Unusual Effect of CO on C_2H_2 Reduction by V Nitrogenase from *Azotobacter vinelandii*

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Nitrogenase catalyzes the reduction of dinitrogen to ammonia as well as the reduction of many similar small molecules containing C, N, and O multiple bonds. One exception to this trend is CO, which is not a substrate but a potent noncompetitive inhibitor of the wild-type enzyme,[1,2] inhibiting the reduction of all substrates, except for protons,[3] which are reduced to H_2. Until now, it has been assumed that the inhibitory effects of CO are similar for both the conventional Mo nitrogenase and the alternative form of the enzyme, V nitrogenase. We report here the surprising result that, at low concentrations, CO acts as a stimulant and not an inhibitor of C_2H_2 reduction to both C_2H_4 and C_2H_6 by *Azotobacter vinelandii* V nitrogenase.

Numerous spectroscopic studies[5−10] have suggested general similarities between the metal clusters of wild-type Mo and V nitrogenase. The amino acid sequences of both enzyme forms also show high homology.[11] It is, therefore, surprising that these two enzyme forms react differently with substrates and inhibitors. For example, at room temperature, Mo nitrogenase reduces C_2H_2 to only C_2H_4, while V nitrogenase[12,13] generates both C_2H_4 and C_2H_6. Another difference is observed during enzymatic turnover in the presence of CO. Under these conditions, Mo nitrogenase generates two different $S = {}^1/_2$ EPR signals,[14−16] while neither signal is detectable when V nitrogenase from *A. vinelandii* is used.[17] These data suggest possible differences in the mechanisms of C_2H_2 reduction and CO inhibition by Mo and V nitrogenase. This paper further investigates these differences by determining the ability of CO to inhibit C_2H_4 and C_2H_6 production from C_2H_2 by V nitrogenase.

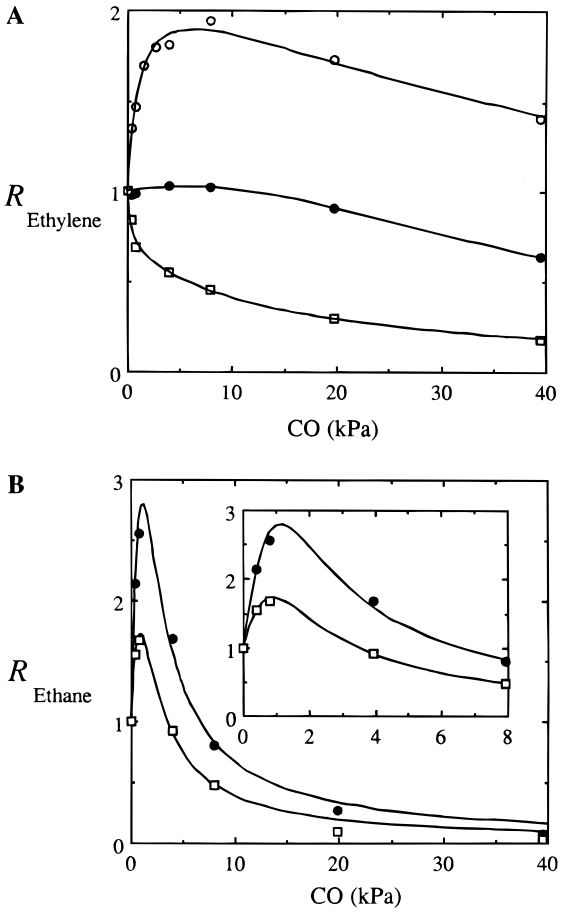

Figure 1. Relative production (R = {enzyme activity in presence of CO}/{enzyme activity without CO}) of C_2H_4 (A) or C_2H_6 (B) from C_2H_2 by *A. vinelandii* V nitrogenase as a function of the external CO pressure. Gas phase (without CO) contained 10% C_2H_2 in Ar. Av1′ was added to degassed MgATP-regenerating solution containing $Na_2S_2O_4$ (20 mM), and the reaction was initiated by the addition of Av2′. Experimental conditions: 15 min incubation, 30 °C. C_2H_4 and C_2H_6 were measured using a Varian (Model 3700) gas chromatograph equipped with a flame ionization detector and a Porapac type T column (C_2H_4) or an activated alumina column (C_2H_6). The molar ratio of Av2′:Av1′ was 1:5 (○, specific activity, 2.4 nmol of C_2H_4 min^{-1} mg^{-1} of Av1′), 1:1 (●, specific activities, 22 nmol of C_2H_4 min^{-1} mg^{-1} of Av1′ and 0.7 nmol of C_2H_6 min^{-1} mg^{-1} of Av1′), and 8:1 (□, specific activities, 68 nmol of C_2H_4 min^{-1} mg^{-1} of Av1′ and 2.4 nmol of C_2H_6 min^{-1} mg^{-1} of Av1′). All listed specific activities refer to product formation in the absence of CO. Specific activity of Av1′ was 550 nmol of H_2 produced min^{-1} mg^{-1} of Av1′. Solid lines: theoretical R values calculated from eq 6. Inset in B: expansion of data and calculated R values for C_2H_6 production at low pressures (0−8 kPa) of CO.

Figure 1 shows the relative production of C_2H_4 and C_2H_6 from C_2H_2 by V nitrogenase as a function of the external CO pressure.[18] These experiments were performed at different rates of electron flux.[19] Since nitrogenase activity requires the component 2 protein (the Fe protein or Av2′) to donate electrons, one at a time, to the component 1 protein (the VFe protein or

(1) Hwang, J. L.; Chen, C. H.; Burris, R. H. *Biochim. Biophys. Acta* **1973**, *292*, 256−270.
(2) Burgess, B. K. In *Molybdenum Enzymes*; Spiro, T. G., Ed.; John Wiley & Sons: New York, 1985; Vol. 7, pp 161−220.
(3) CO inhibiton of H_2 evolution, however, has been observed in a selected number of mutants on the MoFe protein, including the *nif*V⁻ mutant.[4]
(4) Hawkes, T. R.; McLean, P. A.; Smith, B. E. *Biochem. J.* **1984**, *217*, 317−321.
(5) Morningstar, J. E.; Hales, B. J. *J. Am. Chem. Soc.* **1987**, *109*, 6854−6855.
(6) Morningstar, J. E.; Johnson, M. K.; Case, E. E.; Hales, B. J. *Biochemistry* **1987**, *26*, 1795−1800.
(7) Arber, J. M.; Dobson, B. R.; Eady, R. R.; Stevens, P.; Hasnain, S. S.; Garner, C. D.; Smith, B. E. *Nature* **1987**, *325*, 372−374.
(8) Arber, J. M.; Dobson, B. R.; Eady, R. R.; Hasnain, S. S.; Garner, C. D.; Matsushita, H.; Nomura, M.; Smith, B. E. *Biochem. J.* **1989**, *258*, 733−737.
(9) George, G. N.; Coyle, C. L.; Hales, B. J.; Cramer, S. P. *J. Am. Chem. Soc.* **1988**, *110*, 4057−4059.
(10) Chen, J.; Christiansen, J.; Tittsworth, R. C.; Hales, B. J.; George, S. J.; Coucouvanis, D.; Cramer, S. P. *J. Am. Chem. Soc.* **1993**, *115*, 5509−5515.
(11) Joerger, R. D.; Loveless, T. M.; Pau, R. N.; Mitchenall, L. A.; Simon, B. H.; Bishop, P. E. *J. Bacteriol.* **1990**, *172*, 3400−3408.
(12) Dilworth, M. J.; Eady, R. R.; Robson, R. L.; Miller, R. W. *Nature* **1987**, *327*, 167−168.
(13) Dilworth, M. J.; Eady, R. R.; Eldridge, M. E. *Biochem. J.* **1988**, *249*, 745−751.
(14) Davis, L. C.; Henzl, M. T.; Burris, R. H.; Orme-Johnson, W. H. *Biochemistry* **1979**, *18*, 4860−4869.
(15) Lowe, D. J.; Eady, R. R.; Thorneley, R. N. F. *Biochem. J.* **1978**, *173*, 277−290.
(16) Pollock, R. C.; Lee, H.-I.; Cameron, L. M.; DeRose, V. J.; Hales, B. J.; Orme-Johnson, W. H.; Hoffman, B. M. *J. Am. Chem. Soc.* **1995**, *117*, 8686-8687.
(17) Moore, V. G.; Tittsworth, R. C.; Hales, B. J. *J. Am. Chem. Soc.* **1994**, *116*, 12101−12102.

(18) V nitrogenase proteins were purified, and acetylene reduction activity was monitored as previously described.[20] In these experiments, dithionite (20 mM) was used as a reductant. For the data indicated in the figure, the partial pressure of acetylene was 0.1 atm. Data collected at different partial pressures ranging from 0.1 to 0.002 atm showed no substrate dependencies, as expected for noncompetitive interaction. The indicated partial pressure of CO was produced using a Pressure-Lok syringe (Precision Sampling, Baton Rouge, LA) to replace a volume of headspace gas over the reaction mixture with an equal volume of 100% CO (Scott Specialty Gases, Houston, TX) from a CO tank fitted with an Oxysorb cartridge (Messer Griesheim, Valley Forge, PA). Fits to eq 6 with the data were obtained with FFIT 2,2-S, a function-fitting program (A Soft Answer, Macquarie Centre, NSW, Australia).

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Table 1

product	Av2′:Av1′	A (kPa^{-1})	B (kPa^{-1})	C (kPa^{-2})
C_2H_4	8:1	2.0	3.0	0.20
	1:1	0.014	0.004	8.1×10^{-4}
	1:5	1.94	0.87	0.012
C_2H_6	8:1	2.0	0.22	0.50
	1:1	3.1	0.25	0.47

Av1′), the electron flux through the enzyme can be regulated by varying the Av2′:Av1′ ratio. In Figure 1A, the rate of C_2H_4 formation was determined at an Av2′:Av1′ ratio of 8:1, corresponding to high electron flux. These data exhibit what appears to be a typical profile for a single noncompetitive inhibitor. Upon decreasing the component protein ratio to 1:1 (approximating moderate flux), the ability of CO to inhibit C_2H_4 formation decreases, suggesting that the value of the inhibition constant, K_i, has increased. This interpretation becomes suspect when the rate of C_2H_4 formation is monitored at an even lower component ratio (i.e., 1:5, or low flux). Unexpectedly, under this low-flux condition, CO in small concentrations was observed to enhance instead of inhibit C_2H_4 production. Product enhancement at low CO concentrations was also observed (Figure 1B) for C_2H_6. However, unlike the enhancement of C_2H_4, which occurred only under low-flux conditions, enhancement of C_2H_6 was detected at all component ratios tested, including maximum flux (i.e., $\geq$20:1; data not shown). In all of these experiments, CO was not observed to affect total electron flux.

At larger CO concentrations, the relative enhancement of both C_2H_4 and C_2H_6 production decreased, and eventually only product inhibition was observed at high concentrations. From these data, it is obvious that, for both products, the degree of enhancement increased as the flux decreased. These results are the first example of CO acting as an enhancer of product formation by nitrogenase and dramatically differ from the results previously reported[13] for V nitrogenase from *Azotobacter chroococcum*, where, under high flux conditions, CO appeared to induce an identical inhibition of both C_2H_4 and C_2H_6 production. This comparison is even more surprising since sequence alignments (using the MoFe protein structure as a reference) of the 26 nearest-neighbor amino acids to the VFe cofactor in enzymes from both *A. chroococcum* and *A. vinelandii* are identical.[11,22]

The simplest model for explaining the profiles in Figure 1 involves two different sites (labeled 1 and 2) on the enzyme (E) for CO (I) binding. The trend in the data suggests that the enhancement of product formation at low CO concentrations is related to binding at a single (EI_i, $i = 1$ or 2) site, while binding at both sites (EI_1I_2) at higher concentrations induces inhibition. Defining the terms

$$K_1 = [E][I]/[EI_1] \quad (1)$$

$$K_{12} = [EI_1][I]/[EI_1I_2] \quad (2)$$

$$K_2 = [E][I]/[EI_2] \quad (3)$$

$$K_{21} = [EI_2][I]/[EI_1I_2] \quad (4)$$

the general expression for the relative specific activity (R) of C_2H_4 or C_2H_6 production (i.e., activity = 1 when [CO] = 0) is

$$R = \frac{(1 + h_1[I]/K_1 + h_2[I]/K_2)}{(1 + [I]/K_1 + [I]/K_2 + [I]^2/K_1K_{12} + [I]^2/K_2K_{21})} \quad (5)$$

where h_i is called the enhancement factor and represents the relative amount of product generated by enzyme form EI_i compared to the production by the enzyme (E) in the absence of CO. Equation 5 can be simplified to

$$R = (1 + A[I])/(1 + B[I] + C[I]^2) \quad (6)$$

Equation 6 was found to provide an excellent fit to all the data (see Figure 1), yielding the best-fit parameters for the selected data shown in Figure 1 (Table 1).

Because the parameters A, B, and C represent various combinations of the terms h_i, K_i, and K_{ij} as defined above, it is impossible to provide a unique mechanistic explanation for the observed variations in these parameters with flux. However, general statements can be made about the overall data. Recent ^{13}C-ENDOR spectra[16] of the two CO-induced EPR signals in Mo nitrogenase have demonstrated that CO binds sequentially to two sites on the same metal cluster of component 1. Although implied, it has not yet been demonstrated that this CO binding is directly associated with CO inhibition of the Mo enzyme. Our data presented here clearly show that there are likewise at least two different CO binding sites on V nitrogenase that directly influence the enzymology of acetylene reduction. We also show for the first time that CO does not always function as an inhibitor of substrate reduction by nitrogenase but can actually enhance the formation of certain products. Furthermore, the approximate invariance with flux in the values of B and C for C_2H_6 formation suggests that the binding constants for CO are flux independent, such that the differences in the data observed in Figure 1B arise only from variation in the enhancement factor (in the A term) with flux. This simple interpretation, however, does not hold for C_2H_4 formation, where there is no general trend in the variation of the A, B, and C parameters with flux. This latter fact suggests that the mechanisms for C_2H_4 and C_2H_6 formation from C_2H_2 differ (for example, C_2H_4 formation may involve several different forms of the enzyme, each with a different affinity for CO, while C_2H_6 formation may only involve a single form) and, as has been demonstrated many times in the past, again underscores the importance of considering the influence of electron flux in any model of nitrogenase enzymology.

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(19) Different fluxes were used because it has been previously noted[14] that the K_m of C_2H_2 reduction to C_2H_4 by Mo nitrogenase is flux dependent, as is the C_2H_4 inhibition of H_2 evolution.[21]

(20) Hales, B. J.; Case, E. E.; Morningstar, J. E.; Dzeda, M. F.; Mauterer, L. A. *Biochemistry* **1986**, *25*, 7251−7255.

(21) Ashby, G. A.; Dilworth, M. J.; Thorneley, R. N. F. *Biochem. J.* **1987**, *247*, 547−554.

(22) Robson, R. L.; Woodley, P. R.; Pau, R. N.; Eady, R. R. *EMBO J.* **1989**, *8*, 1217−1224.