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DEREPRESSION OF NITRATE REDUCTASE IN THE PRESENCE OF EXCESS AMMONIUM IN A UNICELLULAR ALGA GROWING UNDER CONDITIONS OF PHOSPHATE LIMITATION

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Chemostat cultures of the unicellular alga Cyanidium caldarium have shown that under conditions of phosphate limitation nitrate reductase is completely derepressed even in cells growing in a large excess of ammonium, but that it occurs mainly in a catalytically inactive form. It is hypothesized that phosphate limitation contributes to maintaining intracellular level of glutamine suitable to stimulate inactivation but not repression of nitrate reductase. It is not excluded that in addition to variations in the intracellular level of glutamine, there are other metabolic events of the cell by which repression and inactivation of nitrate reductase could be differently influenced.

It is well established that nitrate reductase of fungi and algae is fully repressed in media containing ammonium (1-4); however, as demonstrated in Anacystis nidulans, nitrate reductase can be freed from ammonium repression in cell systems where the assimilation of ammonium is prevented. Thus, addition of methionine sulphoximine, a powerful inhibitor of glutamine synthetase, to cells of A. nidulans resuspended in the presence of ammonium, brings about derepression of nitrate reductase (4). Like nitrate reductase, nitrogenase also is repressed by ammonium; methionine sulphoximine and methionine sulphone, which inhibit the assimilation of ammonium, cause nitrogenase to be synthesized in the presence of excess ammonium, as observed in the bacteria Azotobacter and Klebsiella (5). Similar observations were made in the cyanobacterium Anabaena cylindrica where even in the presence of ammonium methionine sulphoximine causes not is only nitrogenase synthesis derepressed, but also heterocystis are formed (6).

In the red alga Cyanidium caldarium, as in other algae, nitrate reductase is fully repressed by ammonium; the enzyme, however, is derepressed in continuous cultures where ammonium represents a growth rate-limiting substrate (7).

In the present paper it will be shown that under conditions of phosphate limitation which severely restrict growth, nitrate reductase is fully derepressed even in the presence of a large excess of ammonium.

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Material and Methods

Cyanidium caldarium, strain 0206, was supplied by Prof. T. D. Brock, Wisconsin University. The alga was grown autotrophically at pH 1.9 and at 42°C in continuous light. The composition of the medium, except for phosphate, was as previously reported (8). The alga was grown in chemostat under conditions of phosphate limitation. Continuous cultures were performed in 2 l cylindrical flasks thermostated at 42°C. The flasks were filled with culture medium containing phosphate 0.033 mM, and then inoculated with a few cells. When the maximum growth allowed by the amount of phosphate present was reached fresh medium with the same phosphate concentration was added to the chemostat culture at a dilution rate of 0.25 day^{-1} with the aid of a peristaltic pump, and concomitantly the same amount of culture was removed from the culture vessel. The cultures were magnetically stirred and constantly insufflated with air containing 5 % CO_2. Routine samples were tested for cell density by centrifugation of a definite aliquot of culture in a hematocrit test tube. Throughout the experiments the culture exibited a constant cell density of 2 μl cell/ml culture; no residual phosphate was ever detected in the supernatant medium, which means that this substrate, which was added together with the fresh medium, was immediately and completely utilized by the cells, and that it was a growth rate-limiting nutrient.

To obtain crude extract, the cells, collected by low speed centrifugation and resuspended in phosphate buffer 3 mM, pH 7, were passed through a French-pressure cell, and then centrifuged at 27,000 x g for 20 min. Nitrate reductase was assayed at 30°C in phosphate buffer pH 7.5, 0.05 M, by colorimetric estimation of nitrite formed using dithionite reduced benzyl viologen as electron donor (8). Specific activity is expressed as nmol nitrite produced per min per mg protein. Glutamine synthetase activity was tested by γ-glutamyl hydroxamate transferase assay (9): the specific activity is expressed as μmol hydroxamate produced per min per mg protein. Protein was estimated by the method of Lowry et al. (10).

Results

Specific activity levels of nitrate reductase and glutamine synthetase in cells growing under conditions of phosphate limitation.

Nitrate reductase from C. caldarium can occur in crude extract in two different, well-defined forms, depending on the culture conditions of the cell. Thus nitrate reductase is found in a catalytically active form in cells growing under growth-limiting nitrogen conditions, or up to 60 % of the enzyme can be found in a catalytically inactive form in cells growing under conditions of excess nitrate (11). The inactive enzyme can be activated in vitro by a variety of biochemical treatments (12), one of which consists in heating the crude extract at 55°C for 3 min, then assaying at 30°C. This heat treatment does not effect the benzyl viologen activity of the active enzyme, but it brings about a complete activation of the inactive enzyme which can then be estimated. By measuring nitrate reductase activity comparatively in untreated and in heat-treated extract it is possible to evaluate the per cent of nitrate reductase that occurs in the inactive form. Thus, in the present experiments, the activity of nitrate reductase was assayed both in untreated and in heat-treated extracts.

As shown in Table 1, cells grown in chemostat under conditions of phosphate limitation possess high levels of nitrate reductase activity both in cultures containing a large excess

TABLE 1
Nitrate reductase and glutamine synthetase activities in cells of Cyanidium caldarium grown under different nitrogen and phosphate conditions

Conditions of growth	Specific activity			per cent inactive NR (7)
	GS (4)	NR (5)		
		untreated enzyme	enzyme heated at 55°C 3 min (6)	
NH_4^+ limited plus excess phosphate (1)	2.7	52	61	15
NH_4^+ excess plus phosphate limited (1)	1.2	21	52	59
NO_3^- excess plus phosphate limited (1)	1.0	18	53	66
NH_4^+ excess plus phosphate excess (2)	0.3	2	6.7	70
NO_3^- excess plus phosphate excess (3)	1.2	31	80	61

(1) Phosphate- or ammonium-limited cultures performed in chemostat where the influx medium was supplied at a dilution rate of 0.25 day^{-1}
(2) Chemostat cultures containing all nutrients, including phosphate and ammonium, in excess, and supplied with a fresh medium of the same composition at a dilution rate of 0.25 day^{-1}.
(3) Batch cultures which contained all the nutrients, including phosphate and nitrate, in excess.
(4) Glutamine synthetase: µmol hydroxamate formed per min per mg protein.
(5) Nitrate reductase, benzyl viologen activity: nmol nitrite formed per min per mg protein.
(6) Heating activates the inactive nitrate reductase which can then be estimated.
(7) The per cent of inactive nitrate reductase was estimated from values of nitrate reductase activity found in untreated and in heat-treated extract. The values reported are the average of 20 independent determinations for each growth condition.

of nitrate (20 mM) and in cultures containing a large excess of ammonium (20 mM), as found in heat-treated extracts where all the enzyme present (active plus inactive) is measured. A similar level of activity (about 50 nmol nitrite produced per min per mg protein) was measured in both types of cultures. Comparison of these values with those found in untreated extracts, shows that more than 60 % of nitrate reductase occurred in the inactive form, both in cells growing in nitrate medium and in cells growing in ammonium medium. Glutamine synthetase activity in both types of cells was about 1.2 µmol hydroxamate formed per min per mg protein.

Table 1 also gives values of nitrate reductase and glutamine synthetase activities found in cells growing in chemostat under conditions of ammonium limitation (phosphate in excess), and in cells growing in batch cultures with all the nutrients in large excess and with nitrate

TABLE 2
Variation of nitrate reductase and glutamine synthetase activity levels in cells of C. caldarium grown in chemostat and resuspended in N-free medium or in nitrate-medium

Conditions of incubation of the cells	Specific activity			per cent inactive NR
	GS	NR		
		untreated enzyme	enzyme heated at 55°C 3 min	
Starting cells	1.8	7.5	42	82
1 h incubation in nitrate medium	1.3	18	50	64
1 h incubation in N-free medium	1.5	62	63	0

Cells grown in chemostat under conditions of phosphate limitation with nitrate as the sole nitrogen source (starting cells), were collected, washed and resuspended in a nitrogen-free medium or in a medium containing nitrate (20 mM). Both media contained 0.033 mM phosphate. After 1 h incubation in the same conditions as for growth, the cells were collected and the cell-free extracts tested for nitrate reductase and glutamine synthetase activities.
Specific activities as in Table 1.

as the sole nitrogen source. As can be seen, similar levels of nitrate reductase and glutamine synthetase activities occurred in both types of cells, with the difference, however, that under conditions of excess nitrate, nitrate reductase was up to 60 % inactive, whereas in ammonium-limited cultures it occurred mainly in the fully active form.

In continuous cultures where all nutrients (including ammonium and phosphate) were supplied in excess, nitrate reductase, either the catalytically active or inactive form, was very low or undetectable.

In vivo activation of inactive nitrate reductase upon nitrogen removal.

Cells of C. caldarium grown in chemostat under conditions of phosphate limitation and excess nitrate, whose nitrate reductase was up to 82 % inactive, were collected, washed, resuspended in a nitrogen-free medium or into a nitrate medium (control) both containing 0.033 mM phosphate, and kept under the same conditions as for growth. As can be seen from Table 2, after 1 h incubation the level of glutamine synthetase activity had hardly changed; nitrate reductase activity showed a 20-50 % increases; however the enzyme in the cells resuspended into the nitrate medium was still 64 % inactive, but it was in the totally active form in the cells resuspended in the nitrogen-free medium.

Discussion

The presence of ammonium, an intermediate product of nitrate assimilation, affects the metabolism of nitrate by producing a severe inhibition of nitrate utilization by the cell. As reported previously, in C. caldarium one of the enzymes of the pathway of nitrate

utilization that is profoundly affected by the presence of ammonium is nitrate reductase. In C. caldarium, besides being repressed in the presence of ammonium, nitrate reductase is also rapidly transformed into an inactive species upon the addition of ammonium to cells where the enzyme is derepressed (13). It has been suggested that in C. caldarium, in addition to repression, this inactivation of nitrate reductase also represents a fundamental control mechanism of nitrate utilization (14).

Several reports indicate that the true co-repressor of nitrate reductase is not ammonium per se, but glutamine which derives from ammonium assimilation (15). Glutamine has been found to be involved in the regulation of nitrogenase activity of photosynthetic bacteria (16). Moreover glutamine is the principal effector in regulating glutamine synthetase (17).

Our findings show that under conditions of phosphate limitation nitrate reductase is fully derepressed even in the presence of a large excess of ammonium, with levels of activity similar to those found in the presence of nitrate alone. In the light of the above mentioned reports (15), it may be supposed that under conditions of phosphate limitation which are growth-restricting, the metabolism of ammonium is so severely reduced that. even in the presence of a large excess of ammonium, the internal concentration of glutamine is too low to promote repression of nitrate reductase. An alternative situation may be imagined whereby the actual signal that triggers nitrate reductase repression is not only a suitable level of intracellular glutamine alone, but also a supplementary signal which could arise from an appropriate phosphorous metabolism.

Although the present data are not sufficient to rule out the second hypothesis, the first hypothesis seems to be more plausible because nitrate reductase can be synthesized also when a substrate other than phosphate, for instance ammonium, is the growth rate-limiting nutrient.

Under conditions of phosphate limitation also glutamine synthetase activity is high, which is further support for a low internal level of glutamine; on the other hand this suggests that even if phosphate limitation does not affect the synthesis of glutamine synthetase, it does affect in some way its functionality. Thus, conditions of phosphate limitation may give valuable information about regulation of glutamine synthetase.

Nitrate reductase under conditions of phosphate limitation, however, occurs mainly in the inactive form (up to 66 % or more of the enzyme present was found to be inactive). Therefore it is apparent that under these phosphorous conditions, while the signal for nitrate reductase repression is absent, the signal for nitrate reductase inactivation is still present. A series of observations suggest that glutamine also promotes the mechanism (so far unknown) which brings about inactivation of nitrate reductase (14, 18).

Thus, if glutamine is the actual effector of both repression and inactivation of nitrate reductase, it means that under conditions of phosphate limitation the internal concentration of glutamine is such as to promote inactivation, but not repression, which may reflect the situation existing in cells growing in excess of all nutrients with nitrate as the sole nitrogen source. On the other hand, it can be suggested that in addition to the inactivation signal represented by the internal level of glutamine, there is also a distinct signal for nitrate reductase activation which might be absent from cells maintained under conditions of phosphate limitation. However this second hypothesis can be refuted on the grounds that the removal of nitrogen brings about the reactivation of inactive nitrate reductase. Finally, it cannot be excluded that in addition to intracellular levels of glutamine, there are other metabolic events of the cell by which repression and inactivation of nitrate reductase could be differently influenced.

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