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MOLYBDENUM AND IRON AS CONSTITUENTS OF THE ENZYMES OF THE NITRATE REDUCING SYSTEM FROM *CHLORELLA*

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Abstract—By adding radioactive ^{99}Mo (as molybdate) and ^{59}Fe (as ferrous ion) to a culture of *Chlorella* cells at the moment derepression of the enzymes of the nitrate reducing system was initiated as a consequence of the removal of ammonia from the medium, it could be unequivocally shown that the two metals were incorporated into nitrate reductase and nitrite reductase respectively, remaining associated with the enzymes during purification. After a mild heat treatment of nitrate reductase, exogenous molybdate could be made to interact with the enzyme and to function as electron donor after its chemical reduction with hydrosulfite.

INTRODUCTION

THE ASSIMILATORY nitrate reducing systems from the alga *Chlorella* and from the photosynthetic tissues of higher plants are quite similar. In all cases, the reduction of nitrate to ammonia proceeds in two independent and well characterized enzymatic steps: (1) the reduction of nitrate to nitrite, involving two electrons and catalyzed by the FAD-dependent NADH-nitrate reductase (molecular weight, about 500,000), and (2) the reduction of nitrite to ammonia, involving six electrons and catalyzed by ferredoxin-nitrite reductase (mol. wt. 63,000). In the transfer of electrons from NADH to nitrate, two enzymatic activities participate sequentially, which can be easily and independently assayed although not physically separated: the first, a NADH-diaphorase, and the second, a nitrate reductase proper, or terminal nitrate reductase, which can use reduced flavin nucleotides as electron donor.[1–5]

In *Chlorella*, all the enzymes of the nitrate reducing system are repressed by ammonium ions, but only the second activity of NADH-nitrate reductase, namely nitrate reductase proper, is subjected to a peculiar reversible inactivation promoted *in vivo* by ammonium ion.[6] This second activity, as well as that of nitrite reductase, are inhibited by the addition of cyanide.

It has been firmly established that molybdenum is an essential trace element for algae and higher plants in the process of nitrate assimilation and that the synthesis of nitrate reductase

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depends upon the presence of the metal *in vivo*.[7–12] Notwithstanding, and although molybdenum was early identified by Nicholas and Nason[13] and by Evans and Hall[14] as the metal prosthetic group of nitrate reductase from soybean leaves[15] and was later assumed to be a component of the enzyme from wheat leaves,[16] most workers in the field have so far been unable to find evidence for either its presence or its function as electron carrier in nitrate reductase preparations (sometimes highly purified) from a variety of plants.[3,4,12,16–18] It was also apparent that the enzyme from soybean leaves, in comparison with nitrate reductases from algae and photosynthetic tissues of most higher plants, is rather peculiar, not only with respect to its metal and flavin behaviour, but also to its pyridine nucleotide specificity, NADH and NADPH being equally effective as electron donors.[19]

With respect to the participation of metals in the assimilatory reduction of nitrite by plants, the evidence is rather doubtful.[20] Since nitrite reductase from vegetable marrow,[21] spinach[22,23] and *Chlorella*[1] is inhibited by cyanide (but, by contrast with nitrate reductase, not by azide) and by other more specific iron inhibitors,[24] Hucklesby *et al.* have suggested that the enzyme may be an iron-containing protein.[24]

The purpose of this paper is to report that by adding radioactive ^{99}Mo and ^{59}Fe to *Chlorella* cells at the moment derepression of the enzymes of the nitrate reducing system is initiated by removal of ammonia from the culture medium,[6] it has been possible to demonstrate that molybdenum and iron are respectively associated with the enzymes nitrate reductase and nitrite reductase. Evidence is also presented showing that, under certain conditions, chemically reduced added molybdate can function as electron donor for the enzymatic reduction of nitrate. Preliminary reports of this work have been made previously.[25,26]

RESULTS AND DISCUSSION

Association of Mo with Nitrate Reductase

Table 1 summarizes the nitrate reductase activities and ^{99}Mo radioactivities of different fractions obtained during purification of the enzyme from *Chlorella* cells which had pre-

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TABLE 1. ASSOCIATION OF ^{99}Mo WITH *Chlorella* NITRATE REDUCTASE DURING PURIFICATION

Fraction	Volume (ml)	Total protein (mg)	Total activity (milli-units)	Recovery of activity (%)	Specific activity (milli-units/mg protein)	Purification factor	Total radio-activity (count/min × 10^{-3})	Recovery of radio-activity (%)	Radio-activity / Enzyme activity
I. Crude extract	127	555	2540	100	4·6	1	4066·5	100	1622
II. Calcium phosphate gel eluate	46	217	2920	115	13·5	3	1130·0	28	386
III. Alumina Cγ eluate	9·6	43	2100	82	49	11	102·1	2·5	48
IV. Pooled peak from gel filtration on agarose	13·5	8·2	930	37	113	25	30·3	0·7	32
V. Pooled peak from gel filtration on agarose after association of the enzyme with blue dextran	11·2	1·2	480	19	405	98	8·8	0·2	18

PHYTO 10/7—F

viously incorporated radio-molybdenum under the conditions described in Experimental. It can be seen that there was no apparent correlation between enzyme activity and radioactivity during purification. The final recovery of activity was about 20% whereas the corresponding recovery of radioactivity was hundred times less, i.e. 0·2%. It seems, therefore, that no more than 1% of the total radiomolybdenum was originally associated with nitrate reductase. These figures refer to the crude extract but rather similar values result also if percentages are calculated with respect to the enzyme preparation obtained after the calcium phosphate gel treatment (Fraction II).

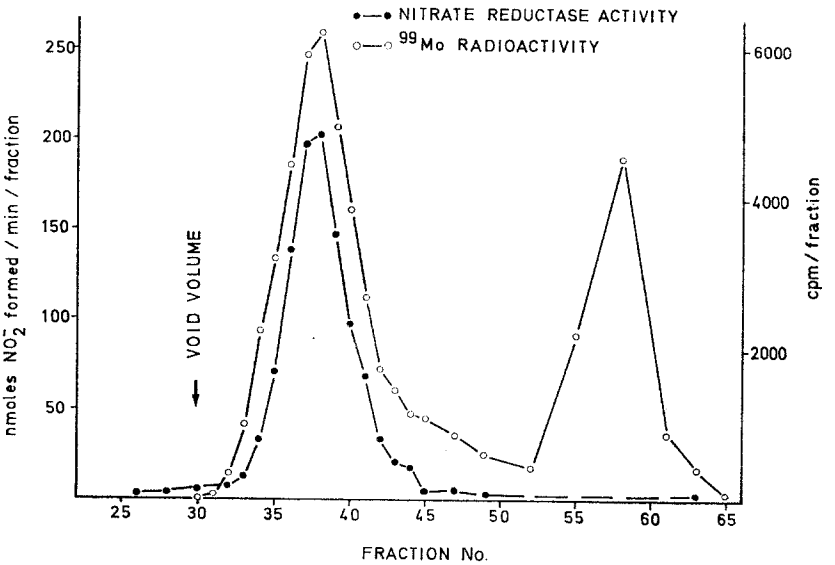

FIG. 1.

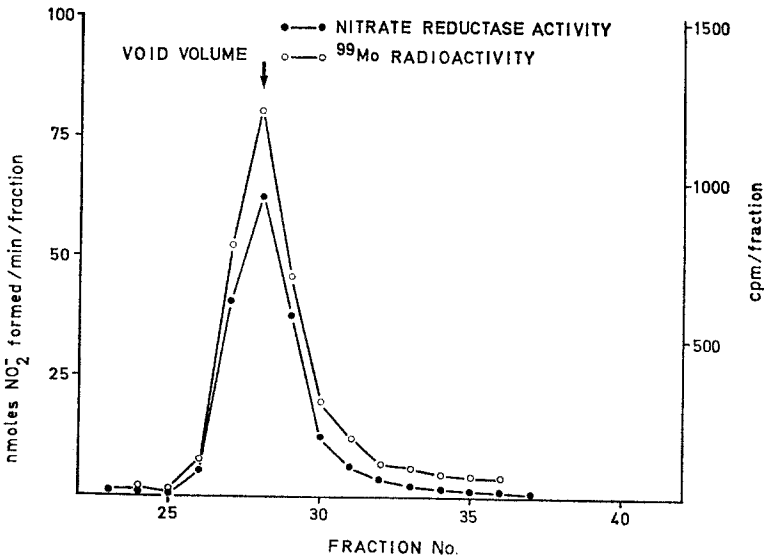

FIG. 2.

It could, however, be unequivocally demonstrated during purification by gel filtration on agarose (Fractions IV and V) that nitrate reductase activity and ^{99}Mo radioactivity were closely interrelated. As can be seen in Fig. 1, a constant ratio of enzyme activity and ^{99}Mo radioactivity with coincidence in peaks was exhibited by the purified fractions which emerged just after the void volume from the agarose column. Figure 2 shows that a similar parallelism between nitrate reductase activity and ^{99}Mo radioactivity became again obvious when the enzyme was further purified by agarose gel filtration after its association with blue dextran (mol. wt. 2×10^6) and came out in the void volume. In contrast with nitrate reductase, nitrite reductase did not appreciably incorporate molybdenum.

Dithionite-reduced Molybdate as Electron Donor for the Enzymatic Reduction of Nitrate

Suggestive evidence concerning the functional role of molybdenum as an electron carrier in the reaction catalyzed by *Chlorella* nitrate reductase was obtained after mildly heating the enzyme at 45° for 5 min, a treatment which although it destroys the NADH diaphorase activity of NADH-nitrate reductase does not affect the activity of nitrate reductase proper as assayed with reduced FMN as electron donor.[3,4] Apparently, the heat treatment promoted the interaction between the enzyme and exogenous molybdate, thus rendering possible the function of the latter as electron donor in the reduced state. By contrast, in soybean nitrate reductase, exogenous reduced molybdate could act as electron donor without any previous treatment of the enzyme.[13]

Association of Fe with Nitrite Reductase

When the experiments just mentioned were repeated with ^{59}Fe instead of ^{99}Mo no clear-cut evidence for the association of iron with nitrate reductase was found. Thus, whereas a certain correspondence seemed to exist between nitrate reductase activity and ^{59}Fe radioactivity in the fractions obtained by gel filtration on agarose (Fig. 3), further purification of

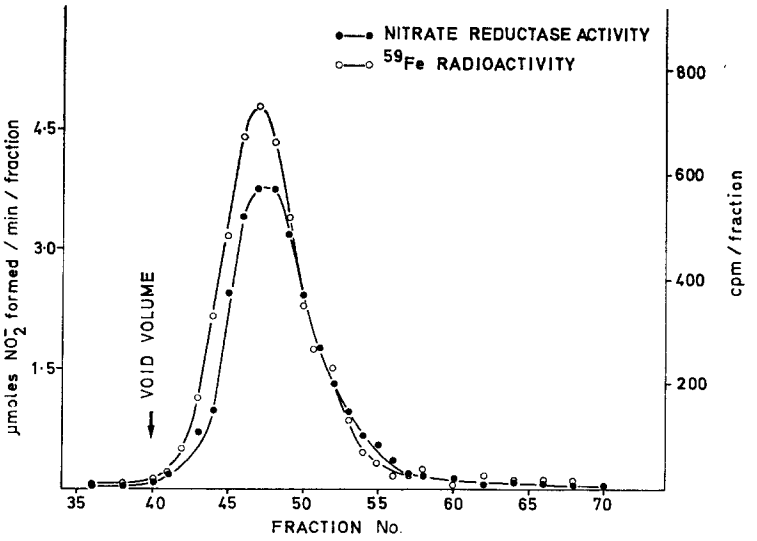

FIG. 3.

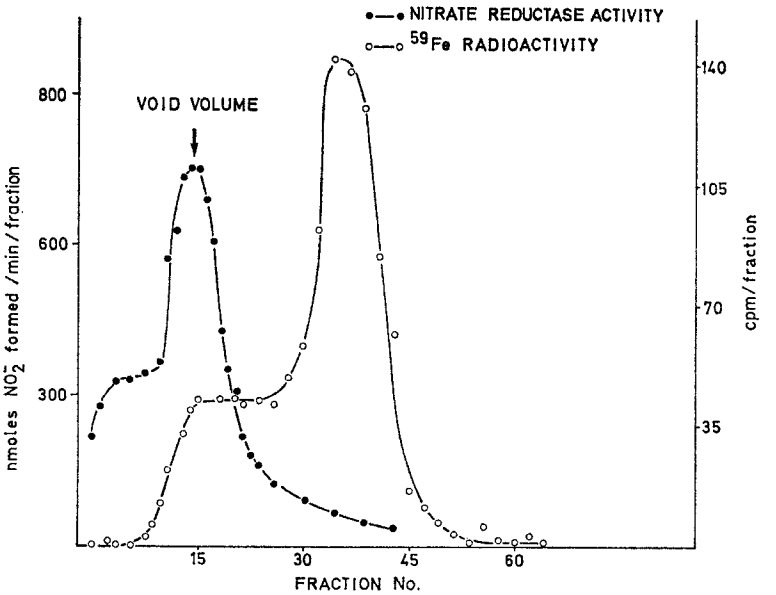

FIG. 4.

the enzyme by the same technique after its association with blue dextran rather indicated that nitrate reductase activity and ^{59}Fe radioactivity were mostly unrelated (Fig. 4).

In contrast with nitrate reductase, the evidence for iron being a constituent of nitrite reductase was well defined. As can be seen in Fig. 5, the fractions obtained by gel filtration with Sephadex G-100 exhibited a close correspondence between nitrite reductase activity and ^{59}Fe radioactivity. On further purification of the enzyme by electrophoresis on polyacrylamide, the parallelism between nitrite reductase activity and ^{59}Fe radioactivity became

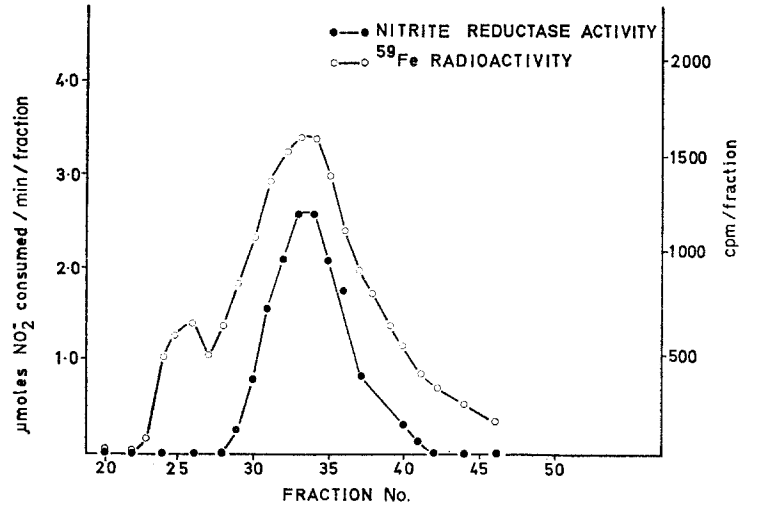

FIG. 5.

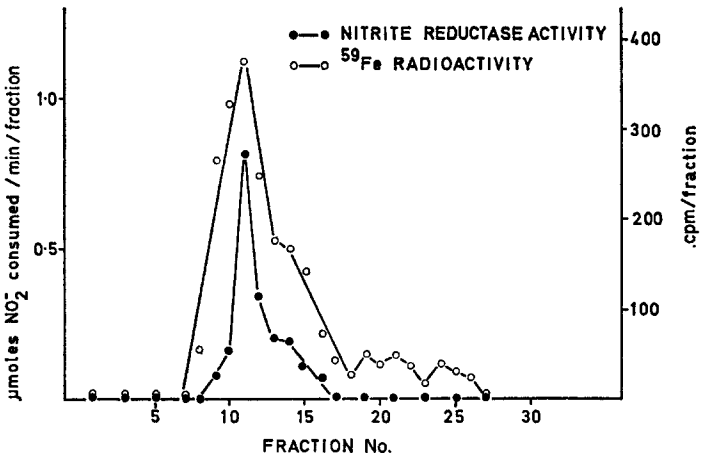

FIG. 6.

even more striking (Fig. 6). These results are in agreement with those previously obtained for nitrite reductase from spinach by colorimetric analysis and atomic absorption indicating that the enzyme contains two atoms of iron per molecule of protein.[26]

TABLE 2. MOLYBDATE REDUCED WITH DITHIONITE AS ELECTRON DONOR FOR NITRATE REDUCTASE FROM *Chlorella*

Addition	NO_2^- formed (nmoles)
None	12
$MoO_4^=$	91
FMN	250

70 µg of nitrate reductase (alumina Cγ eluate) in 0·7 ml of 142 mM potasium phosphate, pH 7·5, were heated at 45° for 5 min. Where indicated, 0·5 µmoles of sodium molybdate or 0·2 µmoles of FMN were added. Other experimental conditions as in the standard assay.

EXPERIMENTAL

Culture of the Alga and Feeding of Labelled Metals

Chlorella fusca Shihira et Kraus (=*pyrenoidosa*) 211–15 from Pringsheim's collection at Göttingen was grown on KNO_3 as described previously,[1] using 5 and 15 l. of culture medium for the Mo and Fe experiments respectively. At the logarithmic phase, the cells were harvested, washed ×2 and transferred into the same original volume of a medium which contained, instead of nitrate, an equivalent amount of N in the form of $(NH_4)_2SO_4$, in order to repress the enzymes of the nitrate reducing system. In the Mo experiments, the metal was excluded from the culture during repression. After 18 hr in the NH_3 medium, the cells were again harvested, washed twice and transferred into the initial nitrate medium, which included now either 2 mc of ^{99}Mo (as molybdate) or 0·7 mc of ^{59}Fe (as ferrous ion). Derepression was subsequently carried out for 4 hr, in order to allow incorporation of the radioactive metals into the *de novo* synthesized enzymes.

Preparation of Cell-free Extracts

About 10 g (Mo experiments) or 30 g (Fe experiments) of alga cells were collected, washed ×2 and broken either by grinding in a mortar with alumina or by homogenizing in a Bühler vibration homogenator with glass beads of 0·3 mm dia. The disrupted cell material was suspended in 5 mM Tris–HCl buffer, pH 7·5, in the proportion of 10 ml/g. fr. wt. of cells, and centrifuged at 20,000 *g* for 30 min. The sediment was discarded and streptomycin sulfate was added to the crude extract up to a final concentration of 7 mM. After allowing to stand for 10 min with occasional stirring, the suspension was centrifuged at 20,000 *g* for 10 min and the resulting supernatant used as starting material for the purification of nitrate reductase and nitrite reductase.

Purification of Nitrate Reductase

The supernatant was treated with calcium phosphate gel in the proportion of 1 mg/mg of protein. The suspension was allowed to stand for 10 min with occasional stirring and then centrifuged at 4000 *g* for 5 min. The pellet was washed by suspending it in 25 mM $NaHPO_4$, pH 7·5, and immediately centrifuged as before. In order to eluate the enzyme adsorbed by the gel, the pellet was thoroughly resuspended in 100 mM $Na_4P_2O_7$, pH 7·0, and allowed to stand for 10 min with occasional mixing. The suspension was centrifuged at 27,000 *g* for 10 min and the sediment discarded. To the calcium phosphate gel eluate (Fraction II), a 2% solution of protamine sulfate adjusted to pH 4·0 with NaOH was added to bring the solution to a final concentration of 0·5%. After 10 min, the suspension was centrifuged at 27,000 *g* for 10 min and the supernatant, which contained the nitrate reductase, was treated with $(NH_4)_2SO_4$ at 40% saturation. The suspension was allowed to stand for 10 min and centrifuged at 27,000 *g* for 10 min. The protein precipitate, which contained the nitrate reductase, was dissolved in 5 mM Tris–HCl buffer, pH 7·5, and treated with alumina Cγ (2 mg per mg of protein). The suspension was allowed to stand for 10 min and then centrifuged at 4000 *g* for 5 min. The pellet was washed by suspending it in 5 mM sodium phosphate, pH 7·5, and immediately centrifuged as before. In order to eluate the enzyme from the gel, the pellet was thoroughly resuspended in 100 mM sodium pyrophosphate, pH 7·0, and, after 10 min, the suspension was centrifuged at 27,000 *g* for 10 min and the sediment discarded. The alumina Cγ eluate (Fraction III) was treated with $(NH_4)_2SO_4$ at 45% saturation and the protein precipitate obtained was dissolved in 2 ml of 50 mM Tris–HCl buffer, pH 7·5, 200 mM NaCl. The concentrated enzyme solution was then applied to a 2·5 × 30 cm agarose column (Bio-Gel A-1·5 m, 100–200 mesh), previously equilibrated with the same Tris–NaCl solution. 2 ml fractions were collected and aliquots were assayed for nitrate reductase activity and for ^{99}Mo or ^{59}Fe radioactivity. The most active fractions were pooled (Fraction IV) and treated with $(NH_4)_2SO_4$ at 45% saturation and the protein precipitate obtained was redissolved in 1 ml of either a 0·2% solution of blue dextran in 100 mM sodium pyrophosphate, pH 7·0, (Mo experiments) or a saturated solution of the dye in the same buffer (Fe experiments). The concentrated enzyme-blue dextran preparation was desalted by passing through a Sephadex G-25 column equilibrated with the same pyrophosphate buffer and was again applied to an agarose column (2·5 × 30 cm and 2·5 × 60 cm, for the Mo and Fe experiments respectively). 2 ml fractions were collected and, after estimation of their enzyme-activity and radioactivity, the most active ones were pooled (Fraction V).

Purification of Nitrite Reductase

The supernatant obtained after the streptomycin sulfate treatment was passed through a DEAE-cellulose bed (3 × 7 cm), previously equilibrated with 50 mM Tris–HCl buffer, pH 8·0, in order to adsorb ferredoxin. Nitrite reductase was eluted with 50 mM Tris–HCl buffer, pH 8·0, 200 mM NaCl. The eluate was treated with $(NH_4)_2SO_4$ at 40% saturation and, after 10 min, the suspension was centrifuged at 27,000 *g* for 10 min. $(NH_4)_2SO_4$ was then added to the resulting supernatant up to 70% saturation. The protein precipitate between 40% and 70% $(NH_4)_2SO_4$ saturation, which contained nitrite reductase, was dissolved in 2 ml of 10 mM Tris–HCl buffer, pH 8·0, 100 mM NaCl and applied to a Sephadex G-100 column (1·5 × 75 cm) previously equilibrated with the same Tris–NaCl solution. 1·6 ml fractions were collected and, after analysis of enzyme activity and radioactivity, the most active ones were pooled and concentrated by treatment with $(NH_4)_2SO_4$ at 70% saturation and subsequently the precipitate was dissolved in 1 ml of the Tris–NaCl solution. The enzyme was further purified in the Shandon preparative polyacrylamide electrophoresis apparatus using a 7·5% acrylamide gel column of 6 cm at pH 9·5. 2 ml fractions were finally collected and analyzed for enzyme activity and radioactivity.

Measurement of Enzyme Activities

Nitrate reductase and nitrite reductase were assayed as previously described[23,27] using, respectively, chemically reduced FMN or methyl viologen as electron donor.

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Analytical Methods

Protein was determined by the method of Lowry *et al.*[28] The radioactivity of ^{99}Mo or ^{59}Fe was automatically estimated, after evaporating the samples to dryness on aluminium planchets, with a continuous gas flow counter, Nuclear Chicago Mod. 4342.

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