Nitrate Assimilation in the Basidiomycete Yeast *Sporobolomyces roseus*

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Ammonium-grown cultures of *Sporobolomyces roseus* developed the capacity to assimilate nitrate when they were incubated in nitrate or nitrogen-free medium. Cycloheximide, 6-methyl purine and antimycin A inhibited this process. Nitrate assimilation required aerobic energy metabolism and was inhibited completely by ammonium. The rapid inhibition of nitrate assimilation by ammonium was not the result of an inhibition of nitrate reductase (NR) activity. Nitrite also inhibited nitrate assimilation. NR in cell-free extracts of *S. roseus* was NADPH-specific and its activity was repressed in cultures containing ammonium and derepressed during nitrogen starvation. Nitrate stimulated the appearance of NR in these cultures. Nitrate assimilation was not limited by apparent (potential) NR activity.

INTRODUCTION

Many autotrophic and heterotrophic micro-organisms are able to assimilate nitrate (Payne, 1973; Brown *et al.*, 1974) and this process has been studied extensively in bacteria, cyanobacteria, algae (Guerrero *et al.*, 1981) and filamentous ascomycetes such as *Aspergillus* (Pateman & Kinghorn, 1976) and *Neurospora* (Garrett & Amy, 1978). Surprisingly, there have been few detailed investigations of nitrate assimilation in yeasts and fewer in basidiomycetes.

In this paper we describe some aspects of the uptake and reduction of nitrate by the basidiomycete yeast *Sporobolomyces roseus*. This species occurs commonly in nature and is a frequent and often abundant member of the phylloplane and atmospheric microflora (Last, 1955; Gregory & Hirst, 1957). Both nitrate and reduced nitrogen are likely to be important sources of nitrogen for phylloplane fungi although, to our knowledge, there have been no published investigations of nitrate assimilation in *S. roseus*. The work described here is part of a larger study of nitrate assimilation in yeasts.

METHODS

Organism and culture. *Sporobolomyces* Y44 was a generous gift from the Department of Botany, University of Bristol, UK. This organism, originally isolated from a phylloplane association at Long Ashton Experimental Station, Bristol, UK, has been identified by us as *S. roseus* as described by Phaff (1970). The liquid mineral medium minus nitrogen and carbon was that described by Ferguson & Sims (1971). Carbon was supplied as glucose (1%, w/v) and nitrogen as NH_4Cl or NaNO_3 (10 mM). Liquid cultures were usually grown for 3 d at 15 °C in 100 ml medium in 250 ml Erlenmeyer flasks in an orbital incubator (110 r.p.m.). Cultures were inoculated from stocks kept on malt extract agar plates.

Organisms were harvested by centrifugation and washed with nitrogen-free medium (with or without 1%, w/v, glucose) or buffer before uptake experiments or enzyme extraction respectively.

Uptake experiments. Inorganic nitrogen uptake was measured by following the disappearance of nitrate, nitrite or ammonium from the medium with zero-time culture densities of 10^8 organisms ml^{-1} . Nitrate was measured in supernatants by the method described by Cawse (1967), nitrite was measured by the Griess-Ilosvay Method as described by Everest *et al.* (1984) and ammonium and intracellular nitrate were measured as described by Cresswell & Syrett (1979). Samples of 5 ml or 10 ml were withdrawn at each sampling time and results were expressed as means of duplicates. The results obtained were reproducible in repeated experiments.

Abbreviation: NR, nitrate reductase.

Nitrate reductase (NR) assays. NAD(P)H-NR activity was measured in cell-free extracts. Cells (approximately 10^{10} organisms) were broken in 10 ml 50 mM-potassium phosphate buffer pH 7.5 containing dithiothreitol (1 mM) and Na_2EDTA (1 mM) by passage through a French pressure cell at 100 MPa. Extracts were clarified by centrifugation at 40000 *g* for 20 min at 3 °C. Assays containing extract (about 500 µg protein), potassium phosphate buffer pH 7.5 (50 µmol), KNO_3 (50 µmol), FAD (2.5 nmol) and NAD(P)H (0.3 mM) were initiated by the addition of NAD(P)H, allowed to proceed for 10 min at 30 °C and terminated by boiling for 2 min. After cooling, residual NAD(P)H was oxidized with phenazine methosulphate (Scholl *et al.*, 1974) and nitrite was estimated.

Terminal-NR (BVH-NR) activity was assayed in cell-free extracts as described above except that benzyl viologen (200 µg), reduced chemically with a sodium dithionite (800 µg)/sodium bicarbonate (800 µg) mixture, replaced NAD(P)H as an electron donor. Assays were terminated by vigorous mixing on a vortex mixer. BVH-NR activity was also measured in frozen/thawed cell preparations. In these experiments duplicate 1 ml samples (10^8 organisms) were centrifuged and resuspended in 50 mM-potassium phosphate buffer pH 7.5, containing 5 µM-flavin adenine dinucleotide and frozen overnight at -20 °C. Suspensions were thawed slowly and assayed for enzyme activity as described above.

Protein determination. Protein in cell-free extracts was determined by the method described by Hartree (1972).

RESULTS

Development of the capacity to take up nitrate

Cultures of *S. roseus* grown with nitrate or nitrite as the sole source of nitrogen were able to take up nitrate immediately when resuspended in fresh medium containing 1 mM- KNO_3 . In contrast, with ammonium-grown cultures there was a lag of 50 min before nitrate uptake commenced (Fig. 1*a*). However, when ammonium-grown cultures were nitrogen-starved with a carbon source (glucose) for 4 h, nitrate was taken up without a lag (Fig. 1*b*). The capacity to take up nitrate immediately was not developed in ammonium-grown cultures that were nitrogen-starved in the absence of a carbon source (Fig. 2*a*) or if cycloheximide (an inhibitor of protein synthesis), 6-methylpurine (an inhibitor of functional mRNA synthesis) or antimycin A (an inhibitor of oxidative energy production) were present during nitrogen starvation (Fig. 2*b*). In these experiments (Fig. 2), the capacity to take up nitrate did develop after a further lag period if glucose was supplied to carbon-starved cells (Fig. 2*a*) or after the removal of inhibitors (Fig. 2*b*).

Some characteristics of nitrate uptake by S. roseus

In these experiments nitrate uptake was investigated with nitrate-grown cultures or ammonium-grown cultures that had been nitrogen-starved, i.e. cultures that had developed the capacity for nitrate uptake.

Little or no nitrate was taken up by cultures incubated in the absence of a carbon source (Fig. 3*a*), under anaerobic conditions (Fig. 3*b*) or in the presence of glucose, air and antimycin A (Fig. 3*c*).

The addition of 1 mM-ammonium to cultures resulted in a rapid and complete inhibition of nitrate uptake (Fig. 4*a*). Ammonium was taken up rapidly by these organisms and nitrate uptake recommenced only when the ammonium concentration of the medium fell to below 0.2 mM. However, these organisms took up nitrate more rapidly after ammonium inhibition had been relaxed [$7.5 \text{ nmol NO}_3^- \text{ min}^{-1} (10^8 \text{ cells})^{-1}$] than they did before ammonium addition [$5.8 \text{ nmol NO}_3^- \text{ min}^{-1} (10^8 \text{ cells})^{-1}$]. The addition of 0.2 mM-nitrite to cultures taking up nitrate resulted in a 43% inhibition of nitrate uptake. In these cultures nitrate and nitrite were taken up concurrently (Fig. 4*b*). Higher concentrations of nitrite (>1 mM), which are toxic to *Sporobolomyces*, prevented nitrate uptake completely (not shown).

We were unable to measure intracellular nitrate in *S. roseus* cells under any conditions.

Nitrate reduction and nitrate assimilation in S. roseus

Nitrate reductase (NR) was measured in cell-free extracts of nitrate-grown cultures (Table 1). The enzyme was active with NADPH as an electron donor but required the addition of flavin adenine dinucleotide for maximum activity; flavin mononucleotide could not replace the latter. The enzyme was not active with NADH as an electron donor whether flavin adenine dinucleotide was present or not. Terminal NR activity, measured with reduced benzyl viologen

Fig. 1. Development of the capacity to take up nitrate. (a) Cultures grown on ammonium (Δ), nitrite ($\bullet$) or nitrate ($\circ$) as sole nitrogen source were transferred to medium containing 1 mM-nitrate. (b) Cultures grown on ammonium were transferred to medium containing 1 mM- KNO_3 : $\circ$, cultures that were nitrogen-starved in nitrogen-free medium for 3 h before nitrate addition; $\bullet$, cultures that received nitrate without prior nitrogen starvation. All cultures contained glucose as a carbon source.

Fig. 2. Effect of glucose and inhibitors on the development of the capacity to take up nitrate during nitrogen starvation. (a) Nitrate uptake was followed in ammonium-grown cultures that were nitrogen-starved for 3 h with ($\circ$) or without ($\bullet$) 1% (w/v) glucose. Both cultures contained glucose immediately before addition of 1 mM-nitrate. (b) Nitrate uptake was followed in ammonium-grown cultures that were nitrogen-starved for 3 h in the absence of inhibitors ($\circ$) or in the presence of 20 μg cycloheximide ml^{-1} (Δ), 5 μg antimycin A ml^{-1} (Δ) or 1 mM-6-methyl purine ($\bullet$). After treatment, cultures were washed in normal nitrogen-free medium and incubated with nitrate and glucose in the absence of inhibitors.

as electron donor, was approximately four times greater than NADPH-NR activity. In some extracts NADPH-NR activity was very labile.

To study the regulation of NR activity in *S. roseus* cultures, we developed a frozen/thawed cell NR assay using reduced benzyl viologen as an electron donor. NADPH was not effective in these assays possibly because frozen/thawed cells are impermeable to it. Cultures grown with ammonia did not contain NR activity but NR activity appeared and increased in these organisms when they were resuspended in nitrate or nitrogen-free medium (Fig. 5a). Moreover, after 4 h, nitrogen-starved cultures contained a NR activity equivalent to 70% of that found in nitrate-grown cultures. In contrast, cultures resuspended in ammonium-nitrate medium showed no increase in NR activity. Fig. 5(b) shows a similar experiment. Here, ammonium-grown cultures were nitrogen-starved before the addition of nitrate or ammonium-nitrate and both nitrate uptake and NR activity were measured. After 4 h of nitrogen starvation, NR activity was $12.5 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$. When nitrate was added to these cultures it was taken up rapidly ($5 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$) and there was a stimulation in the increase of NR activity.

Fig. 3. Effect of glucose and inhibitors on nitrate uptake. Nitrate-grown cultures were resuspended in fresh medium containing 1 mM-nitrate (a) with ($\blacktriangle$) or without ($\triangle$) 1% (w/v) glucose, (b) with glucose under aerobic ($\circ$) or anaerobic ($\bullet$) conditions, (c) with glucose in the absence ($\circ$) or presence ($\blacktriangle$) of antimycin A ($5 \mu\text{g ml}^{-1}$). Anaerobic cultures were incubated in 250 ml Dreschel bottles and bubbled with oxygen-free nitrogen.

Fig. 4. Effect of ammonium and nitrite on nitrate uptake. Nitrate-grown cultures were transferred to fresh nitrate medium: (a) nitrate uptake in the absence of ammonium ($\bullet$); nitrate uptake after the addition (arrow) of 1 mM-ammonium ($\circ$); ammonium uptake ($\triangle$). (b) Nitrate uptake in the absence of nitrite ($\bullet$); nitrate uptake after addition (arrow) of 0.2 mM-nitrite ($\blacksquare$); nitrite uptake ($\square$). All cultures contained glucose as a carbon source.

Table 1. NR activity in cell-free extracts of *Sporobolomyces roseus*

Addition*	NR activity†
NADH	0
NADH + FAD	0
NADPH	1.62
NADPH + FAD	6.46
NADPH + FMN	1.62
BVH	24.85

* BVH, reduced benzyl viologen; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.

† NR activities are expressed as $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

After 3 h these cultures contained a NR activity of $40 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$. During this period the organisms maintained a constant rate of nitrate uptake. When nitrogen-starved cultures received ammonium and nitrate, there was little nitrate uptake [$0.5 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$] although NR activity continued to increase at a rate similar to that which occurred in cultures that did not receive nitrogen.

Fig. 5. Appearance of NR activity in ammonium-grown cultures. (a) Ammonium-grown cultures transferred to nitrogen-free medium (○), 10 mM-nitrate medium (●) or 10 mM-ammonium + 10 mM-nitrate medium (▲). (b) Ammonium-grown cultures transferred to nitrogen-free medium for 3 h before addition (arrow) of 1 mM-nitrate (●), 1 mM-ammonium + 1 mM-nitrate (▲) or nothing (○). Nitrate uptake by cultures that received nitrate only (■) and nitrate + ammonium (□). NR activity was measured in frozen/thawed cells by the terminal NR assay.

Fig. 6. Effect of cycloheximide on nitrate uptake capacity and the development of NR activity in nitrogen-starved cultures. NR activity was measured in an ammonium-grown culture after transfer to nitrogen-free medium (○). After 3 h the culture was divided into two and each received additions (first arrow). One culture received 1 mM-nitrate and NR activity (●) and nitrate uptake (□) were followed. The other culture received cycloheximide (20 µg ml⁻¹) and NR activity was followed for a further 3 h (△). This culture then received 1 mM-nitrate (second arrow) and NR activity (▲) and nitrate uptake (■) were followed. NR was measured in frozen/thawed cells by the terminal NR assay.

In another experiment (Fig. 6) organisms were nitrogen-starved for 3 h, after which one culture received nitrate and the other received cycloheximide. The culture that received nitrate took it up at a rate of 3.2 nmol min⁻¹ (10⁸ cells)⁻¹ and NR activity increased from 9 to 40 nmol min⁻¹ (10⁸ cells)⁻¹. NR activity did not increase in cultures that received cycloheximide. After 3 h with cycloheximide the other culture received nitrate which was taken up immediately at an overall rate of 2.3 nmol min⁻¹ (10⁸ cells)⁻¹, 72% of that by the culture that did

Fig. 7. Effect of nitrogen starvation, carbon starvation and ammonium on NR activity in nitrate-grown cultures. Cultures were transferred to fresh medium containing 10 mM-nitrate (●), 10 mM-ammonium (△), 10 mM-nitrate + 10 mM-ammonium (▲), no nitrogen (○) or 10 mM-nitrate but no glucose (□). NR activity was measured in frozen/thawed cells by the terminal NR assay.

not receive cycloheximide. In contrast, NR activity in the culture with cycloheximide was stimulated little after nitrate addition; the level of NR activity attained was 35% of that in cultures that did not receive cycloheximide.

Fig. 7 shows the effect of nitrogen source and glucose on the maintenance of NR activity in nitrate-grown cultures. At zero-time these cultures contained an NR activity of approximately $10 \text{ nmol min}^{-1} (10^8 \text{ cells})^{-1}$. After 9 h in fresh nitrate medium, NR activity increased by threefold. NR activity also increased in cultures resuspended in nitrogen-free medium but to a much lesser extent. NR activity decreased in cultures resuspended in nitrate medium minus a carbon source. Similarly, NR activity decreased if cultures were resuspended in ammonium medium or medium containing nitrate and ammonium. Decreases in NR activity were similar in these cultures with 50% loss after 4 or 5 h.

DISCUSSION

Ammonium-grown cultures of *S. roseus* did not take up nitrate or contain NR activity. However, the organisms acquired the capacity to assimilate nitrate after a lag period of 50 min in nitrate medium. Nitrate was not essential to 'induce' this capacity since it developed in nitrogen-starved cultures (Fig. 1). This is in contrast to results obtained with filamentous ascomycetes, where the development of the capacity to assimilate nitrate requires induction by nitrate or nitrite (Pateman & Kinghorn, 1976; Garrett & Amy, 1978), but is similar to results obtained with microalgae (Morris & Syrett, 1965; Syrett & Hipkin, 1973; Cresswell & Syrett, 1979) and other species of yeasts such as *Candida utilis*, *C. nitratophila* and *Rhodotorula glutinis* (A. H. Ali & C. R. Hipkin, unpublished results).

In *S. roseus*, development of the capacity to assimilate nitrate during nitrogen starvation required metabolic energy, functional transcription and protein synthesis (Fig. 2) and, once developed, nitrate assimilation was energy-dependent (Fig. 3). One explanation for these results is that a nitrate carrier protein, not present in ammonium cultures, has to be synthesized for nitrate to be assimilated and energy is required for it to function. Unfortunately, we were unable to show an energy-dependent, intracellular accumulation of nitrate against a concentration gradient in *S. roseus* and consequently we have no unequivocal evidence for an active nitrate uptake system like that described for the diatom *Phaeodactylum tricornutum* (Cresswell & Syrett, 1979). An alternative explanation is that nitrate uptake is driven by diffusion along a concentration gradient maintained by the intracellular removal of nitrate by nitrate reduction

(Pateman & Kinghorn, 1976). If this were so, however, one might expect a close coupling between nitrate uptake and nitrate reduction, particularly when nitrate assimilation is perturbed. Two types of perturbation experiments are described here. Firstly, the addition of ammonium to cultures assimilating nitrate resulted in an immediate and almost complete inhibition of nitrate uptake but not a comparable lowering of NR activity (Figs 4 and 5*b*). Ammonium addition did lower the rate of increase in NR activity in such cultures however, and this, presumably, was the result of the inhibition of nitrate uptake. Secondly, the addition of cycloheximide which resulted in a marked inhibition of increases in NR activity resulted in only a small inhibition of nitrate uptake (Fig. 6). Evidently, potential nitrate reduction is not coupled closely with nitrate uptake and, apparently, does not limit nitrate assimilation in *S. roseus*. We suggest that either uptake and reduction are coupled by the supply of reduced pyridine nucleotide which limits actual nitrate reduction *in vivo* and the assimilation of exogenous ammonium makes reducing power unavailable for nitrate reduction, or nitrate uptake and nitrate reduction are two, separate metabolic processes. Pateman & Kinghorn (1976) suggested the possibility of a facilitated diffusion of nitrate coupled with nitrate reduction in fungi. Such a model may encompass most of the alternative mechanisms suggested here. In particular, ammonium inhibition could act at the level of uptake or reduction and nitrite inhibition (Fig. 4*b*) could be explained by competition for binding sites on a translocator.

NR activity in cell-free extracts of *S. roseus* was NADPH-specific and required flavin adenine dinucleotide for maximum activity (Table 1). Thus, this organism resembles filamentous fungi such as *Aspergillus* (MacDonald & Coddington, 1974), *Neurospora* (Pan & Nason, 1978) and *Penicillium* (Renosto *et al.*, 1981) but differs from other yeasts such as *Torulopsis* (Rivas *et al.*, 1973) and *Hansenula* (Zauner & Dellweg, 1983), which contain NR that can utilize electrons from NADH and NADPH. However, NR activity in *S. roseus* differs from that of filamentous fungi and all other yeasts we have studied in being extensively derepressed in nitrogen-starved cultures (Fig. 5). In this respect *S. roseus* resembles some unicellular algae (Syrett & Hipkin, 1973; Hipkin *et al.*, 1983). Studies on the regulation of NR in *S. roseus* (Fig. 7) indicate that it is repressed by ammonium or products of ammonium assimilation. Thus although high NR activities are maintained in nitrogen-free culture, in medium containing ammonium or ammonium and nitrate there is an equal loss of activity; the ammonium effect cannot be simply a result of inducer (nitrate) exclusion. Nitrate also plays a role in the regulation of NR in *S. roseus* since it stimulated increases in NR activity in nitrogen-starved cultures (Figs 5, 6 and 7).

Ammonium repression of NR was also observed when ammonium plus nitrate was added to ammonium-grown cultures (Fig. 5*a*). However, when cultures were nitrogen-starved before addition of ammonium plus nitrate, derepression of NR activity was not prevented (Fig. 5*b*). At present one can only speculate on reasons for this. One possible explanation is that repressor molecules, perhaps glutamine, do not accumulate when ammonium is assimilated rapidly in derepressed, nitrogen-starved cultures. Alternatively, NR activity might continue to increase under these conditions, despite repression, as a result of the assembly of enzyme subunits formed during nitrogen starvation.

Our results indicate that several features of nitrate assimilation in the basidiomycete yeast *Sporobolomyces roseus* distinguish it from other yeasts and filamentous fungi but resemble features described for unicellular algae. This organism is notable for exhibiting a marked derepression of the ability to take up and reduce nitrate after nitrogen starvation.

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