Evidence that the glutamine-stimulated loss of nitrate reductase protein from the yeast *Candida nitratophila* is not the result of inducer exclusion

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Synthesis of nitrate reductase protein and increases in nitrate reductase activity occurred in cultures of the yeast *Candida nitratophila* when they were incubated in medium containing ammonium nitrate. Similar treatment with glutamine plus nitrate resulted in little increase in nitrate reductase activity, in cultures grown previously with reduced nitrogen compounds, and decreases in enzyme activity, in cultures adapted to nitrate. Labelling studies conducted *in vivo* revealed a rapid cessation of *de novo* nitrate reductase synthesis when glutamine was supplied to

nitrate-adapted cultures in the presence of nitrate. Intracellular glutamine concentrations increased rapidly under these conditions and these cultures exhibited high glutamine:glutamate ratios. As nitrate was taken up in the presence of glutamine in these experiments, it is concluded that the glutamine-stimulated inhibition of nitrate reductase synthesis is a consequence of repression and rapid turnover of nitrate reductase mRNA and not inducer (nitrate) exclusion.

INTRODUCTION

Nitrate reductase (NR) (EC 1.6.6.1–3) catalyses an important step in the control of nitrate assimilation in plants and fungi, and occupies a key position in nitrogen metabolism in these organisms. It is a highly regulated enzyme and there is good evidence that its synthesis is controlled at the transcriptional level (Cannons and Hipkin, 1987; Fu and Marzluf, 1987; Johnstone et al., 1990). Genetic studies with *Aspergillus nidulans* and *Neurospora crassa* (Marzluf and Fu, 1989; Scazzocchio and Arst, 1989) suggest that regulation is effected via two circuits, nitrogen metabolite repression and nitrate induction. Similar regulatory circuits may operate in other nitrate-assimilating organisms such as algae and yeasts (Hipkin, 1989; Solomonson and Barber, 1990). When these organisms are grown with reduced nitrogen sources, such as ammonium or glutamine, they usually contain low levels of NR activity compared with organisms grown under inducing conditions (i.e. with nitrate as the sole source of nitrogen). Furthermore, with certain organisms, induction of NR synthesis does not take place when cultures are incubated with both nitrate and a source of reduced nitrogen such as ammonium (Guerrero et al., 1981; Ali and Hipkin, 1985) and this suggests that nitrogen metabolite repression overrides nitrate induction. However, as ammonium inhibits nitrate uptake in many organisms (Syrett and Morris, 1963; Schloemer and Garrett, 1974; Ali and Hipkin, 1985), it is usually not possible to make a clear distinction between an apparent repression of NR synthesis on the one hand and a regulatory response to inducer exclusion on the other. Moreover, in some organisms, NR synthesis apparently takes place in cultures supplied with nitrate and ammonium (Ali and Hipkin, 1986; Oaks et al., 1988).

The object of the study reported here was to investigate the possible significance of inducer exclusion during the apparent repression of NR synthesis by reduced nitrogen metabolites. The yeast, *Candida nitratophila*, is a good organism in which to study this problem as it can assimilate ammonium and nitrate simultaneously under certain conditions (Ali and Hipkin, 1986).

MATERIALS AND METHODS

Organism and culture

Candida nitratophila (556) was obtained from the National Yeast Culture Collection (Norwich, U.K.). Bulk cultures were grown in Difco Yeast Carbon Base medium (11.7 g/l) with nitrate or ammonium supplied at a concentration of 50 mM. In experiments, all nitrogen sources were supplied at a concentration of 10 mM. Other culture conditions were as described previously (Ali and Hipkin, 1986).

NR activity assays

NADH-NR (EC 1.6.6.2) activity was determined in frozen/thawed cell suspensions or in cell-free extracts as described previously (Ali and Hipkin, 1986; Hipkin et al., 1986).

Labelling *in vivo* and immunoprecipitation of ³⁵S-labelled NR

Cultures were grown to late exponential phase in medium containing ammonium as the sole nitrogen source. Cells were harvested, washed by centrifugation (Ali and Hipkin, 1986) and resuspended in fresh medium with no nitrogen source (nitrogen-free) or with nitrate, ammonium, ammonium plus nitrate, glutamine or glutamine plus nitrate as the source(s) of nitrogen (all at 10 mM). Cultures were incubated under these growth conditions for 2 or 4 h. Cultures incubated for 2 h were given 20 µCi of L-[³⁵S]methionine at zero time. Cultures incubated for 4 h were given the same addition, but 2 h after zero time. Extraction, immunoprecipitation and identification of ³⁵S-labelled NR were carried out as described by Cannons and Hipkin (1987).

Purification of NR and preparation of antibodies

NR was purified to electrophoretic homogeneity as previously described (Hipkin et al., 1986) and used to raise antibodies in a rabbit (Cannons et al., 1986).

Abbreviation used: NR, nitrate reductase.

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Nitrate uptake studies

Nitrate uptake was measured by following the disappearance of nitrate from the culture medium. At zero time, culture densities were adjusted to 5×10^7 cells/ml in fresh medium containing 1 mM potassium nitrate in the presence of 10 mM ammonium chloride or 10 mM glutamine. Samples (2.0 ml) were taken at intervals and centrifuged (1000 g) in a bench centrifuge. Nitrate content was determined in supernatants as nitrite. Reduction of nitrate to nitrite was accomplished using pure *C. nitratophila* NR (2.5 pmol) and normal NR assay conditions (Hipkin et al., 1986), except assay incubation times were 60 min.

Amino acid analysis

Amino acids were extracted from frozen/thawed cells into Milli Q water at 70 °C and analysed by h.p.l.c. as described by Flynn and Hipkin (1990).

RESULTS

The effects of ammonium and glutamine on the appearance and disappearance of NR activity in the presence of nitrate

Figure 1 shows the effects of adding nitrate to a culture of *C. nitratophila* which had been adapted to growth with ammonium and glutamine. Initially the culture contained a low level of NR activity. Removal of the reduced nitrogen source (glutamine) and addition of nitrate resulted in a rapid increase in NR activity. Addition of ammonium nitrate under the same conditions stimulated a similar increase in NR activity. In contrast, addition of glutamine plus nitrate only resulted in a small increase in enzyme activity.

Figure 1 Effect of ammonium and glutamine on the appearance of NR activity in a nitrogen-metabolite-repressed culture of *Candida nitratophila*

A culture previously grown in the presence of ammonium was transferred to glutamine medium (●). After 2 h, culture portions received the following additions (arrow): 10 mM nitrate (Δ), 10 mM ammonium plus 10 mM nitrate (▲), 10 mM glutamine plus 10 mM nitrate (○). NR activity (assayed in frozen/thawed cell suspensions) is expressed as nmol/min per ml of culture. Cultures were adjusted to a density of 10^8 cells/ml at zero time.

Figure 2 Effect of ammonium and glutamine on the decrease in NR activity from a nitrate-induced culture of *Candida nitratophila*

A culture was grown with nitrate as the sole nitrogen source until late exponential phase. At zero time, culture portions received the following additions: 10 mM ammonium (●), 10 mM ammonium plus 10 mM nitrate (▲), 10 mM glutamine (Δ), 10 mM glutamine plus 10 mM nitrate (○). Culture densities and NR activity as in Figure 1.

Figure 2 shows the changes in NR activity after the addition of glutamine or ammonium to a nitrate-grown culture of *C. nitratophila*. At zero time, this culture contained high levels of NR activity. Removal of nitrate and addition of ammonium or glutamine resulted in losses of enzyme activity after 2 h and this trend continued for the following 8 h. Addition of glutamine plus nitrate resulted in a similar decrease in NR activity, but NR activity remained high in organisms that received ammonium and nitrate.

Effects of ammonium, glutamine and inducer exclusion on *de novo* synthesis of NR protein

In order to show the effects of ammonium and glutamine on *de novo* synthesis of NR protein, we followed the appearance of ^{35}S -labelled NR by labelling *in vivo*. Figure 3 shows an experiment where NR synthesis was followed in a nitrate-adapted culture which had been given nitrate, ammonium, ammonium nitrate or no nitrogen. Cultures used for these experiments were grown initially on ammonium and then transferred to fresh nitrate medium for 2 h and would have been synthesizing NR protein actively at this time (Cannons and Hipkin, 1987). When such a culture was transferred to fresh medium lacking nitrate (i.e. ammonium or nitrogen-free medium), no labelled NR was detected, indicating that net NR synthesis ceased under those conditions. Both conditions qualify as inducer exclusion treat-

Figure 3 Labelling *in vivo* of NR in the presence of ammonium and nitrate

A culture grown in the presence of ammonium was incubated with nitrate for 2 h and portions were incubated with ^{35}S -labelled methionine for 2 or 4 h with 10 mM nitrate (lanes 1 and 2), 10 mM ammonium (lanes 3 and 4), 10 mM ammonium plus 10 mM nitrate (lanes 5 and 6) or no nitrogen (N-F) (lanes 7 and 8), as indicated. Positions of the molecular-mass markers are indicated.

Figure 4 Effect of nitrate, ammonium and nitrogen-starvation on levels of NR protein

Experimental conditions were as described in Figure 3. NR protein was estimated by rocket immunoelectrophoresis and is expressed on the basis NR protein per total protein. Cultures were treated with nitrate (●), ammonium (○), ammonium nitrate (▲) or no nitrogen (△) after 2 h.

Figure 5 Labelling *in vivo* of NR in the presence of glutamine and nitrate

Conditions were as described in the legend to Figure 3 except culture portions were incubated with 10 mM nitrate (lanes 1 and 2), 10 mM glutamine plus 10 mM nitrate (lanes 3 and 4) or 10 mM glutamine (lanes 5 and 6).

ments. In contrast, incubation with nitrate or ammonium nitrate resulted in the production of new, labelled NR. Moreover, the increased intensity of labelled bands from cultures incubated for 4 h indicated that there were increasing rates of synthesis during the latter part of this period. We also followed the levels of NR

Table 1 Intracellular ammonium and amino acid concentrations in *C. nitratophila*

Cultures previously incubated with nitrate as the sole source nitrogen for 2 h (under growth conditions as described in the Materials and methods section) received additions (10 mM) as indicated. Amino acids and ammonium contents are expressed as pmol/ 10^5 cells.

	Ammonium and amino acid content (pmol/ 10^5 cells)			
	Time (h) ... 0	1	2	4
Nitrate culture				
Ammonium	50	53	69	96
Gln	122	255	231	282
Glu	401	477	453	784
Gln:Glu	0.30	0.53	0.51	0.36
Ammonium plus nitrate culture				
Ammonium	50	52	48	80
Gln	122	358	341	268
Glu	402	543	456	687
Gln:Glu	0.30	0.66	0.75	0.39
Glutamine plus nitrate culture				
Ammonium	50	50	47	80
Gln	122	1054	975	1177
Glu	402	743	452	1130
Gln:Glu	0.30	1.40	2.15	1.04

protein in this experiment by rocket immunoelectrophoresis. The results obtained (Figure 4) show that NR protein decreased when cultures received ammonium chloride or no nitrogen, but increased when they received ammonium nitrate or nitrate.

In contrast, labelled NR was hardly detectable in nitrate-adapted cultures when they received glutamine alone or glutamine plus nitrate (Figure 5) in a separate experiment. As before, however, cultures given nitrate synthesized high levels of labelled NR.

In order to investigate some of the intracellular changes that occurred in these cultures we measured the internal pools of amino acids and ammonium in cells which received additions of nitrate, ammonium plus nitrate, or glutamine plus nitrate. The results obtained are shown in Table 1. Low levels of intracellular ammonium were detected under all conditions and changes in ammonium concentration were small during the first hour after additions. The highest ammonium concentration was detected in the culture which received nitrate only. There was little difference between ammonium levels in the cultures which received ammonium and glutamine. There was a rapid increase in the intracellular concentration of glutamine in the culture that received glutamine plus nitrate, so that after 1 h these cells contained three times more glutamine than those which received ammonium plus nitrate. Increases in intracellular glutamine in the cultures containing nitrate alone and ammonium plus nitrate were rather similar. Intracellular glutamate levels increased in all cultures and increases were greatest in the culture that received glutamine plus nitrate. However, increases in glutamate did not parallel increases in glutamine in this culture. Thus, there was a significant increase in the intracellular glutamine:glutamate ratio after the addition of glutamine, which was not evident in the other cultures.

Uptake of nitrate in the presence of ammonium and glutamine

As *C. nitratophila* will take up ammonium and nitrate simultaneously under certain conditions (Ali and Hipkin, 1986), it

Figure 6 Nitrate uptake by *Candida nitratophila* in the presence of ammonium or glutamine

Nitrate uptake is shown as the disappearance of nitrate (nmol/ml) from medium containing 10 mM ammonium (○) or 10 mM glutamine (△).

could be argued that the difference between ammonium nitrate and glutamine plus nitrate treatments (Figures 3 and 4) was the result of a strong inhibition of nitrate uptake by glutamine. To test this hypothesis we set up an experiment where the effects of ammonium and glutamine on nitrate uptake were compared (Figure 6). The results show that cultures were able to take up nitrate at similar rates irrespective of whether ammonium or glutamine was present.

DISCUSSION

Although early researchers speculated on the roles of induction and repression in the control of nitrate assimilation, it was not until relatively recently that firm evidence for the regulation of NR synthesis at the transcriptional level was obtained (Cheng et al., 1986; Cannons and Hipkin, 1987; Fu and Marzluf, 1987; Johnstone et al., 1990). Nevertheless, the exact roles of nitrogen metabolites such as nitrate or ammonium and glutamine, as inducers and repressors are still understood poorly. In the yeast *Candida nitratophila*, nitrate stimulates net synthesis of NR mRNA and its translation (Cannons and Hipkin, 1987; Hipkin, 1989) and may also play a post-translational role in the regulation of the synthesis of enzymically active NR through the stabilization of the quaternary structure of the enzyme. Thus, although a small amount of NR synthesis may take place in the absence of nitrate (as long as reduced nitrogen is also absent), exclusion of nitrate from cultures adapted to nitrate assimilation results, nevertheless, in a marked inhibition of NR synthesis. To under-

stand the regulation of nitrate assimilation it is important to be able to distinguish between such an effect and the apparent repression of NR synthesis by reduced nitrogen compounds.

Results reported here show that NR synthesis continues in nitrate-adapted cultures when they are supplied with ammonium and nitrate. This is indicated in experiments where enzyme activity was followed, and also where labelling *in vivo* and immunoelectrophoresis were employed to follow changes in new and total NR protein. Experiments with *Arabidopsis thaliana* have also shown that NR activity and NR mRNA levels are high in plants treated with ammonium and nitrate (Crawford and Davis, 1989). Asporogenous yeasts such as *C. nitratophila* assimilate nitrate and ammonium simultaneously (Ali and Hipkin, 1986), and continued NR synthesis under these conditions indicates that ammonium per se is not a repressor of NR synthesis in these organisms. Indeed, evidence from a previous study (Flynn and Hipkin, 1990) suggests that low or decreasing levels of NR activity in *C. nitratophila* correlate with elevated levels of intracellular glutamine. Similarly, other studies with filamentous fungi indicate that the intracellular repression signal is likely to come from a product of ammonium assimilation close to glutamine (Dunn-Coleman et al., 1979; Premakumar et al., 1979). Therefore, it is significant that experiments reported here show that glutamine is a more potent antagonist of NR synthesis than ammonium in yeast, and that this is true irrespective of whether nitrate is also present in cultures or not. Thus, in contrast with treatment with ammonium nitrate, addition of glutamine plus nitrate prevents significant increases in NR activity in nitrate-adapting cultures and also stimulates losses of NR activity similar to those observed when glutamine is the sole source of nitrogen. More strikingly, additions of glutamine and nitrate result in a rapid cessation of *de novo* NR synthesis, as shown by labelling *in vivo*. This correlates with a large and rapid increase in the intracellular concentration of glutamine (Table 1) which is not observed in nitrate- or ammonium plus nitrate-treated cultures. However, since removal of nitrate alone is enough to promote a cessation in NR synthesis, it could be argued that the glutamine plus nitrate effect is merely a form of inducer exclusion resulting from the inhibition of nitrate uptake by glutamine. We have provided evidence that this is not so, as nitrate is taken up at similar rates by cultures containing ammonium or glutamine. We conclude, therefore, that the glutamine inhibition of NR synthesis is not a result of inducer exclusion, and we suggest that it results from a measured response from the cell to the relative magnitude of the intracellular glutamine pool (indicated in Table 1 as the glutamine:glutamate ratio). The rapid effect of glutamine reported here can be explained by repression alone if one argues that NR mRNA is turned over rapidly during repression. Evidence that NR mRNA is turned over rapidly with the onset of nitrogen metabolite repression has been obtained recently for *Neurospora crassa* (Okamoto et al., 1991).

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