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Nitrogenase of *Klebsiella pneumoniae nifV* mutants

Investigation of the novel carbon monoxide-sensitivity of hydrogen evolution by the mutant enzyme

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The MoFe protein of nitrogenase from *Klebsiella pneumoniae nifV* mutants, NifV$^-$ Kp1 protein, in combination with the Fe protein from wild-type cells, catalysed CO-sensitive H_2 evolution, in contrast with the CO-insensitive reaction catalysed by the wild-type enzyme. The decrease in H_2 production was accompanied by a stoicheiometric decrease in dithionite (reductant) utilization, implying that CO was not reduced. However, CO did not affect the rate of phosphate release from ATP. Therefore the ATP/2e ratio increased, indicating futile cycling of electrons between the Fe protein and the MoFe protein. The inhibition of H_2 evolution by CO was partial; it increased from 40% at pH 6.3 to 82% at pH 8.6. Inhibition at pH 7.4 (maximum 73%) was half-maximal at 3.1 Pa (0.031 matm) CO. The pH optimum of the mutant enzyme was lower in the presence of CO. Steady-state kinetic analysis of acetylene reduction indicated that CO was a linear, intersecting, non-competitive inhibitor of acetylene reduction with $K_{ii} = 2.5$ Pa and $K_{is} = 9.5$ Pa. This may indicate that a single high-affinity CO-binding site in the NifV$^-$ Kp1 protein can cause both partial inhibition of H_2 evolution and total elimination of acetylene reduction. Various models to explain the data are discussed.

Nitrogenase, the enzyme that catalyses the conversion of N_2 into NH_3, has been purified from a number of micro-organisms, and consists of two O_2-sensitive iron–sulphur proteins (Eady & Smith, 1979; Lowe *et al.*, 1979). The larger of these proteins (the MoFe protein) has M_r about 220000 and also contains Mo. It is tetrameric, with subunits of two types present in equal numbers. The smaller protein (the Fe protein) has two identical subunits and M_r between 55000 and 67000. Catalytic activity requires both proteins, ATP, a bivalent cation (usually Mg^{2+}), a strong reductant (usually $Na_2S_2O_4$ *in vitro*) and anaerobiosis. In addition to N_2, a number of other small triple-bonded or potentially triple-bonded molecules are reduced by nitrogenase, e.g. acetylene, HCN, methyl isocyanide (Hardy, 1979, and references cited therein). In the absence of such substrates nitrogenase reduces H^+ to H_2. The rate of H_2 production is decreased by other substrates, but electron flux remains apparently constant.

CO inhibits the reduction of all substrates by wild-type nitrogenase, but not the evolution of H_2 (Hwang *et al.*, 1973; Rivera-Ortiz & Burris, 1975; Davis & Wang, 1980). Under CO-inhibited conditions neither reductant utilization (Ljones, 1973) nor ATP hydrolysis (Hardy *et al.*, 1965; Hwang *et al.*, 1973; Davis & Wang, 1980) is affected, and CO is not itself reduced (Ljones, 1973). There is no apparent physiological role for the H_2-evolution reaction of nitrogenase, although Hardy (1979) has proposed that it could be coupled to conventional hydrogenase to scavenge O_2, and so protect nitrogenase.

The MoFe protein from a *K. pneumoniae nifV* mutant (NifV$^-$ Kp1 protein) is specifically defective in the reduction of N_2 and, in contrast with wild-type nitrogenase, H_2 evolution by NifV$^-$ nitrogenase was inhibited by CO (McLean & Dixon, 1981). Since the *nifV* gene is not a structural gene for any of the nitrogenase subunits, it was concluded that the *nifV*-gene product modified the MoFe protein of

Abbreviations used: the MoFe and Fe proteins of wild-type *Klebsiella pneumoniae* nitrogenase are called Kp1 protein and Kp2 protein respectively (Eady *et al.*, 1972), and the MoFe protein from the NifV$^-$ strain is called NifV$^-$ Kp1 protein; a catalytically active mixture of NifV$^-$ Kp1 protein and wild-type Kp2 protein is referred to as NifV$^-$ nitrogenase; *p*CO, partial pressure of CO at s.t.p.

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nitrogenase, specifically enhancing its ability to reduce N_2, and in so doing rendered H_2 evolution insensitive to inhibition by CO. In the present paper we report characterization of the CO-sensitive H_2-evolution reaction of NifV$^-$ nitrogenase and discuss various models that could explain the effect of CO on substrate reduction.

Materials and methods

Growth of organisms and purification of proteins

Klebsiella pneumoniae M5a1 (wild-type) was grown and harvested and the wild-type nitrogenase proteins were purified by methods previously described (Eady *et al.*, 1972; Smith *et al.*, 1976). *K. pneumoniae* strain UNF1613 [UNF107 (*gnd his nif*) pRD253 *nifV2253::Tn7*)] is a derivative of strain UNF3001 (Dixon *et al.*, 1977), constructed by Dr. M. Merrick. It was maintained at −20°C in air in 20% (v/v) glycerol, and grown on minimal agar plates containing trimethoprim (20mg/l) (Dixon *et al.*, 1977) before use. For large-scale cultivation, the organism was first grown at 30°C in air in 20 litres of LB medium (Miller, 1972), and this was used to inoculate 1000 litres of Nitrogen-Free Davis & Mingioli medium (Dixon *et al.*, 1977), containing 1g of arginine hydrochloride/l and bubbled with N_2 (100litres/min) at 30°C. Bacteria were harvested by continuous centrifugation after 24–26h growth, and the cell paste was frozen and stored in liquid N_2 (Baker, 1978). The nitrogenase MoFe protein from the NifV$^-$ strain, UNF1613, was purified as described by Smith *et al.* (1976), except that all buffers were at pH 7.4 ± 0.1. The specific activity of preparations was about 1200nmol of ethylene produced/min per mg of NifV$^-$ Kp1 protein. All nitrogenase preparations were stored at −196°C, at which temperature they were stable for several (at least 4) months.

Assay procedures

Acetylene reduction. The acetylene-reduction activity of the respective nitrogenase components was measured in the presence of an optimal amount of the complementary protein. Specific activities are expressed as nmol of ethylene produced/min per mg of limiting component.

H_2 evolution. H_2 evolution was measured under a gas phase of either Ar or Ar + CO (Eady *et al.*, 1972), in 0.5–8min assays, shaken through 5cm in a reciprocal shaking water bath at approx. 150strokes/min. H_2 was determined on 0.5ml headspace samples by using a Pye–Unicam series 204 gas chromatograph fitted with a thermal conductivity detector with Ar as the carrier gas. CO (Air Products, Hardley, Southampton, U.K.) was diluted, when necessary, in a known volume of Ar in a flask sealed with a rubber closure, and samples were removed by gas-tight syringe and injected into assay bottles of known headspace volume. The partial pressure of CO was calculated and converted into the value at standard conditions [0°C, 101325Pa (1atm)].

ATP hydrolysis. The ATPase activity of nitrogenase was measured in a 1ml assay system containing 10mM-$MgCl_2$ and 10mM-ATP in 25mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/NaOH buffer, pH 7.4, without an ATP-regenerating system. Reaction (5min) was stopped with 0.1ml of 30% (w/v) trichloroacetic acid, and the assay mixture was placed on ice. A 0.5ml gas sample was removed after addition of 0.5ml of ice-cold water, for determination of H_2 in the gas phase. P_i was determined by the method of Ottolenghi (1975).

Dithionite-oxidation assays. The rate of $Na_2S_2O_4$ oxidation was measured at 30°C by monitoring the decrease in A_{359} spectrophotometrically, by using a molar absorption coefficient (ε_{359}) of $500\,M^{-1}\cdot cm^{-1}$ (R. N. F. Thorneley, personal communication). The assay mixture (2ml) was essentially the same as for acetylene-reduction assays (Eady, 1980), except that a dithionite concentration of 3mM was used. Assay constituents (excluding proteins and dithionite) were bubbled with the appropriate gas (Ar or N_2) for 15min before addition of the proteins and dithionite. The MoFe protein was added last to start the reaction after the absorbance had first stabilized. Acetylene was at 13kPa (0.13atm) in Ar. Assays were stopped by addition of 0.5ml of 0.4M-Na_2EDTA, pH 7.4, and gaseous products were determined in 0.5ml headspace samples, which were displaced with 0.5ml of water.

Assays at various pH values. Assay mixtures contained a mixed buffer system of either 25mM-Ada {[(carbamoylmethyl)imino]diacetic acid} / 25mM-Hepes in the range pH 6.12–7.7 or 25mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}/25mM-Hepes in the range pH 7.43–8.56.

Treatment of kinetic data. Data for CO inhibition of H_2 evolution were plotted in a linear form, and constants were derived by unweighted linear regression (see below). The 95% confidence limits were calculated by using standard statistical techniques (Snedecor & Cochran, 1967). Data on CO inhibition of acetylene reduction were first plotted as Hanes–Woolf plots ([S]/v versus [S], from which $K_m^{app.}/V_{max.}^{app.}$ (y-intercept) and $1/V_{max.}^{app.}$ (slope) estimates were obtained by unweighted linear regression. From secondary replots of these parameters against pCO, K_{ii} ($K_{i,intercept}$) and K_{is} ($K_{i,slope}$) were respectively determined. For pure, non-competitive, inhibition K_{ii} and K_{is} are measures of the affinity of the inhibitor, CO, for the enzyme–substrate complex, E–C_2H_2, and the enzyme, E, respectively.

Results

Effect of CO on total electron flow through NifV⁻ nitrogenase

The effect of CO on total electron flow through NifV⁻ Kp1 protein was measured spectrophotometrically by monitoring the rate of oxidation of dithionite ion, which acts as the electron donor *in vitro*. The dithionite-oxidation rate was measured under atmospheres of Ar, Ar + CO, Ar + acetylene and N_2 with purified NifV⁻ Kp1 protein and a 13–33-fold molar excess of wild-type Kp2 protein (Table 1). This rate was essentially the same under Ar, Ar + acetylene and N_2 (Table 1), the absence of any apparent substrate-induced effects being consistent with previous data derived from product formation by NifV⁻ nitrogenase (McLean & Dixon, 1981). Similar results have also been reported for wild-type nitrogenase (Ljones, 1973). In contrast, CO inhibited the rate of dithionite oxidation by 59%, and this was accompanied by a stoicheiometric decrease in H_2 production (dithionite ion is a two-electron donor). These findings indicated that inhibition of H_2 production by CO (McLean & Dixon, 1981) was due to inhibition of electron flow rather than to competition between CO and H_3O^+ for reduction.

Table 1. *Effect of CO and substrates on dithionite oxidation by NifV⁻ Kp1 protein*

Dithionite-oxidation rates were measured spectrophotometrically at 359 nm, under the appropriate gas phase, as described in the Materials and methods section. CO was at 3050 Pa. Assay mixtures contained 0.38 mg of Kp2 protein (sp. activity 1130 nmol of acetylene/min per mg of Kp2 protein) and either 0.0615 mg of NifV⁻ Kp1 protein (Expt. 1) or 0.154 mg of NifV⁻ Kp1 protein (Expt. 2) (sp. activity 684 nmol of acetylene/min per mg of NifV⁻ Kp1 protein). The Kp2-protein/Kp1-protein concentration ratios were 33 (Expt. 1) and 13 (Expt. 2). Assays were stopped after 5 min. N.D., Not determined.

Expt. no.	Gas phase	Dithionite oxidation (nmol/min per mg of Kp1 protein)	H_2 production (nmol/min per mg of Kp1 protein)	No. of replicates
1	Ar	683 ± 46	N.D.	2
	Ar + C_2H_2	683 ± 42	N.D.	2
	N_2	693 ± 6	N.D.	2
2	Ar	620 ± 61	630 ± 33	5
	Ar + CO	255 ± 9	266 ± 8	3

Effect of CO on the ATP/2e ratio for H_2 evolution

ATP hydrolysis during turnover of NifV⁻ nitrogenase was measured with and without 3050 Pa of CO (remainder Ar) in the absence of an ATP-regenerating system (Table 2). A wild-type nitrogenase preparation of similar specific activity to that of the mutant enzyme was used as a control. Electron flow was determined by measuring the H_2 produced in these assays.

No significant change in P_i production (ATP hydrolysis) by NifV⁻ nitrogenase was observed in the presence of CO, in spite of a significant decrease (63%) in H_2 evolution (Table 2). This resulted in an increase in ATP/2e ratio from 4.7 to 12.4 in the presence of CO. In contrast, the ATP/2e ratio for wild-type nitrogenase was unchanged by CO. Thus there was an apparent CO-induced uncoupling of ATP hydrolysis from electron transfer in the mutant enzyme.

There was no significant difference between the ATP/2e ratio for uninhibited NifV⁻ nitrogenase and wild-type nitrogenase, implying that the *nifV* mutation as such does not affect the normal coupling of ATP hydrolysis and electron transfer in the absence of CO.

Effect of CO concentration on H_2 evolution

The specific activity for H_2 evolution decreased to a minimum with increasing *p*CO, indicating that CO

Table 2. *ATP/2e ratio for NifV⁻ Kp1 protein in the presence and in the absence of CO*

The ATP/2e ratio was determined as the ratio of P_i produced to H_2 evolved in 5 min assays in the absence of an ATP-regenerating system, as described in the Materials and methods section. Assay mixtures contained 0.036 mg of NifV⁻ Kp1 protein (sp. activity 1260 nmol of acetylene/min per mg of NifV⁻ Kp1 protein) or 0.041 mg of Kp1 protein (sp. activity 1198 nmol of acetylene/min per mg of Kp1 protein) and 0.43 mg of Kp2 protein (sp. activity 978 nmol of acetylene/min per mg of Kp2 protein). The Kp2-protein/Kp1-protein concentration ratio was 40. ATP/2e ratios were calculated for individual assays and the average value was calculated from these.

Source of Kp1 protein	Gas phase	P_i production (nmol/assay)	H_2 production (nmol/assay)	ATP/2e ratio	No. of replicates
Strain UNF1613 (NifV⁻ Kp1)	Ar	1070 ± 62	227 ± 22	4.7 ± 0.2	4
	Ar + CO	1040 ± 95	84 ± 9	12.4 ± 0.4	4
Strain M5a1 (wild-type)	Ar	1079	246	4.4	1
	Ar + CO	1093	244	4.5	1

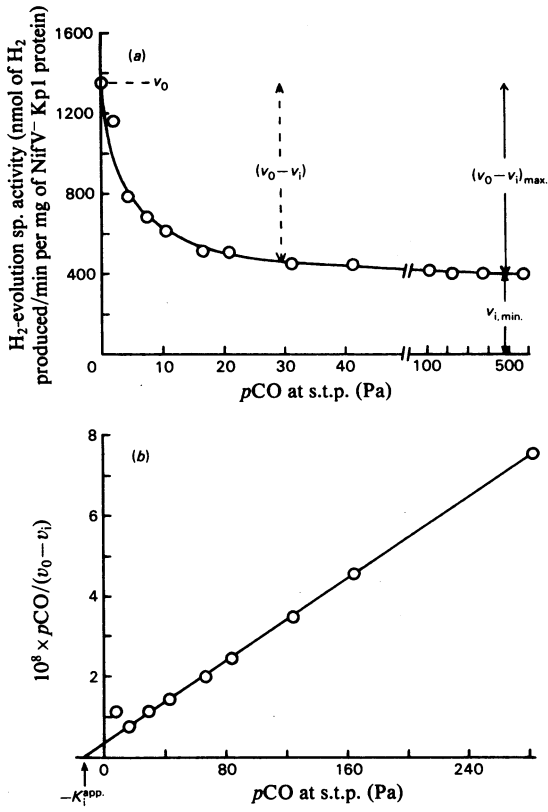


Fig. 1. *Effect of CO concentration on H_2 evolution by NifV⁻ nitrogenase*

H_2 evolution was measured under various partial pressures of CO in Ar. Assay mixtures contained 0.056 mg of NifV⁻ Kp1 protein (sp. activity 1260 nmol of acetylene/min per mg of NifV⁻ Kp1 protein) and 0.223 mg of Kp2 protein (sp. activity 1110 nmol of acetylene/min per mg of Kp2 protein), giving a Kp2-protein/Kp1-protein concentration ratio of 12, and reactions were stopped after 6 min. (*a*) Specific activity for H_2 evolution plotted as a function of *p*CO. (*b*) Determination of $K_i^{app.}$ for CO by using a replot analogous to the Hanes–Woolf form of the Michaelis–Menten equation.

was a partial inhibitor of H_2 evolution by NifV⁻ nitrogenase (Fig. 1*a*). If the difference, v_0-v_i, between the uninhibited rate, v_0, and the rate at a given *p*CO, v_i, was plotted against *p*CO, a curve similar in shape to the Michaelis–Menten (*v*-versus-[S]) plot was obtained. Therefore, by making an appropriate linear replot (Fig. 1*b*), analogous to the Hanes–Woolf form of the Michaelis–Menten equation, the limiting value of the specific activity, $v_{i,min.}$, and of $K_i^{app.}$ could be determined. A linear regression of the replot gave a maximum inhibition of 73% (72–74% with 95% confidence limits), with half-saturation at 3.1 Pa (0.031 matm) CO (<6.9 Pa, with 95% confidence).

The calculated concentrations of dissolved CO in solution at the *p*CO values used, and the degree of inhibition caused, are given in Table 3. These calculations indicated that the free dissolved CO concentration was often below that of the enzyme, and that inhibition could therefore have been under-estimated if CO did not equilibrate rapidly between liquid and gas phase. For this reason, assay systems had a large surface-to-volume ratio, were shaken rapidly, and were run for reasonable periods (6 min). These results indicate a dissociation constant of about 10^{-8} M, assuming that CO is a simple, linear, non-competitive inhibitor of H_2 evolution.

Effect of pH on CO inhibition of H_2 evolution

Changes in proton concentration (pH) alter the catalytic properties of most enzymes, including nitrogenase, and hence analysis of inhibition kinetics at different pH values (substrate H_3O^+ concentrations) was not possible, because of pH-induced changes in parameters ($V_{max.}$, K_m) normally assumed constant in classical Michaelis–Menten analysis. However, since the inhibition by CO was partial, the possible effects of pH on the maximum inhibition were investigated. Rates of H_2 evolution were determined from time courses at pH values in the range pH 6.12–8.56, with and without CO (3050 Pa). At low pH (<pH 6.6) assays became non-linear after a short time, which decreased with pH; for example, at pH 6.12 assays were non-linear after 2 min. Only data from the linear time range were used in constructing Fig. 2. The pH–activity profile of the enzyme was broad with and without CO; however, the pH optima were different for the inhibited and the uninhibited enzyme (Fig. 2*a*). In the absence of CO, the enzyme exhibited a broad peak of activity at pH 6.8–7.5, with a sharp decrease in activity above pH 7.8. The pH–activity profile of the CO-inhibited enzyme gave a peak at pH 6.3–6.8 followed by a gradual decrease with increasing pH. Calculation of the maximum inhibition as a function of pH (Fig. 2*b*) showed that the maximum inhibition was not fixed, but increased smoothly with pH from about 36% at pH 6.12 to 83% at pH 8.56.

Effect of CO on acetylene reduction

Acetylene reduction by wild-type nitrogenase, from all organisms tested, can be completely suppressed by CO (Hwang *et al.*, 1973; Burris, 1979). Acetylene reduction by NifV⁻ nitrogenase can similarly be inhibited by CO (McLean & Dixon, 1981), and the enzymes of *nifV* mutants were also shown to have a similar K_m for acetylene *in vivo* to that of wild-type *K. pneumoniae* enzyme. We therefore tried to determine whether a single class of CO-binding site was responsible for inhibition of

Table 3. *Expected concentrations of dissolved CO and NifV⁻ Kp1 protein during CO-inhibited turnover of NifV⁻ nitrogenase*

The concentration of dissolved CO was calculated from its initial partial pressure in the assay headspace and its solubility of 0.01998 vol. of CO in 1 vol. of water at 101325 Pa (1 atm) at 30°C. The ratio of headspace to liquid volume was such that no correction was necessary for the small amount (<2%) of the total CO bound to NifV⁻ Kp1 protein. The molar concentration of NifV⁻ Kp1 protein was calculated assuming that a fully active pure preparation of NifV⁻ Kp1 protein containing 2 atoms of Mo/molecule has a specific activity for acetylene reduction of 1830 nmol of acetylene/min per mg of NifV⁻ Kp1 protein. The calculation of $K_{diss.}$ assumes that each active site binds CO independently of the other sites:

$$K_{diss.} = \frac{[Kp1][CO]}{[Kp1\text{–}CO]} = \frac{(1-F)[CO]}{F}$$

where F = the fractional saturation of CO sites.

*p*CO (Pa)	[CO] in solution (nM)	Initial [NifV⁻ Kp1] (nM)	Inhibition of H_2 production (%)	Fractional saturation of CO sites (F)	$10^8 \times K_{diss.}$ (M)
2.1	19	175	14	0.194	7.9
4.2	38	175	41	0.569	2.9
7.4	66	175	49	0.681	3.1
10.6	94	175	55	0.764	2.9
16.8	150	175	62	0.861	2.4
20.9	187	175	62	0.861	3.0
31.2	278	175	67	0.931	2.0
41.3	368	175	67	0.931	2.7

both H_2 production and acetylene reduction in NifV⁻ nitrogenase, and whether CO inhibited acetylene reduction partially or totally.

As a preliminary experiment, the K_m for acetylene *in vitro* was determined, with a 12-fold molar excess of Kp2 protein, at acetylene concentrations in the range 18–1600 Pa of acetylene. This gave a $K_m = 566 \pm 73$ Pa (5.73 ± 0.74 matm) of acetylene (mean ± S.D.) for eight determinations, similar to that found with wild-type *K. pneumoniae* nitrogenase under the same conditions (370 Pa). Davis & Wang (1980) claim to have found two very similar K_m values for *K. pneumoniae* nitrogenase *in vitro* [$K_m{}^1 = 69$ Pa (0.7 matm), $K_m{}^2 = 168$ Pa (1.7 matm)], but did not define the ratio of Kp1 protein and Kp2 protein used. Only one K_m value was obtained in the present study because very closely spaced acetylene concentrations were not used.

To study the CO inhibition of acetylene reduction by NifV⁻ nitrogenase, six CO concentrations were used. Three were in the range 4–32 Pa of CO, in which changes in CO concentration caused significant changes in electron flux, and three were in the range 50–190 Pa of CO, in which changes in the *p*CO had little effect on total electron flow (Fig. 1*a*). $K_m^{app.}/V_{max.}^{app.}$ and $1/V_{max.}^{app.}$ values, determined from primary Hanes–Woolf plots, were replotted against *p*CO, from which K_{ii} and K_{is} values were determined on the two sets of data separately and together (Table 4). CO was a linear, intersecting, non-competitive inhibitor of acetylene reduction (Fig. 3). At the lower *p*CO range, no evidence for partial inhibition was observed. The K_{ii} and K_{is} values were similar to the $K_i^{app.}$ for H_2 evolution (Fig. 1*b*), and implied that a single CO-binding site could be responsible for inhibition of both H_2 production and acetylene reduction. Since this high-affinity CO-binding site apparently inhibited acetylene reduction completely, the possible presence of another CO-binding site, with the same, lower, affinity as that found in wild-type Kp1 protein, could not be tested.

Table 4. *Inhibition constants for CO inhibition of acetylene reduction by NifV⁻ nitrogenase*

Inhibition constants (K_{ii} and K_{is}) were derived by unweighted linear regression of secondary replots of $K_m^{app.}/V_{max.}^{app.}$ and $1/V_{max.}^{app.}$ against *p*CO respectively, with use of the data at low and high *p*CO ranges either separately or together. $K_m^{app.}/V_{max.}^{app.}$ and $1/V_{max.}^{app.}$ estimates were first derived from Hanes–Woolf plots of the experimental data by unweighted linear regression.

Range of [CO] (Pa)	K_{is} (Pa)	K_{ii} (Pa)
4.3→31.5	2.2	6.4
48.9→190	2.8	12.0
4.3→190	2.5	9.5

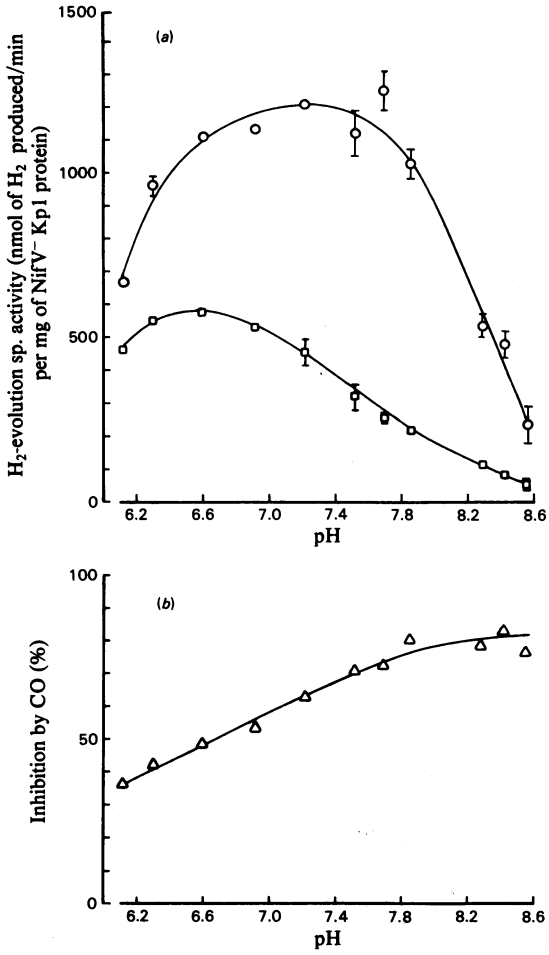


Fig. 2. *Effect of pH on CO inhibition of H_2 evolution by NifV⁻ nitrogenase*

Specific activities for H_2 evolution were determined from time courses in the presence and in the absence of 3050 Pa of CO and plotted as a function of pH (see the Materials and methods section). Assay mixtures contained 0.048 mg of NifV⁻ Kp1 protein (sp. activity 1260 nmol of acetylene/min per mg of NifV⁻ Kp1 protein) and 0.233 mg of Kp2 protein (sp. activity 978 nmol of acetylene/min per mg of Kp2 protein). The Kp2-protein/Kp1-protein concentration ratio was 12. All points were the average of two to four separate determinations, with the exception of pH 6.12 (one determination). (*a*) Effect of pH on H_2 evolution in the presence (□) and in the absence (○) of CO. (*b*) Degree of inhibition by CO plotted as a function of pH.

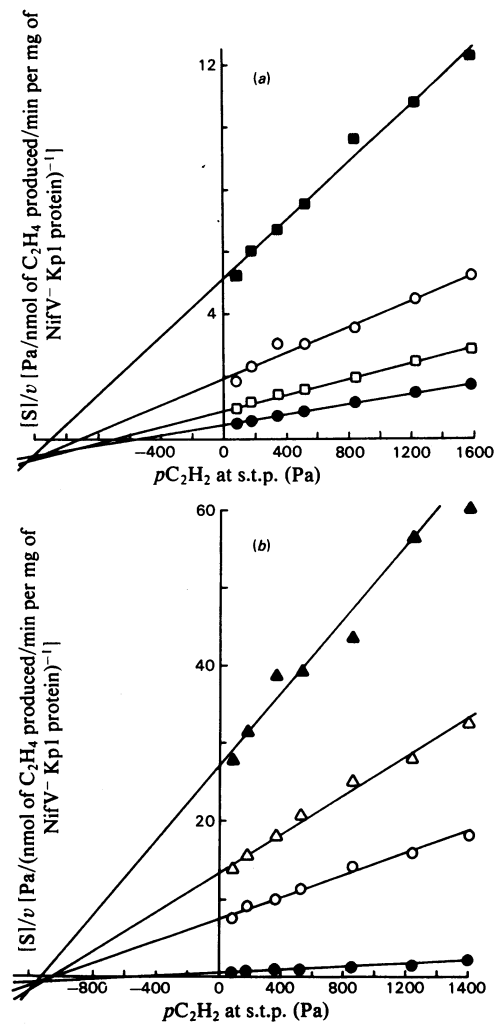


Fig. 3. *Effect of CO on acetylene reduction by NifV⁻ nitrogenase*

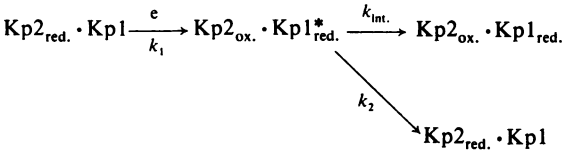
Assays were stopped after 3 min (no CO) or 7 min (with CO), and the assay mixtures contained enzyme as in Fig. 1 legend. (*a*) ●, No CO; □, 4.3 Pa of CO; ○, 10.7 Pa of CO; ■, 31.5 Pa of CO. (*b*) ●, No CO; ○, 48.9 Pa of CO; △, 96.9 Pa of CO; ▲, 190 Pa of CO. Each point is the result of a single assay.

Discussion

The inhibition of H_2 evolution catalysed by NifV⁻ nitrogenase by CO is due to CO-induced inhibition of electron flow through nitrogenase. Dithionite oxidation was inhibited stoicheiometrically with H_2 evolution, indicating that CO was not reduced (Table 1). These assays also showed that the presence of the substrates acetylene and N_2 did not markedly influence the dithionite-oxidation rate, indicating that, like wild-type nitrogenase, electron flow to the enzyme active site is rate-limiting under normal conditions *in vitro*. The degree of inhibition

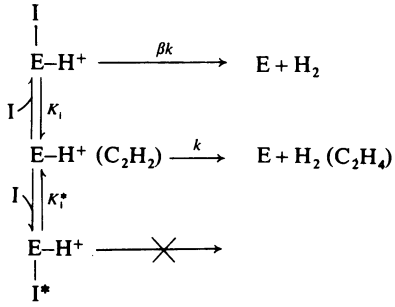
under these conditions (60%) was less than expected (73%) at the given pH (Figs. 1*a* and 1*b*). This may reflect an effect of the low reductant concentration on CO inhibition of H_2 evolution, but this has yet to be investigated.

Hydrolysis of ATP was not detectably affected when H_2 evolution was inhibited by CO (Table 2). Two possible explanations for this are as follows. (*a*) ATP hydrolysis occurred in the absence of electron transfer from Kp2 protein to NifV$^-$ Kp1 protein, in which case the similar values of ATP hydrolysis obtained in the presence and in the absence of CO were fortuitous (uncoupled hydrolysis). (*b*) Coupled hydrolysis continued in the inhibited state. However, CO may have presented a barrier to the transferred electron in reaching the active site or substrate, which could have resulted in back-donation of the electron to Kp2 protein. Re-donation of the same electron to NifV$^-$ Kp1 protein would require further hydrolysis of ATP (and possibly also pre-dissociation of the Kp2-protein · Kp1-protein complex to release ADP and to recharge Kp2 protein with ATP), but would not require further dithionite utilization. Such back-donation or leakage of a transferred electron has previously been suggested by Orme-Johnson *et al.* (1977) as the mechanism of apparent uncoupling of ATP hydrolysis in the wild-type enzyme under certain conditions. The fate of an electron transferred to NifV$^-$ Kp1 protein could thus be governed by two competing rates: one for passage to the active site, $k_{int.}$, and one for leakage to the oxidized Fe protein, k_2:



Under uninhibited conditions, back-donation is presumably insignificant, i.e. $k_{int.} \gg k_2$, and so ATP utilization is at its most efficient.

Investigation of the concentration-dependence of CO inhibition of H_2 evolution indicated that very low concentrations of dissolved CO were effective in the inhibition of NifV$^-$ nitrogenase (Table 3). However, CO was only a partial inhibitor. This raised the possibility that the enzyme preparation was a mixture of wild-type enzyme (CO-insensitive) and mutant enzyme. Two considerations militate against this contention: firstly, if the residual H_2-evolution activity in the presence of CO were due to wild-type enzyme, then it should exhibit the wild-type pH optimum at pH 7.5, whereas a pH optimum near pH 6.7 was observed; secondly, the reversion rate of the Tn7 insertion would be too low to produce a significant proportion of Nif$^+$ cells in our cultures.



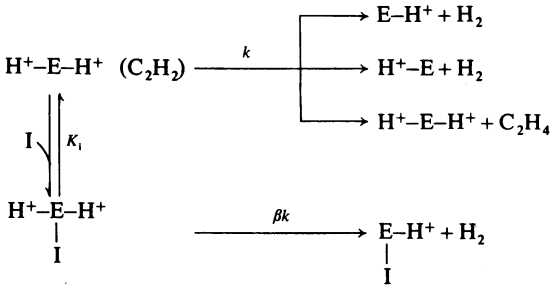
Scheme 1

The enzyme has one H_2-evolution site and two, mutually exclusive, inhibitor sites. Binding of inhibitor at one site (*) prevents H_2 evolution. Acetylene reduction is inhibited by inhibitor binding at either site.

As mentioned above, the NifV$^-$ enzyme gave different pH profiles in the presence and in the absence of a saturating concentration of CO (Fig. 2*a*). This might indicate either that CO increased the K_m for H_3O^+, so that a peak of activity was observed at a lower pH in its presence, or that there were two separated H_3O^+-evolving sites, one of which was CO-insensitive and had a lower pH optimum than the CO-sensitive site. The result of these altered activity profiles was that the degree of inhibition by CO increased with pH (Fig. 2*b*).

The 73% inhibition by CO at pH 7.4 is very close to the maximum inhibition of H_2 evolution by N_2 in the wild-type enzyme at this pH (namely 75%; Rivera-Ortiz & Burris, 1975). Furthermore, the change in the maximum inhibition by CO with pH is similar to the changes, with pH, in the ability of N_2 to inhibit H_2 evolution (i.e. act as a substrate) in wild-type nitrogenase (Hageman & Burris, 1980; R. N. F. Thorneley, personal communication). These interesting parallels between CO and N_2, coupled with the observation that N_2 reduction is specifically defective in the mutant enzyme, might imply that CO binds strongly at or near the defective N_2-reducing site in the NifV$^-$ enzyme, and in so doing mimics some of the effects of N_2 on the wild-type enzyme, without being itself reduced.

The inhibition of acetylene reduction by CO seemed to have inhibition constants similar in magnitude to the $K_i^{app.}$ for inhibition of H_2 evolution. However, acetylene reduction, unlike H_2 evolution, could be totally eliminated by CO. Thus it appeared that a single class of CO-binding site could inhibit both reactions. Here we propose three possible models to explain these phenomena, starting with the premise that the mutant enzyme is saturated with H_3O^+ under normal conditions, by analogy to wild-type nitrogenase.



Scheme 2

The enzyme has two H_2-evolution sites and only one inhibitor site. Binding of the inhibitor prevents H_2 evolution from one site and eliminates acetylene reduction.

In Scheme 1 H_2 is evolved from a single enzyme site, with electron transfer to the active site apparently being rate-limiting (Ljones, 1973; Watt & Burns, 1977; Thorneley & Lowe, 1982). Scheme 1 proposes two inhibitor-binding sites, to which the CO molecule, I, can bind in one of two mutually exclusive modes. Only one of the modes is inhibitory (marked *), whereas the other does not affect H_2 evolution. Processing by the *nifV*-gene product would normally eliminate the inhibition site. The non-inhibitory binding mode is the same as found in wild-type MoFe protein. Saturation of the enzyme with inhibitor will result in partition of the enzyme molecules into populations in the inhibitory or non-inhibitory mode, in proportions depending on the relative values of K_I and K_I^*. The term β defines the ratio of the inhibited to uninhibited rates of H_2 evolution, and is given by:

$$\beta = K_I/(K_I + K_I^*)$$

at saturating *p*CO. This defines the apparent inhibition constant, $K_I^{app.}$, where :

$$1/K_I^{app.} = 1/K_I + 1/K_I^*$$

Since acetylene reduction can be totally inhibited by CO in the wild-type nitrogenase, a separate substrate-reduction site is included, and it is proposed that both binding modes are inhibitory to acetylene reduction. The extra CO-binding site on NifV⁻ Kp1 protein may allow this inhibitor to block total electron flow to the active site(s).

The second model (Scheme 2) proposes that there are two H_2-evolving sites, sites 1 and 2, both present in wild-type Kp1 protein, but that site 1 is in an altered form in NifV⁻ Kp1 protein. These sites compete for the supply of electrons in wild-type Kp1 protein. The inhibitor, I, blocks the action of site 2 only, and so H_2 production can continue from site 1, through which the entire electron flux will now pass. In NifV⁻ Kp1 protein site 1 may not be sufficiently active to dispose of reducing equivalents as fast as they arrive when the other site is blocked. This would potentiate back-transfer of electrons to Kp2 protein and cause the apparent uncoupling of ATP hydrolysis. The defect in site 1 could arise either if it was not saturated with protons at normal pH (pH 7.4), owing to an increase in the K_m for H_3O^+, or if the catalytic rate constant, k', for site 1 was rate-limiting when site 2 was blocked.

The third model is simpler in that it involves single sites for both H_2 evolution and CO binding. In this model (not shown) CO binding at or near the metal atom involved in H_2 evolution (e.g. on the same metal cluster) increases the redox potential of the H_2-evolution site (or the cluster) to the vicinity (probably slightly above) of that of the hydrogen electrode. As a consequence the rate of H_2 evolution would be decreased by CO binding but would be expected to increase with decreasing pH, as observed (Fig. 2). Furthermore, if acetylene reduction occurs on the same cluster, then raising the cluster's redox potential by binding CO could explain the observed complete inhibition of acetylene reduction.

On our data it is not possible to distinguish between these models. Distinguishing between the models in Schemes 1 and 2 would require separation of the effects due to substrate, H_3O^+, concentration from other pH-dependent effects. Evidence for the third model could come from measurements of the redox potentials of the MoFe protein clusters during turnover. No measurements of such potentials have yet been achieved.

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