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COMPARATIVE ASPECTS OF INCORPORATION OF VANADIUM, TUNGSTEN OR MOLYBDENUM INTO PROTEIN OF NITRATE REDUCTASE OF *SPINACEA OLERACEA* L. LEAVES

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SUMMARY

Spinach was grown in molybdenum-deficient sand culture, given radioactively labelled molybdenum, tungsten or vanadium and the protein fractionated for nitrate reductase. Enzymatically active nitrate reductase was formed with molybdenum but not tungsten. Both molybdenum and tungsten were incorporated into protein associated with nitrate reductase or similar but inert protein. Vanadium was not incorporated into any protein and did not promote nitrate reductase activity. Vanadium tended to accumulate in the roots and unlike molybdenum or tungsten was not freely translocated to the leaves.

The specific association between Mo and N assimilation by N_2 fixation or NO_3^- reduction is firmly established[1,2]. The metal is a tightly bound prosthetic component of the proteins in both systems. V can substitute for Mo in N_2 fixation by certain species of *Azotobacter*[3]. The substitution of V for Mo in a growth medium for *Azotobacter vinelandii* OP cells[4,5] resulted in the isolation of protein in the purified nitrogenase fraction having a ratio of 93 % V to Mo and decreased specific activity, binding affinity for acetylene and stability to heat. In contrast, V is unable to modify the Mo requirement of a blue-green alga *Anabaena*[6], or in legume root nodules[7]. W is an antagonist of Mo in N_2 fixation and NO_3^- assimilation by *Azotobacter*, but V appears unable to reverse antagonism by WO_3[8,9]. Inhibition by W of the formation of nitrate reductase activity by Mo in suspended cultures of tobacco XD cells and in intact barley seedlings[10] was shown in spinach[11] to be the formation of an enzymatically inactive W analogue of the enzyme in which it is resistant to removal by dialysis with cyanide or exchange with Mo[12]. It therefore seemed pertinent to test for the possible formation of a V analogue using the same technique.

In the first experiment, Mo-deficient spinach plants were grown as previously described[13] for 6 weeks before being supplied with 200 ml of Mo-free nutrient solution containing 10 μCi ^{49}V and 200 μg V as VO_3. After 24 h the leaves were sampled and extracted protein was purified as for nitrate reductase[13]. There was a continual loss of radioactivity during enzyme purification from the initially low level of 324 counts/1000 s per g fresh wt to zero (background level) in the most purified extract in which the specific activity of the initially low nitrate reductase had increased 25 times. The

radioactivity of the roots, well washed with VO_3 solution to remove extraneous ^{49}V, was 13965 counts/1000 s per g fresh wt confirming previous observations on the accumulation of V in the roots of higher plants[14,15].

TABLE I

COMPARISON BETWEEN ACCUMULATION IN PROTEIN FRACTIONS NORMALLY RICH IN NITRATE REDUCTASE OF V, W AND MO WHEN ADDED TO MO-DEFICIENT SPINACH PLANTS

Nitrate reductase activity (N.R.) expressed as nmoles NO_2 produced/15 min per mg protein. Radioactivity (^{49}V, ^{185}W and ^{99}Mo) expressed as counts/1000 s per mg protein.

Fraction	*Treatment:*	*−Mo*	*V*	*−Mo*	*W*	*−Mo*	*Mo*
		N.R.	^{49}V	*N.R.*	^{185}W	*N.R.*	^{99}Mo
Cell-free extract		8.4	51	10.2	1240	12	5721
$Ca_3(PO_4)_2$ gel eluate		57.7	21	73.4	5331	174	4667
0–50% $(NH_4)_2SO_4$ precipitate		75.6	14	92.5	5511	208	4937
Alumina cγ eluate		191	0	245	18632	421	10228

In the second experiment the carrier V was decreased to 40 μg in 200 ml of Mo-free nutrient solution containing 9 μCi ^{49}V and the plants were left for 48 h before sampling. This treatment led to a 3-fold increase in the foliar V to 944 counts/1000 s per g fresh wt. Table I shows the subsequent purification of nitrate reductase with corresponding radioactivity from these plants compared with effects of equivalent doses of W and Mo given under similar conditions. It is seen that whereas W and Mo accumulate in the fractions rich in nitrate reductase V becomes progressively diluted out. The increase in specific activity of the enzyme during purification was similar for V and W treatments (24 ×) but higher for Mo treatment (35 ×) which has a greater level of enzyme activity due to the inductive effect of Mo. Pre- and post-treatment levels of enzyme activity (nmoles NO_2 produced/15 min per g fresh wt) were 164 and 155 for V, 180 and 149 for W, and 102 and 334 for Mo. In no case did the enzyme activity of the leaves significantly increase as a result of treatment with V. It was therefore clear that no biosynthetically produced V analogue of nitrate reductase was formed under these conditions. This contrasted with W, which induces an enzymically inactive protein[11] and with Mo which is the natural prosthetic metal of the enzyme[13].

The atomic radii of the three metals are 1.29, 1.30 and 1.22 for Mo, W and V, respectively, and the ionic radii of the M^{4+} state are 0.68, 0.68 and 0.64[16] suggesting that the inability of V to form an analogue is related to its size. By analogy, the inability of the W analogue to be enzymically active does not result from altered spatial relationships with the protein but from some other factor, possibly redox potential. Available information[17] shows W to differ significantly from Mo in several redox systems. The results also suggest that nitrate reductase is less flexible around the Mo site of the protein than nitrogenase. In the latter, a V-containing analogue either shows functional but modified properties or a V-containing protein of similar purification characteristics is produced which influences the activity of diminished amounts of nitrogenase. V shows no affinity with nitrate reductase as it is neither incorporated into, nor seems to modify, residual nitrate reductase protein.

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