TUNGSTEN INCORPORATION INTO *AZOTOBACTER VINELANDII* NITROGENASE

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1. Introduction

The inhibitory effect of tungstate (Na_2WO_4) on molybdate metabolism was first reported by Higgins et al. [1] and has been studied in nitrogen-fixing *Azotobacter* [2–5]. The competitive character of this inhibition, together with the chemical similarities of Mo and W, suggest that W can substitute for Mo in biological systems, without, however, exhibiting the characteristic biochemical and catalytic properties of Mo. Recently it was reported [6–8] that W is incorporated into the nitrate reductase complex of spinach and *Chlorella*. The W-containing enzyme was inactive in all the reactions of the nitrate reductase complex except for its NADH-cytochrome *c* reductase activity [6, 7].

In this report we show that W is incorporated into the nitrogenase complex of *Azotobacter vinelandii*. As we have found to be the case for vanadium [5], W is specifically associated with the Fe–Mo protein fraction of nitrogenase. This W-containing protein fraction (Fe–W protein) is inactive in the acetylene reduction, hydrogen evolution, and reductant dependent ATPase activities of nitrogenase.

2. Methods

Azotobacter vinelandii OP (ATCC 13705) was grown in a modified Burk's medium [9] without molybdate. For enzyme purification 80 ℓ of cells were grown in a 100 ℓ steel fermentor with 10^{-4} M Na_2WO_4 (containing about 500 μCi ^{185}W) and 3×10^{-3} M $(NH_4)_2HPO_4$. High concentrations of Na_2WO_4 were used since the Mo contamination in the media, about 2×10^{-8} M [10], increased substantially after autoclaving in the steel fermentor. ^{185}W was assayed by liquid scintillation counting after digestion of the lyophilized samples with NCS solubilizer. Growth rate studies, ethylene determinations, and nitrogenase assay and purification were performed as previously described [5]. Hydrogen was measured with a Varian Model 1532 helium detector gas chromatograph equipped with a molecular sieve 5A column. ATP hydrolysis was assayed by the method of Furchgott and Gubareff [11] as modified by Bulen and LeComte [12].

3. Results

In the presence of ammonia, Na_2WO_4 does not inhibit growth of *A. vinelandii* even at 5×10^{-2} M. The patterns of growth inhibition by Na_2WO_4 were similar when N_2, nitrate, or urea was the sole nitrogen source. A ratio of 200:1 W to Mo resulted in 50% growth inhibition at low Mo concentration (10^{-7} M), while at higher Mo concentrations (10^{-5} M) a 400-fold excess of W was required for 50% inhibition. However, W inhibition of growth was complex since Na_2WO_4 not only lengthened doubling times but lengthened lag phases as well. Very high Na_2WO_4 concentrations (about 5000-fold higher than Mo) were required to suppress all growth when small amounts of ammonia were added to the cultures to reduce the lag phase. This method was used for growing large batches of cultures in the presence of Na_2WO_4: cells were supplied with a limiting amount of ammonia (see Methods) and harvested after the growth curve indicated that ammonia had been exhausted.

Cell-free extracts of such cultures had small but

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Table 1
Purification of Na_2WO_4 grown *Azotobacter vinelandii* nitrogenase.

Preparation	Specific activity*	Specific activity* + 1 mg Fe-Mo protein	(nmoles W/ mg protein)
Centrifuged extract	0.65	15.0	36
Heat treated (60°, 5 min) extract	1.31	30.4	97
Protamine sulfate fraction (PS II)	0.78	2.8	6.0
0.25 M NaCl DEAE chromatography fraction (Fe–W protein)	0.02	N.D.	4.9

* Specific activities given as nmoles acetylene reduced per min per mg protein.

Table 2
Comparison of Fe–Mo protein and Fe–W protein activities*.

Protein	Acetylene reduction	Hydrogen evolution	ATP hydrolysis† (dithionite dependent)
Fe–W protein	3.8	5	30
Fe–Mo protein	520	715	4400

* Activities are expressed as nmoles of product per min per mg protein. All assays performed in presence of 0.3 mg Fe protein.
† ATP hydrolysis corrected for dithionite independent P_i release and ATP hydrolysis during P_i measurement.

significant nitrogenase activity (table 1). This activity was useful in assaying for nitrogenase during purification. The extracts had a normal complement of Fe protein, as indicated from the high activities in the presence of purified Fe–Mo protein (table 1). The extracts had a very high W content (table 1). The intracellular concentration in unbroken cells was about 5×10^{-3} M. Although most of the W was not removed from crude extracts by Sephadex G-25 treatment, it was dialyzable after precipitation of nitrogenase, indicating that the W association with protein might be weak. This observation is similar to that of Notton and Hewitt [8] for nitrate reductase. During purification most of the W remained in the supernatant after precipitation of the nitrogenase components with protamine sulfate. Resolubilization of the nitrogenase was carried out with a limiting amount of cellulose phosphate to preferentially liberate the Fe–Mo protein component. Lack of sufficient Fe–protein therefore accounts for the low activities, with or without Fe–Mo protein, of the PS II fraction. During DEAE cellulose chromatography of the resolubilized material W was specifically associated with the protein band which eluted at 0.25 M NaCl and which normally contained the Fe–Mo protein. Assay of this W-containing protein fraction (Fe–W protein) with Fe protein indicated that acetylene reduction activity was low (about 70% was lost during purification) and that the hydrogen evolution and dithionite-dependent ATPase activities were equally low when compared to the Fe–Mo protein (table 2).

4. Discussion

The W-grown extracts had similar properties to those observed with extracts from cultures grown without Mo or with V [5, 14, 15]. Acetylene reduction activities were low and relatively labile. The Fe protein component was present in normal amounts. Although the nitrogenase of *Azotobacter* is associated in a sedimentable, heat and oxygen resistant complex, the Fe protein reacts with added purified (soluble) Fe–Mo protein. This would indicate that the Fe protein can dissociate from the nitrogenase complex. Dissociation might also be induced by ATP, thereby accounting for the O_2 lability of the Fe protein in crude extracts in the presence of ATP [13].

The W concentrations found in the Na_2WO_4 grown cells were at least 10-fold higher than expected for cells grown under similar circumstances with Na_2MoO_4. Our previous observations [5, 14] showed that when Mo is present in excess in the growth medium it is taken up and, besides being incorporated into nitrogenase, is stored by *Azotobacter* in a protein bound form. Under Mo starvation conditions essentially all available Mo was found in the nitrogenase [5]. The high W concentrations might be accounted for by an unregulated uptake of the metal. Although we show that

W is incorporated into an inactive nitrogenase, it is not clear that this is the primary site of Na_2WO_4 inhibition. Since growth on N_2, $NaNO_3$ or urea is similarly affected by Na_2WO_4, it is possible that Mo uptake, storage, or transport into the enzymes are the functions where W exerts its major inhibitory effect.

The absence in the Fe–W protein of the three major catalytic activities of nitrogenase, substrate reductase, H_2 evolution, and reductant dependent ATP hydrolysis, suggests that Mo is involved in all three reactions. It is of course possible that the larger size of W, when inserted into the protein produces conformational changes which interfere with the nitrogenase reactions. In addition it has not been definitively shown, either in this case or that of nitrate reductase [6, 7], that W is actually incorporated at the same site as Mo. However, we can conclude that all three of the nitrogenase reactions are equally affected by the incorporation of W into the enzyme, suggesting a common active site. We have previously shown [5] that the nitrogenase substrate reduction activity is proportional to the molybdenum content of the enzyme, indicating that the active site contains a single Mo. We suggest, from the results presented here, that this Mo is also directly involved in the ATP hydrolysis and H_2 evolution reactions of nitrogenase.

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References

[1] E.S. Higgins, D.A. Richert and W.W. Westerfeld, Proc. Soc. Exptl. Biol. Med. 92 (1956) 509.
[2] R.F. Keeler and J.E. Varner, Arch. Biochem. Biophys. 70 (1957) 585.
[3] H. Takahashi and A. Nason, Biochim. Biophys. Acta 23 (1957) 433.
[4] W.A. Bulen, J. Bacteriol. 82 (1961) 130.
[5] J.R. Benemann, C.E. McKenna, R.F. Lie, T.G. Traylor and M.D. Kamen, Biochim. Biophys. Acta 264 (1972) 25.
[6] B.A. Notton and E.J. Hewitt, Biochem. Biophys. Res. Commun. 44 (1971) 702.
[7] A. Paneque, J.M. Vega, J. Cardenas, J. Herrera, P.J. Aparicio and M. Losada, Plant Cell Physiol. 13 (1972) 175.
[8] B.A. Notton and E.J. Hewitt, FEBS Letters 18 (1971) 19.
[9] J.R. Benemann, C.W. Sheu and R.C. Valentine, Arch. Mikrobiol. 79 (1971) 49.
[10] C.E. McKenna, Ph.D. dissertation, University of California, San Diego, 1971.
[11] R.F. Furchgott and T. de Gubareff, J. Biol. Chem. 223 (1956) 377.
[12] W.A. Bulen and J.R. LeComte, Proc. Natl. Acad. Sci. U.S. 56 (1966) 979.
[13] M.G. Yates, European J. Biochem. 29 (1972) 386.
[14] C.E. McKenna, J.R. Benemann and T.G. Traylor, Biochem. Biophys. Res. Commun. 41 (1970) 1501.
[15] R.C. Burns, W.H. Fuchsman and R.W.F. Hardy, Biochem. Biophys. Res. Commun. 42 (1971) 353.