ROLE OF MOLYBDENUM AS A CONSTITUENT OF NITRATE REDUCTASE FROM SOYBEAN LEAVES[1,2]

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It has already been shown that the nitrate reductase of soybean leaves which catalyzes the reduction of nitrate to nitrite by reduced diphospho- or triphosphopyridine nucleotide (DPNH or TPNH) is a metallo-flavoprotein with flavin adenine dinucleotide (FAD) as the prosthetic group (1). The metal component of a similar, but TPNH-specific, nitrate reductase from Neurospora (2) has recently been identified as molybdenum (3, 4). Molybdate as well as FAD or the mononucleotide (FMN) were shown to function as electron carriers in the enzymatic transfer of electrons from TPNH to nitrate to yield nitrite (5, 6).

The purpose of this paper is to present similar evidence identifying molybdenum as the metal constituent of nitrate reductase from soybean leaves, and to show that during the enzymatic transfer of electrons from DPNH to nitrate, both flavin and molybdenum function as carriers in the following sequence:

$$\text{TPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

MATERIALS AND METHODS

The crude enzyme extract was obtained from the leaves of 10-day to 5-week-old soybean plants grown in flats containing vermiculite and supplied daily with a complete nutrient solution as previously described (1). The enzyme extract was prepared essentially as described (1) except that the alumina powder was omitted and cysteine and versene were added to the phosphate buffer, each at final concentrations of 10^{-4} M. The clear green supernatant solution resulting from high speed centrifugation was fractionated with calcium phosphate gel and ammonium sulfate in the usual way (1); and the second calcium phosphate gel adsorption eluate was used for most of these studies.

The preparation of the various cofactors, substrates and other materials used in this investigation has already been indicated (1). Reduced flavin ($FADH_2$ or $FMNH_2$) was prepared (6) in Thunberg tubes with hydrosulfite followed by hydrogen gas bubbling and evacuation.

Molybdenum-free nitrate reductase was prepared by successive dialysis against cyanide- and molybdenum-free phosphate-glutathione solutions as described elsewhere (4) except that shorter dialysis times were used. In view of the almost complete loss of activity upon dialysis for 6 or more hours, the soybean enzyme was dialyzed against 10^{-3} M cyanide solution and subsequently molybdenum-free phosphate and glutathione solution for 3 and 2 hours, respectively.

For molybdenum assay, cell-free extracts and the various protein fractions were digested with distilled HNO_3; and molybdenum was determined in suitable aliquots of the cooled and diluted digests by the *Aspergillus niger* assay (4).

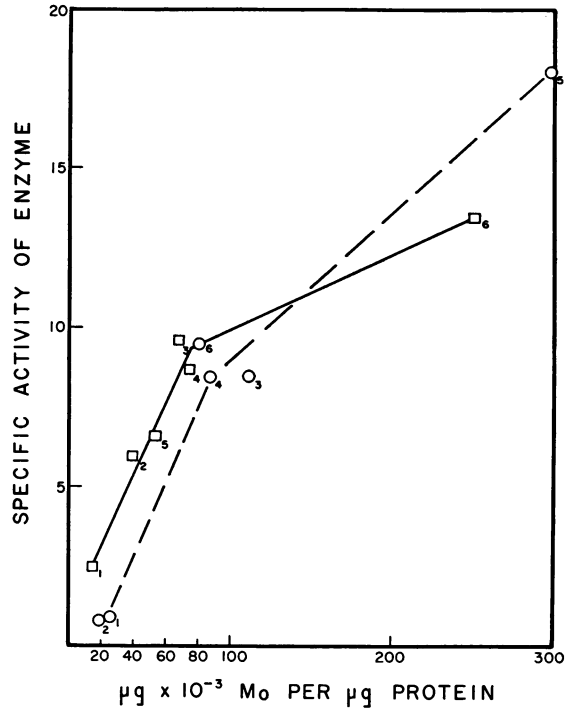

FIG. 1. Relation between molybdenum content and specific activity of nitrate reductase. Circles (○) and squares (□) represent the various protein fractions of two independent fractionations. Subscript 1 is the crude extract, O_2 is the 0 to 40% ammonium sulfate precipitate of the supernatant solution after the first calcium phosphate gel treatment; $\square_2$, $\square_3$ are the first and second eluates, respectively, of the first calcium phosphate gel; O_3 is the eluate of the first calcium phosphate gel; subscript 4 is the 0 to 40% ammonium sulfate supernatant solution of the first calcium phosphate gel eluate; subscript 5 is the 0 to 40% ammonium sulfate precipitate of the first calcium phosphate gel eluate; subscript 6 is the eluate of the second calcium phosphate gel.

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TABLE I

EFFECT OF METALS ON REACTIVATION OF CYANIDE-DIALYZED NITRATE REDUCTASE

TREATMENT	UNITS OF ENZYME ACTIVITY/0.2 ML ENZYME	
	EXPERIMENT I	EXPERIMENT II
I—Undialyzed enzyme ...	7.3	7.1
II—Single dialysis *	6.0	5.2
III—II + cyanide (10^{-3} M)	0	1.6
IV—Successive dialysis **	0	1.0
Added metals		
MoO_3	...	6.2
Na_2MoO_4	...	5.8
$FeCl_3$	...	1.2
$FeSO_4$	...	1.3
$ZnSO_4$	...	1.0
$MnSO_4$	...	1.0
$CoSO_4$	...	1.3
$NiCl_2$	...	1.2
AgCl	...	1.0
$NaWO_4$	...	1.5
$Na_2Cr_2O_7$	...	1.1
Na_3VO_4	...	1.5
$VO_2(NO_3)_2$	...	1.5
$Na_2B_2O_4$	...	1.0

Enzyme assay as described under "Materials and Methods."

* Dialyzed for 3 hrs against 0.1 M phosphate + 10^{-3} M glutathione. All reagents were freed from Mo as indicated (4).

** As for treatment III followed by redialysis for 2 hrs as in treatment II. 0.2 ml enzyme contained 107 μg protein. 1 μg of each metal added to 0.2 ml of enzyme and incubated for 10 min at room temperature before enzyme assay.

Enzyme activity was measured by the colorimetric determination for nitrite as detailed earlier (1). A period of 20 minutes, unless otherwise stated, was generally used for the enzymatic reaction time. Specific activity is expressed as units or 10^{-9} moles of nitrite formed per mg protein. Protein was determined by the Folin method of Lowry et al (7).

RESULTS

Figure 1 shows that there is a direct relationship between specific activity and the molybdenum content per mg protein of the various enzyme fractions. The data represent the results of two independent experiments. The highest activity occurs in the second calcium phosphate gel eluate which also has the greatest molybdenum content. A semiquantitative colorimetric determination for Zn, Cu, Pb, Ni, Co, Hg, Cd, Ga, and Bi, by extraction with dithizone failed to show any consistent relationship between these metals collectively and specific activity.

As shown in table I, a partially purified fraction of nitrate reductase was freed of its metal component by successive dialyses against cyanide- and molybdenum-free phosphate-glutathione solutions for 3 and 2 hours, respectively. The addition of molybdenum trioxide or sodium molybdate reactivated the enzyme to 85 % of the original value. Preincubation in place of molybdenum with other trace metals including Fe^{+++} and Fe^{++}, Zn, Mn, Co, Ni, Ag, W, chromate, V and B, respectively, did not restore the activity of nitrate reductase (table I).

TABLE II

$FMNH_2$ AS AN ELECTRON DONOR FOR THE ENZYMATIC REDUCTION OF NITRATE

REACTION MIXTURES	DPNH FMN KNO_3 ENZYME	KNO_3 − DPNH + $FMNH_2$* ENZYME	KNO_3 − DPNH + $FMNH_2$ MO-FREE ENZYME **	KNO_3 − DPNH + $FMNH_2$ BOILED ENZYME †
mμM NO_2^- formed ..	9.5	8.7	2.0	1.6

Reaction mixtures contained as indicated: 0.3 ml enzyme containing 156 μg protein, 0.24 ml of DPNH (2 μM per ml), 0.24 ml of FMN (0.09 μM/ml), 0.1 ml of 0.1 M KNO_3, and 0.1 M pyrophosphate buffer, pH 7.0 to give a final volume of 3.0 ml. The reactions were started by tipping the other reactants into the reduced flavin in pyrophosphate buffer in a Thunberg tube anaerobically. 0.5 ml of reaction mixture tested for nitrite with sulfanilamide and 1-naphthyl ethylenediamine dihydrochloride.

* Reduced flavin was prepared by reduction with $Na_2S_2O_4$ and H_2 as described (6).

** Mo-free enzyme dialyzed successively in phosphate glutathione cyanide solution as described elsewhere (4).

† Boiled enzyme prepared by heating at 100° C for 10 min was substituted for native enzyme.

TABLE III

DITHIONITE-TREATED MO AS AN ELECTRON DONOR FOR THE ENZYMATIC REDUCTION OF NITRATE

REACTION MIXTURES	DPNH FMN KNO_3 ENZYME	REDUCED MO * − DPNH	REDUCED MO − DPNH − FLAVIN	MO − DPNH	REDUCED MO − DPNH MO-FREE ENZYME	REDUCED MO − DPNH BOILED ** ENZYME
mμM NO_2^- formed	9.5	9.0	9.3	2.0	1.6	1.4

Reaction mixtures contained as indicated: 0.3 ml enzyme containing 205 μg protein, 0.24 ml of DPNH (2 μM per ml), 0.24 ml of FMN (0.09 μM/ml), 0.1 ml of 0.1 M KNO_3, and 0.1 M pyrophosphate buffer pH 7 to give a final volume of 3.0 ml. The reactions were started by tipping the other reagents into the molybdate in phosphate buffer in a Thunberg tube anaerobically. 0.5 ml of reaction mixture tested for nitrite with sulfanilamide and 1-naphthyl ethylenediamine dihydrochloride.

* Reduced molybdate was prepared in Thunberg tubes as indicated (5).

** Boiled enzyme prepared by heating at 100° C for 10 min was substituted for native enzyme.

The results in table II show that reduced flavin can replace TPNH as an electron donor for the enzymatic reduction of nitrate to nitrite. The molybdenum component of the enzyme is necessary for the above reduction as shown by the failure of the molybdenum-free nitrate reductase to mediate the reaction.

Table III indicates that reduced molybdate prepared with $Na_2S_2O_4$ and H_2 can serve anaerobically as an electron donor in the absence of added DPNH and flavin for the enzymatic conversion of nitrate to nitrite. The addition of flavin does not increase the enzymatic rate of nitrate reduction by reduced molybdate.

Discussion

Molybdenum has already been identified as the metal component of Neurospora nitrate reductase (3, 4) and its specific role as an electron carrier elucidated (5, 6). The large volume of evidence pointing to a function of molybdate in nitrate assimilation by higher plants (8) as well as the similarity of nitrate reductase of soybean leaves with that of Neurospora prompted the present investigation concerning the identify of the metal component of the higher plant enzyme. The preceding experiments showing the direct proportionality between molybdenum content and specific activity as well as the specific reactivation of the dialyzed enzyme by molybdenum have identified molybdenum as the constituent of nitrate reductase in the leaves of the soybean plant.

The experiments with reduced flavin and dithionite-treated molybdate, modeled after the more detailed and elaborate experiments with Neurospora (6) (tables II and III), have indicated that the pathway of electron transport in the enzymatic reduction of nitrate by DPNH proceeds through flavin and molybdenum in much the same manner as has been established for Neurospora, namely:

$$\text{DPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

The questions of whether one or more proteins are involved in the above series of reactions is not settled (6). If the enzyme proves to be a single protein, and thus far it has not been possible to demonstrate otherwise by fractionation techiques, it would represent a versatile system in view of its ability to catalyze each of the above reaction steps, very likely at different sites on the enzyme molecule. Other metalloflavin systems such as butyryl-coenzyme A dehydrogenase, DPN-cytochrome c reductase, xanthine oxidase and aldehyde oxidase are presumably single enzymes which catalyze and stepwise transfer of electrons by way of a number of carriers. The latter two systems are molybdoflavoproteins which can be reactivated after removal of molybdenum by molybdenum trioxide and not by molybdate (9, 10). Latimer and Hildebrand (11) state that molybdenum may form compounds having positive oxidation states of 2, 3, 4, 5 and 6. The fact that either $MoO_4^=$ or MoO_3 (both of oxidation state + 6) reactivates the purified nitrate reductase suggests this form as the more oxidized state of the metal in the enzyme. The identity of the less positive oxidation state which the metal assumes during electron transport is not known. By analogy with cytochrome c reductase, polyphenol oxidase, and ascorbic acid oxidase where the iron and copper undergo single electron changes one might speculate that molybdenum is reduced to the + 5 oxidation state.

It may be of interest that the nitrate reductase system does not show an inorganic phosphate requirement in contrast to that reported for xanthine and aldehyde oxidases (9, 10); and that the latter two systems also appear to contain an iron component (10, 12).

Summary

Molybdenum, already established as the metal component of Neurospora nitrate reductase, has also been identified as the metal of nitrate reductase of soybean leaves. This is supported by the proportionality between molybdenum content and specific enzyme activity, as well as the restoration of dialyzed enzyme specifically by MoO_3, or Na_2MoO_4. Other micronutrient elements including Fe, Cu, Mn, Co, Ni, W, V and B, respectively, were without effect. It has also been shown that during the enzymatic transfer of electrons from DPNH to nitrate, flavin and molybdenum function as electron carriers in the following sequence:

$$\text{DPNH} \rightarrow \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

$FMNH_2$ or reduced molybdate (without added flavin) can replace reduced pyridine nucleotides in the enzymatic reduction of nitrate to nitrite.

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PURIFICATION OF CHLOROPLASTS BY A DENSITY TECHNIQUE[1,2,3]

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Since the classical experiments of Granick (6) it has been possible to isolate and purify chloroplasts by differential centrifugation. Recently, it became evident (7) that differential centrifugation is inadequate to separate young small chloroplasts from the small non-plastid particles inferred to be present in leaf homogenates (10). It therefore appeared desirable to develop alternate methods of purifying chloroplasts.

Differential centrifugation separates particles according to size, shape and density, but primarily according to size. Young chloroplasts are closer in size to mitochondria than are mature chloroplasts, and this is the main obstacle to their separation. Another possibility appeared to be the use of the particle densities alone to effect separation. Accordingly, preparations of mixed mature chloroplasts and mitochondria were suspended in 80 to 100 % glycerol solutions in water, and then centrifuged vigorously in the Spinco ultracentrifuge. In this procedure most of the green material, and therefore most of the chloroplasts, were found to float to the top of the tube. Further experiments showed that the viscosity of these solutions could be reduced by using 50 % glycerol by volume and adding sufficient sucrose to make the final preparation 0.4 M in sucrose.

The experiments reported here will deal with the question of the degree of purification achieved with this flotation technique, and its applicability to young chloroplasts.

MATERIALS AND METHODS

The sources of leaf tissues were spinach purchased at a local grocery, tobacco leaves from Turkish tobacco (variety Samsun) grown in six-inch pots in the greenhouse, and primary leaves of red kidney bean plants grown in soil in flats in the greenhouse.

In making preparations of particles, leaves were ground in an Omnimixer at 50 to 60 % of line voltage, in four times their weight M/15 pH 7.0 phosphate buffer made to 0.4 M sucrose. The resulting homogenate was strained through a pad of cheesecloth and glass wool. Centrifugation of this homogenate at 1,000 × g for 12 minutes yielded a pellet containing chloroplasts, some mitochondria, and remains of nuclei. This pellet was resuspended in phosphate-sucrose buffer, and constituted the "crude particle" preparation, in most cases.

For centrifugation in a dense medium, a 2-ml aliquot of the original particle preparation was placed in a 13-ml Spinco centrifuge tube, together with 5.0 ml glycerol and 3.0 ml of a concentrated sucrose solution containing 1.30 gm of sucrose, and the contents of the tube were mixed thoroughly. The fluid in the tube then had a density of 1.17 gm/ml. When the

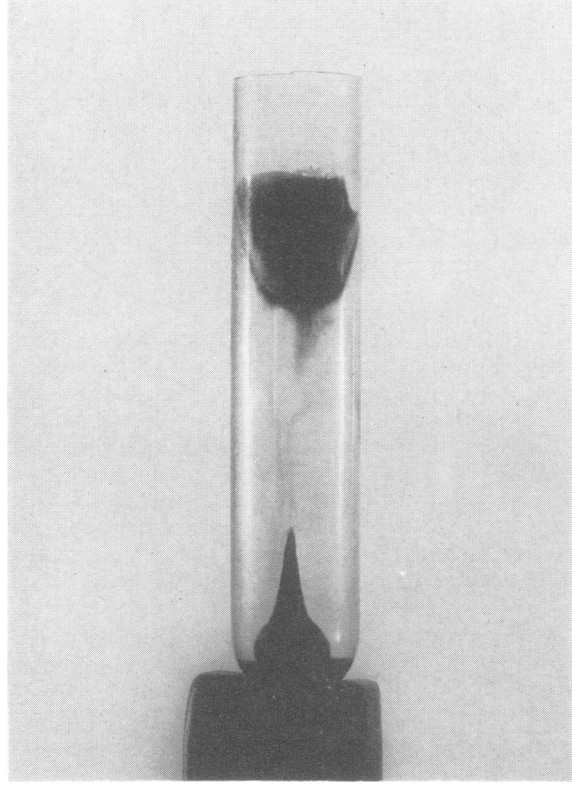

FIG. 1. Centrifuge tube after removal of fluid. Notice the pellet adhering to wall of tube at top, and bottom pellet at the opposite side of the tube.

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