Klebsiella pneumoniae nitrogenase

Mechanism of acetylene reduction and its inhibition by carbon monoxide

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The electron flux through the MoFe-protein of nitrogenase from *Klebsiella pneumoniae* determines the absolute and relative rates of 2H^+ reduction to H_2 and acetylene (C_2H_2) reduction to ethylene (C_2H_4) at saturating levels of reductant ($\text{Na}_2\text{S}_2\text{O}_4$) and MgATP. High electron flux, induced by a high Fe-protein (Kp2)/MoFe protein (Kp1) ratio, favours C_2H_2 reduction. These data can be explained if ethylene, the two-electron reduction product of C_2H_2 , is not released until three electrons have been transferred from Kp2 to Kp1. This explanation is also consistent with a pre-steady-state lag phase for C_2H_4 formation of 250 ms observed when functioning enzyme is quenched with acid. Electron flux through nitrogenase is inhibited by C_2H_2 at high protein concentrations. This is because the association rate between Kp1 and oxidized Kp2 is enhanced by C_2H_2 , leading to an increased steady-state concentration of the inhibitory complex $\text{Kp2}_{\text{ox}}\text{Kp1C}_2\text{H}_2$. This effect is not relieved by CO. Thus CO and C_2H_2 (or C_2H_4) must be bound at the same time to distinct sites, presumably at Mo or Fe centres, on the enzyme.

INTRODUCTION

Klebsiella pneumoniae nitrogenase comprises two metalloproteins, the MoFe protein (Kp1, M_r 224000) and the Fe protein (Kp2, M_r 67000). It catalyses the reduction of N_2 to 2NH_3 , 2H^+ to H_2 and a number of alternative substrates in the presence of a reductant ($\text{Na}_2\text{S}_2\text{O}_4$ *in vitro*) with concomitant hydrolysis of MgATP to MgADP and P_i . The structure and mechanism of nitrogenase have been reviewed by Burgess (1985), Lowe *et al.* (1985), Orme-Johnson (1985), Stephens (1985), Thorneley & Lowe (1985) and Smith *et al.* (1988). Burgess (1985) also gives a comprehensive review of the reduction of additional substrates, including that of C_2H_2 to C_2H_4 (Dilworth, 1966; Schöllhorn & Burris, 1967).

Lowe & Thorneley (1984a,b) and Thorneley & Lowe (1984a,b) developed a comprehensive mechanism for H^+ and N_2 reduction which does not address the question of the reduction of other substrates. The mechanism involves the reversible association of reduced Kp2 with Kp1, followed by the transfer of a single electron from Kp2 to Kp1 and then an obligatory reversible dissociation of oxidized Kp2 from Kp1 before the Kp2 can be re-reduced by dithionite. Such a cycle is repeated eight times to achieve the eight successive one-electron reductions of Kp1 required to convert $8\text{H}^+ + \text{N}_2$ into $\text{H}_2 + 2\text{NH}_3$. H_2 can be released from Kp1 that has been reduced by two, three or four electrons and N_2 binds at the three- or four-electron-reduced level, with concomitant displacement of H_2 . In the present paper we describe the extension of the mechanism to C_2H_2 (acetylene) reduction, which is important for two reasons: first because it is commonly used to assay nitrogenase activity both *in vivo* and *in vitro* (Koch & Evans, 1966; Hardy *et al.*, 1968), and secondly because it sheds light on the chemistry occurring at the substrate-reduction site.

Ashby *et al.* (1987) have shown that C_2H_4 (ethylene), the product of C_2H_2 reduction, is itself slowly reduced to C_2H_6 (ethane) by nitrogenase, although this reaction is inhibited by C_2H_2 . C_2H_4 was also shown to inhibit the total electron flux through nitrogenase and the hydrolysis of MgATP to the same extent. They proposed a scheme that is consistent with the known chemistry of C_2H_2 and C_2H_4 reduction at a metal site. This involves the transfer of a metal hydride and the addition of $2\text{H}^+ + 2\text{e}^-$ to sideways-bound C_2H_2 to give a metal-alkyl intermediate, which can reversibly lose C_2H_4 via a sideways-bound C_2H_4 , regenerating the metal hydride or undergo further re-

duction to yield C_2H_6 . Ashby *et al.* (1987) predicted that C_2H_4 cannot be released from the active site until three electrons have been transferred from Kp2 to Kp1. Here we show that this is indeed the case, and we relate these conclusions to the chemistry of relevant metal complexes. In addition, the effect of CO on C_2H_2 reduction has been studied because, in model complexes, CO, C_2H_4 and N_2 often ligate at the same site, but with various extents of σ - and π -bonding between the ligand and the metal centre. However, CO is a non-competitive inhibitor of N_2 and C_2H_2 reduction on the enzyme (Burgess, 1985), which suggests that it binds at a different site or at a different oxidation level of the same site.

MATERIALS AND METHODS

Nitrogenase

Nitrogenase component proteins were characterized and purified from *Klebsiella pneumoniae* (oxytoca) N.C.I.B. 12204 as described by Thorneley & Lowe (1983). The specific activities of Kp1 and Kp2 at 30 °C were 1750 and 1600 nmol of C_2H_4 produced/min per mg of protein respectively.

Assay procedures

Assays lasting 10 s were performed as described by Thorneley & Lowe (1984b), and longer-time steady-state assays at 23 °C under Ar or Ar/ C_2H_2 (7:3) were performed as described by Lowe & Thorneley (1984b) and terminated by the addition of 0.1 ml of 30% (w/v) trichloroacetic acid.

Pre-steady-state assays were performed as described by Lowe & Thorneley (1984a), modified as described by Lowe *et al.* (1989). Gases (30% C_2H_2 or 10% CO as required) were equilibrated with the contents of the syringe containing ATP.

Reagents

All salts were purchased from BDH Chemicals; biochemicals were from Sigma Chemical Co., Poole, Dorset, U.K. Cylinder Ar and CO were purchased from Air Products, Walton-on-Thames, Surrey, U.K., and C_2H_2 was prepared by the action of water on CaC_2 (calcium carbide).

Computing

This was done on MicroVax II and 3600 computers using National Algorithm Group (NAG) subroutines D02BAF to

solve the differential equations describing the various reaction schemes used. We emphasize that these simulations are not multi-parameter fits to the data, but use independently determined rate constants (Lowe & Thorneley, 1984b). Only two new parameters are introduced in the present paper: (1) a binding constant for C_2H_2 to Kp1, and (2) a binding constant for Kp1 to Kp2 in the presence of C_2H_2 (see below). Up to 10% variation was allowed between experimental determination of protein concentrations and the values used in the simulations to compensate for any inaccuracies in measurements.

RESULTS AND DISCUSSION

Effect of $[Kp2]/[Kp1]$ on C_2H_2 reduction

Eady & Postgate (1974) reported that, at a constant C_2H_2 concentration, a decreased percentage of electron flux is allocated to C_2H_2 reduction as the total electron flux through the MoFe protein is decreased by increasing the MoFe-/Fe-protein ratio ($[Kp1]/[Kp2]$). Such 'titration curves' have never been quantitatively explained. Fig. 1 shows such data obtained under conditions used to determine the rate constants used in the simulations by Lowe & Thorneley (1984a,b). We have simulated this titration curve, as shown by the line through the points of Fig. 1, with the reaction sequence shown in Scheme 1. This is the full scheme of Lowe & Thorneley (1984a) with three additional assumptions: that (1) C_2H_2 binds reversibly to species E_1H and E_2H_2 of the MoFe-protein cycle described by Lowe & Thorneley (1984a) (Scheme 2 of that paper; see the legend to Scheme 1 for the meaning of the symbols E_1H , E_2H_2 , etc.), (2) C_2H_4 is subsequently released from E_3 and E_4 , and (3) all the rate constants of Lowe & Thorneley (1984a) are independent of C_2H_2/C_2H_4 binding. The rate constants used [additional to those given by Lowe & Thorneley (1984a)] are given in Table 1.

Scheme 1 is consistent with the mechanism for C_2H_4 formation from C_2H_2 suggested by Ashby *et al.* (1987), but includes C_2H_2 binding reversibly to E_2H_2 as well as to E_1H . This additional reaction is required to give the dependence of H_2/C_2H_4 shown in Fig. 1 and dominates the shape of the simulated curve; indeed, we cannot unequivocally state from the data of Fig. 1 whether binding to E_1H actually occurs or even whether C_2H_2 can bind to Kp1 in the dithionite-reduced state (E_0). It is, however, essential to our scheme that reduction of Kp1, with C_2H_2 bound, beyond the E_2H_2 level irreversibly commits the protein to forming C_2H_4 .

Fig. 1. Dependence of ratio of $[H_2]$ evolved under Ar to $[C_2H_4]$ evolved under C_2H_2 on $[Kp1]$ at constant $[Kp2]$

$[Kp2]$ was $5 \mu M$. The line is a simulation using Scheme 1.

Scheme 1. Kinetic scheme for C_2H_2 binding and reduction by nitrogenase

Species E_n represent one of two independently functioning halves of the tetrameric ($\alpha_2\beta_2$) Kp1. The subscript n refers to the number of (slow, rate-limiting) Kp1-Kp2 protein-dissociation steps that the half-molecule of Kp1 has undergone. It also indicates the number of electron equivalents that have been transferred from Kp2 relative to the resting (dithionite-reduced) state, E_0 . Each electron transfer is assumed to be followed by a protonation to give the number of association hydrogen atoms (H_n) shown. The sequence $\rightleftharpoons \rightarrow \rightleftharpoons$ represents the reversible association of Kp2 with Kp1, the irreversible transfer of one electron from Kp2 to Kp1 and the reversible dissociation of oxidized Kp2 from reduced Kp1.

Table 1. Rate constants used in conjunction with Scheme 1 for the simulation of Fig. 1

All other rate constants were taken from Lowe & Thorneley (1984a).

Rate constant	Value	Comment
k_{12}	$10^7 M^{-1} \cdot s^{-1}$	Determine K_s for C_2H_2 C_2H_4 release from $E_3HC_2H_4$
k_{-12}	$10^2 s^{-1}$	
k_{14}	$400 s^{-1}$ (fast)	

Effect of C_2H_2 on nitrogenase activity at high protein concentrations

As a preliminary to making rapid-quench measurements to determine the time course of C_2H_4 formation from C_2H_2 , we performed an experiment to check the effect of protein concentration, in the range used for the rapid quench technique, on nitrogenase activity. The results are shown in Fig. 2. The inhibition of C_2H_4 formation from C_2H_2 at high protein concentrations is much greater than that of H_2 evolution under Ar ($\sim 30\%$) predicted, observed and simulated by Thorneley & Lowe (1984b). Any formation of C_2H_6 , the only other possible product, is less than 1% of C_2H_4 formation. This inhibition of total electron flux can only be explained if binding of C_2H_2 and/or a reduction intermediate affects the value of one or more of the Kp1-Kp2 association and dissociation constants [see Scheme 1 and Lowe & Thorneley (1984a)]. The redetermination of all these rate constants in the presence of C_2H_2 is a major study that is beyond the scope of the present paper; we can, however, perform simulations of the time courses for product release using a simplified scheme (Scheme 2). This scheme uses only one rate constant, which defines the rate-limiting step [$k_1 = 6.4 \pm 0.8 s^{-1}$, designated k_{-3} by Lowe & Thorneley (1984a)] of the cycle in which one electron is transferred from Kp2 to Kp1. Inhibition at high protein concentrations is explained by the

[Kp2]/[Kp1] was 4:1. Assays were performed for 10 s. The lines are simulations using Scheme 2. Specific activity is expressed in units of nmol of product produced/min per mg of Kp2.

Scheme 2. Simplified kinetic scheme for C₉H₈ binding and reduction

See the legend to Scheme 1 for an explanation of the symbols E_0 , E_1H , etc. *E represents Kp_1 complexed by Kp_{2ox} . The multiple reactions represented by $\rightleftharpoons \rightleftharpoons$ in Scheme 1 have been simplified by replacing them with a single reaction with rate constant k_1 .

formation of inhibitory $\text{Kp2}_{\text{ox}}\text{Kp1}$ complexes at each Kp1 redox level. The simulations shown in Fig. 2 require two values for the apparent association constants for the binding of Kp2_{ox} to Kp1; in the absence of C_2H_2 , $K_a = (4.0 \pm 0.5) \times 10^3 \text{ M}^{-1}$, and in the presence of C_2H_2 , $K_b = (2.4 \pm 0.3) \times 10^4 \text{ M}^{-1}$ (see Scheme 2). Since we cannot determine the precise concentration of free Kp2_{ox} in these experiments, we have assumed, as a first approximation, that Kp2_{ox} is a constant proportion of the total Kp2 concentration and hence that the ratio of free Kp1 to $\text{Kp2}_{\text{ox}}\text{Kp1}$ is given by the product of $\text{Kp2}_{\text{total}}$ with either K_a or K_b . The effect of this assumption is to force the simulated curves in Fig. 2 to be hyperbolic, which increases their values in the range of about 1–10 μM -Kp1. The true values of K_a and K_b will be greater than those required for the simulations, since the true concentration of free Kp2_{ox} must be less than that of $\text{Kp2}_{\text{total}}$. The enhanced inhibition in the presence of C_2H_2 is therefore a consequence of C_2H_2 stabilizing the $\text{Kp2}_{\text{ox}}\text{Kp1}$ complex. The total electron flux at low protein concentration is independent of C_2H_2 binding, and therefore the 5-fold difference between K_a and K_b is not a consequence of slower dissociation of the $\text{Kp2}_{\text{ox}}\text{Kp1C}_2\text{H}_2$ complex; C_2H_2 presumably enhances the protein association rate. The value of the C_2H_2 binding constant and H_2 -release rates (taken from Lowe & Thorneley, 1984a) only determine the

percentage flux into H_2 and C_2H_4 production at low protein concentrations. The C_2H_2 binding constant ($K_c = 2.5 \times 10^6 \text{ M}^{-1}$) was chosen to fit the ordinate intercepts of Fig. 2. The values of K_a and K_b do not affect the simulation in Fig. 1, since at the low protein concentrations used to obtain these data the concentration of inhibiting complexes is negligible.

Effect of CO on nitrogenase activity at high protein concentrations

The effect of 5% CO on the inhibition of nitrogenase activity at high protein concentrations was investigated. In the absence of C_2H_2 there was no effect of CO on the dependence of H_2 evolution on protein concentration (Fig. 3). In the presence of both C_2H_2 and CO, at low protein concentrations H_2 was the major product, with only about 10% residual C_2H_4 evolution (Fig. 4). However, Fig. 4 also shows that, as the protein

Fig. 3. Dependence of H₂ evolution under Ar on protein concentration in the presence (▲) and absence (▼) of 5% CO

[Kp2]/[Kp1] was 4:1. Assays were performed for 10 s. The line is a simulation using Scheme 2. Specific activity is expressed in units of nmol of H_2 produced/min per mg of Kp2.

Fig. 4. Dependence of C₂H₄ (●) and H₂ (▼) evolution on protein concentration in the presence of 5% CO

[Kp2]/[Kp1] was 4:1. Assays were performed for 10 s. Specific activity is expressed in units of nmol of product produced/min per mg of Kp2. Note that we have not attempted to simulate curves through these data points or those of Fig. 6, since a detailed analysis of CO binding to nitrogenase will require dependencies on relative and absolute component protein concentrations, C_2H_2 and CO. This is beyond the scope of the present paper. However, the dependencies shown in these Figures do allow important conclusions to be drawn (see the text).

concentration is increased, H_2 evolution is inhibited to the same extent as was C_2H_4 evolution in the absence of CO. Thus although C_2H_2 is not being reduced significantly, it still inhibits the total electron flux through Kp1. Therefore C_2H_2 and CO are bound to nitrogenase concurrently. The diversion of reducing equivalents from C_2H_4 formation into H_2 evolution by CO is not due to CO inhibition of C_2H_2 binding. Fig. 4 also shows that, in the presence of CO, the residual C_2H_2 reduction is not inhibited at high protein concentrations; the effect is to change the H_2/C_2H_4 evolution ratio from about 10:1 at $1 \mu M$ -Kp1 to 2:1 at $30 \mu M$ -Kp1. Interestingly, the effect of C_2H_2 in the presence of CO at high protein concentrations is similar to the $NifV^-$ phenotype in which CO inhibits H_2 evolution (McLean *et al.*, 1983).

Pre-steady-state C_2H_4 and H_2 formation under C_2H_2

Fig. 5(a) shows the time courses of C_2H_2 and H_2 formation by nitrogenase in the presence of C_2H_2 and Fig. 5(b) shows the first 2 s of these time courses. H_2 formation is linear after an initial lag of 100 ms, as observed for H_2 in the absence of other substances (Lowe & Thorneley, 1984a); the linear rate is, however, inhibited because of the competing reduction of C_2H_2 to C_2H_4 . C_2H_4 formation is also linear after an initial longer lag of 250 ms. The curves through the points are simulations using Scheme 2, with the same rate constants used for Fig. 2, and show that, although C_2H_2 binds at the E_1H or E_2H_2 levels, C_2H_4 is not formed even on quenching in acid until the E_3 level of reduction; this is

Fig. 5. Pre-steady-state C_2H_4 (●) and H_2 (▼) formation by nitrogenase

Kp1 was $31 \mu M$, with $[Kp2]/[Kp1] = 4:1$. (a) Is the first 10 s of the time course; (b) is the first 2 s of the time course. The lines are simulations using Scheme 2: —, C_2H_4 formation; —, H_2 formation if $E_2H_2C_2H_2$, but not $*E_2H_2C_2H_2$, gives H_2 on quenching; ····, H_2 formation if both $E_2H_2C_2H_2$ and $*E_2H_2C_2H_2$ give H_2 on quenching.

consistent both with the effect of $[Kp2]/[Kp1]$ reported in Fig. 1 above and with the predictions of Ashby *et al.* (1987).

It is not possible to fit the data of Fig. 5(b) unless it is assumed that species $E_2H_2C_2H_2$ gives rise to H_2 on acid quench, whereas $*E_2H_2C_2H_2$ gives neither H_2 nor C_2H_4 and therefore presumably only C_2H_2 . Thus for E_2 with both C_2H_2 and $Kp2_{ox}$ bound, electrons are not transferred from the metal centres to bound protons to give H_2 on quenching in acid.

Effect of CO on pre-steady-state C_2H_4 formation under C_2H_2

Figs. 6(a) and 6(b) show time courses similar to those of Figs 5(a) and 5(b), but with the addition of 5.0 kPa (0.05 atm) of CO. As in the steady-state experiments (Fig. 4), H_2 formation is enhanced at the expense of C_2H_4 formation. The lags in product formation in the presence of CO (Fig. 6) are indistinguishable from those in its absence (Fig. 5). This is different from the reaction of cyanide with nitrogenase, where a lag phase of 3 s occurs before the onset of inhibition of H_2 evolution and formation of the reduction product CH_4 (Lowe *et al.*, 1989).

Mechanistic implications

Scheme 3 shows a mechanism for C_2H_4 formation from C_2H_2 that is consistent with Scheme 2, with the other kinetic data for C_2H_2 reduction by nitrogenase (Burgess, 1985) and with the chemistry of alkyne reduction at mononuclear metal centres (Richards, 1990). It is based on the scheme of Ashby *et al.* (1987) with the addition of intermediate f , which they did not consider, and the reversal of the positions of intermediate g and j , which can be arbitrarily selected (see below). Intermediates a , b , c and d

Fig. 6. Pre-steady-state C_2H_4 (●) and H_2 (▼) formation in the presence of 5% CO

Kp1 was $31 \mu M$, with $[Kp2]/[Kp1] = 4:1$. (a) Is the first 10 s of the time course; (b) is the first 2 s of the time course. No simulations are shown (see the note in Fig. 4).

Scheme 3. Mechanism for reduction of C_2H_2 to C_2H_4

The reduction level, E_n , uses the symbols defined in the legend to Scheme 1.

form part of the H_2 -production cycle of Lowe & Thorneley (1984a). Binding of C_2H_2 at c (to give f) in addition to the binding at b (to give e) is necessary for the successful simulation of the dependence of C_2H_4/H_2 evolution on $[Kp2]/[Kp1]$ described in Fig. 1 and explains why the K_m for C_2H_2 depends on electron flux. f is shown with bound C_2H_2 rather than C_2H_4 , since the pre-steady-state kinetics indicate that it gives C_2H_2 on quenching in acid. The reversible nature of C_2H_4 release (equilibrium $g \rightleftharpoons b$) explains C_2H_4 inhibition of nitrogenase and formation of C_2H_6 as discussed by Ashby *et al.* (1987).

The precise mechanism of the formation of bound C_2H_4 from bound C_2H_2 (f to equilibrium $g \rightleftharpoons j$) remains uncertain. Any intermediates other than g in the conversion of f into j are likely to be present at low concentrations, since protonations would follow rapidly after the slow (rate-limiting) protein dissociation step (conversion of f into g). Chemical model studies (Kashef & Richards, 1989) suggest that the first proton would be transferred to give an intermediate of the form:

If the second proton were transferred to the α -carbon, g would be formed, whereas if it were transferred to the β -carbon, j would be formed after transfer of the metal-bound H to the α -carbon. The elimination of C_2H_4 would occur from g in competition with a slow further reduction to C_2H_6 via j . The conversion of j into b is a classical β -elimination reaction of a metal alkyl to yield an alkane and metal hydride. The mechanism of C_2H_6 formation from C_2H_2 is currently of interest, since both the V-nitrogenase (Dilworth *et al.*, 1988) and certain Mo-nitrogenases with modified FeMo-cofactor-binding sites (Scott *et al.*, 1990) effect this reaction.

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