Biochem J. (1988) 256, 429–432 (Printed in Great Britain)

Molybdenum and vanadium nitrogenases of *Azotobacter chroococcum*

Low temperature favours N_2 reduction by vanadium nitrogenase

Richard W. MILLER* and Robert R. EADY†‡

*Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada,
and †AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.

A comparison of the effect of temperature on the reduction of N_2 by purified molybdenum nitrogenase and vanadium nitrogenase of *Azotobacter chroococcum* showed differences in behaviour. As the assay temperature was lowered from 30 °C to 5 °C N_2 remained an effective substrate for V nitrogenase, but not Mo nitrogenase, since the specific activity for N_2 reduction by Mo nitrogenase decreased 10-fold more than that of V nitrogenase. Activity cross-reactions between nitrogenase components showed the enhanced low-temperature activity to be associated with the Fe protein of V nitrogenase. The lower activity of homologous Mo nitrogenase components, although dependent on the ratio of MoFe protein to Fe protein, did not equal that of V nitrogenase even under conditions of high electron flux obtained at a 12-fold molar excess of Fe protein.

INTRODUCTION

Azotobacter chroococcum has two genetically distinct systems for nitrogen fixation, the well-characterized molybdenum nitrogenase (Mo nitrogenase) (Yates & Planque, 1975) and a recently isolated vanadium nitrogenase (V nitrogenase) (Robson *et al.*, 1986*a*; Eady *et al.*, 1987). A V nitrogenase has also been isolated from *Azotobacter vinelandii* (Hales *et al.*, 1986*a*,*b*) vindicating the suggestion of Bishop *et al.* (1980, 1982) that azotobacters possess an Mo independent nitrogenase. The V nitrogenase of *A. chroococcum* has similar overall requirements for activity to Mo nitrogenase, but differs in that the VFe protein has an additional low-molecular-mass subunit, and has an $\alpha_2\beta_2\delta_2$ subunit structure (Eady *et al.*, 1988*a*) compared with the $\alpha_2\beta_2$ structure of MoFe proteins. The substrate specificity also differs in that C_2H_2 or N_2 do not compete as effectively with H^+ as reducible substrates, and C_2H_6, in addition to C_2H_4, is a product of C_2H_2 reduction (Dilworth *et al.*, 1987, 1988). At 30 °C under N_2 the proportion of electron flux through nitrogenase resulting in the reduction of N_2 (the electron allocation coefficient) for V nitrogenase is 0.5 (Eady *et al.*, 1987), compared with 0.75 for Mo nitrogenase (Simpson & Burris, 1984), the remaining electrons being used for the reduction of H^+. Data presented here show that in contrast to Mo nitrogenase, N_2 remains an effective substrate for V nitrogenase as the assay temperature is decreased from 30 °C to 5 °C. In addition, activity cross-reactions between the components of Mo and V nitrogenase show that this enhanced low temperature activity is associated with the Fe protein of V nitrogenase.

MATERIALS AND METHODS

The Mo nitrogenase component proteins of *A. chroococcum* were purified and assayed as described by Yates & Planque (1975) and the V-nitrogenase components as described by Robson *et al.* (1986*a*) and Eady *et al.* (1987, 1988*b*). H_2 evolution and N_2 reduction were determined by standard methods using sodium dithionite as reductant, as described previously (Eady *et al.*, 1987). The specific activities (nmol of H_2 evolved/min per mg of protein) determined at 30 °C with an optimal amount of the corresponding complementary protein were: $Ac1^{Mo}$, 1734; $Ac2^{Mo}$, 1445; $Ac1^{V}$, 1803; and $Ac2^{V}$, 1763. Activities reported below were determined at specified ratios of Fe protein to MoFe or VFe protein corresponding to non-optimal, but expected physiological ratios of the nitrogenase components. $Ac1^{Mo}$ and $Ac1^{V}$ were not active when assayed in the absence of either Fe protein. Ammonia formation was estimated directly by the method of Corbin (1984) or from the difference in the rate of H_2 production under Ar and N_2. The values determined indirectly were within 10% of the values determined fluorimetrically. In all cases the rates of nitrogenase-catalysed product formation were linear with time, proportional to the MoFe or VFe protein concentration and were not limited by $S_2O_4^{2-}$, indicating that ATP levels maintained by a standard creatine phosphate–creatine

Abbreviations used: For a number of years the abbreviations of Eady *et al.* (1972) for components of Mo nitrogenase have been widely used. In this system Acl denotes the MoFe protein (comonent 1) and Ac2 the Fe protein (component 2) of the Mo nitrogenase of *A. chroococcum*. It is now clear that some azotobacters have three genetically distinct nitrogenase systems (Bishop *et al.*, 1988; Eady *et al.*, 1988*a*) and modification of the abbreviation system is required. We propose to use $Ac1^{Mo}$ and $Ac2^{Mo}$ for the components of Mo nitrogenase of *A. chroococcum* and $Ac1^{V}$ and $Ac2^{V}$ for those of the V nitrogenase. $Ac1^{V}$, therefore, supersedes Ac1*, and $Ac2^{V}$ supersedes Ac2* used previously (Robson *et al.*, 1986*a*; Eady *et al.*, 1987, 1988*b*).

‡ To whom correspondence should be addressed.

Table 1. The effect of temperature on the activity of V nitrogenase and Mo nitrogenase for N_2 and protons as substrates

All activities were measured using a 4-fold molar excess of the corresponding Fe protein of Mo or V nitrogenase at the temperatures indicated. The specific activities (nmol of NH_3 or H_2 formed/min per mg of protein) were determined for MoFe protein ($Ac1^{Mo}$) and VFe protein ($Ac1^{V}$) for N_2 reduction and concomitant H_2 evolution, and for H_2 evolution under Ar as described in the Materials and methods section. The values presented are expressed as a percentage of the rates at 30 °C under these conditions which for $Ac1^{Mo}$ were: NH_3, 436; H_2 under N_2, 296; and H_2 under Ar 1065. Those for $Ac1^{V}$ were: NH_3, 259; H_2 under N_2, 375; and for H_2 under Ar 830. The maximum specific activities of these proteins determined at optimum but non-physiological Fe protein levels are given in the Materials and methods section.

Assay temperature (°C)	Activity (% of specific activity at 30 °C)							
	NH_3		H_2 under N_2		Total e pair flux under N_2		H_2 under Ar	
	$Ac1^{V}$	$Ac1^{Mo}$	$Ac1^{V}$	$Ac1^{Mo}$	$Ac1^{V}$	$Ac1^{Mo}$	$Ac1^{V}$	$Ac1^{Mo}$
30	(100)	(100)	(100)	(100)	(100)	(100)	(100)	(100)
22	–	–	–	–	–	–	64	56
16	30	36	8.7	7.5	20	25	22	25
10	9.1	7.1	5.9	4.6	6.8	6.3	7.7	5.7
7.5	3.8	2.6	4.5	2.9	4.1	2.9	4.1	2.9
5	2.4	0.24	0.57	0.23	1.3	0.37	1.9	0.6

kinase system, or reductant, were not limiting under the conditions of assay.

RESULTS AND DISCUSSION

Extensive kinetic studies of Mo nitrogenase have shown that the rate of electron flux through the enzyme determines the distribution of electrons to the various alternative substrates N_2, H^+, C_2H_2 etc. (see Lowe *et al.*, 1985, for discussion). The effect on nitrogenase activity of decreasing the assay temperature from the usual 30 °C is complex. For example, Watt & Burns (1977) reported that for the Mo nitrogenase of *Azotobacter vinelandii* the activation energy for substrate reduction was higher than for MgATP hydrolysis and at 10 °C the rate of substrate reduction was very low despite continued MgATP hydrolysis. In the case of Mo nitrogenase of *Klebsiella pneumoniae*, decreasing the temperature also curtailed electron flux, but also resulted in the preferential loss of activity towards N_2 as a substrate compared with either H^+ or C_2H_2 (Thorneley & Eady, 1977).

Effect of temperature on electron allocation to N_2 and protons

The effect of temperature in the range 30 °C to 5 °C on the rate of reduction of N_2 and concomitant H_2 evolution, and the rate of H_2 evolution under Ar, was determined for purified Mo and V nitrogenase systems of *A. chroococcum*. Results presented in Table 1 show that as the assay temperature was decreased over this 25 °C range, the specific activity for N_2 reduction by Mo nitrogenase decreased 400-fold compared with 40-fold for V nitrogenase. H_2 evolution concomitant with N_2 reduction showed decreases of 400-fold and 180-fold respectively. The effect of temperature on total electron flux (measured as H_2 evolution under Ar) was 270-fold and 76-fold for Mo and V nitrogenase systems respectively. These results indicate that, in contrast with Mo nitrogenase, N_2 is preferentially reduced by V nitrogenase as the temperature is decreased.

The effect of component ratio on substrate reduction at 5 °C

For both Mo and V nitrogenases when assayed at 5 °C, increasing the ratio of Fe protein to MoFe or VFe protein from 1 to12 markedly increased the electron flux to N_2 or H^+ reduction (Fig. 1). An approximately linear increase towards both substrates was observed with Mo nitrogenase with a 10-fold increase in activity occurring over this range of component ratios. In contrast, a saturation curve was observed for N_2 as a substrate for V nitrogenase with little increase in the rate of N_2 reduction occurring above a component ratio of a 4-fold excess of Fe protein. In addition, the specific activity of V nitrogenase with N_2 or H^+ as substrate was 2–3-fold higher than that of Mo nitrogenase in the lower range of component ratios. These data are consistent with V nitrogenase achieving the appropriate redox level for N_2 reduction at a lower electron flux than is required by Mo nitrogenase. This behaviour, which has also been observed at 30 °C (Eady *et al.*, 1987) may be an important factor in allowing N_2 reduction by V nitrogenase to continue over a wider temperature range than Mo nitrogenase.

The efficiency of N_2 reduction by V nitrogenase at 5 °C is highest at component ratios of $Ac1^{V}$ and $Ac2^{V}$ below 4 (Fig. 1), ratios which are probably in the physiological range. This is a consequence of the rate of proton reduction increasing with component ratios greater than 4, while N_2 reduction is already at a maximum at a 4-fold excess of $Ac2^{V}$ over $Ac1^{V}$.

The effect of the origin of the Fe protein on substrate reduction efficiency

The components of Mo and V nitrogenase of *A. chroococcum* are sufficiently similar to form functional hybrid nitrogenases (Robson *et al.*, 1986*a*; Eady *et al.*, 1987, 1988*b*). Such activity cross-reactions show that the characteristic substrate-reduction patterns of V nitrogenase, i.e., C_2H_6 formation from C_2H_2 (Dilworth *et al.*,

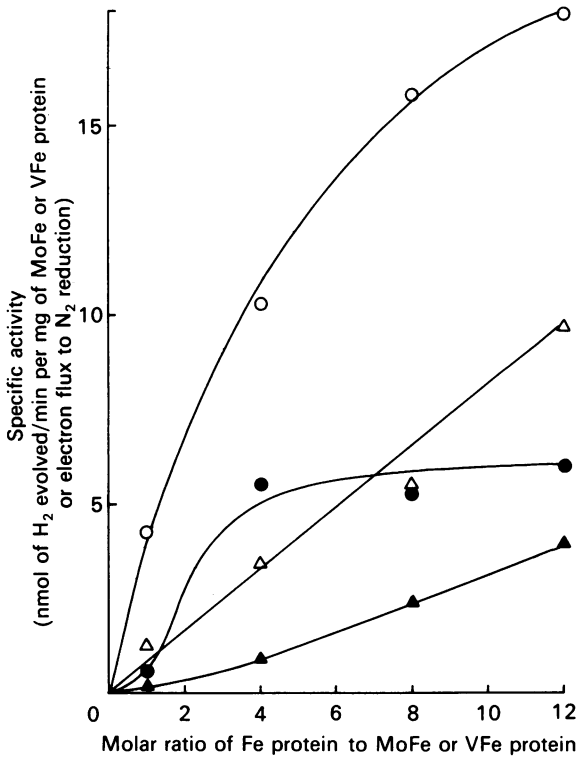

Fig. 1. Effect of the ratio of nitrogenase components on H_2 evolution under Ar and electron allocation to N_2 as a substrate at 5.2 °C

Nitrogenase activity at 5.2 °C was measured as H_2 evolution under Ar or under N_2 after 1 h incubation as described in the Materials and methods section. Assays contained $Ac1^{Mo}$ (0.8 μM) or $Ac1^{V}$ (0.65 μM) and were complemented with $Ac2^{Mo}$ or $Ac2^{V}$ as appropriate, at the molar ratios indicated. Electron pair allocation to N_2 reduction was calculated from the difference in H_2 produced under N_2 compared with Ar. Specific activities for H_2 evolution under Ar were: (○), $Ac1^{V}$; (△), $Ac1^{Mo}$; those for e pairs utilized for N_2 reduction were: (●), $Ac1^{V}$; (▲), $Ac1^{Mo}$.

Table 2. Dinitrogen reduction by $Ac1^{Mo}$ and $Ac1^{V}$ at 30 °C and 10 °C when complemented with either $Ac2^{Mo}$ or $Ac2^{V}$

Specific activities of $Ac1^{Mo}$ and $Ac1^{V}$ were measured in duplicate assays containing a 4-fold molar excess of Fe protein under the conditions of Table 1. NH_3 was measured fluorimetrically by the method of Corbin (1984) and H_2 by gas chromatography.

Protein combination	Product	Specific activity (nmol of product formed/min per mg of protein) 10 °C	30 °C
$Ac1^{Mo}$–$Ac2^{Mo}$	NH_3	31.3±0.8	437±32
	H_2 under N_2	13.6±0.3	296±4.9
	Total 2e flux	60.6±1.7	950±50
$Ac1^{Mo}$–$Ac2^{V}$	NH_3	43.9±5.0	377±14
	H_2 under N_2	27.77±0.6	361±23
	Total 2e flux	93.52±7.8	926±3.5
$Ac1^{V}$–$Ac2^{V}$	NH_3	23.4±2.1	259±8.0
	H_2 under N_2	21.1±1.1	375±18
	Total 2e flux	56.2±1.0	764±30
$Ac1^{V}$–$Ac2^{Mo}$	NH_3	14.0±0.2	162±1.4
	H_2 under N_2	13.9±0.5	233±10
	Total 2e flux	35.0±0.4	476±11

1987) and relatively high rates of H_2 evolution in the presence of N_2 or C_2H_2 (Eady *et al.*, 1987) are a characteristic of the VFe protein.

To establish if the differential effect of temperature on the activity of V nitrogenase when compared with Mo nitrogenase was due to some property of the VFe protein, the activity of both systems, and also the active hybrid $Ac1^{Mo}$–$Ac2^{V}$, and $Ac1^{V}$–$Ac2^{Mo}$ systems were compared at 10 °C and 30 °C. Data for the reduction of N_2 and concomitant H_2 evolution were obtained at a 4-fold molar excess of either $Ac2^{Mo}$ or $Ac2^{V}$ and are shown in Table 2. The higher proportion of electrons directed to H_2 evolution under an atmosphere of N_2 at 30 °C by V nitrogenase compared with Mo nitrogenase (Eady *et al.*, 1987) still occurred at 10 °C in assays containing $Ac1^{V}$. For $Ac1^{Mo}$, although the total electron flux at 30 °C was similar whichever Fe protein was used in the assay, the total electron flux and the activity towards N_2 was significantly higher at 10 °C when assayed with $Ac2^{V}$ compared with $Ac2^{Mo}$. In contrast, the total electron flux through the $Ac1^{V}$–$Ac2^{Mo}$ system was attenuated by approx. 40% compared with $Ac1^{V}$–$Ac2^{V}$ at both temperatures. However, at 10 °C the total electron flux and activity towards N_2 was greatest when $Ac2^{V}$ was used in the assay (Table 2). These data clearly indicate that, when assayed at 10 °C, the specific activity of both $Ac1^{V}$ and $Ac1^{Mo}$ is highest when assayed with $Ac2^{V}$, and that under these conditions it is the Fe protein associated with V nitrogenase which confers the property of more effective N_2 reduction at low temperatures.

The overall mechanisms of substrate reduction by Mo and V nitrogenase, although not yet compared in detail, are likely to be similar since hybrid cross-reactions are active, a situation not always observed with Mo nitrogenase components purified from different organisms (see Eady & Postgate, 1974). The observed differences in temperature dependence on activity may reflect the difference in activation energy of the rate-limiting reaction in nitrogenase turnover. For Mo nitrogenase of *Klebsiella pneumoniae* the rate-limiting step at 23 °C has been identified as the rate of dissociation of the nitrogenase protein complex, $Kp2_{ox}$ $(MgADP)_2$–$Kp1_{red}$, following electron transfer from the Fe protein to the MoFe protein (Thorneley & Lowe, 1983). Our observation that it is the Fe protein which confers the altered temperature behaviour of V nitrogenase would suggest that, provided the rate-limiting step is unchanged at 10 °C, the rate of dissociation of $Ac2^{V}{}_{ox}$ $(MgADP)_2$ from either $Ac1^{Mo}$ or $Ac1^{V}$ protein should be faster than the rate of dissociation of $Ac2^{Mo}{}_{ox}$ $(MgADP)_2$.

$Ac2^{Mo}$ and $Ac2^{V}$ have very similar physicochemical properties. The polypeptide sequences differ in only 32 out of 289 amino acid residues (Robson *et al.*, 1986*b*) and both contain a [4Fe4S] centre (see Eady *et al.*, 1988*b*; Yates & Planque, 1975) of similar redox potential and rate of reduction by $SO_2^{\cdot-}$ (Bergström *et al.*, 1988). This close similarity would suggest that a programme of

site-directed mutagenesis to improve the efficiency of Mo nitrogenase by changing $Ac2^{Mo}$ for higher efficiency at low temperatures might be feasible.

The contribution made by organisms fixing N_2 via V nitrogenase to global nitrogen cycling is not known. Although the availability of Mo or V regulates the expression of Mo-independent nitrogenase systems in azotobacters (see Bishop *et al.*, 1988), the V nitrogenase could potentially make a significant contribution at low temperature under conditions of Mo limitation or where regulation by Mo was lacking, if our observations are applicable *in vivo*.

It has been shown that bacteroids formed in the *Rhizobium meliloti* alfalfa symbiotic system can maintain nitrogenase activity (including N_2 reduction as determined from the difference in the rate of H_2 evolution under Ar and N_2) to lower temperature than does Mo nitrogenase when assayed *in vitro* (Miller *et al.*, 1986). It has been proposed that this difference is due to a higher adenylate energy charge being developed in bacteroids as the temperature is lowered, resulting in an increase in nitrogenase activity (Miller *et al.*, 1986). However, in view of the data reported here, this phenomenon could arise from differences in the behaviour of the Fe protein *in vivo* compared with isolated Mo nitrogenase. Further work is required to clarify this possibility.

We thank Dr. R. Robson for useful discussions, Marie Eldridge for skilled technical assistance, Dr. B. E. Smith for constructive comments on the manuscript and Beryl Scutt for typing.

REFERENCES

Bergström, J., Eady, R. R. & Thorneley, R. N. F. (1988) Biochem. J. **251**, 165–169

Bishop, P. E., Jarlenski, D. M. L. & Hetherington, D. R. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 7342–7346

Bishop, P. E., Jarlenski, D. M. L. & Hetherington, D. R. (1982) J. Bacteriol. **150**, 1244–1251

Bishop, P. E., Premakumar, R., Joerger, R. D., Jacobson, M. R., Dalton, D. A., Chisnell, J. R. & Wolfinger, E. D. (1988) Proc. 7th Int. Symp. Nitrogen Fixation, Cologne (Bothe, H. J. & Newton, W. E., eds.), pp. 71–79, Fisher Verlag, Stuttgart

Corbin, J. L. (1984) Appl. Environ. Microbiol. **47**, 1027–1030

Dilworth, M. J., Eady, R. R., Robson, R. L. & Miller, R. W. (1987) Nature (London) **327**, 167–168.

Dilworth, M. J., Eady, R. R. & Eldridge, M. (1988) Biochem. J. **249**, 745–751

Eady, R. R. & Postgate, J. R. (1974) Nature (London) **249**, 805–810

Eady, R. R., Smith, B. E., Cook, K. A. & Postgate, J. R. (1972) Biochem. J. **128**, 655–675

Eady, R. R., Robson, R. L., Richardson, T. H., Miller, R. W. & Hawkins, M. (1987) Biochem. J. **244**, 197–207

Eady, R. R., Robson, R. L., Pau, R. N., Woodley, P., Lowe, D. J., Miller, R. W., Thorneley, R. N. F., Smith, B. E., Gormal, C., Fisher, K., Eldridge, M. & Bergström, J. (1988*a*) Proc. 7th Int. Symp. Nitrogen Fixation, Cologne (Bothe, H. & Newton, W. E., eds.), pp. 81–86, Fisher Verlag, Stuttgart

Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Lowe, D. J. (1988*b*) Biochem. **256**, 189–196

Hales, B. J., Case, E. E., Morningstar, J. E., Dzeda, M. F. & Mauterer, L. A. (1986*a*) Biochemistry **25**, 7251–7255

Hales, B. J., Langosch, D. & Case, E. E. (1986*b*) J. Biol. Chem. **261**, 15301–15306

Lowe, D. J., Thorneley, R. N. F. & Smith, B. E. (1985) in Metalloproteins Part 1: Metal Proteins with Redox Roles (Harrison, P. M., ed.), pp. 207–247, Macmillan, London

Miller, R. W., Al-Jabore, A. & Berndt, W. B. (1986) Can. J. Biochem. **64**, 556–564

Robson, R. L., Eady, R. R., Richardson, T. H., Miller, R. W., Hawkins, M. & Postgate, J. R. (1986*a*) Nature (London) **322**, 388–390

Robson, R. L., Woodley, P. & Jones, R. (1986*b*) EMBO J. **5**, 1159–1163

Thorneley, R. N. F. & Eady, R. R. (1977) Biochem. J. **167**, 457–461

Thorneley, R. N. F. & Lowe, D. J. (1983) Biochem. J. **215**, 393–405

Simpson, F. & Burris, R. H. (1984) Science **244**, 1095–1097

Watt, G. D. & Burns, A. (1977) Biochemistry **16**, 264–270

Yates, M. G. & Planque, K. (1975) Eur. J. Biochem. **60**, 467–476

Received 17 May 1988/accepted 30 June 1988