THE ROLE OF TUNGSTEN IN THE INHIBITION OF NITRATE REDUCTASE ACTIVITY IN SPINACH (SPINACEA OLERACEA L.) LEAVES

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SUMMARY: Molybdenum-deficient plants transferred to $^{185}WO_4^{=}$, incorporate W in protein fractions, normally associated with nitrate reductase (NR.). Plants grown with Mo do not accumulate W in NR. After disc electrophoresis, purified fractions show maximum radioactivity in the expected area of NR. No enzyme was induced by $WO_4^{=}$. NADH-diaphorase was high in Mo deficient and in $WO_4^{=}$ treated plants and decreased after induction of NR. by Mo. Inhibition of NR. induction by W results from the formation of a tungstoprotein analogue, lacking NR. activity but active as a diaphorase. Super-induction of NADH-diaphorase by W reflects increased nitrate accumulation. NADH-diaphorase patterns in purified extracts change in distribution and abundance in response to Mo or W treatments.

INTRODUCTION: Tungsten is a competitive inhibitor of molybdenum utilisation by Aspergillus niger (1) and Azotobacter vinelandii (2) in the presence of nitrate as a nitrogen source and inhibits nitrate assimilation and nitrogen fixation by Azotobacter (3). Molybdenum is specific and essential in vivo for the induction of nitrate reductase (E.C. 1.6.6.1.)* in molybdenum-deficient plants either by root uptake (4) or by infiltration into leaf fragments (5, 6). The enzyme formation is concluded to involve de novo protein synthesis (6, 7) and molybdenum is incorporated during induction into the protein associated with the enzyme activity (8).

Heimer, Wray and Filner (9) showed inhibition by tungstate of production of NR. activity in suspended cultures of tobacco XD cells and in intact barley shoots, which was overcome by molybdate. They could not differentiate between (a) non-specific toxicity of tungstate; (b) inhibition by tungstate of molybdate incorporation into NR. apoenzyme; or (c) inhibition of the formation of

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* Abbreviations : NR. = Nitrate Reductase : $BV^{\cdot}$ = Reduced Benzyl Viologen
pNT = p-nitroblue tetrazolium chloride :
DCPIP = Phenol-indo-2:6-dichloro-phenol.

the apoenzyme. Wray and Filner (10) favoured the idea that the NR. apoenzyme is induced by nitrate in barley even in the presence of tungstate but is functional only with respect to associated 8S $NADH_2$-cytochrome c reductase activity. The unpurified solution used to grow the seedlings contained sufficient molybdenum as impurity to support maximum NR. formation. They were however unable to show unequivocal ^{185}W labelling of the 8S cytochrome c reductase when induced by nitrate in the presence of sufficient tungstate to antagonise the molybdenum requirement for NR. activity. We have compared the effects of transferring molybdenum deficient or normal spinach plants to $^{185}WO_4^=$ or $^{99}MoO_4^=$ solutions with reference to NR. formation and the distribution of labelled proteins, NR. and its associated and other NADH-diaphorase proteins after disc electrophoresis. Evidence is presented for the formation of a tungsto-protein analogue of NR. having neither NADH nor BV NR. activities but retaining NADH-diaphorase activity.

MATERIALS AND METHODS: Spinach were grown in sand culture either with complete nutrient or molybdenum free nutrients (11) with addition of 0.00002 ppm Mo. Plants were transferred with minimum root damage to solutions containing $^{185}WO_4^=$ or $^{99}MoO_4^=$.in otherwise molybdenum free nutrient solutions as described elsewhere (8). The protein was fractionated up to the post alumina C_γ stage (8, 12), assayed for NADH.NR. (13), NADH-diaphorase (14) and total protein (15, 16) and subjected to electrophoresis using the standard 5% acrylamide, pH 9.5 system (17) but omitting the sample and spacer gels (18). Gels were stained for (a) total protein with naphthalene Black, (b) NADH-NR. (19), (c) NADH-diaphorase (20), and (d) BV -NR. by 15 min anaerobic incubation with 15% reduced BV at pH 7.5 (21) before demonstration of enzymically produced nitrite. Radioactivity in 2 mm slices of gel was determined by dissolving the gel (22), plating and counting on an automated system (23).

RESULTS: NR. activity. The stepwise purification of NR. from lowMo NO_3 plants which had been transferred to tungstate solution is shown in Table 1. In frac-

TABLE 1

Purification of nitrate reductase from lowMo NO_3 plants transferred to ^{185}W

	Nitrate Reductase*	Protein+	Radioactivity^x
Crude extract	154	10,200	31,600
$CaPO_4$ supern.	0	6,000	14,400
PO_4 wash	3	700	2,000
P_2O_7 eluate	101	750	10,400
0-50% NH_4SO_4	86	490	7,500
Alumina C supern.	4	265	600
Dil P_2O_7 wash	15	15	50
P_2O_7 eluate	17	40	4,000

* Results expressed as muM NO_2 produced/15 mins/g. fr. wt. Activity before transfer : 180

+ " " " ug/g F. wt.

x " " " c/400 secs/g F. wt.

$CaPO_4$ gel prepared according to Dixon and Webb (26).

tions which would normally be rich in NR. there was, after initial removal of large amounts of surplus radioactivity, probably in the ionic form, an association of tungstate with the enzyme. There was no net enzyme production after transfer of the lowMo NO_3 plants to the tungstate solution. When normal amounts of molybdenum and associated NR. were present as in normalMo NO_3 plants (Table 2) there was no such association between tungstate and enzyme and the radioactivity declined continually as the enzyme was purified. Although twice the amount of tungsten was taken up by the normalMo NO_3 plants as compared to the lowMo NO_3 plants during the same period, the most highly purified fraction from the normal plants contained a negligible amount of radioactivity. The ratio of enzyme activity to radioactivity changed one thousand fold from 0.0043 in Table 1 to 4.35 in Table 2 for the most purified fraction. When lowMo NO_3 plants were transferred to a solution containing radioactive molybdate the enzyme activity increased from 10 to 188 units per g. F. Wt.

The results of electrophoresis of the most purified fractions (Fig. 1)

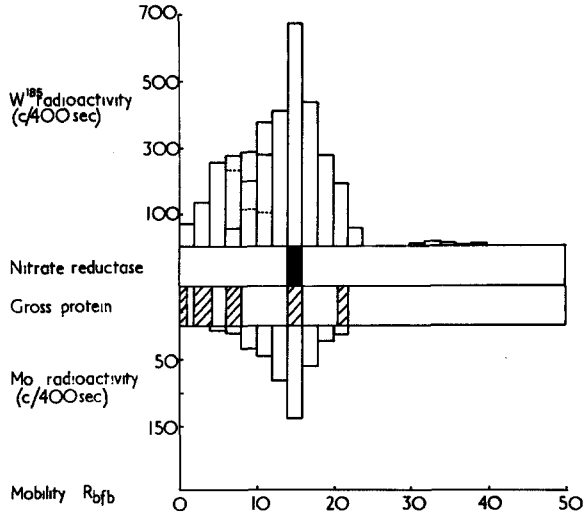

Fig. 1. Radioactivity of acrylamide gel slices obtained from purified extracts of lowMo NO_3 plants transferred to $^{185}WO_4^=$ or $^{99}MoO_4^=$ showing association of peak with nitrate reductase region and typical gross protein pattern.

TABLE 2

Purification of nitrate reductase from +Mo NO_3 plants transferred to ^{185}W

	Nitrate reductase*	Protein+	Radioactivity x
Crude extract	605	16,000	61,000
$CaPO_4$ supern.	48	11,700	50,500
PO_4 wash	5	730	530
P_2O_7 eluate	237	870	3,000
0-50% NH_4SO_4	186	570	85
Alumina C supern.	5	230	25
Dil. P_2O_7 wash	0	270	0
P_2O_7 eluate	87	55	20

* Results expressed as muM NO_2 produced/15 mins/g F. Wt. Activity before transfer : 92

+ " " " ug/g F. Wt.

x " " " c/400 secs/g F. Wt.

$CaPO_4$ gel prepared according to Dixon and Webb (26).

show that the peak of tungsten radioactivity obtained after electrophoresis of purified protein from lowMo NO_3 plants transferred to tungstate solution occurred in the same region as NR. having the same mobility from both normalMo NO_3 plants transferred to tungstate and lowMo NO_3 plants which had NR. induced by transfer to radioactive molybdate solution. The skew distribution of the tungsten radioactivity in the gel observed from 3 experiments may indicate either some instability of the protein during electrophoresis or the presence of a separate overlapping fraction which was not obvious in the almost normal distribution of molybdenum shown here and in other experiments (8). The peak of molybdenum radioactivity was also coincident with NR. activity which migrates as a single band with respect to both $NADH_2$ and BV as electron donors (8). Several protein bands which were not associated with the peaks of tung-

TABLE 3

NADH diaphorase and nitrate reductase from purified extracts of transferred spinach plants

	LowMo NO_3 → -Mo	LowMo NO_3 → ^{185}W	LowMo NO_3 → ^{99}Mo
NADH diaphorase*	6.67	5.15	4.28
NADH Nitrate reductase+	541	388	1314
Ratio NR/diaphorase	81	75.3	307

* Results expressed as change in O.D. units of DCPIP/min/mgm protein

\+ " " " muM NO_2 produced/15 mins/mgm protein.

sten or molybdenum radioactivity showed the specificity of the association of both metals with the NR. region. The purified proteins containing radioactive tungsten or molybdenum were dialysed overnight at 2°C against a phosphate buffer pH 7.5 containing 5 x 10^{-4}M EDTA with or without unlabelled molybdate. In no case was there any loss of radioactivity and no increase in NR. activity of the tungsten protein. Purified enzyme showed negligible loss of activity under control conditions although inhibition by cyanide was freely reversible.

NADH-diaphorase activity. LowMo NO_3 plants were transferred (a) to molybdenum free nutrient, (b) to molybdenum free nutrient with added tungstate or (c) to complete nutrient with molybdate. The NR. was purified after 24 hrs. The results (Table 3) show that when lowMo NO_3 plants were transferred to tungstate (b) there was no increase in total diaphorase content compared to controls (a), there was moreover no change in the low NR. level or NR./diaphorase ratio. In lowMo NO_3 plants transferred to complete nutrient (c) the diaphorase level fell below that of both (a) and (b), whereas NR. increased and the NR./diaphorase ratio increased four-fold.

When cauliflower plants are grown in sterile cultures with low molybdenum and ammonium sulphate instead of nitrate (Gundry, Notton and Hewitt, unpublished work) their NR. activity is negligible and total diaphorase is also very low. Infiltration of excised leaf tissues (6) with nitrate alone results in production of diaphorase activity, whereas molybdate and nitrate are needed simultaneously to allow simultaneous induction of NR. which occurs without any further increase in diaphorase produced by nitrate alone. Plants grown without adequate molybdenum accumulate nitrate (24) and addition of tungstate to barley also causes nitrate accumulation (10). NR. is low under both these conditions, but the NR.-associated and total diaphorase activity remains high. Accumulated nitrate disappears very rapidly following addition of molybdenum (25, 26). Nitrate seems therefore to be an inducer of the NADH-diaphorase moiety of the NR. system even in the absence of the prosthetic metal.

The diaphorase patterns obtained after electrophoresis of the purified

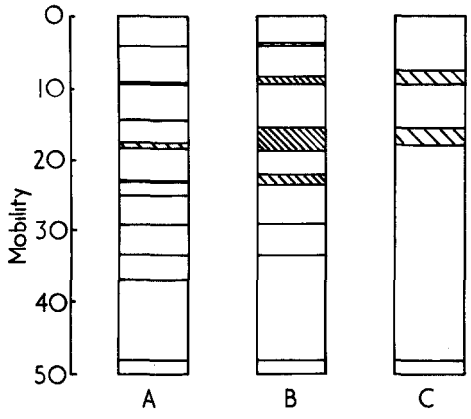

Fig. 2. NADH-Diaphorase patterns obtained by p-nitro-tetrazolium stain of purified nitrate reductase preparations: (A) lowMo NO_3; (B) lowMo NO_3 transferred to $WO_4^=$; and (C) lowMo NO_3 transferred to $MoO_4^=$. Intensity of bands indicated by intensity of hatching.

spinach NR. (Fig. 2) changed when a lowMo NO_3 plant was transferred to tungstate or molybdate. Up to nine separate diaphorase bands were still visible at this stage for the control plants transferred only to molybdenum free solution. After transfer to tungstate six were still present but only two or three could be detected after plants were transferred to molybdate. The increase in intensity in the diaphorase band corresponding with NR. which is observed on transferring to molybdate or especially to tungstate is possibly compensated by the reduction in the number of the other diaphorases, since an approximate equality remains between the different treatments in terms of overall DCPIP diaphorase activity (Fig. 2 and Table 3). The addition of the metal may therefore cause a redistribution of some diaphorases which then correspond in mobility with the NR. system.

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