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Nitrous Oxide Degradation by Cobalamin-Dependent Methionine Synthase: Characterization of the Reactants and Products in the Inactivation Reaction†

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ABSTRACT: Cobalamin-dependent methionine synthase catalyzes the remethylation of homocysteine to form methionine, using methyltetrahydrofolate as the primary methyl donor. The enzyme is susceptible to inactivation by the anaesthetic gas, nitrous oxide, through either short-term exposure to high levels or chronic exposure to low levels of this agent. We have studied the chemical reaction wherein the bound cobalamin prosthetic group of the enzyme from *Escherichia coli* catalyzes the degradation of nitrous oxide. By poising the enzyme at low ambient potentials in an electrochemical cell, the concentration of enzyme in the highly reactive cob(I)alamin state can be controlled, and the observed rate of inactivation is directly dependent upon the amount of enzyme in this redox state. The inactivation consumes both nitrous oxide and electrons, while nitrogen gas is evolved. The inactivation process is also directly dependent upon the proton concentration, but the effect of pH was found to be on the enzyme, revealing a redox-linked base presumed to be on or near the cobalamin. The chemical reaction between enzyme-bound cob(I)alamin and nitrous oxide that leads to inactivation is therefore independent of pH. In a single turnover experiment, where enzyme-bound cob(I)alamin is generated from methylcobalamin by methyl transfer to the substrate homocysteine, the cob(I)alamin decays to cob(II)alamin, suggesting that the degradation of nitrous oxide involves one-electron reduction of nitrous oxide. We propose that the inactivation chemistry is likely to be a one-electron reduction of nitrous oxide, which leads to the formation of a highly reactive oxidant, such as hydroxyl radical, and subsequent enzyme damage.

Nitrous oxide (N_2O), or laughing gas, is an anaesthetic agent that is currently in relatively common use. Under some circumstances, however, it has been demonstrated to precipitate a set of serious side effects, and these include megaloblastic anemia and a subacute combined degeneration of the spinal cord. The megaloblastic anemia, associated with severe bone marrow depression, was first observed in patients who were being continuously treated with nitrous oxide over the course of several days to relieve the psychological strain brought on by tetanus-induced paralysis (Lassen et al., 1956). Megaloblastic changes in red blood cells were later more directly correlated with continuous exposure to nitrous oxide in humans (Amess et al., 1978). In the same year, a case study of dentists who abused nitrous oxide over periods of months to years through intermittent, recreational use was reported (Layzer, 1978), and these patients demonstrated a neurological disorder similar to the subacute combined degeneration of the spinal cord associated with vitamin B_{12} deficiency. Deacon et al. (1978) investigated the effect of nitrous oxide on the two vitamin B_{12}-dependent enzymes in rat and reported that the anaesthetic gas inhibits methionine synthase, but not methylmalonyl-CoA mutase. A broad range of subsequent investigations supports the basic hypothesis that nitrous oxide selectively inactivates methionine synthase and that the observed physiological sequelae are consistent with this inactivation [for overviews, see Chanarin et al. (1985) and Metz (1992)].

The development of an understanding of the biochemical basis for susceptibility to nitrous oxide has been a slow process. Given that nitrous oxide directly affects the activity of one of the two cobalamin-dependent enzymes in humans, the primary suspect from a chemical standpoint would be methionine synthase, because this enzyme utilizes the highly reduced cob(I)alamin state. Highly reduced transition metals, including the cob(I)alamin form of vitamin B_{12}, have been shown to catalyze the reductive degradation of nitrous oxide [Banks et al., 1968; Blackburn et al., 1977; also see Vaughan et al. (1987)]. Methylmalonyl-CoA mutase is not thought to require such a highly reduced catalytic intermediate, since this enzyme binds adenosylcobalamin and performs chemistry best rationalized as being initiated by the adenosyl radical, while the cobalt remains as the relatively inert cob(II)alamin [reviewed by Retey (1982)]. Yet despite a wealth of experimental information on the effects of nitrous oxide *in vivo*, the direct demonstration that methionine synthase is inactivated *in vitro* has been limited to a communication showing that turnover by the mammalian enzyme or the purified enzyme from *Escherichia coli* is irreversibly inhibited under an atmosphere of nitrous oxide (Frasca et al., 1986). Frasca et al. also reported that the *E. coli* enzyme carried out approximately 3900 methyl transfers from CH_3-H_4folate[1] to homocysteine under an atmosphere of nitrous oxide before becoming inactivated; turnover is likely to play a role in protecting the enzyme from

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetate, sodium salt; E_m, electrochemical midpoint potential; E_{m7}, electrochemical midpoint potential at pH 7; Hcy, L-homocysteine; CH_3-H_4folate, (6-*R*,*S*)-5-methyltetrahydrofolate monoglutamate; mV, millivolts; SHE, standard hydrogen electrode; Tris, tris(hydroxymethyl)aminomethane.

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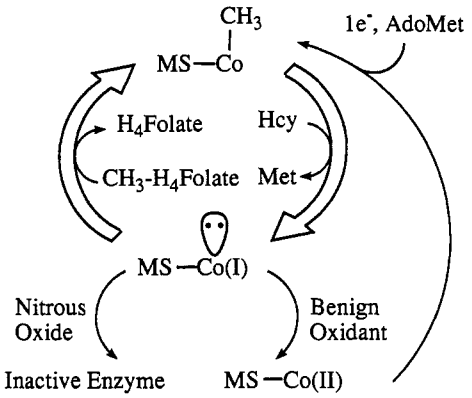


FIGURE 1: Turnover scheme for methionine synthase. The enzyme cycles in turnover between the cob(I)alamin and methylcobalamin forms of the prosthetic group, catalyzing methyl transfer from CH_3-H_4folate to homocysteine. Oxidation of the cob(I)alamin disables the enzyme, but activity can be recovered by reductive methylation involving one electron and *S*-adenosylmethionine. Interception of cob(I)alamin by nitrous oxide can irreversibly inactivate the enzyme.

inactivation, since nitrous oxide must compete for cob(I)-alamin with the chemical remethylation by CH_3-H_4folate. Spectrally, the inactivated *E. coli* enzyme retained bound cob-(II)alamin, although partial degradation of the prosthetic group was also observed.

Cobalamin-dependent methionine synthase from *E. coli* is mechanistically very similar to the enzyme from mammalian sources [reviewed in Taylor and Weissbach (1973)] and Banerjee and Matthews (1990)] and is available in highly purified form in sufficient quantities to permit detailed studies of the mechanism of inactivation by nitrous oxide. The studies reported in this article have been performed with the enzyme from *E. coli*, but there is every reason to believe that the mechanism of inactivation of the mammalian enzyme by nitrous oxide will be similar.

Figure 1 presents a summary of the mechanism through which methionine synthase catalyzes the remethylation of homocysteine to form methionine [reviewed by Taylor and Weissbach (1973) and Banerjee and Matthews (1990)]. Because the enzyme utilizes the highly reduced and oxidatively unstable cob(I)alamin species, the enzyme periodically becomes oxidized by one or two electrons to the cob(II)alamin or cob(III)alamin species, respectively. The enzymatic turnover is therefore susceptible to the presence of oxidants, and methionine synthase functions best in an environment of low oxygen tension. Solutions saturated in nitrous oxide present an unfavorable environment for this enzyme, both because N_2O is a potent oxidant (Latimer, 1938) and because it possesses moderate solubility in aqueous solution (25 mM at 25 °C; Weast, 1987). Under most circumstances, oxidation of the cobalamin is benign, and the enzyme has a mechanism for the return to catalysis by reductive methylation involving *S*-adenosylmethionine [see Banerjee et al. (1990) and references cited therein]. Oxidation of the enzyme-bound cob-(I)alamin by nitrous oxide has been proposed to lead to inactivation, but no direct evidence for either the chemical reaction between the two species or the degree to which the proposed oxidation is coupled to inactivation of the enzyme has been reported.

Two general models have been proposed [see Frasca et al. (1986)] to explain how methionine synthase becomes inactivated by nitrous oxide, and the stoichiometries for the chemical reactions that could lead to inactivation are outlined in eqs 1 and 2. Both involve chemical degradation of nitrous oxide by cob(I)alamin, but they predict different pathways to enzyme inactivation. In the first model (eq 1), the products derived from the degradation of nitrous oxide are benign, and the generation of enzyme-bound cob(III)alamin is at the center of proposals for inactivation. Cob(III)alamin could potentially form kinetically stable complexes with strong ligands donated by the enzyme, and two-electron oxidation of the bound cobalamin would be the key event preceding loss of activity. This model implies that two electrons are transferred from the cob(I)alamin to nitrous oxide and that two protons are taken up in the process. The second model to explain inactivation invokes the same reactants, but it requires a different stoichiometry of electron transfer and proton uptake in the reaction (eq 2). It suggests that a one-electron reduction of nitrous oxide, a commonly used method of generating hydroxyl radicals by pulse radiolysis (Spinks & Woods, 1990), allows for the formation of a highly reactive intermediate like a hydroxyl radical. Note that radical recombination of the stable cob(II)alamin and the highly reactive hydroxyl radical unifies the two mechanisms (eq 3), but still allows for the escape of a potentially damaging species during the reduction of nitrous oxide.

$$\text{enz–cob(I)alamin} + N_2O + 2H^+ \rightarrow \text{enz–cob(III)alamin} + H_2O + N_2 \quad (1)$$

$$\text{enz–cob(I)alamin} + N_2O + H^+ \rightarrow \text{enz–cob(II)alamin} + HO^\bullet + N_2 \quad (2)$$

$$\text{enz–cob(II)alamin} + HO^\bullet + H^+ \rightarrow \text{enz–cob(III)alamin}\cdot H_2O \quad (3)$$

The goal of this work was to characterize the process by which cobalamin-dependent methionine synthase degrades the anaesthetic gas nitrous oxide. Specifically, we sought to substantiate the proposal that nitrous oxide intercepts the highly reactive cob(I)alamin, an intermediate essential to turnover that distinguishes methionine synthase from other enzymes that utilize the adenosylcobalamin prosthetic group. If the cob(I)alamin group is becoming oxidized, what is the cobalamin product, and what are the products derived from nitrous oxide? Specifically, is the cobalamin becoming oxidized to the cob(III)alamin state, a two-electron oxidation, yielding an oxidized prosthetic group that stably captures a protein ligand to give an exchange-inert complex? Or does the inactivation process require an alternate reductive degradation of nitrous oxide, leading to the formation of an oxidant that damages the protein or the prosthetic group proximal to the cobalt? Finally, what is the effect of pH on the inactivation chemistry, i.e., is the inactivation catalyzed by protons? Protons clearly play a different role in each of the models proposed for the inactivation process. This initial characterization is intended to lay the groundwork for the chemical characterization of the effects of nitrous oxide inactivation on both the enzyme and the cobalamin prosthetic group itself (Drummond & Matthews, 1994).

MATERIALS AND METHODS

Materials. Triquat was the kind gift of Professor Stephen Ragsdale at the University of Nebraska, and the 5-deazaflavin-3-methanesulfonic acid was provided by Professor Vincent Massey at the University of Michigan. Methyl viologen dihydrochloride was purchased from the Sigma Chemical Co., as were the other chemical reagents used in this work.

Purification of Methionine Synthase. Recombinant methionine synthase (MetH) from *E. coli* K-12 strain DH5αF'/p4B6.3 was overproduced and purified as previously reported (Banerjee et al., 1989). The enzyme was isolated with the enzyme-bound prosthetic group present as a mixture of cob(II)alamin, cob(III)alamin, and methylcobalamin forms. When enzyme in the methylcobalamin form was required, the enzyme was reductively methylated in the presence of *S*-adenosylmethionine (AdoMet) as described by Luschinsky et al. (1992). For most of the experiments described here, the methylcobalamin was demethylated with homocysteine in the presence of dithiothreitol to yield the enzyme-bound cob(II)alamin form, as previously described (Drummond et al., 1993b).

Purification of Gases. Argon (99.99%) was scrubbed by passage over a heated copper-containing column as previously described (Matthews et al., 1974). Nitrous oxide (United States Pharmaceuticals grade) was bubbled through a solution of sodium dithionite (0.9 M) in sodium hydroxide (3.3 M) to remove traces of oxygen [see Fieser (1924)]. Anthraquinone-2-sulfonic acid was omitted from this modification of Fieser's solution to prevent excessive foaming in the gas bubbler.

Measurement of the Rate of Electrochemical Inactivation. The anaerobic electrochemical titration cell of Harder et al. (1989) was used whenever methionine synthase was poised at a given potential. Potentials were maintained with a potentiostat (Model CV-27 from Bioanalytical Systems, West Lafayette, IN). The inactivations were carried out on solutions of 1 μM methionine synthase in 100 mM potassium phosphate buffer containing 100 mM potassium chloride and 500 μM mediator dye at the potentials given in the legend for Figure 4. Methyl viologen (E_{m7} = –440 mV *vs* the SHE) was used as the mediator when potentials between –400 and –480 mV were desired, while triquat (E_{m7} = –540 mV) was used for potentials below –480 mV. In each experiment, the enzyme solution was prepared in the cell on ice and stirred gently over the course of 45 min at ambient temperature as the atmosphere was exchanged for nitrous oxide or argon. At 5-min intervals, the cell was taken through seven cycles of partial evacuation followed by replacement with the oxygen-free gas. The solution was allowed to warm to room temperature before the cell was poised at the desired potential; functionally, the cell required approximately 3 min to approach equilibrium. Samples were withdrawn through a port fitted with a rubber septum at the specified times, and they were stored at room temperature and assayed upon the completion of the experiment.

Dependence of Inactivation Rate on pH. Each inactivation experiment was performed in large part as described above, except that the potential was maintained at –450 mV *vs* the SHE and 500 μM methyl viologen was used as the mediator. Each experiment contained 50 mM potassium phosphate, 50 mM Tris buffer, 100 mM KCl, and 1 μM methionine synthase as above, but 200 μM homocysteine was also included in each experiment to increase the extent of inactivation. Homocysteine did not affect the rate of inactivation, but it did decrease the residual activity following the inactivation, presumably by demethylating *in situ* the small amount of methylated enzyme present.

Measurement of the Enzyme-Catalyzed Rate of Nitrous Oxide Reduction. Each rate determination was carried out at pH 6.6 in a volume of 900 μL at the final concentrations given. Methionine synthase (0–5 μM) was buffered in 100 mM potassium phosphate containing 100 mM KCl, 25 mM EDTA, 100 μM methyl viologen, and 5 μM 5-deazaflavin-3-sulfonic acid. The enzyme solution was transferred to a spectrophotometric cell, and the gaseous atmosphere was exchanged for nitrous oxide at ambient temperature, as described above for the electrochemical inactivation protocol. The absorbance of the cell contents was adjusted to zero at 600 nm to remove the small contribution of the enzyme at this wavelength, and the enzyme and mediator dye were photochemically reduced by irradiation with a Sun Gun lamp (650 W, 120 V) in a 2-L Pyrex beaker filled with water at room temperature. The beaker acts as an ultraviolet filter, while the water acts as a heat sink to maintain the temperature of the cell. Because the solubility of nitrous oxide varies greatly with temperature, fluctuations over the course of the experiment can lead to the formation of gas bubbles that interfere with spectral monitoring of the experiment. The contents of the cell were partially reduced by irradiation at a setting of 60 V for 30 s, the cell contents were gently mixed, and the irradiation was repeated. At higher enzyme concentrations, where the rate of mediator decay was more rapid, a third burst of light for 15 s was added. The cell was placed in a spectrophotometer maintained at 25 °C, and the oxidation of the methyl viologen radical cation was monitored at 600 nm.

Spectrophotometric Determination of the Cob(II)-/Cob(I)-alamin Equilibrium Midpoint Potential (E_m). A concentrated stock of methionine synthase in the cob(II)alamin state was prepared as previously described by treatment of the enzyme with homocysteine in the presence of dithiothreitol (Drummond et al., 1993b). The buffer and the thiols were removed by three cycles of concentration of the solution in a Centricon 30 microconcentrator (Amicon) at 4 °C, followed by replacement of the buffer with 5 mM potassium phosphate buffer (pH 6.6) containing 1 mM EDTA. For the measurement of the E_m at each pH value, the lightly buffered stock was diluted to a final volume of 900 μL of 100 mM potassium phosphate buffer containing 35 μM methionine synthase, 100 mM KCl, 25 mM EDTA, 100 μM methyl viologen, and 5 μM 5-deazaflavin-3-sulfonic acid and adjusted to the pH value reported. The phosphate buffer stocks were prepared by mixing 1 M stocks of potassium dihydrogen phosphate and potassium monohydrogen phosphate to approximate the desired pH value at the concentration of the experiment and then diluting this stock to a final concentration of 200 mM phosphate buffer after the addition of potassium chloride to 200 mM and EDTA to 50 mM. Final adjustments on each 2× stock were then made with 1 N HCl to give the desired pH on a standardized pH meter (Corning Model 125). The enzyme-containing solution was placed in an anaerobic spectrophotometric cell, the atmosphere was replaced with argon at 4 °C, and then the system was reduced by visible light irradiation through a Pyrex beaker at 25 °C as described above. The anaerobic solution was reduced by visible irradiation in cycles of 60-s exposure at a rheostat setting of 40 V to give ~50% reduction of the methyl viologen, based on the visible absorbance at 600 nm, and then exposed for 30 s at a rheostat setting of 60 V for 2–3 flashes to achieve ~70% reduction. Spectra were recorded after each irradiation and at 5–20-min intervals after the system approached equilibrium. Because cob(I)alamin is unstable in aqueous solution, reducing protons to hydrogen gas (Tackett et al., 1963), the contents of the cell slowly oxidize, and this property of the system was employed to effectively titrate the reduced, equilibrated system.

Calculation of the Midpoint Potential from the Spectra. The extinction coefficients at 474 nm for cob(I)alamin (5100 M^{-1} cm^{-1}) and cob(II)alamin (11 000 M^{-1} cm^{-1}) bound to methionine synthase at pH 7.2 (Banerjee & Matthews, 1990)

and the reduced methyl viologen radical cation at 600 nm (13 600 M^{-1} cm^{-1}; Mayhew, 1978) were used to determine the concentration of each species in redox titrations of 35 μM enzyme and 100 μM methyl viologen. The cob(II)-/cob(I)-alamin couple was monitored at 474 nm, where methyl viologen contributes little absorbance, while the methyl viologen concentration was monitored at 600 nm. Because methyl viologen dimerizes at higher concentrations (K_d = 1 mM; Thorneley, 1974), small discrepancies exist between the predicted and measured absorbances at each concentration, and an empirical plot of A_{600} *vs* A_{474} (nm) was constructed for methyl viologen to correct for these small differences. Both the E_m of methyl viologen (E_{m7} = –440 mV *vs* the SHE) and of the dimerization equilibrium are independent of pH. The reduced methyl viologen concentration was used to determine the ambient potential of the cell [E_h; see Clark (1960)] from the Nernst equation, and the concentration of each cobalamin form was estimated after the contribution of methyl viologen at 474 nm was subtracted.

Two additional minor corrections were made. First, during the photoreduction process, approximately 10% of the methyl viologen mediator was damaged in each photochemical reduction cycle, and the effective total methyl viologen concentration in equilibrium with the enzyme was estimated to be 90 μM. In control experiments without enzyme, photochemical poising of the cell using the same protocol as that used to equilibrate the enzyme yielded approximately 90% reduction of the mediator. Following reoxidation of the cell and exchange of the atmosphere for argon, the same photoreduction protocol yielded ~90% of the reduction obtained in the first photoreduction cycle, and similar results were obtained in subsequent runs. Second, in titrations containing methionine synthase, a small but significant increase in absorbance at 474 nm was observed when oxygen was introduced into the cell following each experiment, even though the blue methyl viologen radical cation was already completely oxidized. The final enzyme concentration was calculated on the basis of the final enzyme absorbance before oxygen was admitted into the cell; this treatment has little effect on the absolute value of the midpoint potentials, but prevents skewing in the slopes of the Nernst plots at high ambient potentials.

RESULTS

Oxidation to Cob(III)alamin Does Not Lead to Inactivation. The stoichiometry of a commonly proposed model to explain nitrous oxide inactivation of methionine synthase was presented in eq 1. Basically, oxidation of cob(I)alamin by two electrons has been proposed to precede capture of the resulting cob(III)alamin by a ligand donated by the enzyme. Although we have been unable to demonstrate a direct, two-electron oxidation of cob(I)alamin by nitrous oxide, we wanted to establish whether or not the formation of cob(III)alamin bound to the enzyme would lead to inactivation. Without specifying the exact nature of the sixth cobalamin ligand, the activity of enzyme in the cob(III)alamin form has been demonstrated. First, the E_m of the cob(III)-/cob(II)alamin couple was determined by Banerjee et al. (1990) to be +273 mV at pH 7.2, and although some activity was reportedly lost during the titration, the bulk of the enzyme remained active after oxidation. In Figure 2, a spectrum of the cobalamin product is shown when enzyme-bound cob(I)alamin is exposed to oxygen. The spectrum of the cobalamin product is essentially identical to that of free aquocobalamin, with characteristic absorption maxima at 350 and 525 nm (Dolphin, 1971). The enzyme retained at least 95% of the initial activity following this oxidation, and the cobalamin could be converted to the characteristic cob(II)alamin form in a slow reduction ($t_{1/2}$ = 7.5 min) with excess homocysteine (2 mM) in an anaerobic environment. Minimally, this demonstrates that a cob(III)alamin species may be generated, bound to methionine synthase, without loss of activity. This is consistent with the requirement that methionine synthase must be able to survive interception by oxidants during turnover and argues that there is no inherent reason why a two-electron oxidation should lead to inactivation of the enzyme.

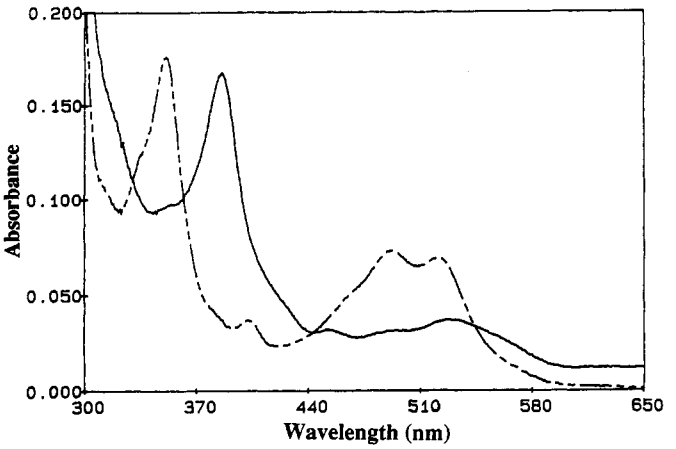


FIGURE 2: Reaction of enzyme-bound cob(I)alamin with oxygen. Methionine synthase in the cob(I)alamin form was generated by treating bound methylcobalamin with the substrate homocysteine under an argon atmosphere. Immediately upon admission of oxygen to the cell, the spectrum of bound cob(I)alamin (—), with a characteristic absorbance at 385 nm, is converted to a spectrum (– – –) with an absorbance maximum at 350 nm, consistent with bound aquocob(III)alamin.

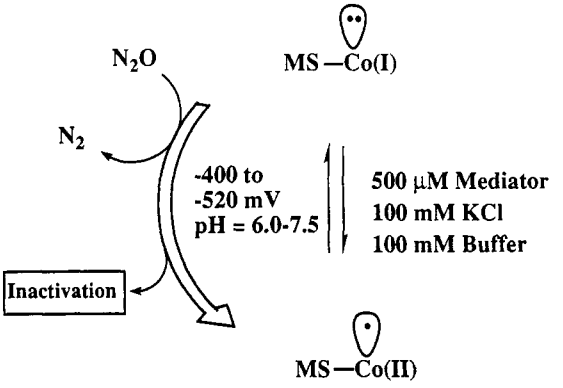


FIGURE 3: Strategy for the electrochemical inactivation of methionine synthase. The enzyme-bound cob(II)alamin prosthetic group is equilibrated with cob(I)alamin by poising at defined potentials. Nitrous oxide interception of cob(I)alamin may lead to either a benign oxidation or an inactivation event, but oxidized cobalamin bound to active enzyme can be reequilibrated with cob(I)alamin. Either methyl viologen or triquat was used as the mediator of electron transfer between a gold electrode and the cobalamin.

Electrochemical Inactivation of Methionine Synthase. Studies of the inactivation of methionine synthase have been hampered by the inability to inactivate the enzyme on a relatively large scale. We first sought to develop methods for generation of the cob(I)alamin state in the absence of substrates, largely to avoid the protection from oxidation that turnover affords the cob(I)alamin intermediate. By poising the enzyme under a defined atmosphere in an electrochemical cell and at a given potential (Figure 3), the relative amounts of enzyme in the cob(II)- and cob(I)alamin states can be directly calculated from the experimentally determined E_m

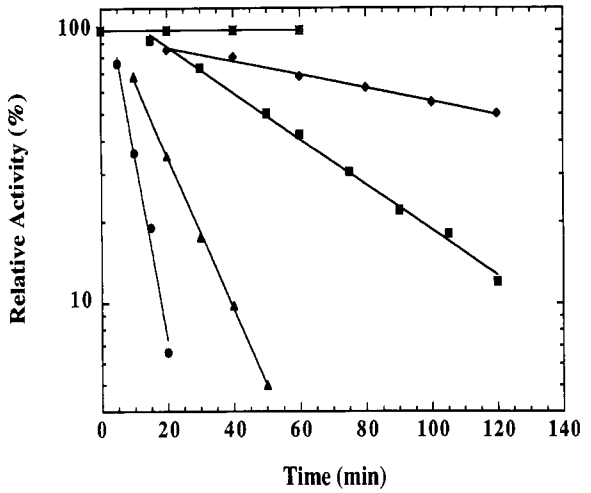


FIGURE 4: Dependence of the rate of methionine synthase inactivation by N_2O on the oxidation state of the cobalamin. Methionine synthase containing bound cob(II)alamin was poised in an electrochemical cell under nitrous oxide at the following potentials: −510 (●), −480 (▲), −430 (■), and −400 mV (◆) *vs* the SHE. No inactivation was observed over the course of the experiment when the cobalamin was methylated (×), or when the atmosphere was replaced by argon at −510 mV (○). At the end of each inactivation experiment, the enzyme displayed a residual activity of approximately 10% of the initial activity, and this was subtracted before the relative activity was determined.

using the Nernst equation. The enzyme-bound cob(II)-/cob(I)alamin couple was first determined by Banerjee et al. (1990) to be −526 mV at pH 7.2 in an identical electrochemical cell (Harder et al., 1989). After an anaerobic solution of methionine synthase containing bound cob(II)alamin was poised over a range of defined potentials, electron paramagnetic resonance was used to quantitate the amount of enzyme in each oxidation state at 20 or 100 K. We have repeated the midpoint potential determination using a spectrophotometric titration at 25 °C, and our value (E_{m7} = −463 mV) has been used to estimate the amount of cob(I)alamin in each experiment reported here. As given in Figure 3, equilibration of the enzyme at low potentials in the cell allows for multiple cycles of nitrous oxide reduction by cob(I)alamin until the enzyme becomes inactivated. When high concentrations of methionine synthase (40 μM) were inactivated, characterization of the gaseous head space above the inactivation experiment by gas chromatography followed by mass spectrometry showed that nitrogen had been formed when compared to controls lacking enzyme (data not shown).

Figure 4 summarizes the results of poising the enzyme containing bound cob(II)alamin at varying potentials under argon or nitrous oxide. The stability of enzymatic activity was first established under an inert atmosphere of argon to demonstrate that the low-potential environment provided by the reduced mediator was not harmful. When the enzyme was poised at −510 mV, bringing most of the enzyme into the cob(I)alamin state, the activity was stable over the course of the experiment. When the argon was replaced with nitrous oxide, activity was lost in a pseudo-first-order fashion, and the rate of this process was dependent upon the applied potential. For example, when a 1.0 μM solution of the enzyme was poised at −400 mV, the concentration of enzyme predicted to be in the cob(I)alamin state was 0.09 μM and the enzyme slowly inactivated. As the potential was lowered, bringing more enzyme into the cob(I)alamin form, the rate of inactivation increased. Further, the rate of inactivation can be directly correlated with the amount of enzyme in the cob(I)alamin state (Figure 5), implying that this species is indeed

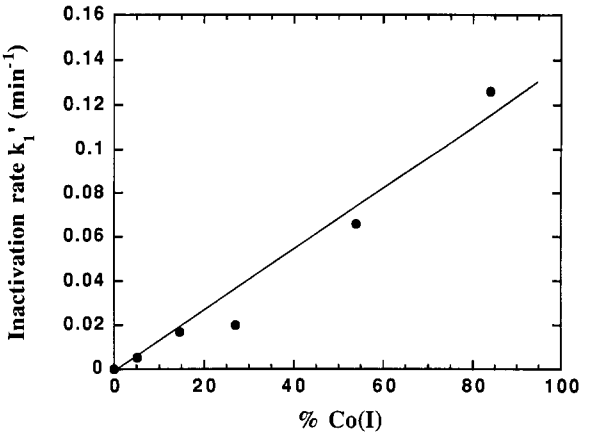


FIGURE 5: Replot of inactivation rates as a function of enzyme in the cob(I)alamin state. On the basis of the E_m determined in this work, the fractions of the total enzyme calculated to be in the cob(I)alamin state at pH 7.2 at each applied potential were plotted against the pseudo-first-order rate constants for the inactivation reported in Figure 3.

a participant in the inactivation process. From this replot, the rate of inactivation when all of the enzyme is in the cob(I)alamin state (and in the absence of substrates) is estimated to be 0.13 min^{-1}. Finally, when the cobalamin prosthetic group was methylated before the enzyme was poised under a nitrous oxide atmosphere, effectively preventing access to the cob(I)alamin state, no activity was lost over the course of the experiment.

Because a different number of protons is predicted to be taken up by each of the proposed inactivation stoichiometries (eqs 1 and 2), the effect of pH on the inactivation process was studied. If the rate-limiting step in the inactivation is the chemical degradation of nitrous oxide, and if this step involves the uptake of protons (i.e., the reaction is catalyzed by protons), the dependence of the inactivation reaction on the pH could aid in the distinction of the two stoichiometries. It should be noted that, in the degradation of nitrous oxide by free cob(I)alamin to nitrogen gas and water (Blackburn et al., 1977), and in pulse radiolysis experiments where nitrous oxide is reduced to form hydroxyl radicals by solvated electrons (Schmidt & Ander, 1969), the degradation either shows a substoichiometric dependence on pH or is independent of pH. A reasonable but less informative finding would be that the rate of nitrous oxide degradation by methionine synthase was independent of buffer pH, as seen in the model studies, and any effect of the pH on the rate of inactivation would have to be assigned to a protonation event on the enzyme affecting the concentration of cob(I)alamin present at the applied potential.

Buffer pH Affects the Rate of Inactivation. Figure 6 shows the dependence of the rate of inactivation on the buffer pH over 1.5 units, which is equivalent to a 30-fold change in the proton concentration. The experimental data were fit with the theoretical slope predicted if the rate of inactivation were dependent upon a single proton. The experimental fit is excellent, strongly supporting single proton involvement in the inactivation over the pH range studied, but the mechanism by which the proton exerts the effect is not yet clear. For example, rather than catalyzing the degradation of nitrous oxide, the proton could instead be altering the redox properties of the enzyme. As previously shown in Figure 4, the inactivation is dependent upon the concentration of enzyme in the cob(I)alamin state; if the cob(II)-/cob(I)alamin redox couple is associated with the obligatory binding of a proton, as shown in eq 4, a similar theoretical dependence on the pH

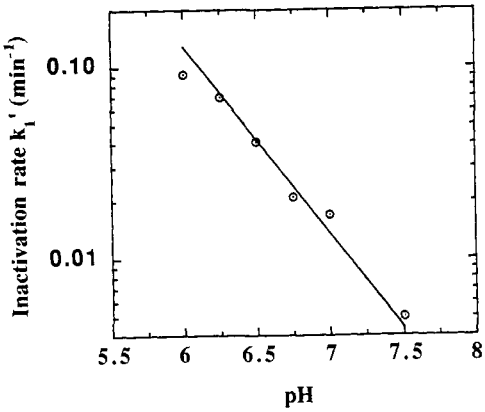


FIGURE 6: Dependence of the inactivation rate on hydrogen ion concentration. Each inactivation was performed under nitrous oxide at –450 mV *vs* SHE. The circles represent the experimental rates of inactivation, while the line through the data is the theoretical slope calculated on the assumption that the observed rate of inactivation is dependent on the uptake of a single proton.

of the buffer would be expected. Such redox-linked protonations have been observed for other proteins that undergo changes in oxidation state [e.g., lipoamide dehydrogenase; see Matthews and Williams (1976)].

enz–cob(II)alamin–B: $+ H^+ + 1e^- \leftrightarrow$

enz–cob(I)alamin–BH^+ (4)

Cob(II)-/Cob(I)alamin Midpoint Potential Is Dependent upon pH. In order to distinguish between the two possible roles proposed for the proton, the E_m was determined as a function of pH. This is a difficult undertaking, because the E_m of the enzyme [–526 mV at pH 7.2, determined by Banerjee et al. (1990)] is unusually low. If the redox couple is pH-dependent, an increase of 59 mV in the midpoint potential for each pH unit decrease is predicted when a single proton is coupled to the reduction, and at lower pH values the cob(I)alamin state should be more easily attained. We chose to study the E_m by equilibrating the enzyme at varying potentials with a redox mediator that could be monitored spectrally, and we began with oxidized enzyme and mediator in an anaerobic cell under argon. The contents of the cell were then reduced by visible irradiation in the presence of EDTA and catalytic amounts of a 5-deazaflavin-3-methanesulfonic acid (Massey et al., 1978; Massey & Hemmerich, 1978), and these experiments were performed over part of the range of pH values where the electrochemical inactivation was measured. As described below, the E_{m7} determined for the cob(II)-/cob(I)alamin couple in this work (–463 mV *vs* the SHE) was obtained using considerably lower concentrations of enzyme and electrochemical mediator than those used in the experiments of Banerjee et al. (1990), and the determination was performed at the temperature (25 °C) where the inactivation experiments were carried out. It is not clear why the absolute value for the midpoint potential differs from that of the previous work, although physical effects such as dimerization of the oxidized enzyme at the high concentrations used in the previous work could have lowered the apparent midpoint potential.

The constraints on the choice of mediator are great. Its electrochemical midpoint should be pH-independent, and it should be capable of reducing methionine synthase to cob(I)alamin over the working range attainable by photoreduction. Spectrally, it must possess little absorption where the cob(II)- and cob(I)alamin absorption spectra possess a large absorption difference, and there must be a wavelength where the absorbance of the dye can be readily correlated with the

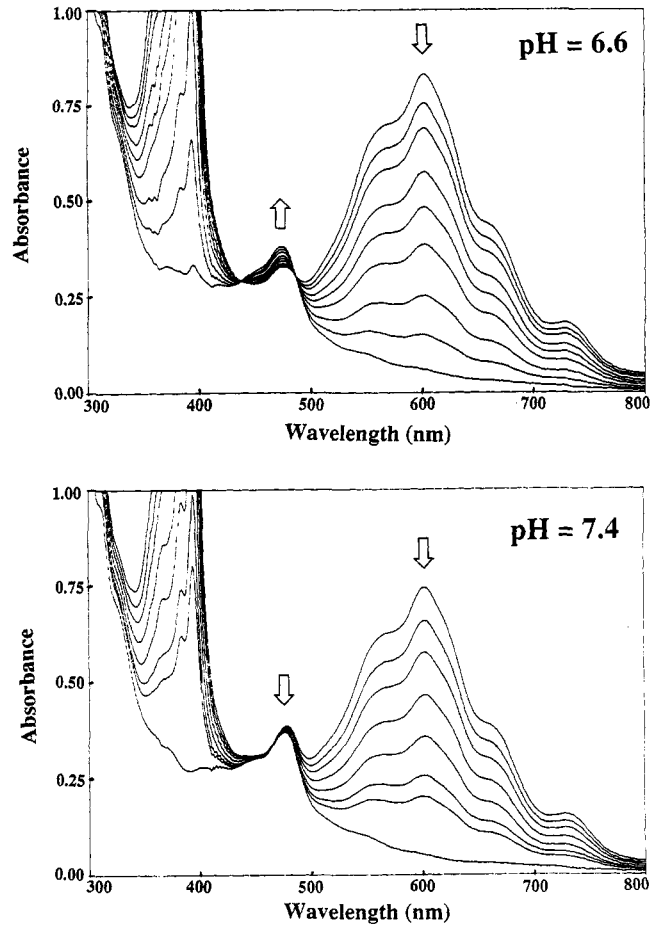


FIGURE 7: Representative spectra demonstrating how the E_m of methionine synthase was determined as a function of pH. The mixture of 100 μM methyl viologen and 35 μM enzyme was initially photoreduced and then allowed to oxidize slowly (see the text). The key feature to note is the presence or absence of cob(I)alamin oxidation to cob(II)alamin, measured at 474 nm, as the cell potential increases. Panel A (top) shows the equilibration of cob(II)alamin with the methyl viologen mediator at pH 6.6 as the system is slowly oxidized. The methyl viologen radical cation was monitored 600 nm, while the cob(II)-/cob(I)alamin couple was monitored at 474 nm. Oxidation of cob(I)alamin to cob(II)alamin, as assessed by an increase in the absorbance at 474 nm, occurs along with the oxidation of the electrochemical mediator. The mediator concentration serves to report the ambient potential of the cell. Panel B (bottom) shows the same oxidation at pH 7.4, and here little oxidation of the cobalamin is observed.

ambient potential of the system using the Nernst equation. Methyl viologen, with a pH-independent E_{m7} of –440 mV *vs* the SHE, was the only available mediator that functioned reliably in the photoreduction, and this limited us to studying the E_m over approximately 1 unit of pH. From a practical standpoint, the mediator functions as an electrochemical buffer, and its utility is greatest near its own midpoint potential. It should be noted that no enzymatic activity was lost in the process of reductively equilibrating the enzyme with methyl viologen using the photochemical technique described in this work.

The results of equilibrating the enzyme in the presence of a slight excess of methyl viologen are presented in Figure 7A,B. The reduction of cob(II)- to cob(I)alamin was monitored at 474 nm while the methyl viologen radical cation was monitored at 600 nm, and the concentrations of each of the species were calculated on the basis of reported extinction coefficients, as described in the Materials and Methods section. Qualitatively, the spectra reveal that cob(II)alamin is readily reduced by methyl viologen at the low end of the pH range

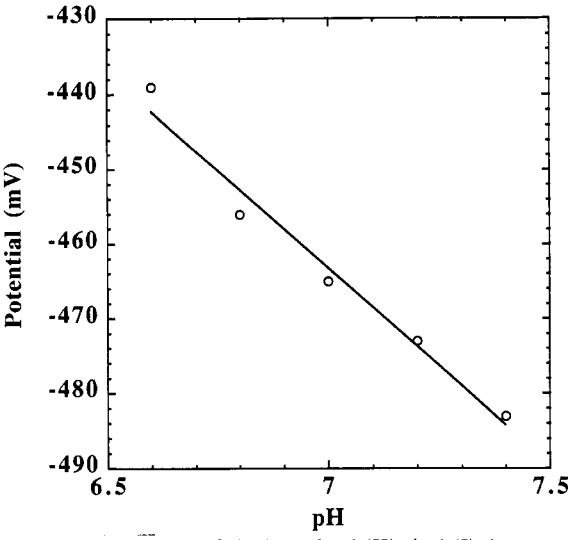


FIGURE 8: Dependence of the bound cob(II)-/cob(I)alamin E_m on ambient pH. A theoretical slope for binding of a single proton concomitant with reduction by one electron would be −59 mV per pH unit change; the experimental slope was −53 mV in this experiment.

(6.6), while little reduction is observed at the upper end (pH 7.4). Following an E_m determination of the cob(II)-/cob(I)alamin redox potential in pH increments of 0.2 unit over this range, Figure 8 shows the dependence of E_m on the pH. From the plot we estimate an E_{m7} of −463 mV *vs* the SHE, and the slope of the plot (−53 mV) is consistent with a single proton binding the enzyme concomitant with reduction to cob(I)alamin, as presented in eq 4. The observation that the enzyme inactivates more readily as the pH is lowered is therefore rationalized as a direct effect on the concentration of enzyme-bound cob(I)alamin at −450 mV, rather than on the rate of enzyme-catalyzed nitrous oxide degradation.

The Apparent Product of Cob(I)alamin Reaction with Nitrous Oxide Is Cob(II)alamin. We wanted to characterize the oxidation state of the cobalamin product formed when cob(I)alamin degrades nitrous oxide. The visible absorbance spectrum of the two-electron-oxidized, enzyme-bound cob(III)alamin resembles that of the free aquocob(III)alamin (Dolphin, 1971; and Figure 2 above), and it is readily distinguished from that of cob(II)alamin. Figure 9 shows the decay of cob(I)alamin bound to methionine synthase under an atmosphere of nitrous oxide. Enzyme-bound cob(I)alamin was generated by methyl transfer from methylcobalamin to the substrate homocysteine (500 μM), and here no mediators or reducing agents were present other than the homocysteine thiols. The spectral product, formed in an isosbestic decay at a characteristic rate of 0.12 ± 0.01 min^{-1}, has the absorbance properties of cob(II)alamin. When the experiment was performed under an atmosphere of argon, a similar product is observed, but at a much slower rate (2.6×10^{-3} min^{-1}). The decay of cob(I)alamin could not be directly coupled to the generation of nitrogen gas or other potential breakdown products due to the small amount formed in a single turnover of 8 μM methionine synthase. Nonetheless, no evidence for cob(III)alamin (two-electron-oxidized, bound aquocobalamin) was seen in this experiment. The possibility that cob(III)alamin is the initial product in the decay and that it is rapidly reduced to cob(II)alamin seems very unlikely due to the slow rate of this process, as described above.

Enzyme-Catalyzed Degradation of Nitrous Oxide Consumes Electrons. Essential to each of the models for inactivation is the premise that nitrous oxide is reductively degraded by methionine synthase. We therefore wanted to

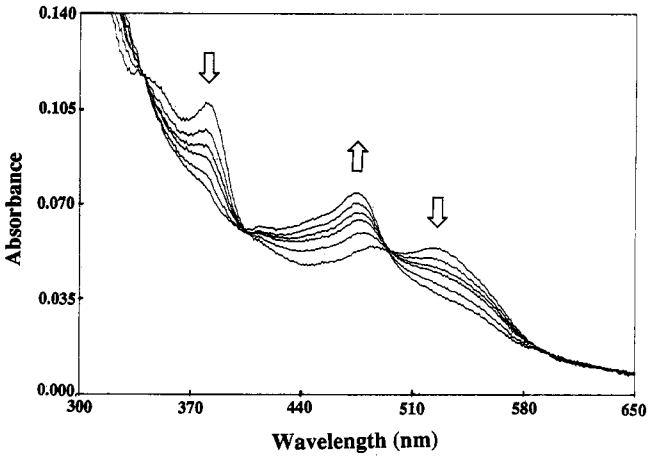


FIGURE 9: Decay of enzyme-bound cob(I)alamin under an atmosphere of nitrous oxide. Bound cob(I)alamin, generated by the demethylation of methylcobalamin (8 μM) by homocysteine (500 μM), has a characteristic absorption maximum at 385 nm. Under an oxygen-free atmosphere of nitrous oxide, this species decays to a cob(II)alamin species, characterized by the absorbance maximum at 474 nm.

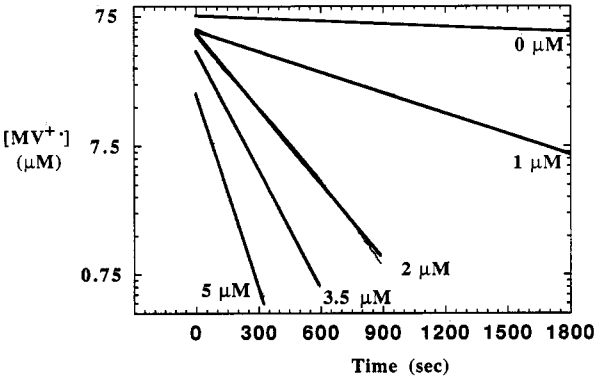


FIGURE 10: Methionine synthase catalyzes the transfer of electrons from the one-electron-reduced methyl viologen radical cation (MV^+) to nitrous oxide. Enzyme with bound cob(II)alamin at concentrations of 0–5 μM was equilibrated with photochemically reduced methyl viologen in buffer saturated with N_2O (~25 mM). Straight lines drawn through the data points reflect the oxidation of one-electron-reduced methyl viologen. Time zero represents the initiation of data collection, approximately 2 min after photoreduction was complete.

demonstrate that methionine synthase is capable of transferring electrons to nitrous oxide and establish whether or not the process is catalytic. As shown in Figure 10, varying concentrations of methionine synthase with bound cob(II)alamin (1–5 μM) were allowed to equilibrate with reduced methyl viologen at a low potential. This was achieved by the photoreduction of a large excess of methyl viologen mediator (100 μM) under N_2O, and the conversion of the blue methyl viologen radical cation to colorless, oxidized methyl viologen was monitored at 600 nm as an assay for methionine synthase-catalyzed electron transfer to nitrous oxide. The pH of the experiment was chosen to be 6.6, so that the electrochemical midpoint of the enzyme was the same as that of methyl viologen, and in this way the ratio of oxidized to reduced enzyme is the same as that of the oxidized to reduced mediator at equilibrium. An expression that relates the concentration of the enzyme in the cob(I)alamin state to the characteristic absorbance of reduced methyl viologen (A_{600}) is presented in eq 5. This is readily derived from the Nernst expression for the cobalamin redox couple at equilibrium with the methyl viologen redox couple, where K = [cobalamin (total)]/[methyl viologen (total)]ϵ_{600}, and ϵ_{600} is the extinction coefficient for the methyl viologen radical cation at 600 nm.

$$\frac{d[\text{cob(I)alamin}]}{dt} = K\frac{dA_{600}}{dt} \quad (5)$$

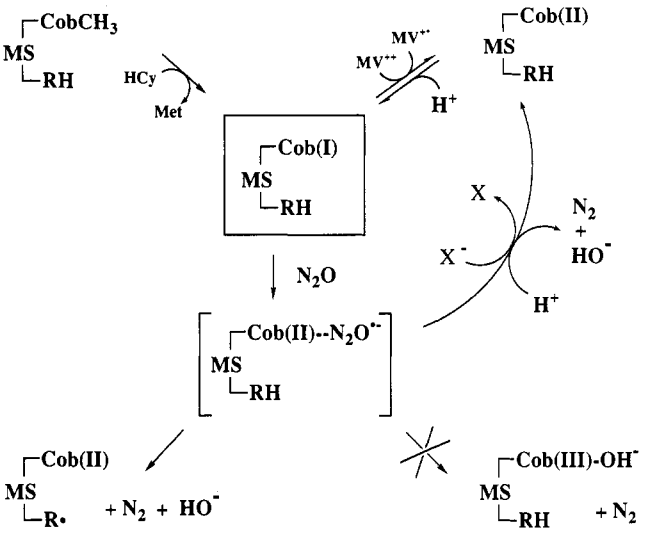
This experiment shows that the rate of oxidation of the methyl viologen mediator is directly dependent upon the concentration of methionine synthase in the individual experiment. In Figure 9, we demonstrate that the cob(I)alamin form of methionine synthase is rapidly oxidized in the presence of N_2O, and we have also shown that the oxidation of enzyme-bound cobalamin is ~50-fold slower in buffers saturated with argon. Thus, we conclude that the enzyme catalyzes the reduction of nitrous oxide using electrons derived from methyl viologen. As described above, the concentration of enzyme in the cob(I)alamin state can be directly related to the concentration of the reduced methyl viologen mediator, and the rate of electron transfer to nitrous oxide can also be directly related to the concentration of enzyme with bound cob(I)-alamin. We infer that the electron transfer is independent of the concentration of reduced mediator, because the decay of the reduced mediator remains pseudo-first-order over at least the first 95% of the mediator oxidation. Because the nitrous oxide concentration in aqueous solution is high (25 mM) relative to the cobalamin and does not change appreciably over the course of these experiments, it can be treated as a constant, as given in eq 6. From a replot of the data (not shown), we have determined that the rate of transfer is linearly dependent upon the enzyme concentration, and from these data we calculate a pseudo-first-order rate constant k_1' for electron transfer from cob(I)alamin to nitrous oxide of 0.12 ± 0.02 min^{-1}. These data also show that electron transfer is catalytic and that the reduction of nitrous oxide under these conditions does not modify the enzyme in a way that prevents further reduction. This is true even after the enzyme becomes inactivated.

$$\frac{d[\text{cob(I)alamin}]}{dt} = k_2[N_2O][\text{cob(I)alamin}] = k_1'[\text{cob(I)alamin}] \quad (6)$$

What Is the Fate of the Oxygen Derived from Nitrous Oxide? No conclusive evidence for the chemical nature of the oxygen-containing product derived from nitrous oxide has been obtained, either in model studies or in this work. We propose, on the basis of characterization of the damage sustained by methionine synthase following nitrous oxide inactivation in the electrochemical cell (Drummond & Matthews, 1994), that reductive degradation of nitrous oxide can lead to specific modifications of the enzyme. In brief, a C-terminal 37.2-kDa domain that binds AdoMet (Drummond et al., 1993a) and is responsible for the reductive methylation of the enzyme-bound cob(II)alamin, sustains most of the damage following electrochemical inactivation, although minor damage to the cobalamin can also be demonstrated. Without proof of the identity of the species derived from oxygen, the data in this study are consistent with the formation of a damaging oxidant when the enzyme degrades nitrous oxide and becomes inactivated. We conclude that the stoichiometry of inactivation is most consistent with that given in eq 2, where a reactive species is generated from nitrous oxide, and we propose that hydroxyl radical is a reasonable candidate for this species.

DISCUSSION

Our goal in this work was to investigate the chemical mechanism by which cobalamin-dependent methionine synthase from *E. coli* degrades nitrous oxide and becomes inactivated. Figure 11 summarizes the results of our investigations and is presented as a working model for the discussion that follows.



(Inactivation Pathway)

FIGURE 11: Proposal for the reductive degradation of nitrous oxide by cob(I)alamin bound to methionine synthase. The cob(I)alamin form was generated either by demethylation of methylcobalamin or by equilibration of the cob(II)alamin form with a mediator dye such as methyl viologen (MV). Cob(I)alamin reductively degrades nitrous oxide in a pH-independent reaction that appears to involve the transfer of a single electron to form cob(II)alamin and an uncharacterized product. X^- in the figure represents a species, such as homocysteine or the methyl viologen radical cation, that is capable of donating a second electron to the reduction of nitrous oxide. Inactivation of the enzyme is associated with oxidation of the enzyme, presumably by one electron (Drummond & Matthews, 1994).

Our first objective was to identify the reactive cobalamin species that participates in the inactivation event, and three lines of evidence strongly implicated cob(I)alamin as the reactive intermediate that reductively degrades nitrous oxide with the concomitant oxidation of the cobalamin. First, methionine synthase inactivated at a rate that was linearly dependent upon the concentration of enzyme that possessed the cob(I)alamin oxidation state. When the prosthetic group bound to the methionine synthase was in the cob(II)- or cob-(III)alamin oxidation state, or when it was methylated, no inactivation was seen under an atmosphere of nitrous oxide. Second, when enzyme-bound cob(II)alamin was equilibrated with an excess of reduced methyl viologen mediator under an atmosphere of nitrous oxide, the cobalamin catalyzed the transfer of electrons from one-electron-reduced methyl viologen to nitrous oxide, and the rate of this process was directly dependent upon the concentration of enzyme in the reduced cob(I)alamin state. Third, when cob(I)alamin was generated from methylcobalamin under an atmosphere of nitrous oxide and the cobalamin oxidation state was monitored spectrally, the cobalamin was oxidized to cob(II)alamin.

In order to understand the mechanism through which methionine synthase becomes inactivated, it is essential to establish whether the reduction of nitrous oxide by enzyme-bound cob(I)alamin proceeds by one or two electrons. We have demonstrated that two-electron oxidation of the enzyme-bound cob(I)alamin to the cob(III)alamin state does not lead to a loss of activity. Furthermore, no evidence for oxidation of the enzyme to cob(III)alamin during the reaction with nitrous oxide has been obtained, and enzyme-bound cob(III)-alamin is not reduced by enzyme in the cob(I)alamin state.

Thus, our data strongly suggest that the enzyme reacts with nitrous oxide by single-electron transfer. If one electron were transferred to N_2O, this species could decompose to a transient, reactive intermediate such as hydroxyl radical or nitric oxide. The hallmark of this reaction pathway is the oxidative modification of the cobalamin or the oxidation of the enzyme proximal to the cobalamin. The latter model is most consistent with the results obtained in this work, as discussed below, and the accompanying article defines a set of modifications that occurs to methionine synthase during the inactivation described here.

Indirect support for a one-electron transfer from cob(I)-alamin to nitrous oxide comes from the demonstration that methionine synthase catalyzes electron transfer from the methyl viologen radical cation to nitrous oxide. In this type of experiment, the strong absorbance of the methyl viologen radical cation in the visible range was used to monitor the rate of electron transfer to nitrous oxide. The enzyme-bound cob-(I)alamin intermediate, formed upon equilibration of the starting cob(II)alamin with one-electron-reduced methyl viologen mediator, transfers electrons to nitrous oxide at a rate of 0.12 ± 0.02 min^{-1}. This rate is experimentally indistinguishable from the rate at which enzyme-bound cob-(I)alamin decays isosbestically to cob(II)alamin under an atmosphere of nitrous oxide (0.12 ± 0.01 min^{-1}), consistent with the proposal that nitrous oxide is accepting one electron from cob(I)alamin. What is difficult to rationalize is the nature of the one-electron-reduced product derived from nitrous oxide during the electron-transfer experiment, since radical species such as hydroxyl radical, derived from N_2O, are likely to require the input of a second electron to give products that are ultimately stable (see Figure 11). A potential source of this second electron is EDTA, which could undergo decarboxylation and generate a low-potential electron, formaldehyde, and ethylenediaminotriacetic acid (Massey et al., 1978).

The rates measured above for electron transfer to nitrous oxide provide a context for interpreting the rate of inactivation measured in the electrochemical cell. We have demonstrated that the electrochemical inactivation requires the enzyme to be in the cob(I)alamin state. In the absence of substrates this inactivation occurs at a rate of 0.13 min^{-1}, similar to the rate of one-electron oxidation of cob(I)alamin by nitrous oxide observed spectrally. These data are consistent with an inactivation stoichiometry wherein cob(I)alamin reduces nitrous oxide by one electron. This is the stoichiometry presented earlier in eq 2, and it predicts that nitrogen gas and a reactive oxidant like hydroxyl radical are formed as products in the inactivation reaction. That the rate of inactivation is the same as the rate of electron transfer to N_2O argues for a close coupling of nitrous oxide reduction and enzyme inactivation in the electrochemical cell.

We found that the electrochemical equilibrium between the enzyme-bound cob(II)- and cob(I)alamin species behaved as if the binding of a single proton was coupled to the formation of the cob(I)alamin state. We have inferred from this result that the rate-limiting step in the inactivation reaction, presumed to be the reductive degradation of nitrous oxide by the cob(I)alamin, is independent of pH. This conclusion does not agree with either formal stoichiometry for inactivation, presented earlier in eqs 1 and 2, but it is consistent with the pH independence of the reductive degradation of nitrous oxide by free cob(I)alamin in model studies and pulse radiolytic generation of hydroxyl radical from nitrous oxide (Blackburn et al., 1977; Schmidt & Ander, 1969).

While the identity of the basic residue that becomes protonated upon reduction of the cobalamin has not been identified, it might reasonably be contributed by either the cobalamin or the enzyme. If it is derived from the cobalamin, one candidate is the dimethylbenzimidazole nitrogen that is the lower axial ligand for the cobalt under most circumstances. When the free cob(II)-/cob(I)alamin couple was measured (Lexa & Saveant, 1983), the potential was found to be pH-independent near neutrality, but protonation of the dimethylbenzimidazole occurred concomitant with reduction at slightly lower pH values (the apparent pK for protonation of this base is 4.7). One model for the pH dependence of the inactivation observed in this work is that binding of the cobalamin to the enzyme alters the pK of the dimethylbenzimidazole and that this group becomes protonated upon reduction. Alternatively, the enzyme might contribute a basic side-chain moiety near the cobalamin. In either case, the protonation of a base proximal to the cobalamin is a means through which the enzyme could facilitate the reductive methylation of cob(II)alamin, since such a protonation would reduce the thermodynamic barrier to one-electron reduction of the cobalt.

Our experiments indicate that enzyme inactivation by nitrous oxide does not prevent methionine synthase from catalyzing the reduction of nitrous oxide, which is consistent with the evidence presented in the accompanying article that the cobalamin prosthetic group is largely undamaged when inactivated enzyme is reisolated. However, the catalytic generation of nitrogen gas during nitrous oxide reduction must lead to the simultaneous generation of oxygen-containing products. It is surprising that the enzyme does not suffer extensive damage, particularly polypeptide chain cleavage, under conditions where many equivalents of a damaging oxidant might be generated. We have not attempted to characterize the products of catalytic nitrous oxide oxidation by methionine synthase, but we suspect that they are intercepted by components present in the reaction mixture such as EDTA.

The coupling of enzyme inactivation to nitrous oxide reduction is dependent on the experimental conditions. During inactivation in the electrochemical cell, the extrapolated rate of inactivation of cob(I)alamin (0.13 min^{-1}) is identical to the measured rate of decay of enzyme-bound cob(I)alamin to cob(II)alamin under nitrous oxide (Figure 10). While this leads us to conclude that inactivation of the enzyme in the electrochemical cell occurs during a single cycle of nitrous oxide reduction, the cob(II)alamin enzyme reisolated after the single turnover experiment shown in Figure 10 had lost less than 5% of its activity. One might rationalize the protection from inactivation as being due to the presence of homocysteine or methionine, bound to the enzyme when cob-(I)alamin is generated. These oxidatively sensitive compounds might protect the enzyme from oxidative damage by trapping a reactive species. However, no protection was afforded by either of these amino acids at millimolar concentrations in the electrochemical inactivation.

We rationalize the difference in inactivation outcomes by considering the multidomain nature of methionine synthase. Methionine synthase is a monomeric structure of 136.1 kDa that can be proteolyzed into a 97.4-kDa domain that retains the cobalamin and a C-terminal 37.7-kDa domain (Drummond et al., 1993b). The larger domain is able to carry out catalysis once it has been reductively methylated, while the C-terminal 37.7-kDa domain binds the *S*-adenosylmethionine (AdoMet) that contributes a methyl group to the cobalamin. From a

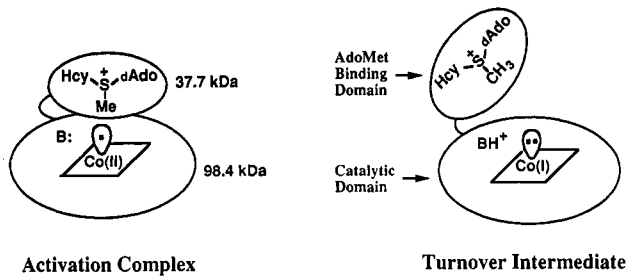


FIGURE 12: Domain structure of methionine synthase. The 37.7-kDa domain that binds AdoMet must approach the cobalamin for reductive activation, but it must be excluded from the primary catalytic cycle. Inactivation may depend upon the proximity of this domain to the cobalamin, where reductive degradation of nitrous oxide occurs.

functional standpoint, the AdoMet binding domain must be excluded from catalysis following activation to prevent a futile methylation cycle, since AdoMet is synthesized from methionine and ATP (Cantoni & Durell, 1957). In the absence of substrates, the AdoMet binding domain must approach the cobalamin in order to transfer a methyl group for activation (Figure 12). We have found that this domain is a primary site of modification when the enzyme loses activity, implying that it is indeed close to the cobalamin when nitrous oxide degradation results in enzyme inactivation (Drummond & Matthews, 1994). However, when the enzyme is cycling between methylcobalamin and cob(I)alamin, excluding the AdoMet binding domain, the putative oxidant released from nitrous oxide might not have access to the C-terminal domain and might escape without damaging the enzyme.

In summary, the experiments reported here support the model that methionine synthase becomes inactivated when the highly reduced cob(I)alamin state becomes oxidized by nitrous oxide to produce nitrogen gas and a damaging oxidant. The apparent cobalamin product is cob(II)alamin, and no evidence for the formation of a cob(III)alamin species has been observed, nor does it appear that the formation of cob(III)alamin on the enzyme alone is sufficient to produce inactivation. From a formal standpoint, one-electron reduction of nitrous oxide by cob(I)alamin is expected to lead to the highly reactive hydroxyl radical or its equivalent, and this species may lie on the pathway between nitrous oxide degradation and methionine synthase inactivation.

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