Biochem. J. (1988) 254, 469–475 (Printed in Great Britain)

Reversible and irreversible effects of nitric oxide on the soluble hydrogenase from *Alcaligenes eutrophus* H16

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The effects of NO on the H_2-oxidizing and diaphorase activities of the soluble hydrogenase from *Alcaligenes eutrophus* H16 were investigated. With fully activated enzyme, NO (8–150 nM in solution) inhibited H_2 oxidation in a time- and NO-concentration-dependent process. Neither H_2 nor NAD^+ appeared to protect the enzyme against the inhibition. Loss of activity in the absence of an electron acceptor was about 10 times slower than under turnover conditions. The inhibition was partially reversible; approx. 50% of full activity was recoverable after removal of the NO. Recovery was slower in the absence of an electron acceptor than in the presence of H_2 plus an electron acceptor. The diaphorase activity of the unactivated hydrogenase was not affected by NO concentrations of up to 200 μM in solution. Exposure of the unactivated hydrogenase to NO irreversibly inhibited the ability of the enzyme to be fully activated for H_2-oxidizing activity. The enzyme also lost its ability to respond to H_2 during activation in the presence of NADH. The results are interpreted in terms of a complex inhibition that displays elements of (1) a reversible slow-binding inhibition of H_2-oxidizing activity, (2) an irreversible effect on H_2-oxidizing activity and (3) an irreversible inhibition of a regulatory component of the enzyme. Possible sites of action for NO are discussed.

INTRODUCTION

The soluble NAD^+-linked hydrogenases (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2) from the hydrogen bacteria *Alcaligenes eutrophus* H16 and *Nocardia opaca* 1b are both tetramers of molecular mass approx. 200 kDa and consist of four non-identical subunits. Both have similar catalytic activities, structures and prosthetic group complements (Schneider *et al.*, 1984; Schneider & Piechulla, 1986). In the tetrameric holoenzyme form these hydrogenases catalyse the oxidation of H_2 coupled to the reduction of NAD^+ or a variety of artifical electron acceptors (Schneider & Schlegel, 1976; Schneider *et al.*, 1984). The *N. opaca* enzyme can be dissociated into two dissimilar dimers. One dimer (56 kDa and 27 kDa subunits) exhibits hydrogenase activity in the presence of H_2 and certain artificial electron acceptors, but not NAD^+. The other dimer (63 kDa and 31 kDa subunits) exhibits NADH dehydrogenase (diaphorase) activity and is inactive in H_2 oxidation. Only when the two dimers are recombined is the ability to reduce NAD^+ with H_2 restored (Schneider *et al.*, 1984). The hydrogenase-active dimer from *N. opaca* 1b is thought to contain two Ni atoms and one [4Fe-4S] centre. The diaphorase-active dimer is thought to contain one FMN molecule, two [4Fe-4S] centres and one [2Fe-2S] centre (Schneider *et al.*, 1984). Studies with mutants indicate that the prosthetic group distribution in *A. eutrophus* H16 soluble hydrogenase is similar to that in *N. opaca* 1b hydrogenase (Hornhardt *et al.*, 1986). The roles of these redox centres in H_2 oxidation have not been fully elucidated.

The few studies of the inhibition of the soluble hydrogenase from *A. eutrophus* H16 have only revealed limited information about its catalytic mechanism. This enzyme is unique among hydrogenases in general in that it is the only one not shown to be sensitive to CO (Schneider *et al.*, 1979). Egerer & Simon (1982) characterized dicoumarol as an inhibitor of electron transfer between the flavin moiety of the hydrogenase and NAD^+ and artificial electron acceptors. Schneider & Schlegel (1981) investigated the role of O_2 and superoxide radicals on the stability of the enzyme, and other investigators have considered the effects of chaotropic agents, proteolysis and other conditions on the inactivation of the similar soluble hydrogenase from *A. eutrophus* Z1 (Gruzinskii *et al.*, 1977; Pinchukova *et al.*, 1979; Popov *et al.*, 1984, 1986).

In the present paper we describe the results of experiments investigating the effects of NO on *A. eutrophus* H16 soluble hydrogenase. NO was first characterized as a potent inhibitor of hydrogenase activity in cell-free extracts of *Proteus vulgaris* (Krasna & Rittenberg, 1954). Inhibition of the $^2H/^1H_2O$ exchange reaction by low concentrations of NO (2 μM in solution) was partially reversible, whereas it became irreversible at high concentrations (20 μM). Tibelius & Knowles (1984) also characterized NO as a potent irreversible inhibitor of hydrogenase activity in whole cells of *Azospirillum brasilense*. More recently, Berlier *et al.* (1987) have used sensitivity to inhibition by CO, NO and nitrite as a diagnostic method for differentiating between three classes of hydrogenases isolated from *Desulfovibrio* species.

MATERIALS AND METHODS

Purification of soluble hydrogenase

The soluble hydrogenase from *A. eutrophus* H16 was purified as described in the preceding paper (Hyman *et al.*, 1988). The low specific activity (24 μmol/min per mg of protein) was primarily due to prolonged storage of the enzyme (see Hyman *et al.*, 1988). However,

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the enzyme used was of consistent specific activity, which facilitated comparison of activities between experiments and with the results presented in Hyman *et al.* (1988).

Activity assays

Unless otherwise stated, all assays were carried out as described in the preceding paper (Hyman *et al.*, 1988).

Manipulation and quantification of NO

NO is rapidly oxidized in the presence of O_2; therefore direct quantification of low concentrations of NO by gas chromatography is difficult. Furthermore, the low concentrations of NO used in the present study are close to the resolution limits (200 μl/l) reported for this technique (Frunzke & Zumft, 1984). In view of these problems, we chose to use a calibrated standard mixture of 1 ± 0.08% NO in He (Liquid Carbonic, Chicago, IL, U.S.A.). Estimates of maximal values for NO gas-phase concentrations were made by quantifying the added He with a Shimadzu 8-A gas chromatograph (Kyoto, Japan) fitted with a thermal conductivity detector. The gas chromatograph was fitted with a 4.9 m × 3.1 mm stainless-steel column containing molecular sieve 5A and was operated with a column temperature of 25 °C, a detector temperature of 70 °C and a detector current of 70 mA. Ar was used as carrier gas at a flow rate of 20 ml/min. All chromatograms were quantified with a Shimadzu CR-3A integrator.

Before use, the NO/He mixture was passed through a Dreschel bottle containing aq. 5 M-KOH to remove any contaminating NO_2. The gas mixture was then passed through an N_2-filled 160 ml stoppered vial with a needle vent. After 30 min the NO/He mixture had displaced the N_2, and this vial was used as a stock for experiments requiring the addition of low concentrations of NO. Transfers of gas from the stock were made with glass-barrelled gas-tight syringes. For experiments requiring a concentration of NO higher than 1%, a cylinder of pure NO was used (purity > 99%) (Liquid Carbonic). The gas from this cylinder was treated in the same way as the NO/He mixture. Estimates of NO concentrations in solution were determined by using the α value of 0.047 for NO solubility in water at 20 °C (Dean, 1985).

Gases other than NO were also of high purity (> 99.99%) and were obtained locally. Residual O_2 was stripped from these gases by passage through a heated Cu-based catalyst (R3-11; Chemical Dynamics Corp., South Plainsfield, NJ, U.S.A.). All other chemicals used were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.).

RESULTS

Kinetics of inhibition of H_2 oxidation by NO

Our initial investigations of NO inhibition made use of enzyme that had been fully activated for H_2-oxidizing activity (20 h at 4 °C in the presence of 50 μM-NADH and 101 kPa H_2). When H_2-dependent Methylene Blue reduction was measured in the presence of 45–150 nM-NO (in solution), the initial activity declined over the first 30–90 s to a lower and nearly constant activity (Fig. 1*a*). As the NO concentration was raised, the rate of loss of activity increased and the level of residual activity decreased. The non-linearity of the control incubation is due to both a substrate (Methylene Blue) limitation and

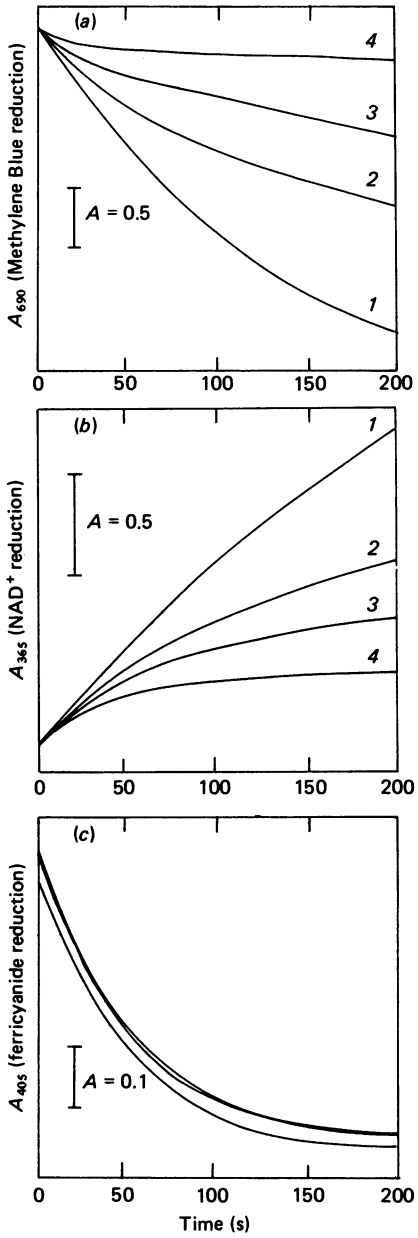

Fig. 1. Inhibition of H_2 oxidation by NO under turnover conditions

(*a*) Effect of NO on H_2-dependent Methylene Blue reduction. Cuvettes (12.8 ml) contained 3 ml of buffer plus 150 μM-Methylene Blue and 101 kPa H_2. The cuvettes also received 0 μl (curve *1*), 30 μl (curve *2*), 50 μl (curve *3*) or 100 μl (curve *4*) of 1% NO in He. The cuvettes were then shaken to equilibrate the liquid and gas phases. Reactions were initiated by addition of 15 μl samples of activated enzyme solution (101 kPa H_2 plus 50 μM-NADH, 20 h; 1 mg of total protein/ml). (*b*) Effect of NO on H_2-dependent NAD^+ reduction. Cuvettes were prepared as above except that NAD^+ (10 mM) was used in place of Methylene Blue. The cuvettes also received 0 μl (curve *1*), 5 μl (curve *2*), 10 μl (curve *3*) or 20 μl (curve *4*) of 1% NO in He. Reactions were initiated by the addition of 50 μl of enzyme activated as above. (*c*) Effect of NO on diaphorase activity. Cuvettes contained 3 ml of buffer plus 0.6 mM-NADH, 0.6 mM-ferricyanide and 101 kPa N_2. Cuvettes received 0 μl, 50 μl or 100 μl 1% NO in He. Reactions were initiated by addition of 50 μl of enzyme activated as for (*a*).

an inactivation that occurs upon exposure of this enzyme to oxidized Methylene Blue over the time course of the assay (results not shown).

For hydrogenase exposed to NO, when residual activity as a function of time was fitted to an exponential (over the first 40 s; $r > 0.99$), an apparent first-order process was revealed. The analysis, taking into account the 5 s mixing time before initiation of the spectrophotometer run, provided initial rates of 15, 16 and 14 nmol of H_2/min per μl of enzyme and $t_{\frac{1}{2}}$ values of 38, 20 and 9.1 s for 45 nM-, 75 nM- and 150 nM-NO (in solution) respectively. The same rate of inhibition of H_2-dependent Methylene Blue reduction was observed for a constant NO concentration if enzyme was activated with either NADH plus 101 kPa H_2 or NADH alone (N_2 gas phase).

When NO was present during assays of H_2-dependent NAD^+ reduction with saturating NAD^+ concentrations (K_m 0.8 mM, assay concentration 10 mM-NAD^+), inhibitions similar to those seen in the Methylene Blue-reduction assays were observed (Fig. 1*b*). However, lower concentrations of NO were required to inhibit H_2 oxidation in these assays than in the Methylene Blue-reduction assays. Analysis of the data shown in Fig. 1(*b*) revealed that the loss of activity in each NO-containing incubation was an apparent first-order process ($r > 0.99$). Initial rates of 13, 12 and 11 nmol of H_2/min per μl of enzyme and $t_{\frac{1}{2}}$ values of 47, 36 and 23 s were obtained for enzyme assayed with 8 nM-, 16 nM- and 31 nM-NO (in solution) respectively. If the NAD^+ concentration was lowered to 0.6 mM, the half-life of activity in the presence of 31 nM-NO was still 23 s (average of three estimates). This suggests that there is no competition between NO and NAD^+.

The highest concentration of NO used to inhibit Methylene Blue reduction (150 nM-NO; Fig. 1*a*) had no effect on the diaphorase activity of the enzyme over the time course of the assay (Fig. 1*c*). These results indicate that the effects of NO at this concentration and over this time period (200 s) are specific for the H_2-oxidizing portions of the enzyme.

The results shown in Fig. 1 demonstrate that low concentrations of NO rapidly inhibit H_2 oxidation by activated hydrogenase under turnover conditions. A subsequent experiment considered the effects of NO on activated enzyme incubated under non-turnover conditions (i.e. absence of an electron acceptor). Enzyme activated for 20 h in the presence of 50 μM-NADH and 1 kPa, 2.5 kPa, 5 kPa, 10 kPa and 30 kPa H_2 exhibited initial rates of H_2 oxidation of 7.0, 8.5, 10.8, 12.1 and 13.3 nmol of H_2/min per μl of enzyme respectively (85 μM-Methylene Blue, 101 kPa H_2). When a constant concentration of NO (140 nM in solution) was added to the incubation vials, H_2-dependent Methylene Blue-reducing activity was again lost in a time-dependent and apparently first-order process. The $t_{\frac{1}{2}}$ values were 1.2, 1.5, 1.2, 1.4 and 1.7 min for enzyme activated with 1 kPa, 2.5 kPa, 5 kPa, 10 kPa and 30 kPa H_2 respectively. The similar half-lives indicate that the rate of inhibition is independent of the H_2 concentration used to activate the enzyme and that there is no competition between NO and H_2. The H_2 concentrations present during both the activations and inhibitions bracketed the reported K_m for H_2 of 37 μM (approx. 5 kPa) for this enzyme (Schneider & Schlegel, 1976). Thus with activated hydrogenase the inhibition by NO occurs approx. 10 times faster under turnover conditions than in the absence of an electron acceptor for a given concentration of NO.

We attempted to determine the effect of NO on active hydrogenase in the absence of a reductant and in the presence of the oxidant, Methylene Blue. Enzyme activated with H_2 plus NADH was transferred to Methylene Blue assay cuvettes containing N_2. Upon exhaustion of the small amount of H_2 carried over with the enzyme sample (i.e. no further reduction of Methylene Blue, about 10 s), NO (to 150 nM in solution) was added to each cuvette. At various times (up to 200 s), turnover was re-initiated by addition of 1 ml of H_2-saturated Methylene Blue solution (150 μM) and the rate of H_2-dependent Methylene Blue reduction was determined. In the absence of NO the enzyme progressively lost activity (30% after 50 s, 75% after 200 s), owing to the oxidizing conditions. The presence of NO did not affect this loss of activity. However, upon addition of H_2 a time-dependent loss of activity, similar to that shown in Fig. 1(*a*), was still observed. For a given concentration of NO, the rate constant for the loss of activity was the same whether the enzyme was assayed directly in the presence of NO (as in Fig. 1) or after initial oxidation by Methylene Blue, as described above.

Time-dependent effects of NO on diaphorase activity

Unlike H_2-oxidizing activity, the diaphorase activity of this hydrogenase does not require activation. In contrast with the potency of NO as an inhibitor of H_2 oxidation, Table 1 shows that the diaphorase activity of the enzyme is considerably less sensitive to NO. Only a 10% loss of activity was observed when unactivated enzyme was incubated for up to 1 h in the presence of 10 kPa NO (200 μM-NO in solution). In contrast, the reductively activated enzyme was sensitive to inhibition by NO even at pressures as low as 0.1 kPa (2 μM in solution). This loss of activity was slow relative to the effects of equivalent concentrations of NO on the H_2-oxidizing activity. A gradual decline in the diaphorase activity of both the activated and the unactivated enzyme was observed over the time course of the 4 h experiment.

Reversibility of NO inhibition

The reversibility of NO inhibition was investigated under conditions where activated NO-treated enzyme was assayed for H_2-oxidizing activity with or without the prior removal of NO. Time courses of activity for enzyme pre-exposed to NO in the absence of an electron acceptor and then transferred to assay cuvettes without NO are shown in Fig. 2. For both H_2-dependent NAD^+ reduction (Fig. 2*a*) and Methylene Blue reduction assays (Fig. 2*b*), the activity of the NO pre-exposed enzyme was initially (first 10 s) very low, which indicated that the enzyme was still inhibited. Over the following 30 s the activity increased and then became constant. The extent of the activity recovered was dependent on the concentration of NO to which the enzyme had previously been exposed; higher concentrations of NO resulted in lower recoveries of activity. In no case was the recovery of activity complete. The lowest NO concentration tested yielded a recovered activity that was only 21% of the control. In separate experiments quantities of NO equivalent to those carried with the enzyme from the incubation to the assay did not inhibit the enzyme to the same extent as in the reversibility experiments. Because the recovery of activity occurred in both Methylene Blue reduction and

Table 1. Effect of NO on diaphorase activity

Samples (0.5 ml) of anaerobic enzyme solution (1 mg of total protein/ml) placed in the inner chamber of two 13.8 ml incubation vials were treated for 20 h at 4 °C with either 101 kPa H_2 plus 50 μM-NADH (activated enzyme) or 101 kPa N_2 alone (unactivated enzyme). Portions (100 μl) of both of these enzyme samples were then transferred to 13.8 ml anaerobic incubation vials containing various concentrations of NO in either 101 kPa H_2 (activated enzyme) or 101 kPa N_2 (unactivated enzyme). In all cases the NO was added as an overpressure. At 20 min intervals, 20 μl portions of enzyme were removed and assayed for residual diaphorase activity as described in the Materials and methods section.

Conditions	Time (min)	Diaphorase activity (μmol of NADH/min per mg of enzyme) Unactivated enzyme	Activated enzyme
No NO	0	52.9	50.7
	20	56.9	42.8
	40	51.8	43.7
	60	48.4	43.0
	240	45.2	44.6
0.1 kPa NO	0	49.5	46.9
	20	49.7	36.8
	40	46.9	32.4
	60	46.3	30.9
1 kPa NO	0	47.1	42.4
	20	44.7	33.8
	40	42.6	27.1
	60	43.0	24.0
10 kPa NO	0	44.4	40.1
	20	43.6	18.5
	40	43.3	15.7
	60	40.7	15.3

NAD^+ reduction assays, it was clearly distinct from the autocatalytic activation that occurs in the presence of NAD^+ and H_2, but not in the presence of Methylene Blue and H_2.

Fig. 3 shows the time course for the recovery of activity when activated enzyme was initially exposed to NO under non-turnover conditions (absence of electron acceptor), followed by the removal of the NO by purging the enzyme incubation vial with H_2. In the control incubation the removal of H_2 resulted in a progressive loss of approx. 30% of the initial activity, which was subsequently fully recovered after exposure to H_2 (see Hyman *et al.*, 1988). In the NO-containing incubation over 95% of the initial activity was lost within 20 min. Treatment with H_2 resulted in a progressive recovery of activity to about 50% of the pre-inhibited rate. In contrast with the relatively rapid rate of recovery of activity shown in Fig. 2, this recovery of activity took over 2 h to complete. A further experiment showed that even after 20 h no more than 50% of the initial activity was recovered (Table 2). The extent of the recovery of activity was similar whether or not H_2 was present during the inhibition phase and was not dependent on the H_2 oxidation assay (Methylene Blue or NAD^+ reduction).

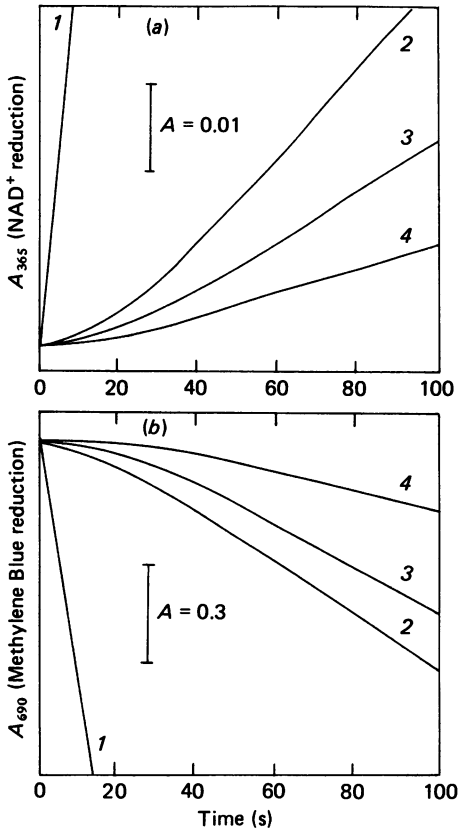

Fig. 2. Reversibility of NO inhibition under turnover conditions

Samples (100 μl) of hydrogenase (1 mg of total protein/ml) were activated with 101 kPa H_2 plus 50 μM-NADH for 20 h, then incubated in 13.8 ml anaerobic incubation vials (101 kPa N_2) containing 0 μl (curve *1*), 100 μl (curve *2*), 250 μl (curve *3*) or 500 μl (curve *4*) of 1% NO in He which was added as an overpressure. After 30 min portions of each enzyme sample were assayed for (*a*) H_2-dependent NAD^+ reducing activity and (*b*) H_2-dependent Methylene Blue-reducing activity. Sample sizes were 15 μl for (*a*) and 50 μl for (*b*).

Effect of NO pre-exposure on the activation of enzyme

The experiments described above suggest that NO inhibition of this enzyme involves both a reversible and an irreversible component. To differentiate further between these two forms of NO inhibition, we considered the effects of NO treatment on the ability of the enzyme to be activated for H_2 oxidation. Exposure of the unactivated enzyme to NO resulted in a distinct loss of ability of the enzyme to be activated by a variety of treatments (Fig. 4). Activation of the NO-treated enzyme with $Na_2S_2O_4$ and 101 kPa H_2 resulted in an initial burst of activity over the first 1 h, followed by a progressive loss of H_2-dependent Methylene Blue-reducing activity. A more stable activation was achieved when NO-treated enzyme was activated with NADH plus 101 kPa H_2. However, in both cases the maximal level of activation achieved was no more than 35% of the control enzyme activated with $Na_2S_2O_4$ plus 101 kPa H_2. NO pre-treatment also considerably decreased the rate at which the enzyme could be activated with H_2 alone, although it apparently did not completely inhibit this process.

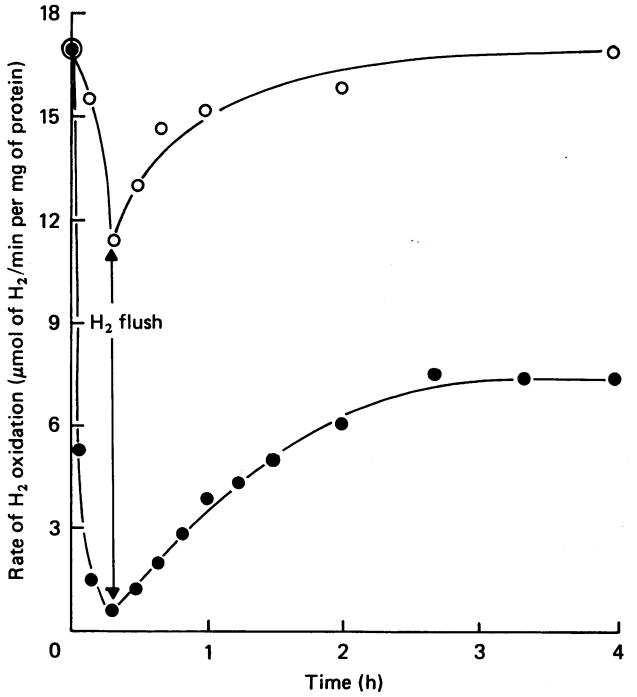

Fig. 3. Reversibility of NO inhibition under non-turnover conditions

Hydrogenase (1 mg of total protein/ml) was activated with 101 kPa H_2 plus 50 μM-NADH for 20 h. Enzyme samples (200 μl) were placed in 13.8 ml anaerobic incubation vials containing either 101 kPa He (○) or 1% NO in He (●) at $t = 0$. Samples (10 μl) were removed at regular intervals and residual H_2-dependent Methylene Blue-reducing activity was determined as described in the Materials and methods section. After 20 min both vials were purged continuously with O_2-free H_2 (50 ml/min) and the sampling was continued.

NO treatment of the unactivated hydrogenase greatly decreased the ability of the enzyme to respond to H_2 when in the presence of NADH. When hydrogenase was activated with NADH plus H_2 (Table 3) following NO treatment, the rate of Methylene Blue reduction was only 33% higher than when activated in the presence of NADH alone. No increase in activity was observed with H_2 plus NADH compared with NADH alone if H_2-dependent NAD^+ reduction was measured. In contrast, the Methylene Blue-reduction activity of control enzyme was stimulated approx. 5-fold by inclusion of H_2 plus NADH during the activation relative to NADH alone. Similarly, the NAD^+-reduction activity was stimulated nearly 4-fold by inclusion of H_2 in the activation. The effects of NO pre-exposure on the level of subsequent activation of H_2-oxidizing activity were similar whether enzyme was exposed to 20 μM-NO (in solution) for 10 min or 1 h (results not shown).

The diaphorase activity measured for enzyme treated with NO followed by activation of the H_2-oxidizing activity was not appreciably different from that for enzyme without NO pretreatment (Table 3).

DISCUSSION

We have presented evidence that suggests that NO has three modes of action on *A. eutrophus* soluble hydro-

Table 2. Reversibility of NO inhibition of activated enzyme

Samples (200 μl) of anaerobic enzyme solution (1 mg of total protein/ml) activated for 20 h at 4 °C (101 kPa H_2 plus 50 μM-NADH] were placed in separate 13.8 ml anaerobic incubation vials containing 101 kPa H_2. NO (200 μl) was added to one vial. After 20 min (designated as time 0 below) both vials were purged with a continuous stream of O_2-free H_2 (50 ml/min). After 3 h the various activities of both enzyme samples were assayed as described in the Materials and methods section and the H_2 stream was discontinued. After storage at 4 °C for 17 h the various activities of both enzyme samples were assayed again.

		Activity (μmol of substrate/min per mg of enzyme)		
Treatment	Time (h)	H_2-dependent Methylene Blue reduction	H_2-dependent NAD^+ oxidation	Diaphorase activity
Control	0	15.9	11.8	41.9
+NO	0	0	0	41.9
Control	3	14.0	10.0	41.3
+NO	3	4.9	3.4	35.6
Control	20	14.1	8.7	39.9
+NO	20	6.2	3.6	41.2

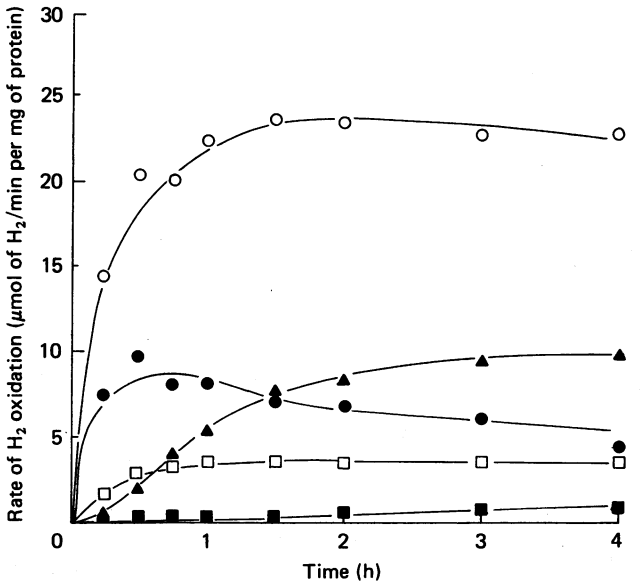

Fig. 4. Effect of NO treatment of unactivated enzyme on activation of H_2-oxidizing activity

Samples (500 μl) of unactivated enzyme (1 mg of total protein/ml) were placed in 13.8 ml anaerobic incubation vials containing either 101 kPa N_2 (○ and □) or 1% NO in He (●, ■ and ▲). After 30 min both vials were evacuated and equilibrated with N_2. Samples (0.1 ml) of both enzyme treatments were then transferred to 13.8 ml anaerobic incubation vials with the following gas phases and additions: ○ and ●, 101 kPa H_2 plus $Na_2S_2O_4$ to 1 mM; □ and ■, 101 kPa H_2, no additions; ▲, 101 kPa H_2 plus NADH to 50 μM. At the indicated times samples were removed from each incubation and the H_2-dependent Methylene Blue reducing activity was determined (see the Materials and methods section).

Table 3. Effect of NO treatment on the ability of hydrogenase to be activated by H_2 and NADH

Anaerobic enzyme solution (1 ml; 1 mg of total protein/ml) was placed in a 13.8 ml anaerobic incubation vial containing a gas phase of 1 % NO in He. After 30 min the enzyme sample was repeatedly evacuated and equilibrated with O_2-free N_2. Samples (100 μl) of this NO-pretreated enzyme were then transferred to 13.8 ml anaerobic vials for subsequent activation at 4 °C for 20 h under the conditions described below. After 20 h the various activities of the individual enzyme samples were assayed. The untreated sample was prepared in the same way, except without exposure to NO.

	Activity (μmol of substrate/min per mg of enzyme)		
Activation conditions	H_2-dependent Methylene Blue reduction	H_2-dependent NAD^+ reduction	Diaphorase activity
NO-treated			
101 kPa N_2 alone (no NADH)	0	0.2	46.0
101 kPa N_2 + 50 μM-NADH	2.7	2.7	42.0
101 kPa H_2 + 50 μM-NADH	3.6	2.4	41.9
Untreated			
101 kPa N_2 + 50 μM-NADH	3.2	2.9	42.1
101 kPa H_2 + 50 μM-NADH	17.0	10.7	39.3

genase. These are (1) a potent time-dependent reversible inhibition of the H_2-oxidizing activity, (2) an irreversible effect on H_2-oxidizing activity, and (3) an irreversible effect on the unactivated enzyme that limits its ability to be activated. The evidence for each of these effects is discussed below.

The inhibition of H_2 oxidation by NO has the characteristics of a slow-binding inhibition (Figs. 1 and 2). The dominant feature of this form of inhibition is that the equilibrium between free enzyme (E), inhibitor (I) and the inactive enzyme–inhibitor complex (EI) is achieved slowly relative to substrate turnover. Morrison (1982) and others have described two mechanisms for this form of inhibition. Both mechanisms predict a first-order loss of activity arising from the low rate of formation of an inactive EI complex from E and I. The two mechanisms can be differentiated by their respective effects on the initial rates in activity progress curves.

The presence of low concentrations of NO inhibited H_2-dependent Methylene Blue reduction in an NO-concentration- and time-dependent fashion (Fig. 1). The inhibition resulted in a constant residual activity, despite an excess of inhibitor relative to enzyme. Our analysis suggested that NO had no effect on the initial rate of reaction. This is characteristic of Mechanism-1-type slow-binding inhibition where E and I combine directly (without an intermediate isomerization) to give inactive EI complex (Morrison, 1982). The constant residual activity in the NO-treated samples directly implies that an equilibrium is reached between E, I and EI, and therefore that the inhibition is reversible. Enzyme pre-exposed to NO exhibited a time-dependent increase in activity when transferred to turnover conditions (Fig. 2). We conclude that this time-dependent increase in activity is due to the dissociation of an inactive EI complex to yield free and active enzyme.

Although the results in Figs. 1 and 2 demonstrate a qualitative adherence to the behaviour expected of a slow-binding inhibitor (Morrison, 1982; Sculley & Morrison, 1986), there are several important considerations that prevent a more quantitative kinetic analysis. Firstly, we found no evidence of competition between NO and H_2 or NAD^+. However, the kinetic mechanisms for slow-binding inhibitors developed by Morrison and others all assume a competition between inhibitor and substrate. The second complication is that a tacit assumption behind present kinetic theories of slow-binding inhibition is that full recovery of activity is to be expected from simple dissociation of the inactive EI complex. Although NO inhibition was partially reversible when NO-treated enzyme was transferred to assay cuvettes without prior removal of the NO (Fig. 2), the extent of recovery of activity did not approach 100 %. This suggests that some factor other than simple dissociation limits the reversibility. The reversibility of NO inhibition of the activated enzyme after NO was removed by flushing with H_2 was limited to approx 50 % of full activity (Fig. 3 and Table 1). The inability to recover H_2-oxidizing activity fully indicates that there is an irreversible component to the inhibition as well. These results suggest that the forward rates of inhibition characterized in Fig. 1 are actually combined rates for two inhibitory processes, one of which is reversible, the other irreversible. Whether these two forms of inhibition occur at the same or different sites on the enzyme is not yet apparent.

The rates of inhibition and recovery of activity depended upon whether the enzyme was under turnover or non-turnover conditions. Activated hydrogenase was inhibited about 10 times faster under turnover conditions than in the presence of H_2 and the absence of an electron acceptor. When activated hydrogenase was exposed to the oxidant Methylene Blue in the absence of H_2, no NO-dependent inhibition was observed over 200 s. Although this experiment is complicated by the loss of activity in the presence of oxidant alone, the results are consistent with the need for turnover conditions to achieve maximal rates of inhibition. Perhaps NO preferentially and reversibly binds to a component of the enzyme that is generated more frequently under turnover than under non-turnover conditions (no acceptor present). For example, a redox centre may be more susceptible to attack by NO as it cycles from reduced to oxidized forms during turnover (H_2 oxidation).

The predominant biochemical use of NO in the past has been in ligand-binding studies of haem proteins. Kinetic studies using NO strictly as an inhibitor have been restricted to non-O_2-utilizing enzymes such as nitrogenase (Meyer, 1981) and hydrogenase (Krasna & Rittenberg, 1954). These, and other studies (Reddy *et al.*, 1983), have suggested that the inhibitory effects of NO arise from the destruction of [4Fe-4S] centres present in these enzymes. It is possible that the same is true for the soluble hydrogenase. NO treatment of the unactivated hydrogenase gave rise to an apparently irreversible effect that resulted in a loss of some of the enzyme's ability to

achieve maximal activation and to respond positively to H_2 in the presence of NADH. Because NO was relatively ineffective at inhibiting the diaphorase activity of this enzyme in the unactivated state, neither the flavin nor the [2Fe-2S] centre on the diaphorase side of the enzyme is a candidate for the site of this irreversible NO effect. These two prosthetic groups represent the minimum redox components required for NADH dehydrogenase activity. Furthermore, the level of activity of hydrogenase activated with NADH alone was very similar for both NO-pretreated and untreated enzyme (see Table 2). After NO pre-exposure, hydrogenase retained both its ability to be activated by NADH alone and to oxidize H_2. If the minimum redox components required for H_2 oxidation are Ni and the [4Fe-4S] centre of the hydrogenase active dimer (Hornhardt *et al.*, 1986), then these must also be unaffected by NO pretreatment. (It seems likely, however, that one or both of these components are the sites of the reversible inhibition, possibly through the formation of an unstable redox-state-dependent complex with NO.) In contrast, pre-exposure of hydrogenase to NO resulted in a loss of the enzyme's ability to respond to H_2 during activation in the presence of NADH plus H_2. These observations are consistent with the selective and irreversible destruction of a regulatory component of this enzyme. Cammack *et al.* (1982) suggested that an Fe-S centre may fulfil a regulatory role in this soluble hydrogenase. In view of the known effects of NO on [4Fe-4S] centres in other enzymes (see above), and the reasonable elimination of some of the other prosthetic groups as possible sites of this effect of NO, it seems plausible that one of the [4Fe-4S] centres in the diaphorase dimer fulfils this regulatory role and is the site of the irreversible NO effect on activation.

The present understanding of the role of the redox components in the soluble NAD^+-reducing hydrogenases is limited. The use of inhibitors represents a powerful means of probing the role of such components. A further kinetic study of the effects of NO on the activities of the holoenzyme in addition to the individual dimers of dissociated forms of this enzyme (or the enzyme from *N. opaca* 1b) may well enable the sites of the slow-binding reversible/irreversible inhibition and the irreversible effect on activation to be discriminated and their combined effects more clearly understood. Furthermore, the use of NO as an inhibitor of these enzymes is especially appealing for e.p.r. studies because of the paramagnetic qualities of NO and the proposed interaction between NO and e.p.r.-active components such as Fe-S centres.

Received 9 September 1987/19 February 1988; accepted 17 May 1988

This work was supported by U.S. Department of Agriculture Competitive Grant no. 84-CRCR-1-1466 and by Department of Education Grant no. DE-FG03-84ER132571.

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