Regulation of Nitrate Reductase Synthesis in the Yeast *Candida nitratophila*

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Immunoelectrophoresis, using antibodies raised against homogeneously pure nitrate reductase (NR; EC 1.6.6.2), was used to study the regulation of NR synthesis in *Candida nitratophila*. Extracts from ammonium-grown cultures did not contain cross-reacting material, but cross-reacting material was detected in cultures transferred to nitrogen-free and nitrate medium. The appearance of NR activity in these extracts appeared to be the result of *de novo* protein synthesis. The possible role of nitrate in the stimulation of active NR synthesis is discussed. Losses of NR activity that occurred in nitrate-grown cultures transferred to ammonium medium correlated with decreases in cross-reacting material.

INTRODUCTION

Assimilatory nitrate reductase (NR) is a highly regulated enzyme in eukaryotic microorganisms. In fungi such as *Aspergillus* and *Neurospora*, high NR activity is found in cultures only when they are grown with nitrate or nitrite, in the absence of ammonium (Kinsky, 1961; Sorger, 1965; Garrett, 1972). This has led to a general consensus that NR is 'induced' by nitrate or nitrite and 'repressed' by ammonium or a product of ammonium assimilation. Moreover, increases in NR activity that occur in nitrate-adapting cultures of *Neurospora crassa* appear to result from *de novo* synthesis of NR (Bahns & Garrett, 1980; Amy & Garrett, 1979). In contrast, in the green alga *Chlorella*, NR synthesis appears to be derepressed in nitrogen-starved, ammonium-grown cultures (Hipkin & Syrett, 1977) and there is evidence that repressed cultures contain NR precursors (Funkhouser *et al.*, 1980). Recent research into the regulation of nitrate assimilation in yeasts in our laboratory has shown that these organisms resemble unicellular algae in some respects. Thus, ammonium-grown cultures of *Sporobolomyces roseus* (Ali & Hipkin, 1985) and other yeasts, including *Candida* spp. (Ali, 1985), acquire the capacity to assimilate nitrate after a short period of nitrogen starvation.

In this paper we present results bearing on the role of nitrate in regulation of the synthesis of NR in ammonium-grown cultures of the yeast *Candida nitratophila*. The structure and properties of highly purified preparations of this enzyme are described in the preceding paper (Hipkin *et al.*, 1986).

METHODS

Organism and culture. *Candida nitratophila* (NCYC 556) was grown as described by Hipkin *et al.* (1986). Ammonium medium contained 10 mM-NH₄Cl, nitrate medium contained 10 mM-NaNO₃ and ammonium nitrate medium contained 10 mM-NH₄NO₃. Nitrogen-free medium was normal medium minus a nitrogen source. Cultures were washed with nitrogen-free medium between transfers. Zero-time culture densities were set to 10⁸ organisms ml⁻¹.

Preparation of cell-free extracts. These were prepared in extraction buffer (50 mM-potassium phosphate, pH 7.5, containing 5 µM-FAD) as described by Hipkin *et al.* (1986).

Abbreviations: BVH, reduced benzyl viologen; FAD, flavin adenine dinucleotide; NR, nitrate reductase.

Preparation of frozen/thawed cells. Samples of culture (0.5 ml) were harvested in a bench centrifuge and resuspended in 1 ml extraction buffer and frozen at -16°C for 16–24 h. The frozen suspension was thawed slowly at room temperature.

Nitrate reductase (NR) (EC 1.6.6.2) assays. NADH-NR activity was assayed in cell-free extracts (Hipkin *et al.*, 1986) and by a frozen/thawed, whole cell assay. In the whole cell assay, 1 ml suspensions of frozen/thawed cells were incubated with $50\text{ }\mu\text{mol KNO}_3$ for 5 min at 30°C before the reaction was started with the addition of $0.5\text{ }\mu\text{mol NADH}$. Reactions were allowed to proceed for 10 min and then terminated by boiling. Residual NADH was oxidized by the addition of phenazine methosulphate (Scholl *et al.*, 1974) and nitrite was estimated as described by Hipkin *et al.* (1986). In this assay a single freeze/thaw cycle renders the yeast cells permeable to NADH, nitrate and nitrite. NADH-NR activity by this assay was expressed as $\text{nmol min}^{-1} (\text{ml culture})^{-1}$. For the *in vitro* assay, NR activity was expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

Protein determination. Protein was measured by the method of Bradford (1976), using Bio-Rad protein reagent.

Preparation of antiserum. A 2000-fold purified NR preparation (homogeneous by SDS gel electrophoresis) was used to immunize a male New Zealand White rabbit. Approximately $150\text{ }\mu\text{g}$ NR protein in 50% (v/v) Freund's complete adjuvant was injected subcutaneously into the shaved hindquarters of the rabbit at multiple sites. This procedure was repeated at 2 week intervals on alternate sides of the rabbit until a total of four injections had been given. Then, 10 d after the final injection, approximately 8 ml blood was collected from the lateral vein of the ear. The blood was allowed to clot and then centrifuged at 40000 g . The supernatant, stored at -20°C , served as the antibody preparation.

Immunological techniques. Antibody reaction was tested by Ouchterlony double diffusion (Ouchterlony, 1953) and titration against NR activity.

Crossed immunoelectrophoresis was done in 0.8% (w/v) agarose gels (25 ml) on horizontal plates ($10 \times 10\text{ cm}$) with barbitone buffer (30 mM, pH 8.4) as the electrolyte. In the first dimension, $20\text{ }\mu\text{l}$ crude extract (about $200\text{ }\mu\text{g}$ protein) and/or purified NR (about $0.8\text{ }\mu\text{g}$ protein) were electrophoresed for 40 min at 25 mA (250 V) at 4°C in gel lacking antibodies. For tandem crosses, two wells were cut in the gel at the cathode end, 5 mm apart. In the second dimension proteins separated in the first dimension were electrophoresed into agarose gel containing 0.35% NR antiserum for a further 2–3 h under the above conditions. Gels were washed with 0.15 M-NaCl for 24 h, washed four times with distilled water over a similar period, dried and stained with Coomassie brilliant blue.

Rocket immunoelectrophoresis was done using the horizontal electrophoresis system described above in 0.8% (w/v) agarose gels (25 ml) containing 0.35% NR antiserum. The gel was electrophoresed at 30 mA for 2 h followed by 20 mA for 4 h at 4°C . Gels were washed and stained as described above.

RESULTS

Appearance and disappearance of NR in C. nitratophila

Preliminary experiments showed that NADH-NR activities measured in frozen/thawed cells of *C. nitratophila* were highest in cultures grown with nitrate as the sole source of nitrogen. In cultures grown on reduced nitrogen sources such as urea, hypoxanthine or L-amino acids (e.g. glutamine, glutamic acid, asparagine, aspartic acid) NR activity was never more than 10% of the activity measured in nitrate-grown cultures, and was usually less than 4%. Cultures grown with ammonium as sole nitrogen source did not contain measurable NR activity. In contrast, cultures grown with ammonium plus nitrate or ammonium-grown cultures that had been nitrogen-starved for 3 h contained 25% and 20%, respectively, of the NR activity of nitrate-grown cultures (results not shown). Fig. 1 shows the appearance of NR activity in ammonium-grown cultures of *C. nitratophila*; it increased slowly during an initial 2 h period of nitrogen starvation and then increased rapidly after the addition of 10 mM-nitrate. Addition of cycloheximide with the nitrate, or 30 or 60 min later, resulted in a rapid inhibition of the increase in NR activity.

NR activity was lost from nitrate-grown cultures under various conditions, e.g. transfer of organisms to nitrate medium lacking a carbon source, transfer to nitrogen-free medium or transfer to ammonium medium (results not shown). Losses of NR activity under these conditions were similar, approximately 50% of the original NR activity being lost after 5 h. When nitrate-grown cultures were transferred to ammonium nitrate medium there was little change in NR activity for the initial 4 h, after which it decreased at a rate similar to that observed with the ammonium-grown culture. NR activity increased in cultures resuspended in fresh nitrate medium; after 4 h activity had approximately doubled. Thereafter, NR activity decreased.

Fig. 1. Effect of cycloheximide on the appearance of NADH-NR activity in *C. nitratophila*. An ammonium-grown culture was washed, transferred to nitrogen-free medium for 120 min, and then supplied with 10 mM-KNO₃ (arrowed). Cycloheximide (20 µg ml⁻¹) was added after 120 min (▲), 150 min (△) or 180 min (●); ○, no cycloheximide added. NADH-NR activity was measured by the frozen/thawed cell assay. Zero-time cell densities were 10⁸ cells ml⁻¹.

Fig. 2. Effect of anti-(NR) serum on NR activity. Immunological titration of NR. Samples of pure NR were incubated with increasing amounts of anti-(NR) serum at 30 °C for 30 min and then at 4 °C for 2 h. Residual NADH-NR activity (●), BVH-NR activity (▲) and cytochrome *c* reductase activity (■) were then assayed. The 100% activities were as follows [nmol min⁻¹ (mg protein)⁻¹]: NADH-NR, 373; BVH-NR, 350; cytochrome *c* reductase, 383. Null serum had no effect on NR activity. Inset: Ouchterlony double immunodiffusion gel containing 20 µl of (a) pure NR and (b) anti-(NR) serum.

Regulation of NR activity in *C. nitratophila*

Immunological techniques were used to investigate whether changes in NR activity that occurred under various conditions in *C. nitratophila* cultures correlated with changes in levels of NR protein. Double diffusion of rabbit NR-antibodies against pure NR in Ouchterlony gels gave a strong precipitin band (Fig. 2, inset). Further, titration experiments showed that the antiserum strongly inhibited the NADH-NR, BVH-NR and cytochrome *c* reductase activities

Fig. 3. Crossed immunoelectrophoretic analysis of NR in *C. nitratophila*. (a) Crossed immunoelectrophoresis of pure NR (800 ng); (b) tandem crossed immunoelectrophoresis of pure NR (800 ng) vs cell-free extract (200 μ g protein) from a nitrate-grown culture; (c) crossed immunoelectrophoresis of cell-free extract (200 μ g protein) from an ammonium-grown culture; (d) tandem crossed immunoelectrophoresis of pure NR (800 ng) vs cell-free extract (200 μ g protein) from an ammonium-grown culture. Precipitation bands were detected after washing in saline and then staining with Coomassie blue and subsequent destaining.

of the enzyme (Fig. 2). Presumably the antibodies recognized and reacted at multiple sites on the enzyme molecule. Preimmune serum had no effect on NR activity.

The appearance of NR activity in ammonium-grown cultures after transfer to nitrogen-free or nitrate medium could result from activation of inactive protein, assembly of precursor protein or a net synthesis of active protein. If activation or assembly were involved then it should be possible to detect cross-reacting material in ammonium-grown cultures by using NR antibodies. To test this, cell-free extracts from ammonium-grown cultures were subjected to crossed immunoelectrophoresis. A single hump-shaped precipitin band was obtained when pure NR was electrophoresed into the antibody-containing gel (Fig. 3a). A tandem cross of pure NR and crude extract from a nitrate-grown culture produced a contiguous, double-humped precipitin band, without spurs (Fig. 3b). This indicated identity between pure NR and NR protein in crude extract, as expected. However, extract from an ammonium-grown culture did not produce

Fig. 4. Appearance and disappearance of NR protein in *C. nitratophila*. (a) An ammonium-grown culture was washed, nitrogen-starved for 2 h and then supplied with 10 mM-KNO₃. (b) A nitrate-grown culture was washed and transferred to ammonium medium. At each time point indicated samples of cell-free extract were assayed for NADH-NR activity and also subjected to rocket immunoelectrophoresis. □, NR catalytic activity [nmol min⁻¹ (mg NR protein)⁻¹]; ▲, NR specific activity [nmol min⁻¹ (mg total protein)⁻¹]; ■, NR protein [μg NR protein (mg total protein)⁻¹]. The 100% values were as follows for (a) and (b) respectively: NADH-NR catalytic activity, 48.3 and 59.2 μmol min⁻¹ (mg NR protein)⁻¹; NADH-NR specific activity, 350 and 450 nmol min⁻¹ (mg total protein)⁻¹; NR protein, 7.38 and 7.6 μg NR protein (mg total protein)⁻¹.

a precipitin reaction (Fig. 3c) and a tandem cross of pure NR and extract from an ammonium-grown culture only produced one precipitin hump (Fig. 3d).

Quantitative analysis of the increase in NR protein in nitrogen-free and nitrate-grown cultures was obtained by rocket immunoelectrophoresis. Preliminary experiments using pure NR protein indicated that there was an acceptable, linear relationship between rocket height and amount of NR protein (not shown). Using this sensitive technique it was possible to investigate whether increases in NR activity were correlated with increases in NR protein. Ammonium-grown organisms were nitrogen-starved for 2 h before addition of nitrate. Cell-free extracts were prepared from these cultures and samples were assayed for NADH-NR activity and subjected to rocket immunoelectrophoresis. Several experiments were done and each gave similar results; Fig. 4(a) shows the results of a typical experiment. No cross-reacting material was detected in zero-time samples. Subsequently during nitrogen starvation, NR protein was detectable after the first hour but did not increase thereafter until nitrate was added. However, the specific activity of NADH-NR activity increased over the whole 2 h period of nitrogen starvation. After nitrate addition, NR protein and NADH-NR activity increased rapidly. Thus, before nitrate addition, the catalytic activity of NR cross-reacting material increased slowly, whereas after nitrate addition it attained a higher and constant value rapidly.

When a nitrate-grown culture was resuspended in ammonium medium there was a gradual loss of NADH-NR activity, which correlated with decreases in cross-reacting material measured by rocket immunoelectrophoresis (Fig. 4b). The catalytic activity of NR cross-reacting material under these conditions showed fluctuation but little overall change.

DISCUSSION

The results presented here indicate that NR activity is repressed in *C. nitratophila* cultures grown with ammonium, or other reduced nitrogen sources, as long as nitrate is absent. Moreover, since significant amounts of NR activity can be measured in nitrogen-starved

cultures and may be sustained for at least 5 h in the absence of nitrate (Ali, 1985), this suggests that derepression plays a role in the regulation of NR in *C. nitratophila*. However, nitrate must also play a prominent role in the regulation of NR in this organism since its addition to nitrogen-starved cultures resulted in a large increase in NR activity and cultures grown with ammonium and nitrate contained about 25% of the fully induced activity of nitrate-grown cultures. Under these conditions there must be a complex balance between nitrate stimulation of NR activity and nitrogen metabolite repression.

Increases in NR activity that occur when ammonium-grown cultures are nitrogen-starved or transferred to nitrate medium could result from regulatory events at the transcriptional, translational or post-translational level. In algae, such as *Chlorella*, post-transcriptional control has been postulated (Hipkin & Syrett, 1977) and there is evidence that ammonium-grown cultures synthesize NR precursor protein, which is assembled when cultures are transferred to nitrate medium (Funkhouser *et al.*, 1980, 1983; Funkhouser & Ramadoss, 1980). In contrast, results obtained from immunological studies here show that precursor NR protein was absent from ammonium-grown cultures of *C. nitratophila*. Similar conclusions were drawn by Amy & Garrett (1979) for *N. crassa* NR. In fungi, it appears that translation of mRNA, at least, limits the production of active NR in ammonium-grown cultures. Our results show, however, that *C. nitratophila* differs from *N. crassa* in that removal of ammonium alone results in the synthesis of protein, which cross-reacts with NR antibodies, although addition of nitrate stimulates a larger increase in this protein. However, it is significant that the catalytic activity of NR (i.e. activity based on a unit NR protein basis) was higher and reached a steady state rapidly in cultures that received nitrate. We suggest that during nitrogen starvation NR is derepressed but its activity is limited by the assembly of the active enzyme. We would argue, therefore, that addition of nitrate stimulates both the net synthesis and assembly of active enzyme. In this model, nitrate plays a post-translational role in the assembly and stabilization of NR. Similarly, it has been suggested that nitrate stabilizes the formation of active NR in *Chlamydomonas* (Hipkin *et al.*, 1980, 1985) and plays a post-transcriptional role in the regulation of active NR in *Neurospora* (Sorger *et al.*, 1974).

Loss of NR from nitrate-grown cultures of *C. nitratophila* occurs when the organisms are deprived of nitrogen or carbon or when they are resuspended in ammonium medium. Similar results have been obtained with other fungi (Lewis & Fincham, 1970; Hynes, 1973). Results shown here indicate that losses of NR protein that occur in ammonium cultures are not rapid and do not involve an overall change in catalytic activity. This suggests that enzyme inactivation is not responsible for decreases in activity but results from a cessation of enzyme synthesis, dilution and protein turnover.

The role of transcriptional control in the regulation of NR synthesis remains largely unknown. However, recent experiments by us show that ammonium-grown cultures of *C. nitratophila* do not contain poly A⁺ mRNA that translates *in vitro* into the 95 kDa subunit of NR, whereas nitrogen-starved and nitrate-adapting cultures do (A. Cannons & C. R. Hipkin, unpublished).

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