# NITROGENASE FROM VANADIUM-GROWN AZOTOBACTER: ISOLATION, CHARACTERISTICS, AND MECHANISTIC IMPLICATIONS

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SUMMARY: A nitrogenase, designated V-$N_2$ase, was extracted from Azotobacter vinelandii OP grown on $N_2$ in medium supplemented with V in place of Mo. V-$N_2$ase was similar to Mo-$N_2$ase (from Mo-grown cells) in purification procedure, activity requirements, reactions catalyzed, and biphasic Arrhenius plot. V-$N_2$ase was less active, less stable to heat and in storage, and had a slightly lower activation energy than Mo-$N_2$ase.

Comparison of V- with Mo-$N_2$ase revealed differences throughout the $N_2$ase reaction sequence from initial substrate binding to electron transfer and product release, specifically including 1) decreased affinity for reducible substrates and CO, 2) enhanced allocation of electrons to $H_3O^+$ at the expense of substrate reduction, and 3) decrease in the $C_3H_6$:$C_3H_8$ ratio in acrylonitrile reduction.

The similarities of V- and Mo-$N_2$ase suggest the substitution of V for Mo in $N_2$ase; the differences provide the first basis for implicating Mo directly in $N_2$ase catalysis and suggest an active site function for Mo.

Mo has long been associated nutritionally with $N_2$ fixation (1,2) and was recently shown to occur in the ratio of 2 atoms Mo/molecular weight of ca. 270,000 daltons after repeated recrystallization of the Mo-Fe protein of nitrogenase ($N_2$ase) (3). In the present work a $N_2$ase, designated V-$N_2$ase, was purified from an Azotobacter species capable of assimilating V in place of Mo during growth on $N_2$ (4) and was used to elucidate Mo function. Comparisons of the characteristics and reactivity of V-$N_2$ase with Mo-$N_2$ase (from Mo-grown cells) supports the concept that Mo is functional at the active site of $N_2$ase.

METHODS: Azotobacter vinelandii OP (ATCC 13705) was grown on a modified Burke's medium supplemented with 1 μg Mo/liter as $Na_2MoO_4 \cdot 2H_2O$ (3) or 1 mg V/liter as $Na_3VO_3 \cdot 16H_2O$. Stock cultures were maintained in shake flasks by daily serial transfer using 2.5% inoculum for Mo cultures and, to obtain comparable growth, 10% for V cultures. Media supplemented with neither Mo nor V failed to support growth after 5 daily subcultures using 10% inoculum. Twenty-liter cultures of both Mo- and V-grown cells were grown, harvested, stored, extracted and purified through the resolubilization (PS-2) step

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by procedures described earlier for Mo-$N_2$ase (3,5), except the 60° V-$N_2$ase incubation was reduced from 10 to 5 min. to preserve activity. The Fe, V and Mo were measured by comparing intensity of emission spectral lines to standard reference plates with a Bausch & Lomb Large Littrow Emission Spectrometer. Mo was also determined according to (6). Protein was estimated according to the Biuret method (7) and enzyme activities were measured as described in (3,8,9).

RESULTS: The specific activities of V-$N_2$ase preparations were consistently less than their Mo-$N_2$ase counterparts; nevertheless, approximately the same fold purification is achieved at each step with both enzymes. The V-$N_2$ase was less stable to heating at 60°, and on standing at room temperature it lost all activity in about two weeks, whereas the purified Mo-$N_2$ase remained fully active during comparable storage. The $N_2$ase activities of preparations obtained during purification are indicated in Table I.

TABLE I. PURIFICATION OF V- AND Mo-$N_2$ase

| Preparation | Activity | | Purification | | Recovery | |
|---|---|---|---|---|---|---|
| | V | Mo | V | Mo | V | Mo |
| | Units/mg protein | | Fold | | % of Units | |
| Extract | 16 | 69 | 1.0 | 1.0 | 100 | 100 |
| PS-1 | 20 | 72 | 1.2 | 1.0 | 100 | 84 |
| △ 60° | 39 | 179 | 2.4 | 2.6 | 68 | 73 |
| PS-2 | 67 | 308 | 4.2 | 4.5 | 52 | 62 |

Purification (3,5) consisted of precipitation of nucleic acids by protamine sulfate (PS-1), followed by heating to 60° (△ 60°) 5 min. for V-$N_2$ase and 10 min. for Mo-$N_2$ase, then by precipitation of enzyme with excess protamine sulfate and resolubilization with cellulose phosphate (PS-2). Activity of extracts in terms of units/gm wet wt. cells was 1500 and 7320 for V- and Mo-cells, resp.; 1 unit of activity = 1 nmole $H_2$ evolved $min.^{-1}$.

Although traces of Mo were detectable in the purified V-$N_2$ase, the levels were sufficiently low to render quantitative estimation difficult, but a maximum of 10% of the activity in the V preparations was due to Mo-$N_2$ase contamination. Mo, V and Fe emission spectrographic analyses indicated atomic ratios of $Mo_{1-5}V_{20-100}Fe_{400-2000}$.

Like Mo-$N_2$ase (10,11), V-$N_2$ase required reductant, ATP and $MgCl_2$ for activity. It reduced all $N_2$ase substrates tested, including $N_2$, $C_2H_2$, acrylonitrile, propionitrile and acetonitrile, and evolved $H_2$ concurrently with these reductions or exclusively in the absence of added reducible substrate. The reduction of only $N_2$ was inhibited by $H_2$; inhibition by CO was observed where tested, i.e., with $N_2$, $C_2H_2$ and acrylonitrile reductions. V-$N_2$ase was assayed with the same reaction

mixture used routinely (3) for Mo-$N_2$ase and was saturated at the levels of ATP (5 mM) and $Na_2S_2O_4$ (20 mM) in this mixture, though $K_M$ values were not determined. Unless stated otherwise V-$N_2$ase values are compared with Mo-$N_2$ase values obtained concurrently using purified (PS-2) preparations.

V-$N_2$ase coupled activated electrons to added reducible substrates only 25-35% as effectively as Mo-$N_2$ase, and accordingly allocated a higher fraction of these electrons to $H_3O^+$ for $H_2$ evolution (Table II). V-$N_2$ase was similar to Mo-$N_2$ase, however, in the recently described $D_2O$ enhancement of electron allocation to nitriles, such as acrylonitrile (12) and acetonitrile (8).

TABLE II. ELECTRON ALLOCATION IN V- AND Mo-$N_2$ase

| Substrate | Products | % of Electrons allocated to Substrate* | |
|---|---|---|---|
| | | V-$N_2$ase | Mo-$N_2$ase |
| $N_2$ | $NH_3$ | 25 | 70 |
| $C_2H_2$ | $C_2H_4$ | 35 | 98 |
| $CH_2CHCN$ | $C_3H_6$, $C_3H_8$ | 5 | 23 |
| $CH_2CHCN$ (in $D_2O$) | $C_3H_3D_3$, $C_3H_3D_5$ | 9 | 37 |

*Balance of electrons allocated to $H_3O^+$.

TABLE III. REDUCTION AND CO INHIBITION KINETICS IN V- AND Mo-$N_2$ase

| Preparation | $N_2$ Reduction | | | | $C_2H_2$ Reduction | | | |
|---|---|---|---|---|---|---|---|---|
| | $K_M$ atm $N_2 \times 10^2$ | | $K_I$ atm CO$\times 10^5$ | | $K_M$ atm $C_2H_2 \times 10^3$ | | $K_I$ atm CO$\times 10^5$ | |
| | V | Mo | V | Mo | V | Mo | V | Mo |
| 1 | 26 | | | | | | | |
| 1 | 19 | 18 | | | | | | |
| 2 | 28 | 16 | 76 | * | 24 | 5 | 54 | 8 |
| 3 | 19 | 16 | 30 | 5 | | | | |
| 3 | | 16 | | 10 | | | | |
| 4 | 29 | 15 | 62 | 3 | 16 | 5 | 19 | 7 |
| Average | 24 | 16 | 56 | 6 | 20 | 5 | 36 | 8 |
| Literature (9,15,17) | | 16 | | 29 | | 4 | | 31 |

*Inhibition not competitive.

Michaelis and CO inhibition constants were obtained for $N_2$ and $C_2H_2$ reductions by four V- and Mo-$N_2$ase preparations (Table III). The

V-$N_2$ase values were consistently higher, with the least effect apparent in the $K_M$ values for $N_2$ reduction. Inhibition by CO was clearly competitive in V-$N_2$ase reactions, but the competitive nature of inhibition in the comparable Mo-$N_2$ase reactions was not always clearly demonstrable, particularly at CO levels >0.0001 atm. In the present work Mo-$N_2$ase showed greater sensitivity to CO than observed previously (9), and was consistently more sensitive to CO than V-$N_2$ase. For acrylonitrile reduction a $K_M$ greater than 10 mM, the value previously determined with Mo-$N_2$ase (12), was indicated, but a more exact value could not be established. The ratio of $C_3H_6$:$C_3H_8$ formed in the reduction of acrylonitrile was consistently ca. 50% less with V-$N_2$ase than with Mo-$N_2$ase (Table IV). This difference was also observed with whole cells which were incubated under Ar:$O_2$ (80:20) in media supplemented with 10 mM acrylonitrile; analyses made after 18 hrs. showed $C_3H_6$:$C_3H_8$ ratios of 4.9 for Mo-cells and 1.5 for V-cells.

TABLE IV. COMPARISON OF ACRYLONITRILE REDUCTION BY V- AND Mo-$N_2$ase

| Preparation | $CH_2CHCN$ (mM) | Reaction Solvent | Products* $C_3H_6$ | $C_3H_8$ | $\frac{C_3H_6}{C_3H_8}$ |
|---|---|---|---|---|---|
| V-$N_2$ase | 10 | $D_2O$ | 19.9 | 6.1 | 3.2 |
| | 10 | $H_2O$ | 10.3 | 3.3 | 3.1 |
| | 20 | $H_2O$ | 35 | 14 | 2.5 |
| Mo-$N_2$ase | 10 | $D_2O$ | 592 | 88 | 6.8 |
| | 10 | $H_2O$ | 390 | 56 | 6.9 |
| | 20 | $H_2O$ | 1087 | 154 | 7.1 |

*nmoles, in addition to $H_2$; reactions incubated to completion.

Arrhenius plots were prepared from specific activity values of $C_2H_2$ reduction by V-$N_2$ase and Mo-$N_2$ase over the range 10-35° (Fig. 1). The activation energy for the Mo-$N_2$ase, calculated from Figure 1, is 14 and 35 kcal/mole above and below the critical temperature of ca. 20°, respectively, in agreement with previously determined values (9,13). The characteristic inflection at ca. 20° is also apparent with the V-$N_2$ase curve, but the activation energies are calculated to be slightly less, 10 kcal/mole above 20° and 30 below.

DISCUSSION: The formation in V cells of a V analog of the Mo-Fe protein - possibly differing only in metal content - is the most direct interpretation of the comparisons described; the isolation and characterization of a possible V analog will be reported elsewhere. The parallel behavior

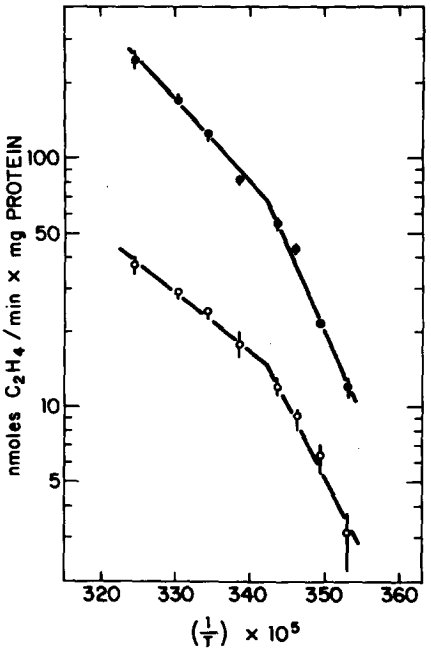


Figure 1. Arrhenius plot of $C_2H_2$ reduction activity of Mo-$N_2$ase (•) and V-$N_2$ase (o). Reaction mixtures of 1-2 ml contained (in μmoles/ml) 5 ATP, 30 creatine phosphate, 5 $MgCl_2$, 20 $Na_2S_2O_4$, and (in mg/ml) 0.1 creatine kinase and 1.5-2.6 mg Mo-$N_2$ase or 3.0-6.0 mg V-$N_2$ase; pH, 7.2; atmosphere, 10% $C_2H_2$ in Ar; incubation time, 4-10 min.

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of the two $N_2$ases through an almost identical purification sequence bespeaks a considerable degree of equivalence, as do the similarities in reaction requirements, reactions catalyzed, and activation energies, including the highly specific inflection in Arrhenius curves at ca. 20°. Instability induced by the presence of V in a Mo site(s) could account for the uniformly poorer performance of V-cells and preparations relative to their Mo counterparts, as observed in cell growth, specific activity, heat tolerance and storability comparisons. The slightly lower activation energy indicated for V-$N_2$ase could also stem from the destabilizing effect of such a modification.

The kinetic data implicate Mo in substrate binding, particularly with $C_2H_2$ and acrylonitrile, but less so with $N_2$. Involvement in product dissociation may be inferred from the altered ratio of acrylonitrile reduction products which presumably reflects the ease of $C_3H_6$ release from $N_2$ase; the substantially lower $C_3H_6$:$C_3H_8$ ratio found for V-$N_2$ase suggests that $C_3H_6$ dissociates from V-$N_2$ase less readily than from Mo-$N_2$ase, resulting in more $C_3H_6$ undergoing further reduction to $C_3H_8$.

Thus, product release as well as substrate complexation may be modified with V-$N_2$ase, implicating Mo through the complete sequence of reducible substrate-enzyme interaction. A similarly extensive function for Mo is indicated in a recent inorganic model for $N_2$ase (16).

The existence of more than a single reducible substrate binding site, implied by the relatively slight effect of V-$N_2$ase on $N_2$ complexation, is in general agreement with conclusions drawn from inhibition analyses (14), and supports a recently advanced mechanism (11) in which $N_2$ is proposed to bind initially at an $H_2$-sensitive Fe site, subsequently bridging to a Mo site, while other reducible substrates (e.g., $C_2H_2$) complex only at the Mo site; the present data indicate CO binding at the Mo site but do not preclude other CO-sensitive sites. The proposed mechanism places Mo at the terminus of the electron activation system, serving as the site of added substrate reduction and as either the $H_2$ evolution site or closely coupled to that site; Mo would thus be intimately associated with electron allocation, a function supported by the present work.

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