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Nitrous Oxide Inactivation of Cobalamin-Dependent Methionine Synthase from *Escherichia coli:* Characterization of the Damage to the Enzyme and Prosthetic Group†

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ABSTRACT: Nitrous oxide, or laughing gas, is an anaesthetic agent that inactivates cobalamin-dependent methionine synthase. This enzyme uses the highly reactive, enzyme-bound cob(I)alamin oxidation state of the prosthetic group to effect methyl group transfer from 5-methyltetrahydrofolate to homocysteine to form tetrahydrofolate and methionine. The cob(I)alamin is capable of reductively degrading nitrous oxide, and here we characterize the modifications that occur to the *Escherichia coli* enzyme following electrochemical inactivation. Methionine synthase was inactivated on a milligram scale by equilibrating enzyme containing bound cob(II)alamin with a reduced electrochemical mediator to give the reactive cob(I)alamin state under an anaerobic atmosphere of nitrous oxide. The primary damage occurs to a 37.2-kDa domain that binds *S*-adenosylmethionine (AdoMet), and inactive enzyme can no longer be reductively methylated using AdoMet. The damage is oxidative, and it includes the covalent addition of the mediator, triquat, to the enzyme selectively at valine 1177, as well as the formation of a covalent cross-link between peptides containing the only two cysteines within this domain. Spectrally, the prosthetic group bound to inactive enzyme resembles cob(II)alamin, although some loss in absorbance is apparent. When the enzyme was reconstituted with [^{57}Co]cobalamin and the inactivation repeated, the cobalamin was recovered unmodified in ~75% yield, but two products derived from the cobalamin were also observed. We interpret the finding of oxidatively modified products as strong evidence that reductive degradation of nitrous oxide releases a potent oxidant, presumably hydroxyl radical or its equivalent, that is capable of modifying sites proximal to the cobalamin.

The symptoms of cobalamin deficiency have been associated with both prolonged administration of nitrous oxide and repeated exposure to this anaesthetic agent [see references in Drummond and Matthews (1994)]. Of the two cobalamin-dependent enzymes in humans, nitrous oxide has been demonstrated to selectively inactivate methylcobalamin-dependent methionine synthase (Deacon et al., 1978). No direct effect on the adenosylcobalamin-dependent methylmalonyl-CoA mutase has been observed, although prolonged exposure to nitrous oxide has been reported to alter cobalamin metabolism and subsequently disrupt the activity of the mutase as well (Kondo et al., 1981). The reason nitrous oxide does not inactivate methylmalonyl-CoA mutase is likely the fact that methylmalonyl-CoA mutase does not utilize the highly reduced cob(I)alamin state. Instead, homolysis of the adenosyl–cobalt bond produces an adenosyl radical that initiates a set of reactions best explained as substrate rearrangements via free radical intermediates (Retey, 1982). The two activities may still be related; inactivation of methionine synthase by nitrous oxide has been shown to produce cobalamin analogs in rats that are released into the bloodstream (Kondo et al., 1981), and interaction of these analogs with methylmalonyl-CoA mutase has been proposed to connect methionine synthase inactivation with disruption of the mutase activity.

Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from CH_3-H_4folate[1] to homocysteine to produce methionine and H_4folate [for reviews, see Taylor and Weissbach (1973) and Banerjee and Matthews (1990)]. This enzyme does make use of the reactive cob(I)alamin oxidation state of the prosthetic group, and this highly reduced intermediate renders the enzyme sensitive to oxidants. As shown in Figure 1 of the preceding article in this issue (Drummond & Matthews, 1994), methionine synthase cycles in catalysis between the methylcobalamin and cob(I)alamin forms, alternately accepting a methyl group from CH_3-H_4folate and donating it to homocysteine. If the cob(I)alamin form of the enzyme is intercepted by an oxidant, catalytically incompetent cob(II)alamin or cob(III)alamin forms of the enzyme are produced. The return of these enzyme forms to the catalytic cycle requires a reductive methylation, in which AdoMet rather than CH_3-H_4folate serves as the methyl donor. We have previously shown that the AdoMet binding site is located in a C-terminal 37.7-kDa fragment that can be generated by proteolysis of the native 136.1-kDa enzyme with trypsin (Drummond et al., 1993b) and in an overlapping 37.2-kDa fragment generated by further digestion and lacking four additional residues at its N-terminus. The N-terminal 98.4-kDa portion of the enzyme retains the binding site for methylcobalamin, and it catalyzes the conversion of

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CH_3-H_4folate, (6-*R*,*S*)-5-methyltetrahydrofolate monoglutamate; E_m, electrochemical midpoint potential; H_4folate, (6-*R*,*S*)-tetrahydrofolate monoglutamate; Hcy, L-homocysteine; HPLC, high-pressure liquid chromatography; LysC, lysyl endopeptidase C from *Achromobacter* (protease I).

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CH_3-H_4folate and homocysteine to H_4folate and methionine. However, once the cob(I)alamin intermediate is oxidized, this domain can no longer be reductively methylated using AdoMet.

In the accompanying article (Drummond & Matthews, 1994), we report our studies that characterize the reductive degradation of nitrous oxide by the reactive cob(I)alamin bound to methionine synthase. We sought to distinguish between a mechanism for inactivation that involved two-electron oxidation of the cob(I)alamin by nitrous oxide to give a bound cob(III)alamin that could not be reactivated and a mechanism involving one-electron oxidation of the cobalamin with concomitant formation of a damaging oxidant. Our results strongly support the latter model, where cob(I)-alamin transfers one electron to nitrous oxide and the enzyme catalyzes repeated degradations of nitrous oxide even after enzyme inactivation. These results also imply that the cobalamin itself is not the primary site of damage following inactivation, at least in the short term, but rather that the protein structure proximal to the cobalamin becomes modified. In this work, we characterize the extent of the cobalamin damage and identify specific sites of covalent enzyme modification. We have found that N_2O-inactivated methionine synthase is characterized by oxidative modifications to a 37.2-kDa C-terminal domain that binds AdoMet, a domain that must have access to the cobalamin during the reductive methylation of methionine synthase (Drummond et al., 1993b). Digestion of methionine synthase with catalytic amounts of trypsin gave good yields of this domain, as well as the complementary 98.4-kDa domain that retained the bound cobalamin. We previously developed an HPLC separation of peptides from the AdoMet binding domain that were generated by proteolytic digestion with LysC and characterized these peptides by mass spectrometry (Drummond et al., 1993a), and we have now applied this approach to rapidly localize modifications that are a result of nitrous oxide inactivation.

EXPERIMENTAL PROCEDURES

Source of Materials. Triquat dibromide was a gift from Professor Stephen Ragsdale at the University of Nebraska. Methyl viologen and other chemical reagents were purchased from the Sigma Chemical Co. LysC was purchased from Waco Biochemicals.

Purification of Methionine Synthase. Recombinant methionine synthase (MetH) from *Escherichia coli* K-12 strain DH5αF′/p4B6.3 was overproduced and purified as previously reported (Banerjee et al., 1989).

Isolation and LysC Digestion of the 37.2-kDa C-Terminal Domain. Methionine synthase was cleaved with trypsin following arginine 899 to release the AdoMet binding domain as previously reported (Drummond et al., 1993a). This work also describes the subsequent digestion of the domain by the lysine-specific endoprotease LysC to give fragments that can be separated by HPLC and characterized by mass spectrometry or amino acid sequencing.

Synthesis of Methyl[^{57}Co]Cobalamin. [^{57}Co]Cyanocobalamin (10.5 μCi) was purchased from Amersham and diluted with unlabeled cyanocobalamin to give 2.0 mL of a 22 μM stock in water. This solution was prepared in a cuvette, covered with Parafilm, and gently bubbled with argon gas for 25 min to remove oxygen, and the cobalamin was reduced with sodium borohydride (38 μL of a 20 mg/mL stock, 10 mM final concentration). Methyl iodide (3.1 μL of a 1% solution in ethanol) was then added, and the methylation was followed spectrally over the course of 60 min; the reaction was complete within 30 min, and excess methyl iodide was decomposed by the borohydride over the remainder of the incubation. The excess borohydride was quenched by the addition of potassium phosphate buffer (150 μL of a 1 M stock, pH 7.2). The experiment provides a 20 μM, buffered stock of [^{57}Co]-methylcobalamin (5.25×10^5 dpm/nmol). Methylcobalamin is light-sensitive when not bound to the apoenzyme, and the subsequent reconstitution steps were carried out in near-darkness.

Apoenzyme Preparation and Reconstitution. A modification of the method of Taylor (1970) was used to generate apoenzyme from holoenzyme. The cobalamin from methionine synthase was released by denaturing the enzyme in 6 M urea in the presence of homocysteine, which was added to demethylate any residual bound methylcobalamin. Methionine synthase (33.6 μM in a final reaction volume of 1250 μL) was incubated at 37 °C for 15 min in a mixture of 33 mM potassium phosphate buffer at pH 7.2 containing 6 M urea, 20 mM dithiothreitol, and 1 mM homocysteine. The light brown solution was loaded onto a 1 × 10 cm column of G-75 gel filtration resin (Pharmacia) that had been equilibrated in 20 mM phosphate buffer at 4 °C, and 1 mL fractions were collected by eluting the column with the equilibration buffer. Fractions containing protein were identified by their absorbance at 280 nm, and the third and fourth milliliters of eluate had most of the apoprotein. These fractions were combined, frozen in an acetone bath containing dry ice, and lyophilized to dryness. The solid apoenzyme and buffer mixture was redissolved by adding the entire 2.2 mL of radiolabeled methylcobalamin stock maintained at 4 °C. The solution was kept on ice for 30 min, allowed to warm to room temperature, and then incubated at 37 °C for 10 min. After cooling on ice, the solution was again passed over a G-75 column equilibrated with 20 mM phosphate buffer. Red fractions containing holoenzyme, eluting in the early fractions ahead of free methylcobalamin, were combined and concentrated to 1.1 mL of 7 μM holoenzyme (20% yield). The methylcobalamin was converted to cob(II)alamin by treatment with homocysteine and dithiothreitol, as previously described (Drummond et al., 1993b).

Electrochemical Inactivation of Methionine Synthase by Nitrous Oxide. The general procedure for electrochemical inactivation has been described in the accompanying article (Drummond & Matthews, 1994). For the electrochemical inactivation using the reconstituted, radiolabeled cobalamin, 1 μM enzyme initially in the cob(II)alamin state was poised at −510 mV *vs* the standard hydrogen electrode for 30 min under an atmosphere of nitrous oxide. Triquat (1 mM) was used as the electrochemical mediator ($E_m = -540$ mV; Salmon & Hawkridge, 1980), and the inactivation was performed in potassium phosphate buffer (pH 7.2) containing 100 mM potassium chloride and 200 μM homocysteine to demethylate any residual methylcobalamin. A companion reaction where the nitrous oxide was replaced by argon was performed as a control.

Characterization of Inactive Enzyme by Two-Dimensional Gel Electrophoresis. This analysis was performed as described by O'Farrell (1975), as modified by Blumenthal et al. (1976). Peptides were visualized by staining with Coomassie blue dye.

Separation of Cobalamins by HPLC. Following electrochemical poising under nitrous oxide or argon, aquocobalamin was released from the enzyme by denaturation in 6 M urea. The addition of potassium cyanide (150 μM final concentration) resulted in more efficient release of the prosthetic group as cyanocobalamin. Typically, 100 μL of 1 μM methionine synthase was treated with 200 μL of 9 M urea in

the presence or absence of cyanide and incubated for 15 min at 37 °C, and then 100-μL injections were made directly onto a reverse-phase HPLC column (Ultrasphere ODS 5 from Beckman). A two-solvent system was used to separate the cobalamins, on the basis of the method of Binder et al. (1982). Solvent A was PicB7 (5 mM heptanesulfonic acid at pH 3.5, purchased from Waters), and solvent B was methanol. The column was equilibrated with 80% PicB7 and 20% methanol at a flow rate of 1.0 mL/min for 30 min before the sample was injected. The solvent mixture was maintained for 5 min before a linear gradient to 50% methanol was run over 10 min, and this mixture was then maintained for 15 min. The initial solvent system was then reestablished with a linear gradient over 1 min. Using this program, cyanocobalamin eluted at 17 min, homocysteinylcobalamin at 18 min, aquocobalamin at 20 min, and methylcobalamin at 22 min. Radiolabeled samples were coeluted with standard samples to support the validity of the retention time assignments.

RESULTS

A requirement for the study of modifications to either the cobalamin or methionine synthase following N_2O inactivation is the ability to generate reasonable quantities of inactivated enzyme for study. Because the inactivation event is a relatively rare event compared to turnover, only catalytic quantities of inactivated enzyme have been available until now. In the absence of substrates, the cobalamin prosthetic group can be equilibrated between the cob(II)- and cob(I)alamin oxidation states by electrochemically poising the enzyme at chosen potentials in the absence of oxygen. A potentiostat was used to control the applied potential, and an organic mediator dye such as triquat or methyl viologen was included to mediate electron transfer between the working electrode that is the source of electrons and the enzyme (Harder et al., 1989). The activity of the enzyme was stable under nitrogen or argon, and in this way the midpoint potential of the cobalamin couple was measured recently (Banerjee et al., 1990). When enzyme-bound cobalamin was poised at varying potentials under an atmosphere of nitrous oxide, enzymatic activity was lost at a rate dependent upon the concentration of enzyme in the cob(I)alamin state (Drummond & Matthews, 1994). In these experiments, methionine synthase catalyzes the reductive degradation of nitrous oxide. By maintaining the potential at a fixed value, electrons that were irreversibly lost to the reductive degradation of nitrous oxide are replaced, and this strategy of "redox cycling" allows for multiple degradations of nitrous oxide by the bound cob(I)alamin. It is independent of whether or not the degradation is a benign oxidation or leads to enzyme inactivation, as long as the cobalamin is not damaged in such a way that precludes reduction to a reactive cobalt(I) state. As described in the Experimental Procedures section, we have electrochemically inactivated nanomole quantities of methionine synthase.

Do Modifications Occur to the Cobalamin? The most accessible feature of cobalamin-dependent methionine synthase is the absorbance spectrum of the prosthetic group. Figure 1 shows the characteristic spectrum of enzyme-bound cob(II)alamin before nitrous oxide inactivation and the spectrum obtained following inactivation to 10% of the initial activity. The sample buffer was replaced by ultrafiltration following inactivation to remove a low concentration of a yellow side-product that formed as the electrochemical mediator decomposed. Qualitatively, the spectrum of inactivated enzyme resembles that of cob(II)alamin, although ~15% of the characteristic absorbance at 475 nm has been lost. This

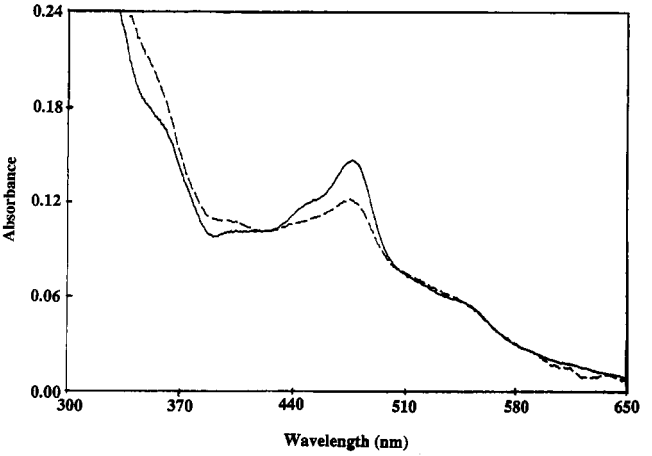

FIGURE 1: Spectra of the cobalamin prosthetic group bound to methionine synthase before (—) and after (– –) electrochemical inactivation.

may indicate that the cobalamin product formed in the reaction between cob(I)alamin and nitrous oxide is cob(II)alamin and that the inactivation proceeds by a one-electron transfer to nitrous oxide. Because of the strongly reducing environment of the electrochemical cell, the initial formation of cob(III)alamin followed by one-electron reduction to cob(II)alamin cannot be ruled out in this experiment. However, in the characterization of the chemistry by which cob(I)alamin reacts with N_2O, no evidence for this pathway was seen when the reaction was monitored spectrally (Drummond & Matthews, 1994).

In order to confirm the identity of the cobalamin product, the inactivation was repeated with radiolabeled cobalamin. Methionine synthase apoenzyme was prepared by a modification of the method of Taylor (1970), and the enzyme was reconstituted with methylcobalamin containing ^{57}Co. Reconstitution of the apoenzyme in our hands gave 20–56% reconstitution with cobalamin, and this recovery was paralleled by 20–56% recovery of the initial activity. Following conversion of the methylcobalamin to cob(II)alamin by treatment with homocysteine, the enzyme was electrochemically inactivated and denatured in 6 M urea at 37 °C for 15 min, and the cobalamin products were examined by high-pressure liquid chromatography (HPLC). This treatment converted the brown, enzyme-bound cob(II)alamin to the red color characteristic of free aquocob(III)alamin, and the major product coeluted with aquocobalamin on the HPLC instrument. This was compared with enzyme prepared in the same manner and poised at the same potential under argon, and again the major product released coeluted with the expected aquocobalamin. The recovery of cobalamin could be increased by including potassium cyanide in the denaturations (Figure 2), providing a strong ligand for the cobalamin to prevent association with ligands donated by the enzyme. The major cobalamin product coeluted with the expected cyanocobalamin in each case. These results are consistent with the spectral evidence suggesting that the cobalamin was not the predominant site of modification in the inactivation.

While cobalamin damage was not the primary reason for the loss of activity, two new cobalamin products were observed in 5–10% yields. Additionally, approximately 15% of the label was not recovered as a discrete product, but instead was spread out over the HPLC trace or remained bound to the enzyme. The remaining 75% of the cobalamin was recovered in the HPLC fractions where unmodified aquocobalamin eluted or where cyanocobalamin eluted following treatment of the

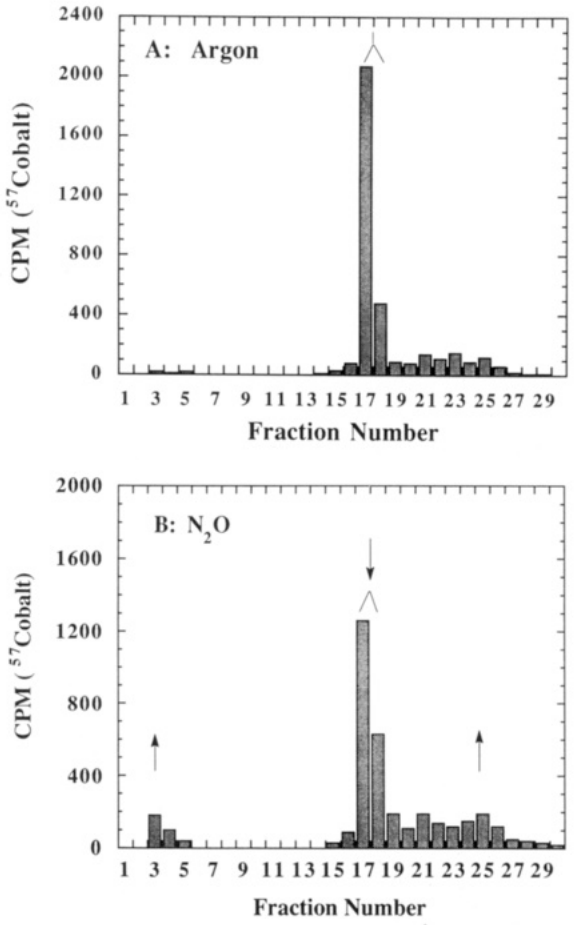

FIGURE 2: HPLC traces of [^{57}Co]cobalamin isolated from methionine synthase after poising under argon (A) or N_2O (B). The elution of standard cobalamins is described in the Experimental Procedures section; cyanocobalamin elutes in fractions 17 and 18. The experiment performed under argon (A) shows that most of the radioactivity can be reisolated as cyanocobalamin upon denaturation in the presence of cyanide. The radioactivity spread out over fractions 19–29 in this trace was also observed before electrochemical poising. Following inactivation under nitrous oxide, ~75% of the radioactivity was reisolated as cyanocobalamin as compared to the argon control. The upward arrows (B) label the fractions where additional radioactivity appeared that was present at greater levels than in the argon control.

denatured enzyme with potassium cyanide. The structures of the two defined products that retained the radioisotope, eluting in fractions 3 and 25 (Figure 2), could not be readily determined. However, they coeluted with the two major products formed when free cob(II)alamin was oxidatively modified by treatment with ascorbic acid in an aerobic environment (Nazhat et al., 1989), suggesting that they are oxidatively modified corrins or free cobalt complexes released following modification. The partial loss of the characteristic absorbance at 475 nm described in Figure 1 is consistent with the observation here that ~25% of the cobalamin is damaged in the inactivation, relative to the recovery of cobalamins in the argon control.

Why Is Methionine Synthase Inactive? Given that inactivated methionine synthase possesses largely unmodified cobalamin that is in the turnover-incompetent cob(II)alamin form, it requires reductive methylation by AdoMet to reenter catalysis. In order to assess whether or not the cobalamin could be converted to the turnover-competent methylcobalamin by AdoMet, both nitrous oxide-inactivated methionine synthase and an equivalent, active control reaction, manipulated in an identical way under argon, were poised at a low potential with AdoMet in a standard protocol for methylation (Luschinsky et al., 1992). The cobalamin bound to active enzyme was converted to methylcobalamin, while the cobalamin bound to the nitrous oxide-inactivated enzyme was essentially unchanged (data not shown). One characteristic of inactive enzyme is therefore the inability to transfer a methyl group from AdoMet to the cobalamin, a step required before the primary catalysis of methyl transfer from CH_3-H_4folate to homocysteine may occur.

Enzyme Becomes Covalently Modified in the Domain That Binds AdoMet. Because the prosthetic group is not the primary site of modification, this implies that the protein structure also suffers oxidative modification. Localization of damage in a monomeric structure of 136.1 kDa is a daunting task, but the loss of the capability to reductively methylate the cobalamin with AdoMet suggests a starting point. A structural model for this enzyme has recently been described (Drummond et al., 1993b), and a key feature of the model is the localization of both the binding of AdoMet and the reductive activation of cob(II)alamin to a 37.7-kDa domain at the carboxyl terminus. Digestion with catalytic amounts of trypsin divides the enzyme into a 98.4-kDa domain that retains the cobalamin and is capable of carrying out the primary catalytic cycle and a 37.7-kDa domain that binds AdoMet. While one-dimensional, denaturing polyacrylamide gels give no evidence of damage to the primary amino acid sequence, a two-dimensional analysis shows a clear distinction between active enzyme from the control experiment and the enzyme inactivated under N_2O. The horizontal dimension in this analysis (Figure 3) is an isoelectric focusing of the polypeptides, and this separates the tryptic fragments on the basis of charge.

Figure 3 primarily shows the fragments derived from limited tryptic digestion of active enzyme from the control experiment performed under argon. The essential features are the large, catalytic domain of 98.4 kDa near the top of the gel and two prominent C-terminal fragments just above the inset box. The larger, more basic C-terminal fragment on the left includes residues 897 through the carboxyl terminus (37.7 kDa), while the less basic fragment on the right has additionally lost residues 897–900 (KKPR). The inset box contains the same two C-terminal fragments from the N_2O-inactivated enzyme, aligned with the corresponding fragments from the control experiment. The arrows show that these domains migrate as if they are more basic in overall charge in comparison with the control domains. Not shown is that the remainder of the fragments from the inactivated enzyme can be overlaid with the corresponding fragments derived from catalytically active enzyme.

Following tryptic digestion, the native, charge-modified AdoMet binding domain was separable from unmodified material on the anion-exchange column used to purify the enzyme. In a typical run (Figure 4), 60–75% of the enzyme had 37.2- and 37.7-kDa domains with altered charge, and this material was our primary target for study. That this charge addition is associated with the inactivation is substantiated by two observations. Methionine synthase with bound methylcobalamin, poised under the same nitrous oxide atmosphere at the same potential, does not lose activity (Drummond & Matthews, 1994). No evidence for a change in the overall domain mass or charge was seen, suggesting that the inactivation is dependent upon the enzyme possessing the bound cob(I)alamin oxidation state and that the modification is associated with nitrous oxide degradation by the cobalamin. Further, the charge modification occurs as the enzyme inactivates; no further change in the overall charge, or in the

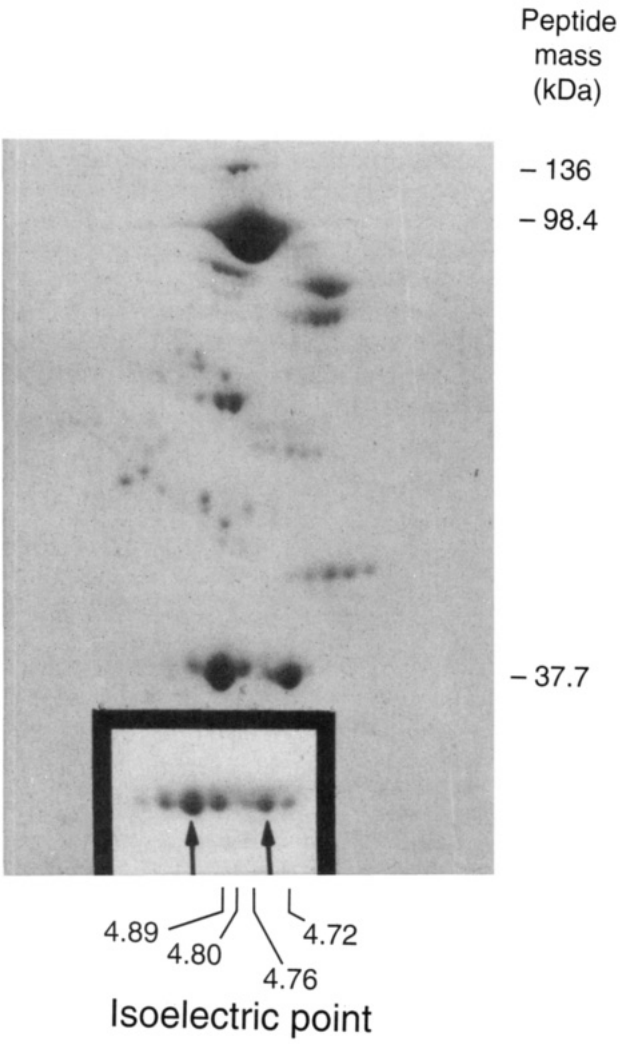

FIGURE 3: Two-dimensional polyacrylamide gel electrophoretic analysis of unmodified methionine synthase following limited tryptic digestion. Separation in the horizontal dimension is by isoelectric focusing in the presence of 6 M urea, and the calculated isoelectric points of defined peptide fragments of methionine synthase (characterized by electrospray mass spectrometry and N-terminal sequencing) are shown. Separation in the vertical dimension is by electrophoresis in a polyacrylamide gel in the presence of sodium dodecyl sulfate, and the masses of characterized peptide fragments of methionine synthase have been indicated to the right of the figure. The inset box shows the two C-terminal domains of ~37 kDa derived from the corresponding digestion of enzyme inactivated by nitrous oxide. The vertical alignment reflects the left-shifting of these fragments, indicating an apparent overall increase in the positive charge of these fragments in this analysis.

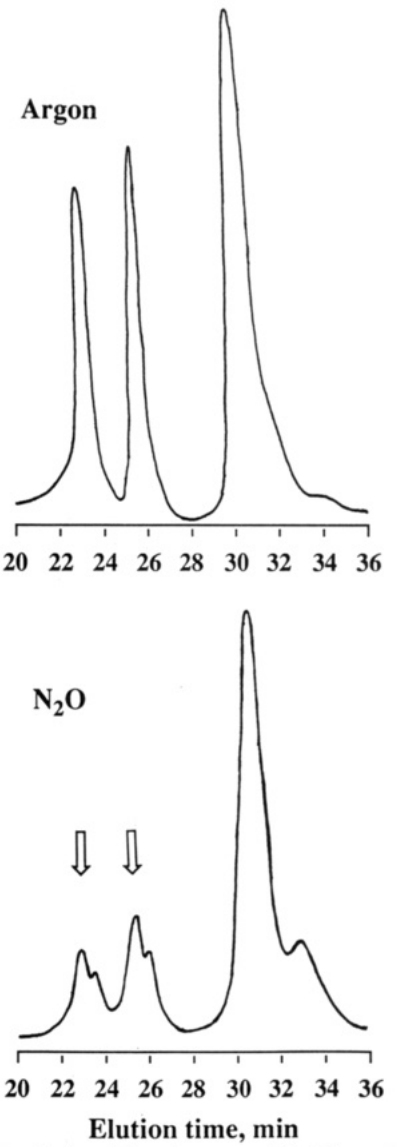

FIGURE 4: Resolution of the charge-modified C-terminal domains by anion-exchange chromatography. The top panel shows the resolution of three fragments derived from limited tryptic proteolysis of methionine synthase of the control poise under argon. In order of elution and increasing anionic charge, they have been localized as residues 896–1200 (37.7 kDa), 901–1200 (37.2 kDa), and 1–895 (98.4 kDa). Following N_2O inactivation (lower panel), ~60–75% of the enzyme suffered a charge modification in the C-terminal domains that allowed the modified domains, labeled with arrows, to be separated. Undigested methionine synthase is prominent in the nitrous oxide-inactivated trace following the 98.4-kDa domain.

ratio of altered to unaltered fragments, is observed upon prolonged poising of the inactivated enzyme.

Identification and Localization of the Modifications. Our primary tool for the definition of modifications was electrospray mass spectrometry. The smaller, 37.2-kDa domain was evaluated first and compared with the domain of an enzyme submitted to an identical control poise performed under argon. The mass of the domain, predicted from the updated protein sequence (Drummond et al., 1993a), was 37 191 Da. The experimentally determined mass of purified methionine synthase, digested and analyzed, was 37 205 Da. With an experimental error of ~0.05% (~19 Da), this value was in agreement with the deduced sequence. The domain mass from the argon control experiment was 37 216 Da, and we rationalize the slightly high mass values as due to partial oxidation of sensitive residues on this domain, since no effort was made to protect the domain from oxidation in the experimental workup. The mass of the nitrous oxide-inactivated domain, determined to be 37 423 Da, provided an immediate explanation for the added positive charge. The mass increase is consistent with the addition of the partially reduced electrochemical mediator triquat, a species that is monocationic.

In order to localize the modification to a specific residue, the modified and control domains were subjected to proteolysis with LysC, an enzyme that specifically cleaves polypeptides on the C-terminal side of lysine residues. This procedure, performed under denaturing conditions, provided essentially complete digestion. Good resolution of the resultant peptides using an HPLC instrument was achieved on a reverse-phase (C_{18}) column, and the traces for the argon control and nitrous oxide-inactivated products are presented in Figure 5. The purified peptides were then resubjected to electrospray mass

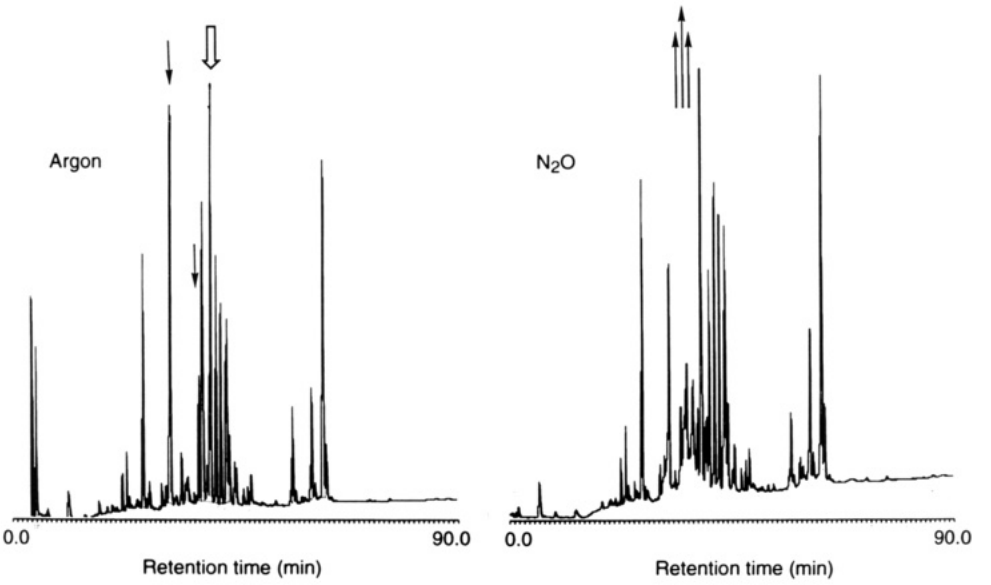

FIGURE 5: HPLC traces of peptides derived from the 37.2-kDa C-terminal domain. Proteolytic digestion of the argon control and nitrous oxide-inactivated enzyme with LysC generated a family of defined fragments, and most of the peptides were not modified in the process. Downward arrows indicate a reduction in the amount of a peptide, while upward arrows indicate the formation of new peptides following inactivation. The peptide containing the valine that became modified is marked with a broad arrow.

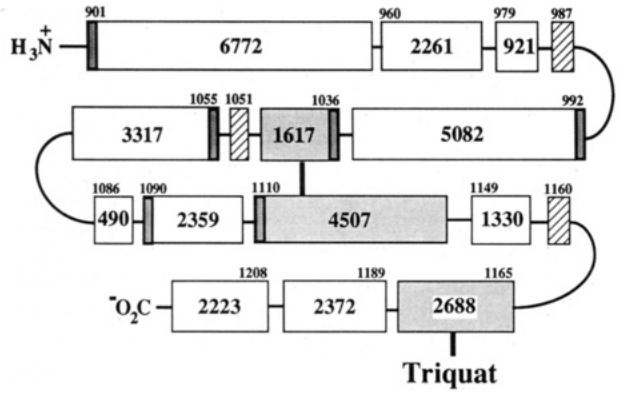

FIGURE 6: Peptide map of the C-terminal 37.2-kDa domain showing sites of modification. A peptide near the C-terminus became covalently modified by the mediator triquat, while a covalent cross-link formed between the only two peptides containing cysteinyl residues in the domain. The boxes indicate the masses of the isolated proteolytic fragments, and the positions of the fragments within the primary sequence were confirmed by amino acid sequencing. Three small peptides (hatched boxes) were not identified in this analysis.

spectrometric analysis to identify damage that led to mass changes. Most of the peptides were unchanged in the process, suggesting that the modifications were specific. The differences that were found between the two traces fell into two categories. First, two peptides, which were formed in equal amounts, could be identified by mass and N-terminal sequence analysis as derivatized by triquat as described here. The second modification was the formation of a covalent cross-link between the two peptides that each contain a cysteine, which is consistent with the oxidative formation of a disulfide bond between these two peptides. The global localization of these changes in the primary sequence is given in Figure 6.

Nitrous oxide inactivation in the presence of triquat results in the appearance of two new peptides, each with a mass of 2884 Da. This mass is equivalent to the mass of the peptide running from leucine 1165 to lysine 1188 (2688 Da), plus the parent mass of triquat (198 Da), less 2 Da. The identity of each of these peptides was confirmed by Edman degradation through as many cycles as possible, and this included the entire 24 amino acid sequence of one modified peptide. The sequence obtained for the parent peptide (1), isolated from the control reaction, was identical to that deduced from the DNA sequence. The two modified sequences (2 and 3) are presented here, and each of the modified peptides contains a degradation cycle in which no amino acid is present where valine 1177 should be found. This suggests that triquat has covalently added to this residue in each case and that this change does not prevent the N-terminal derivatization and peptide bond hydrolysis required for the chemical sequencing procedure. Although we have not directly demonstrated that triquat is the molecule that was added to these peptides, the changes in mass of the C-terminal domain and the modified peptides, and in the net charge of the C-terminal domain, are consistent with this conclusion.

parent peptide:

LTESF AMWPG AS V SG WYFSH PDSK (1)

modified peptide 1:

LTESF AMWPG AS **X** SG WYFSH PDSK (2)

modified peptide 2:

LTESF AMWPG AS **X** SG W (3)

Our working model to explain the addition of triquat to valine 1177 brings together our recent work characterizing the conditions under which methionine synthase degrades nitrous oxide to become inactivated (Drummond & Matthews, 1994) with the nature of the chemical alterations found in this work. We reported that the inactivation requires the prosthetic group to be in the cob(I)alamin state and that this species appears to transfer one electron to nitrous oxide, leaving bound cob(II)alamin. We proposed that the data were most consistent with the formation of a damaging oxidant such as hydroxyl radical, a diffusible species capable of modifying a number of sites on the enzyme and the cobalamin. Our results here showing multiple products in the inactivation are also consistent with this model, but the most convincing signature of this species may be the covalent coupling of triquat to the unreactive valine residue. Hydroxyl radicals are able to abstract hydrogen atoms from unactivated carbon–hydrogen

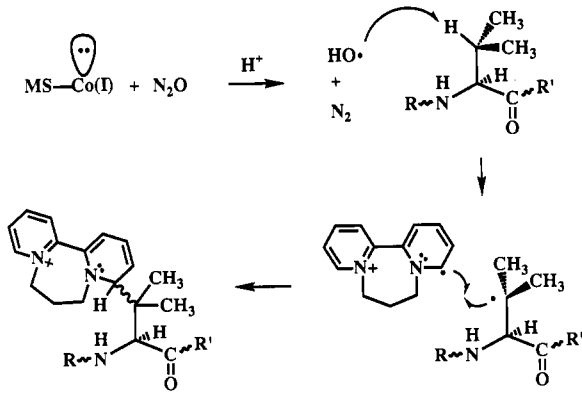

FIGURE 7: Mechanistic proposal for the covalent addition of triquat to a valyl residue. Generation of hydroxyl radical from nitrous oxide could abstract a hydrogen atom from the protein to leave a protein radical. Condensation with one-electron-reduced triquat mediator radical would then produce a covalent adduct with the enzyme, generating a separable, diastereomeric pair of products.

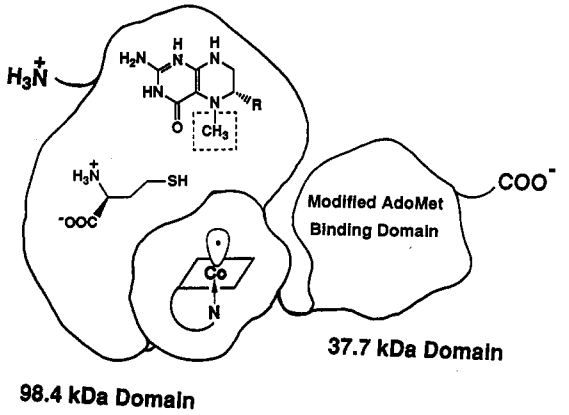

FIGURE 8: Structural model for inactivated methionine synthase. The C-terminal, AdoMet binding domain must approach the cob(II)alamin for reductive activation of the enzyme. Degradation of nitrous oxide by cob(I)alamin modifies this 37.7-kDa domain in the absence of AdoMet.

bonds (Spinks & Woods, 1990), and the valyl side chain presents two sites where such an abstraction would produce a relatively stable radical. Once such a radical was formed, as shown for β-hydrogen atom abstraction in Figure 7, coupling with a second radical such as the one-electron-reduced mediator would produce a covalently bound, cationic derivative where a new stereocenter is created in the triquat moiety. This coupling would produce a separable, diastereomeric pair of peptides, derivatized at the same site and possessing identical masses. Chemically, bond formation between even sterically hindered radical pairs is a reasonable reaction [see Carey and Sundberg (1984)], and the bulky nature of the individual species here may further support this reaction pathway. We cannot eliminate the possiblity that the formation of two modified peptides of identical mass but different chromatographic properties might result from the formation of two different regioisomers of the peptide by hydrogen atom abstraction from the α- and β-positions of the chain of Val 1177.

The second modification to the AdoMet binding domain was the formation of a covalent cross-link between two peptides within this domain. As previously shown in Figure 5, the intensity of the two peptides that each contain one cysteine is diminished. These are labeled with the narrow, downward arrows in the HPLC run characterizing peptides from active enzyme. A new peptide, the center peak of the three labeled peptides in the HPLC run characterizing the N_2O-inactivated enzyme, accounts for these peptides. First, the mass of this new peptide (6124 Da) is equivalent to the mass sum of the two peptides, shown in Figure 6, that contain amino acids 1036–1050 (1617 Da) and 1110–1148 (4507 Da). In order to form a covalent cross-link, such as the formation of a disulfide bond, protons will be lost in the bond formation. Two protons will be lost in the case of disulfide formation, but the predicted mass of the oxidized product (6122 Da) is still within the experimental error of this set of measurements [see Drummond et al. (1993b)]. Amino-terminal sequencing of this new peptide yielded two residues in each degradation cycle, and these correspond to a mixture of the predicted amino acid sequences through 10 cycles. While the formation of a disulfide bond between the two cysteines can certainly account for the covalent coupling of these peptides, other oxidative cross-links could easily be proposed. The characterization of this interpeptide coupling is currently in progress.

DISCUSSION

The goal of this work was to characterize the damage that occurs to cobalamin-dependent methionine synthase following inactivation by nitrous oxide. To summarize our findings, inactivated enzyme suffers oxidative modification, primarily to the protein structure, although damage to the cobalamin is also observed. The nature of the damage suggests that nitrous oxide is reductively degraded to release an oxidant, modifying sites proximal to the cobalamin. The ability to identify multiple products may imply that the oxidant is not completely specific in its site of addition, and not all of the enzyme has been evaluated yet for modifications. The apparent addition of triquat to the unreactive valyl side chain is at least consistent with the formation of a radical species from nitrous oxide that transiently produces an enzyme radical, as discussed here.

The damage to methionine synthase reported in this work provides insight into the structure of methionine synthase, a monomeric enzyme of 136.1 kDa. Figure 8 summarizes previous work [Drummond et al. (1993b) and references cited therein] wherein binding of the prosthetic group and substrates was assigned to the mosaic of individual domains that make up this enzyme. In order to access the reactive cob(I)alamin state for the inactivation experiments described here, we began with enzyme possessing the bound cob(II)alamin oxidation state. The next step in catalysis for this species is reductive methylation by AdoMet, and the cob(II)alamin must interact with the 37.7-kDa C-terminal domain that binds this substrate. The observation of damage to the C-terminal domain reported here strongly supports a model where the cobalamin binding domain must lie close in space to the AdoMet binding domain, at least during reductive methylation. More specifically, the modification of valine 1177 and the cross-linking of the two cysteine-containing peptides upon inactivation place constraints on the location of these amino acid residues with respect to each other and to the cobalamin. The derivatized valine residue may provide a marker for a residue proximal to the cobalt when the two domains interact, while the cross-linked peptides provide an indication that these two peptides must approach each other in the native structure. Whether or not the cross-linking of the peptides represents the formation of a disulfide bond remains to be established.

In order to generate large enough quantities of N_2O-inactivated methionine synthase for study, an electrochemical technique was used to reduce the enzyme to the cob(I)alamin

state in the absence of substrates. Under an atmosphere of nitrous oxide, the enzyme inactivated only when the cob(I)-alamin oxidation state could be achieved, and the rate of inactivation was directly dependent upon the concentration of enzyme in this state (Drummond & Matthews, 1994). When triquat was used as a mediator, transferring electrons to and from the enzyme, it became attached covalently and selectively to valine 1177 in the C-terminal domain in a manner that correlates with enzyme inactivation. However, this clearly is not the mechanism through which the enzyme becomes inactivated *in vivo*, since triquat is not a physiological species. Inactivation in an electrochemical cell in the presence of methyl viologen, rather than triquat, also leads to loss of enzyme activity, but in this case no charge modification occurs and the methyl viologen is not cross-linked to the 37-kDa domain, as assessed by mass spectrometry. In this work, triquat appears to capture a reactive enzyme species, and it has served as a reporter of where a reactive site existed, at least transiently, on the enzyme. One might speculate that, if protein modifications were initiated by the abstraction of a hydrogen atom by a species like hydroxyl radical, a reactive protein radical might propagate by abstracting hydrogen atoms from sites, such as thiol residues, that generate more stabilized radicals. The physiological inactivation event, and the inactivation of the enzyme in the presence of methyl viologen, may require the addition of a separate molecule as yet uncharacterized or may result from radical propagation.

When methionine synthase is isolated and assayed in crude extracts from mammals exposed to N_2O, residual activity is commonly observed [Koblin et al. (1982) and references cited therein]. This could reflect the presence of more than one form of methionine synthase, including one that is not inactivated by nitrous oxide. This work provides another alternative for this residual activity, since purified enzyme was routinely reduced to ~5–10% of the initial activity by electrochemical inactivation. The primary site of damage appears to be the 37.7-kDa domain responsible for reductive activation of the cobalamin (Drummond et al., 1993b), rather than the 97.4-kDa domain that binds cobalamin and is capable of effecting turnover following reductive methylation. In this model, the residual activity could be the result of normal methyl transfer from CH_3-H_4folate to homocysteine by an unmodified domain or subunit, where the overall production of methionine is greatly reduced by the inability to efficiently recover from periodic oxidation of the cob(I)alamin. This model makes a set of testable predictions, including one that suggests that the 97.4-kDa domain of the inactivated enzyme should be capable of turnover if it were to be chemically methylated.

One of the tenets of the working hypothesis given here is that a species like hydroxyl radical is formed when nitrous oxide becomes degraded by methionine synthase. It is reasonable to ask whether or not such a species could be demonstrated by trapping with an appropriate radical trap. Unfortunately, many of the commonly used traps that covalently capture hydroxyl radical to produce stable free radicals are themselves unstable in the reducing medium present in the inactivation. These include DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) and TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy free radical). Each of the stable species that we have used to look for protection from inactivation in turnover have failed to protect, and the substrates homocysteine (1 mM) and CH_3-H_4folate (250 μM) have not offered any protection in the electrochemical inactivation. Each of these species is oxidatively sensitive and might be expected to offer some ability to scavenge oxidants, but they bind to the 98.4-kDa catalytic domain. This is again consistent with the localization of damage to the C-terminal, AdoMet binding domain rather than the catalytic domain (refer to Figure 8). The best candidates for protection might instead be analogs of *S*-adenosylmethionine. It should be noted that, in our previous work characterizing the chemistry of this inactivation reaction, the rate of inactivation closely paralleled the rate of transfer of electrons from cob(I)alamin to nitrous oxide. This suggests that a trapping species would have to be exceptionally effective to intercept a degradation reaction closely coupled to the inactivation.

Finally, the formation of products derived from the cobalamin in the inactivation of methionine synthase is relevant to the *in vivo* experiments performed in mammals. Kondo et al. (1981) reported that cobalamin analogs appeared in the blood of rats following prolonged exposure to nitrous oxide. They suggested that nitrous oxide inactivation of methionine synthase could lead to the release of these analogs in the blood and that this release could inhibit other cobalamin binding proteins. Our electrochemical inactivation of the *E. coli* enzyme produced cobalamin analogs over the course of the inactivation. We have also recently shown that the inactive enzyme is capable of continued catalytic degradation of nitrous oxide (Drummond & Matthews, 1994). Inactive enzyme containing bound, unmodified cobalamin is potentially still a target for oxidative modification by nitrous oxide. Thus while the cobalamin is not the primary site of damage in this work, our results are consistent with the observation that cobalamin analogs are released from methionine synthase during nitrous oxide exposure.

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REFERENCES

Banerjee, R. V., & Matthews, R. G. (1990) *FASEB J. 4*, 1450–1459.

Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P., & Matthews, R. G. (1989) *J. Biol. Chem. 264* (23), 13888–13895.

Banerjee, R. V., Harder, S. R., Ragsdale, S. W., & Matthews, R. G. (1990) *Biochemistry 29*, 1129–1135.

Binder, M., Kolhouse, J. F., Van Horne, K. C., & Allen, R. H. (1982) *Anal. Biochem. 125*, 253–258.

Blumenthal, R. M., Reeh, S., & Pederson, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3743–3747.

Carey, F. A., & Sundberg, R. J. (1984) in *Advanced Organic Chemistry Part A: Structure and Mechanisms*, pp 625–690, Plenum Press, New York.

Deacon, R., Perry, J., Lumb, M., Chanarin, I., Minty, B., Halsey, M. J., & Nunn, J. F. (1978) *Lancet ii*, 1023–1024.

Drummond, J. T., & Matthews, R. G. (1994) *Biochemistry* (preceding article in this issue).

Drummond, J. T., Ogorzalek Loo, R. R, & Matthews, R. G. (1993a) *Biochemistry 32*, 9282–9289.

Drummond, J. T., Huang, S., Blumenthal, R. M., & Matthews, R. G. (1993b) *Biochemistry 32*, 9290–9295.

Frasca, V., Riazzi, B. S., & Matthews, R. G. (1986) *J. Biol Chem. 261* (34), 15823–15826.

Harder, S. R., Feinberg, B. A., & Ragsdale, S. W. (1989) *Anal. Biochem. 181*, 283–287.

Koblin, D. D., Waskell, L., Watson, J. E., Stokstad, E. L. R., & Eger, E. I. (1982) *Anesth. Analg. (N.Y.) 61*, 75–78.
Kondo, H., Osborne, M. L., Kolhouse, J. F., Binder, M. J., Podell, E. R., Utley, C. S., Abrams, R. S., & Allen, R. H. (1981) *J. Clin. Invest. 67*, 1270–1283.
Luschinsky, C. L., Drummond, J. T., Matthews, R. G., & Ludwig, M. L. (1992) *J. Mol. Biol. 225*, 557–560.
Nazhat, N. J., Golding, B. T., Alistair Johnson, G. R., & Jones, P. (1989) *J. Inorg. Biochem. 36*, 75–81.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Retey, J. (1982) in B_{12} (Dolphin, D., Ed.) Vol. 2, pp 357–379, Wiley-Interscience, New York.
Salmon, R. T., & Hawkridge, F. M. (1980) *J. Electroanal. Chem. 112*, 253–264.
Spinks, J. W. T., & Woods, R. J. (1990) in *An Introduction to Radiation Chemistry*, pp 284–285, John Wiley & Sons, New York.
Taylor, R. T. (1970) *Arch. Biochem. Biophys. 137*, 529–546.
Taylor, R., & Weissbach, H. (1973) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 1, pp 121–165, Academic Press, New York.