REVERSIBLE CYANIDE INHIBITION OF SPINACH (*SPINACEA OLERACEA L.*) NITRATE REDUCTASE AND NON-EXCHANGEABILITY *IN VITRO* OF PROTEIN BOUND MOLYBDENUM AND TUNGSTEN

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1. Introduction

Nicholas and Nason [1] identified molybdenum as the metal constituent of nitrate reductase of soybean by dialysis against cyanide, which supposedly removed the metal, followed by dialysis against purified phosphate buffer and finally reactivation of the intact enzyme specifically with molybdate. Attempts to repeat these results with enzymes obtained from different plants, including spinach, have been unsuccessful [2–5]. Nevertheless, contrary to previous doubts [4, 6], nitrate reductase of spinach is now shown [7] to be a molybdoprotein. Tungsten inhibits the production of nitrate reductase in barley [8] but still allows the induction by nitrate of an associated NADH-cytochrome *c* reductase protein [9]. The inhibitory effect of Tungsten [8] is explained by the production *in vivo* of an inactive tungstoprotein analogue of nitrate reductase [10]. We now report that inhibition of the spinach enzyme by cyanide [4] is reversible without removal of molybdenum from the enzyme. Cyanide is unable also to remove ^{185}W labelled tungsten from the protein produced when tungstate is given to molybdenum deficient plants. Moreover, exchange *in vitro* during dialysis between protein bound and free molybdenum or tungsten is insignificant in spite of evidence for occurrence of this reaction *in vivo* [7, 11].

2. Materials and methods

Spinach plants were grown in sand culture with a low molybdenum (0.0001 ppm) nutrient solution as previously described [7] using methods appropriate to micronutrient investigations [12]. After 6 weeks growth the plants were given 450 ml of molybdenum free nutrient solution containing 100 μCi ^{185}W and 36.4 μg tungsten as tungstate. After 24 hr the plants were harvested and the protein was fractionated [13] using the weak nitrate reductase already produced under conditions of limited molybdenum supply [7] as a marker. Enzyme activity, radioactivity and protein were determined as previously described [7]. Phosphate buffers were freed of molybdenum by the copper sulphide precipitation method [14] and water was estimated to contain negligible molybdenum (0.00005 ppm) as shown from biological assays [12]. Dialysis tubing was pretreated [1] first in 0.05 M phosphate buffer pH 7.5 containing 1 mM EDTA and 1 mM cysteine hydrochloride for 48 hr and then without cysteine for 24 hr.

3. Results and discussion

Table 1 shows the co-fractionation of nitrate reductase and tungstoprotein from low molybdenum plants which had been given tungstate for 24 hr before sampling and confirms [10] the specific tendency of tunsgten to replace molybdenum in nitrate reductase. The weak nitrate reductase activity in these plants (16 units, table 1) results from the uptake of the limited amount of molybdenum in the nutrient solution necessary to prevent death at the seedling stage. It resembles that present in similar plants not given tungsten. When molybdenum is given to deficient plants for 16 hr the resultant enzyme formation has

Table 1
Purification of nitrate reductase and associated concentration of ^{185}W in protein from tungstate treated low molybdenum plants.

Fraction	Specific activity[a]				Radioactivity[b]	Radioactivity / Enzyme activity
	W Treated	Controls				
Crude extract	15.8	19.7[c]	70[d]	140[e]	252	5.25[f]
$Ca_3(PO_4)_2$ supernatant	0				169	
P_2O_7 eluate of $Ca_3(PO_4)_2$ gel	145				903	6.23
0–50% $(NH_4)_2SO_4$ ppt	204				1324	6.49
P_2O_7 eluate of alumina c	605	541[c]	3000[d]	6000[e]	4249	7.02

[a] Specific activity as nmoles NO_2 produced/15 min/mg protein.
[b] Radioactivity as counts/400 sec/mg protein.
[c] Low Mo plant untreated.
[d] Low Mo plant given Mo for 16 hr.
[e] +Mo healthy plant.
[f] Ratio calculated for W activity after subtraction of W activity in first supernatant.

Table 2
Effect on activity of dialysis of normal nitrate reductase in the presence of cyanide or tungsten.

Treatment	Enzyme activity[a]	
	Cyanide experiment	Tungsten experiment
Undialysed prepartion held at 4° for 24 hr	–	5900
Dialysis against 0.1 M PO_4 buffer 1 mM EDTA and 10 mM cyanide pH 7.5 for 3 hr	784	–
Subsequent redialysis against 0.1 M PO_4 buffer and 1 mM EDTA pH 7.5 for 18 hr after cyanide treatment above	4368	–
Control dialysed against 0.1 M PO_4 buffer 1 mM EDTA pH 7.5 for 24 hr	4200	5967
Control dialysed against 0.1 M PO_4 buffer 1 mM EDTA and 10 μM tungstate pH 7.5 for 24 hr	–	5817

[a] Enzyme activity as nmoles NO_2 produced/15 min/mg protein.

Table 3
Dialysis of purified enzyme concentrated from tungstate treated plants against 1 mM potassium cyanide and redialysis against molybdenum.

Treatment	Enzyme activity[a]	Radioactivity ^{185}W [b]
Initial solution	605	4249
Undialysed solution[c] held at 4° for 16 hr	567	4221
for 21 hr	452	3959
Dialysis against 0.05 M PO_4 1 mM EDTA pH 7.5 for 16 hr	605	3805
Dialysis against 0.05 M PO_4 1 mM EDTA, 1 mM KCN pH 7.5 for 16 hr	162	4000
Further dialysis against 0.05 M PO_4 buffer 1 M EDTA pH 7.5 for 5 hr	358	3500
Further dialysis against 0.05 M PO_4 buffer, 1 mM EDTA and 10 μM MoO_4 pH 7.5 for 5 hr	351	3500

[a] Enzyme activity as nmoles NO_2 produced/15 min/mg protein.
[b] Radioactivity as counts/400 sec/mg protein.
[c] This preparation was held in an open vessel for 21 hr out of contact with the cysteine-treated sealed dialysis sac.

an activity of about 70 units in crude extracts, concentrating to about 3000 in the purified fraction (table 1). In plants grown with adequate molybdenum the comparable fractions have about double these activities and concentrate molybdenum in the purified protein [7].

Cyanide inhibition of normal spinach nitrate reductase was concentration dependent up to 0.5 mM with K_i approx. 10 μM for a non-competitive reaction. Inhibition was established in about 1 min [15] and did not increase when enzyme and cyanide were incubated for 2 hr before assay. Enzyme activity which was inhibited 90% by 0.5 mM cyanide was restored to the value of control preparations by further dialysis for 18 hr at 4° against purified phosphate buffer (table 2, col. a), without addition of molybdenum. Dialysis of similar preparations against a solution containing 10 μM tungstate (table 2, col. b) showed no loss in activity compared to controls dialysed without tungstate or untreated.

A 3 ml portion of the most purified fraction of enzyme from the tungstate treated plants of table 1 was dialysed for 16 hr at 4° against 3 l of 0.05 M phosphate buffer pH 7.5 containing 1 mM EDTA either without, or with initially, 1 mM cyanide which was added also to the enzyme inside the dialysis sac (table 3). The enzyme activity after cyanide dialysis with precautions to maintain the concentration during the 16 hr period was decreased by 75% compared with the control without cyanide. Radioactivity did not however decrease significantly showing that tungsten was not removed from the tungstoprotein by cyanide, in spite of the inhibition of the enzyme.

The effect of molybdenum on enzyme activity after treatment with the cyanide and on the tungsten labelled protein was examined. Reversal of the cyanide inhibition (table 3) was accomplished as in the previous experiment by further dialysis against fresh phosphate buffer. The inhibited preparation was also redialysed in the presence of 10 μM molybdate. There was no further enhancement of activity compared with cyanide removal alone and no decrease in radioactivity of the protein bound tungsten. The reversal of cyanide inhibition was therefore independent of the presence of ionic molybdenum and there was no loss of labelled tungsten by exchange with molybdenum in solution.

A sample of nitrate reductase labelled with ^{99}Mo by a procedure comparable to that previously described [7] was dialysed against unlabelled molybdate (10 μM) for 16 hr without any change in the radioactivity of the protein. A purified preparation obtained from normal molybdenum grown plants was dialysed in the presence of ^{185}W labelled tungstate for 16 hr at 4°. The ionic tungsten was then removed by passing the protein through 100 col. vol. of G-25 Sephadex. There was no loss of enzyme activity compared with control preparations and no gain in radioactivity. This showed that no exchange had occurred and that the sustained activity was not due to partial formation of an active tungsten analogue. In previous work [7] there was no exchange between ^{99}Mo and a functioning enzyme system during 16 hr at 20°.

4. Conclusions

The restoration of activity of cyanide treated enzyme after the removal of cyanide by plain buffer purified to remove molybdenum and the retention of radioactivity in the tungsten protein show that cyanide inhibition of nitrate reductase is not due to the dissociation of the metallocyanide complex from the protein. The rapid development of the inhibited state, the low effective cyanide concentration and reversibility jointly indicate the formation of a loosely bound complex between metallo-protein and cyanide from which the cyanide is freely dissociable with complete restoration of enzyme activity and without loss of prosthetic metal. Tungsten is apparently similarly firmly bound to apoenzyme and does not exchange with molybdenum *in vitro*. This contrasts with the demonstration of apparent exchange between tungsten and molybdenum *in vivo* when gross protein synthesis is inhibited by cycloheximide in tobacco callus cultures [15] and with the possibility that exchange may occur *in vivo* between enzyme bound and excess cellular molybdenum in spinach [7]. The demonstarted removal of molybdenum by cyanide from the *Neurospora* enzyme [16] and its postulated removal by cyanide from the soybean enzyme [1] or by ammonia but not by cyanide from the *Vicia* enzyme [5] compared with non-removal from spinach indicates the differences in the stability constants of the chelation systems in different nitrate reductase proteins.

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