Nitrogenase of *Klebsiella pneumoniae*

DISTINCTION BETWEEN PROTON-REDUCING AND ACETYLENE-REDUCING FORMS OF THE ENZYME: EFFECT OF TEMPERATURE AND COMPONENT PROTEIN RATIO ON SUBSTRATE-REDUCTION KINETICS

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Non-linear rates of acetylene reduction and concomitant H₂ evolution were observed for the nitrogenase of *Klebsiella pneumoniae* at 10°C. A lag phase of 1–4 min, dependent on the ratio of Mo–Fe protein to Fe protein present, occurred before linear rates of acetylene reduction were achieved. A complementary burst phase for concomitant H₂ evolution in the presence of acetylene was also observed. When the proton was the only reducible substrate present, linear rates of H₂ evolution were observed. N₂ was a poor substrate under these conditions. Similar lag and burst phases occurred at 30°C, but only when a large molar excess of Mo–Fe protein with respect to Fe protein was present. The results at 10°C show that the binding of acetylene to the enzyme stimulates electron flow, but that these electrons, which initially reduce protons, can only reduce acetylene after a lag phase that cannot be accommodated in the turnover time calculated under steady-state conditions.

Klebsiella pneumoniae nitrogenase consists of two proteins: a tetrameric protein, Kp1* or Mo–Fe protein, mol.wt. 220000, and a dimeric protein, Kp2 or Fe protein, mol.wt. 67000. Both proteins, in an anaerobic environment, MgATP and a source of electrons, probably ferredoxin or flavodoxin *in vivo* and sodium dithionite *in vitro*, are required for activity. The enzyme will reduce N₂ to NH₃, acetylene to ethylene and protons to H₂. Each of the substrate reductions is accompanied by the hydrolysis of MgATP to MgADP (see Zumft & Mortenson, 1975). Different forms of the enzyme capable of reducing only protons, protons plus acetylene and protons plus N₂ have been identified in studies with a heterologous nitrogenase consisting of the Mo–Fe protein from *K. pneumoniae* and the Fe protein from *Clostridium pasteurianum* (Smith *et al.*, 1976a, 1977). Linear rates of product formation were only obtained after extensive lag phases (10 min for ethylene production and 35 min for NH₃ production) that could not be accommodated within the turnover time of the enzyme. No

lag phase was observed for H₂ evolution. These results were attributed to different forms of the enzyme being capable of reducing the three different substrates and the lag phases representing slow activation reactions necessary before acetylene or N₂ reduction was possible. Since these effects were obtained with a hybrid enzyme with component proteins from different organisms, it could be argued that they have little relevance to the situation *in vivo* within a single organism. We now report the observation of similar effects with the homologous enzyme from *K. pneumoniae* under certain conditions of temperature and ratio of component proteins.

Materials and Methods

Growth of organism and purification of nitrogenase proteins

K. pneumoniae M5a1 was grown and the nitrogenase proteins from this organism purified by established methods (Eady *et al.*, 1972; Smith *et al.*, 1976b). These methods yielded nitrogenase proteins with the following specific activities in the acetylene-reduction assay: Kp1 protein, 1600–2000; Kp2 protein, 1100–1500 nmol of ethylene produced/min per mg of protein. The mol.wts. used were: for Kp1 protein, 220000, and for Kp2 protein, 67000.

* Abbreviations: The nitrogenase proteins from various organisms are denoted by a capital letter indicating the genus, a lower-case letter indicating the species and either the number '1' indicating Mo–Fe protein or '2' indicating Fe protein. Kp is *Klebsiella pneumoniae*, Cp is *Clostridium pasteurianum*, Av is *Azotobacter vinelandii*, Ac is *Azotobacter chroococcum*. E.p.r., electron paramagnetic resonance.

Assay procedures

The production of ethylene from acetylene, H_2 from H_3O^+ and NH_3 from N_2 by nitrogenase was measured by established assay procedures (Eady *et al.*, 1972), with the following changes. An increased $Na_2S_2O_4$ concentration of 30mM and a pH of 7.4 were used. Addition of premixed solutions of Kp1 and Kp2 proteins in the appropriate ratio by a single syringe was used to initiate the reaction. The addition of protein and replacement in the thermostatically maintained shaker bath at 10 or 30°C took 5 ± 1 s. A similar time was taken for assay termination by syringe addition of 0.1 ml of 30% (w/v) trichloroacetic acid. In time-course experiments, a number of assays stopped after various times were used rather than periodic gas sampling from a single assay, to avoid underestimation of the gaseous products owing to slow equilibration between liquid and gas phases.

Results

The effects of temperature and ratio of component proteins on various substrate reductions have been investigated and are summarized in Table 1.

Ethylene production

Time courses for the amount of ethylene produced at three component ratios at 10°C are presented in Fig. 1. In all cases a linear rate of ethylene production was only obtained after a lag phase. The length of the lag phase varied from 1 to 4 min depending on protein

Fig. 1. Effect of component-protein ratio on the time course of acetylene reduction at 10°C

The assay conditions were as described in the text. The broken lines are an extrapolation of the linear section of the curves, and the abscissa intercepts of these lines were used as a measure of the lag phase. In all three curves $[Kp2 \text{ protein}] = 0.9 \mu M$; ■, $[Kp1 \text{ protein}] = 0.2 \mu M$; ●, $[Kp1 \text{ protein}] = 0.9 \mu M$; ▲, $[Kp1 \text{ protein}] = 2.6 \mu M$. The data are summarized in Table 1.

Table 1. Effect of temperature and component-protein ratio on substrate-reduction kinetics

Substrate reduction was measured as described in the Materials and Methods section at the temperatures and component-protein ratios indicated. Steady-state rates refer to the linear region of the substrate-reduction time course. The lag period of acetylene reduction was obtained by extrapolation of the linear rate to the time abscissa.

Temp.	[Protein Kp1] (μM)	[Protein Kp2] (μM)	Protein ratio ([Kp1]:[Kp2])	Steady-state rates of product formation (nmol/min per mg of Kp2 protein)					Acetylene reduction	
				Ethylene	H_2 under acetylene	Total (ethylene+ H_2)	H_2 under argon	NH_3	H_2 under N_2	Total electrons ($\frac{2}{3}NH_3 + H_2$ under N_2)
10°C	2.6	0.9	2.9:1.0	66	7	73	31	<12	28	28-46
	0.9	0.9	1.0:1.0	91	3	94	35	<5	29	29-36
	0.2	0.9	1.0:4.5	12.5	<0.3	12.5-12.8	7.4	<5	4.5	4.5-11
30°C	2.0	2.2	1.0:1.1	1187	92	1279	1572	371	684	1241
	2.0	0.22	9.0:1.0	1397	185	1582	1797	148	1387	1610
	2.0	0.09	22:1.0	1068	401	1469	1555	<42	1521	1521-1584
									Observed lag (s)	Calc. turnover time (s)
									240	13
									120	9
									~60	70
									<20	0.77
									70	0.66
									240	0.86

ratio (Table 1). The abscissa intercept of an extrapolation of the linear section of the reaction progress curve was used as a measure of the lag phase. Similar effects were obtained at 30°C, but only at molar ratios of Kp1 to Kp2 protein in an excess of 1:1 (Table 1). Under these conditions the length of the lag phase varied from about 20s to 4min. Lags of less than 30s were difficult to measure, owing to the difficulty in starting and stopping assays at such short times. The steady-state rates of ethylene production after the lag phase are consistent with more detailed titration curves at 30°C, which indicate maximal activity at a Kp1/Kp2 protein ratio close to 1:1 (Eady *et al.*, 1972).

Concomitant H₂ evolution during ethylene production

Results for H₂ evolution under conditions that induce pronounced lag phases for ethylene production at 10°C and 30°C are presented in Figs. 2 and 3. In both cases a 'burst phase' of H₂ evolution, complementary to the lag phase for ethylene production, was observed before a constant rate of H₂ evolution was established. This burst phase must be a catalytic process, since its amplitude is much greater than could be obtained from a single enzyme turnover. The broken

Fig. 2. Time courses for acetylene reduction and concomitant H₂ evolution at 10°C

The assay conditions were as described in the text with [Kp1 protein] = 2.6 μ M and [Kp2 protein] = 0.9 μ M. $\circ$, Ethylene production; Δ , H₂ production. The broken line was obtained by addition of the amounts of ethylene and H₂ produced and represents the total electron flow through the enzyme. The lag phase for ethylene production was complemented by a burst phase for H₂ evolution resulting in a constant electron flow. Similar bursts for H₂ evolution were obtained for the other two protein ratios used at 10°C in Fig. 1 for acetylene reduction. The specific activities for H₂ evolution presented in Table 1 were calculated from the slopes of the linear sections of the H₂ evolution curves after completion of the burst phase.

Fig. 3. Time courses for acetylene reduction and concomitant H₂ evolution at 30°C

The assay conditions were as described in the text with [Kp1 protein] = 2.0 μ M; [Kp2 protein] = 0.09 μ M. $\circ$, Ethylene production; Δ , H₂ production. The broken line was obtained by addition of the amounts of ethylene and H₂ produced and represents the total electron flow through the enzyme. Other details are as described in Fig. 2. Data at this and other protein ratios are summarized in Table 1.

lines in Figs. 2 and 3 represent the total electron flow obtained by summation of the amounts of H₂ and ethylene produced at a given time. These lines exhibit no significant lag or burst, clearly showing that total electron flow was constant over the times amenable to experiments using conventional assay techniques. The total electron-flow plots at other protein ratios, with one exception, showed no detectable lags or bursts. This was expected, since the turnover times based on the Kp2 protein concentration and the rate of total electron flow were less than 13s (Table 1). The one exception was at 10°C with an excess of Kp2 over Kp1 protein, when the turnover time was calculated to be 70s. In this case a lag phase of about 60s was observed for both acetylene reduction and total electron flow.

H₂ evolution under argon

Results for H₂ evolution under argon at 10°C and 30°C are presented in Fig. 4 and Table 1. Linear rates of H₂ evolution were observed with no detectable lag or burst phases. Only slight inhibition (approx. 10%) of H₂ evolution was observed when the Kp1/Kp2 protein ratio was increased from 1:1 to 3:1 at 10°C (Table 1). This contrasts with the situation for ethylene production, in which 30% inhibition was observed (Table 1). At 30°C both H₂ evolution and ethylene production show a decrease in activity of less than 10% on increasing the Kp1/Kp2 protein ratio from 1:1.1 to 22:1.

H₂ evolution under N₂, and NH₃ formation

At both 10°C and 30°C, linear rates of H₂ evolution under N₂ were observed with no detectable lag or

Fig. 4. Effect of component-protein ratio on the time course for H_2 evolution under argon at $10^\circ C$

The assay conditions were as described in the text with $[Kp2 \text{ protein}] = 0.9 \mu M$ and with $\blacksquare$, $[Kp1 \text{ protein}] = 0.2 \mu M$; $\bullet$, $[Kp1 \text{ protein}] = 0.9 \mu M$; $\blacktriangle$, $[Kp1 \text{ protein}] = 2.6 \mu M$. The broken line refers to the data points at $[Kp1 \text{ protein}] = 0.9 \mu M$. The specific activities for H_2 evolution obtained from the slopes of the lines are given in Table 1. No lag or burst phases were observed in these experiments.

burst phases. At $10^\circ C$ the steady-state rates were very similar to those determined under argon. The amounts of NH_3 produced at $10^\circ C$ were so low that only upper limits are quoted in Table 1. The scatter in the data did not allow the detection of either lag or burst phases, and it was concluded that under these conditions N_2 is a poor substrate. At $30^\circ C$, as the ratio of $Kp1/Kp2$ protein was increased, the rate of N_2 reduction decreased, with a corresponding increase in concomitant H_2 evolution, the total electron flow remaining constant. At the highest ratio used, the amount of NH_3 produced was at the limit of detection, indicating that N_2 was a poor substrate under these conditions.

Discussion

Nitrogenase will reduce a number of small triple-bonded substrates, including N_2 , azide, acetylene, cyanide and isocyanide. In addition, protons are reduced to H_2 , a reaction which is only partially suppressed in the presence of other reducible substrates. CO inhibits the reduction of all substrates except protons (Hardy & Burns, 1968). Mutual-competitive-inhibition studies with the various alternative substrates and CO have established that multiple or modified sites exist for different reducible substrates (Hwang *et al.*, 1973). A significant advance was the identification of different forms of nitrogenase capable of reducing protons but not acetylene (Smith *et al.*, 1976a), or acetylene and not N_2 (Smith *et al.*,

1977), by using a heterologous enzyme consisting of $Kp1$ and $Cp2$ proteins. With the heterologous $Kp1$ - $Cp2$ enzyme complex, a lag phase for acetylene reduction which could not be accommodated in the turnover time was observed. No such lag phase was observed for H_2 evolution under argon. Our data above show that a similar situation can occur for the homologous nitrogenase formed from $Kp1$ and $Kp2$ proteins at $10^\circ C$ or at $30^\circ C$ with an excess of $Kp1$ protein over $Kp2$ protein.

At $10^\circ C$ and $30^\circ C$ at a variety of component protein ratios, the turnover times based on the concentration of limiting protein were calculated to be less than 13 s. Hence the observed lags of up to 4 min for ethylene production must be associated with an activation process that does not re-occur during the steady-state linear phase of ethylene production. No such activation is required for the enzyme to evolve H_2 , since linear rates of H_2 evolution were observed under argon (Fig. 4). The burst phase for H_2 evolution in the presence of acetylene, during which the rate of H_2 evolution gradually falls as the rate of acetylene reduction increases means that electron flow is constant with time (Figs. 2 and 3). After activation, electrons that would otherwise reduce protons, are redirected to reduce acetylene. At $10^\circ C$ the total electron flow under acetylene (ethylene plus H_2 produced) was increased by up to 170% at high $Kp1/Kp2$ protein ratios relative to the total electron flow under argon (H_2 only produced). Initially all the electrons are directed into H_2 evolution, and only after the lag phase into ethylene production. At $10^\circ C$, where increased electron flow under acetylene was most evident, the initial detectable rate of H_2 evolution under acetylene was more than twice the linear steady-state rate of H_2 evolution under argon. We conclude that binding of acetylene to one or both of the nitrogenase component proteins stimulates electron flow through the enzyme. These additional electrons can only be used for acetylene reduction after the activation reaction associated with the lag phase.

Evidence for an acetylene-induced conformation change in dithionite-reduced $Kp1$ protein has previously been obtained from e.p.r. studies (Smith *et al.*, 1973). The increased electron flow induced by acetylene at $10^\circ C$ may be due to acetylene perturbing a temperature-sensitive conformational change. A conformational change occurring at about $15^\circ C$ in $Cp1$ protein has been deduced from an e.p.r. study of CO inhibition (W. Orme-Johnson, personal communication) and from the temperature dependence of steady-state data for acetylene reduction by *K. pneumoniae* nitrogenase at low protein concentrations (Thorneley *et al.*, 1975).

The redistribution of electrons between acetylene- and proton-reducing sites could be explained by the presence of two sites for H_2 evolution. One of these

two sites, after the activation process, is capable of reducing acetylene. It is presumably this site which is susceptible to CO inhibition (Hardy & Burns, 1968). An alternative explanation would involve one site for H_2 evolution and another for acetylene reduction. The presence of acetylene would induce a conformational change that results in increased electron flow into the H_2 evolution process; another slower conformational change (associated with the lag phase) would then have to occur before acetylene was reduced and ethylene released. The second conformational change, which is responsible for the lag phase and does not occur during turnover in the steady-state, could be regarded as an electron-gating mechanism that redirects electrons from the hydrogen- to the acetylene-reducing site. Slow transitions relative to turnover time are an increasingly common feature of enzyme systems and may be of regulatory importance. The term 'hysteresis' has been suggested by Frieden (1970) to describe such phenomena.

Our data illustrate a serious limitation in the use of the continuous-dithionite-oxidation assay which monitors total electron flow for nitrogenase (Ljones & Burris, 1972). The complementary lag and burst phases for ethylene and H_2 production respectively result in the total electron flow being constant, and hence these phases would not be observed with the dithionite oxidation assay. Indeed, Shah *et al.* (1975) have dismissed possible lags in ethylene formation as a gas-sampling artifact and have erroneously quoted the linearity of the dithionite-oxidation assay in support of their dismissive argument. However, we do agree, as discussed in the Materials and Methods section of the present paper, that a series of single stopped assays is much more reliable than successive sampling of a single assay during a time-course experiment. The complementary lag and burst phases that we report for ethylene and H_2 production respectively, which together result in constant electron flow, make it exceedingly unlikely that our observations are a gas-sampling artifact.

The lack of inhibition for acetylene reduction observed at 30°C as the Kp1/Kp2 protein ratio was increased is different from previously reported data (Eady *et al.*, 1972). We attribute this, in part, to the failure to take into account the extent of the lag phase in the earlier work, but more probably to the use of proteins of a higher specific activity in the present study.

The results presented in Table 1 are relevant to the interpretation of acetylene-reduction data collected to estimate nitrogen fixation in the field. At certain component-protein ratios, and more significantly at 10°C, acetylene-reduction assays greatly exaggerate the ability of purified nitrogenase to reduce N_2 to NH_3 . Division of the rate of ethylene formation by a factor of 3 does not give an accurate estimate of

nitrogen reductase activity. Under N_2 , a large number of the electrons available for reduction go into the H_2 evolution process. Schubert & Evans (1976) came to the same conclusion using assays both *in situ* and with excised roots of nodulated nitrogen-fixing symbionts, including soya beans. However, they assumed in their calculations that the rate of H_2 evolution under argon gives the total electron flux that would occur in the presence of other reducible substrates, i.e. N_2 or acetylene. They calculated their rates of N_2 reduction from the difference between rates of H_2 evolution under a mixture of argon, O_2 and CO_2 , and in air. Since we showed above that the total electron flux increases in the presence of acetylene and probably also in the presence of N_2 , the type of calculation done by Schubert & Evans (1976) to estimate N_2 -fixation rates would not be valid under the conditions of our experiments.

The ambient soil and assay temperature could be important factors in estimating nitrogen-fixation rates *in vivo*. The present observations may help to explain the differences in estimates of nitrogen-fixation rates when data from $^{15}N_2$ - and acetylene-reduction assays are used (Hardy *et al.*, 1973).

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