondria did not

Table 2. Levels of NADPH-NR, MVR-NR and FMNH₂-NR in isolated mitochondrial fractions

Mitochondrial fractions were prepared from untreated cells, from cells incubated for 30 min with 10 mM nitrite at pH 4.5 or 2 mM DNP at pH 5.0, and from cells incubated for 120 min in presence of $40 \mu\text{g cycloheximide ml}^{-1}$ after nitrite or DNP removal. Values represent the mean of three independent experiments $\pm$ SD.

	Enzyme activity [nmol nitrite min^{-1} (mg protein) $^{-1}$]				
	Control cells	30 min with 10 mM NO ₂ ⁻	120 min after NO ₂ ⁻ removal	30 min with 2 mM DNP	120 min after DNP removal
MVR-NR	60 ± 5	396 ± 10	78 ± 5	453 ± 11	80 ± 10
NADPH-NR	< 1	< 1	< 1	< 1	< 1
FMNH ₂ -NR	1 ± 0.2	19 ± 2	0.8 ± 0.3	21 ± 3	21 ± 0.2

Fig. 4. Reversible inactivation of NR by proton ionophores. Nitrate-grown cells were resuspended in YNBGN buffered with 25 mM MES/Tris at pH 5.0 and incubated for 30 min at 30 °C after which 2 mM DNP (a) or 0.2 mM CCCP (b) was added to the cell suspension. After 30 or 20 min, respectively, cells were resuspended in YNBGN, free of proton ionophores, containing 40 µg cycloheximide ml⁻¹. The levels of NADPH-NR (▽) and MVR-NR (■) were determined. Experiments were repeated five times without significant differences; results shown are from a single experiment.

exhibit NADPH-NR activity. The highest levels of MVR-NR were associated with the mitochondrial fraction from cells incubated with nitrite; these cells did not present NADPH-NR activity. Likewise, the restoration of NADPH-NR activity, after nitrite removal from the cell suspension, is linked to NR dissociation from mitochondria.

Inactivation of NR by proton ionophores

Since nitrite is a weak acid (pK_a 3.2) and its effect on NR inactivation was dependent on external pH, it could act on the cells as a proton ionophore. We therefore tested the effect of the proton ionophores 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on NR activity *in vivo*. The addition of 2 mM DNP or 0.2 mM CCCP to cells grown in the presence of nitrate resulted in inactivation of NADPH-NR activity. Enzyme activity was restored when the proton ionophores were removed and the cells were incubated in YNBGN in the presence of 40 µg cycloheximide ml⁻¹ (Fig. 4).

As in the case of nitrite, the degree of NADPH-NR inactivation by DNP and CCCP was dependent on the external pH (Fig. 2b). Fractionation of crude membrane from cells incubated with either DNP or CCCP showed that the activity of MVR-NR was associated with mitochondria (Table 2).

Solubilization of inactive NR associated with mitochondria

When mitochondria isolated from cells treated with either nitrite or DNP were incubated with 11.6 mM Triton X-100 at pH 10.0, about 30% of MVR-NR was solubilized. Incubation of mitochondria with 500 mM Na₂SO₄ at

pH 9.5 solubilized about 15% of MVR-NR. These were the highest levels of solubilization obtained using different pH values and Triton X-100 or Na₂SO₄ concentrations. The enzyme solubilized *in vitro* did not recover the activity shown with NADPH as electron donor.

DISCUSSION

The inactivation of the *H. anomala* NR by nitrite could act as a short-term control of NR activity. Thus, this mechanism could play a role similar to the inactivation of glutamine synthetase by ammonium in *Escherichia coli* (Stadtman, 1990). In both cases, the presence of an alternative nitrogen source inactivates an enzyme involved in nitrogen assimilation.

The effects of heat shock (Siverio *et al.*, 1993) and proton ionophores on NR were comparable to the effects caused by nitrite – inactivation and cosedimentation of NR with the mitochondrial fraction – possibly because the three agents produce some common signal. A similar situation has been reported in *Saccharomyces cerevisiae*, where phosphorylation and inactivation of fructose biphosphatase can be achieved both by glucose addition and by addition of proton ionophores (Mazón *et al.*, 1982).

The mechanism of inactivation of NR remains unknown so far. However, the inactivation by nitrite seems to be achieved through its uncoupler effect on the cells, since at alkaline pH low inactivation was observed and, also, DNP and CCCP caused the inactivation of NR. The inactivation could be brought about by phosphorylation by a cAMP-activated kinase, since proton ionophores increase the levels of cAMP (Mazón *et al.*, 1982). Alternatively, since heat shock and uncouplers have the same effect on NR activity, the denaturation–renaturation of NR with or without involvement of heat-shock proteins (Gething & Sambrook, 1992; Mager & Moradas-Ferreira, 1993) may be responsible for the process of NR inactivation and insolubilization.

The inactivation of *H. anomala* NR in the early-stationary phase (Minagawa & Yoshimoto, 1984; C. González & J. M. Siverio, unpublished) seems to be different from that reported here, since it is irreversible and the enzyme remains in the soluble fraction.

Cosedimentation of NR and mitochondria in *H. anomala* was reported by Pichitony & Méténier (1967), although the results of Zauner & Dellweg (1983) and Minagawa & Yoshimoto (1983) contradicted this finding. We have found that in cells incubated with nitrite or proton ionophores, or subjected to heat shock, NR always cosediments with the mitochondrial fraction. Also restoration of NADPH-NR activity always paralleled the disappearance of NR from the particulate fraction and its appearance in the soluble fraction. Since NR from tobacco cells attaches to membranes in different stages of the cell cycle (Horau *et al.*, 1991), binding of inactive NR to mitochondria is an attractive hypothesis. In fact a case in which a reversible inactivation is accompanied by binding to an organelle has been reported for the interferon-induced protein kinase p68 (Dubois *et al.*, 1991). However

our present evidence is not sufficient to permit a definitive conclusion. We cannot reject the possibility that aggregation of the protein is responsible for its inactivation, as we could only achieve partial solubilization. The possible physical interaction between NR and mitochondria requires further experimentation.

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