Effect of nitrogen source on the levels of nitrate reductase in the yeast *Hansenula anomala*

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Levels of nitrate reductase (NR) protein in *Hansenula anomala* and *Hansenula wingei* were determined using specific antiserum raised against the enzyme from *H. anomala*. Extracts from nitrate-grown cells contained NR protein, while in those from cells grown on ammonium, glutamine or peptone, no cross-reacting material could be observed. Enzyme activity correlated with the levels of cross-reacting material. When nitrate was used as nitrogen source, NR was always present, even in cultures with ammonium, glutamine or peptone, although in these cases both the levels of activity and protein were lower. NR activity was consistently two to four times higher in cells grown in glucose than in cells grown in ethanol. Nitrate was required for NR induction, and deprivation of nitrate from nitrate-grown cells resulted in a rapid loss of NR activity.

Introduction

Some yeasts, such as *Hansenula anomala*, are able to use nitrate as nitrogen source (Barnett *et al.*, 1984). The main points of regulation in the nitrate assimilatory pathway appear to be the nitrate uptake system and the nitrate reductase (NR). In contrast with the situation for filamentous fungi (Pateman & Kinghorn, 1976; Dunn-Coleman *et al.*, 1984; Wray & Kinghorn, 1989), the regulation of NR by nitrogen and carbon sources has been little studied in yeast. So far (Ali & Hipkin, 1985, 1986; Cannons *et al.*, 1986; Jones *et al.*, 1987; Hipkin, 1989) it has been shown that NR appears in cells cultured in nitrate-containing media and is absent in ammonium-cultured cells.

Several questions remain unanswered concerning the regulation of NR in yeast. It is not clear whether nitrate is an inducer and the reduced nitrogen sources are repressors (nitrogen metabolite repression) or if both mechanisms are operative, and whether the carbon sources also play a role in the regulation of activity levels. In addition, the possible existence of mechanisms that could inactivate NR reversibly or irreversibly has not been carefully examined.

In this work we show that in *H. anomala*, nitrate is an inducer of NR, while the role of nitrogen metabolite repression is unimportant. Effects of nitrate deprivation and glucose are also described.

Methods

Organism and growth conditions. *Hansenula anomala* (CECT 1112) was obtained from the Colección Española de Cultivos Tipo (Valencia, Spain). *Hansenula wingei* (CBS 2432) and *Hansenula polymorpha* (CBS 4732) were kind gifts from J. A. Barnett (Norwich, England). The yeasts were grown at 28 °C with shaking in a liquid medium containing 0.17% yeast nitrogen base without amino acids and ammonium sulphate (YNB, Difco), 2% (w/v) glucose or ethanol as carbon source, and either 50 mM-sodium nitrate, 50 mM-ammonium chloride, 25 mM-glutamine or 2% (w/v) Bacto peptone (Difco) as sole nitrogen source.

Preparation of cell-free extracts and NR assay. The cells were stored at –20 °C until used. Approximately 50 mg of cells (wet weight) were mixed with 200 µl extraction buffer (100 mM-potassium phosphate, pH 7.4, 20 µM-FAD, 1 mM-EDTA and 1 mM-PMSF) and 1 g glass beads (0.5 mm diameter) and vortexed for 90 s. After 3 min on ice, 300 µl extraction buffer were added, and the mixture was vortexed again for 10 s. The mixture was then centrifuged for 5 min at 3000 g, and the supernatant used for assay. NR was assayed in a final volume of 1 ml, containing 50 mM-potassium phosphate buffer, pH 7.4, 50 µl extract (approximately 0.10–0.15 mg protein), 20 mM-sodium nitrate and 0.2 mM-NADPH, plus 10 mM-potassium sulphite as nitrite reductase inhibitor. The assay mixture was incubated at 30 °C for 15 min, and the reaction stopped with 100 µl 1 M-sodium hydroxide and 100 µl 1 M-zinc sulphate. The mixture was centrifuged at 3000 g for 5 min and nitrite was determined in the supernatant (Snell & Snell, 1949).

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Abbreviations: NR, nitrate reductase; YNB, yeast nitrogen base without amino acids and ammonium sulphate.

Fig. 1. Specificity of anti-NR antiserum. (a) Crude extract, 10 μ g protein from nitrate-grown cells subjected to SDS-PAGE and transferred to nitrocellulose. NR was determined on the nitrocellulose filter with 1:2000 anti-NR antiserum and antirabbit IgG 1:5000 conjugated to peroxidase. (b) Crude extract, 10 μ g protein subjected to PAGE in non-denaturing conditions. NR was localized on the nitrocellulose filter with the anti-NR antiserum and by the activity on the gel (■).

Reduced methyl viologen: nitrate reductase activity was determined as above but using 0.2 mM-reduced methyl viologen instead of NADPH.

Preparation of anti-NR antiserum. NR was purified to homogeneity as described by Zauner & Dellweg (1983). The antiserum was prepared following Bailey (1984). Approximately 40 μ g pure NR in 0.5 ml 50% (v/v) Freund's complete adjuvant was injected subcutaneously, three times at two week intervals, to immunize male New Zealand white rabbits. The blood was collected three weeks after the last injection, and once clotted was centrifuged at 5000 g for 20 min; the supernatant was divided into 0.5 ml samples and stored at -20°C until used as anti-NR antiserum.

PAGE and Western blot. Extracts were made as described above with the addition of the following protease inhibitors: 0.25 mg ml^{-1} trypsin inhibitor, 0.1% apotrinin and 10 mM-benzamidine. Both denaturing and non-denaturing PAGE were done as described by Smith (1987). Transfer of proteins to nitrocellulose membranes and immunodetection of NR were done as described by Winston (1987). Anti-NR antiserum diluted 1:2000 and antirabbit IgG diluted 1:5000 conjugated to peroxidase were used.

Determination of NR activity in non-denaturing PAGE. The gel was cut into 2 mm sections, and NR activity was determined as indicated above with reduced methyl viologen as substrate.

Protein concentration was measured according to Bradford (1976).

Results

Characterization of the antiserum raised against nitrate reductase

Titration of the anti-NR antiserum showed that the antiserum was able to inactivate NR (data not shown). The specificity of the antiserum was determined by Western blot, performing the electrophoresis in denaturing and non-denaturing conditions. A band of 52 kDa corresponding to the NR subunits, along with other minor bands, was observed after electrophoresis in denaturing conditions (Fig. 1*a*). In samples subjected to non-denaturing PAGE, NR mobility was determined by activity and by Western blot. The same mobility was

observed for the band detected by Western blot and by NR activity (Fig. 1*b*).

Nitrate reductase activity and protein in cells grown on different nitrogen and carbon sources

The levels of NR in *Hansenula anomala*, *H. polymorpha* and *H. wingei* grown in different nitrogen and carbon sources are shown in Fig. 2. The cells were grown overnight at 30 °C with shaking and harvested at mid-exponential phase. In our experimental conditions, the NR activity in *H. anomala* grown on nitrate was three times higher than that measured in the other species tested. NR activity was also consistently 2–4 times

Fig. 2. Levels of NR protein and activity in *H. anomala* (*H.a.*), *H. wingei* (*H.w.*) and *H. polymorpha* (*H.p.*). Cells were grown in YNB and glucose or ethanol as carbon source and different nitrogen sources: (A) 50 mM-nitrate, (B) 50 mM-ammonium, (C) 25 mM-glutamine, (D) 2% (w/v) Bacto peptone (Difco), (E) 50 mM-nitrate + 50 mM-ammonium, (F) 50 mM-nitrate + 50 mM-glutamine and (G) 50 mM-nitrate + 2% (w/v) peptone. (a) Levels of NR protein in cells grown in glucose, determined by Western blot. Crude extract, 10 µg protein, subjected to non-denaturing PAGE and transferred to nitrocellulose. (b) NR activity. Thin bars indicate SD (*n* = 3), protein levels are from a single experiment.

Fig. 3. Appearance of NR. Ammonium-grown *H. anomala* was resuspended at 10 mg ml⁻¹ in YNB-glucose with different nitrogen sources. (a) Levels of NR protein in cells resuspended in (A) 50 mM-nitrate or (B) 25 mM-glutamine + 50 mM-nitrate determined by Western blot. Crude extract, 10 μg (A) and 20 μg (B) protein subjected to non-denaturing PAGE and transferred to nitrocellulose. (b) NR activity. Symbols: Δ, nitrogen-free medium; ▽, 50 mM-nitrate; ◇, 50 mM-nitrate + 50 mM-ammonium; ●, 50 mM-nitrate + 25 mM-glutamine; ▼, 50 mM-nitrate + 40 μg cycloheximide ml⁻¹. Experiments were repeated three times without significant differences; results shown are from a single experiment.

higher in cells grown on glucose than in ethanol-grown cells. When the yeasts were grown in nitrate-containing media in the presence of ammonium, glutamine or peptone, NR was only partially repressed. Levels of NR protein were determined in *H. anomala* and *H. wingei* by Western blot. NR protein was not detected in cells grown in ammonium, glutamine or peptone (Fig. 2). A close correlation was observed between levels of NR activity and NR protein.

Appearance of nitrate reductase

In Fig. 3 the kinetics of appearance of NR activity in *H. anomala*, as well as the levels of NR protein under different conditions, are shown. Only nitrate was able to induce NR activity, and that occurred even in the presence of 50 mM-ammonium or 25 mM-glutamine. In a nitrogen-free medium, NR activity was absent after 6 h incubation. The addition of 40 μg cycloheximide ml⁻¹ to nitrate-containing medium inhibited the appearance of NR activity. Levels of NR protein were always tightly

correlated with NR activity. In *H. wingei*, nitrate from 10 mM in a medium containing 50 mM-ammonium was able partially to induce NR (Fig. 4), in good agreement with the results shown in Fig. 2. Under the same experimental conditions, the levels of induction were much higher in *H. anomala*.

Disappearance of nitrate reductase activity

The disappearance of NR activity and NR protein under different conditions is shown in Fig. 5. The rates of disappearance of NR activity in nitrogen-free, nitrogen-free + cycloheximide, ammonium, ammonium + cycloheximide and glutamine media were all similar. However, in cells resuspended in nitrate + cycloheximide, the rate of NR activity disappearance was much lower than in the cases described above.

As expected from Fig. 2, in cells that were resuspended in a medium with ammonium + nitrate or glutamine + nitrate, NR activity only decreased to approximately 50% of the activity in cells resuspended in nitrate. Again,

Fig. 4. Effect of nitrate concentration on appearance of NR in the presence of ammonium. Ammonium-grown *H. anomala* (filled bars) and *H. wingei* (open bars) were resuspended at 10 mg ml⁻¹ in medium containing 50 mM-ammonium + different concentrations of nitrate. NR activity was determined 2 h after transfer. Thin bars indicate SD ($n = 4$).

Western blots showed that NR protein and activity were tightly correlated.

Nitrate-grown cells were washed and resuspended in ammonium medium until NR activity was reduced by 50%. When transferred again to nitrate medium, with or without cycloheximide, they did not recover NR activity in the presence of cycloheximide (Fig. 6).

Discussion

In the yeasts studied in this work, *H. anomala*, *H. wingei* and *H. polymorpha*, neither NR protein, determined in *H. anomala* and *H. wingei*, nor NR activity was present in cells grown in ammonium, glutamine or peptone. However, NR was present in *H. anomala*, and *H. wingei* incubated or grown in nitrate + ammonium, nitrate + glutamine and nitrate + peptone (Figs 2, 3 and 5). It can therefore be concluded that the nitrogen metabolite repression produced by ammonium or products of its assimilation is overcome by the inducing effect of nitrate. On the other hand, in these media no NR activity was detected in *H. polymorpha*. *H. anomala* showed NR activity only in the presence of nitrate, in contrast to *Sporobolomyces roseus* (Ali & Hipkin, 1985) and *Candida nitratophila* (Ali & Hipkin, 1986; Cannons *et al.*, 1986) where NR was present in nitrogen-free medium.

Fig. 5. Disappearance of NR. Nitrate-grown *H. anomala* was resuspended at 10 mg ml⁻¹ in YNB-glucose with different nitrogen sources. (a) Levels of NR protein in cells resuspended in: A, ammonium; B, glutamine; C, nitrate + ammonium; D, nitrate + glutamine. Crude extract (A, 10 µg; B, C, D, 20 µg) was subjected to non-denaturing PAGE and transferred to nitrocellulose for Western blotting. (b) NR activity. Symbols: ▽, 50 mM-nitrate; ◇, 50 mM-nitrate + 50 mM-ammonium; ▼, 50 mM-nitrate + 40 µg cycloheximide ml⁻¹; □, 50 mM-ammonium; ■, nitrogen-free medium; ▲, nitrogen-free medium + 40 µg cycloheximide ml⁻¹; △, 50 mM-ammonium + 40 µg cycloheximide ml⁻¹; ●, 25 mM-glutamine; ○, 25 mM-glutamine + 50 mM-nitrate. Experiments were repeated five times without significant differences; results shown are from a single experiment.

The levels of NR protein present under different conditions (Figs 2, 3 and 5) and the observed effect of cycloheximide on NR activity (Fig. 6) show that NR activity correlated with NR protein and that the appearance and disappearance of NR activity was associated with synthesis and loss of NR protein.

H. anomala, *H. wingei* and *H. polymorpha* grown in glucose possess levels of NR activity around 2–4 times higher than cells grown in ethanol (Fig. 2). These levels of induction are similar to those produced by glucose on the glycolytic enzymes in yeast (Maitra & Lobo, 1971; Entian *et al.*, 1984).

Fig. 6. Effect of cycloheximide on appearance of NR. Nitrate-grown *H. anomala* was resuspended at 10 mg ml^{-1} in YNB-glucose containing 50 mM -ammonium ($\square$) until NR activity reached approximately 50% of the initial level. At the time indicated (arrow) cells were washed with water (discontinuity in the scale) and transferred to YNB-glucose + ammonium and YNB-glucose + 50 mM -nitrate with (∇) or without ($\circ$) $40 \mu\text{g}$ cycloheximide ml^{-1} . Experiments were repeated three times without significant differences; results shown are from a single experiment.

In *H. anomala* grown in nitrate and transferred to nitrate + cycloheximide, the rate of disappearance of NR activity was much lower than in cells transferred to nitrogen-free medium or nitrogen-free + cycloheximide (Fig. 5). Since cycloheximide suppresses synthesis of NR, these results suggest that nitrate improved the resistance of NR to proteolysis. Cycloheximide does not seem to inhibit any proteolytic system involved in the NR degradation, as in cells transferred to a nitrogen-free medium with or without cycloheximide, the rate of loss of NR activity was similar.

The characterization of the NR from *H. anomala* with the anti-NR antiserum by Western blot suggests a subunit mass of 52 kDa in SDS-PAGE for the NR subunits, in accordance with Zauner & Dellweg (1983). However, nitrate reductases from many other organisms, including yeast, have subunit masses of around 100 kDa (Hipkin, 1989). In this work, subunits were prepared in the presence of protease inhibitors and the enzyme is active under non-denaturing conditions, and so 52 kDa seems to be the true mass of NR subunits of *H. anomala*. Cloning of the NR gene from *H. anomala* will be necessary to resolve this problem definitively.

In contrast to the results reported here for *H. wingei* (Fig. 4), Jones *et al.* (1989) reported that ammonium or glutamine prevented the induction by nitrate of NR. They

also reported levels of NR activity twice as high as those obtained in this work. These differences could be due to the strain used, as well as to the methods.

In conclusion, the results obtained here, along with those already reported (Hipkin, 1989), show that in yeast no inactive NR is present in ammonium-, glutamine- or peptone-grown cells. This contrasts with observations in algae and plants (Funkhouser *et al.*, 1980; Funkhouser & Ramadoss, 1980; Oaks *et al.*, 1988). On the other hand, the appearance and disappearance of NR is tightly correlated with the synthesis and loss of NR protein. This process, in *H. anomala*, is mainly regulated by nitrate, with less participation from nitrogen metabolite repression.

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