Methyl Transfer from Methylcobalamin to Thiols. A Reinvestigation†

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ABSTRACT: The methyl transfer from methylcobalamin to thiols has been reinvestigated. By use of methylcobalamin selectively enriched with ^{13}C in the methyl moiety, the methyl transfer to thiols was followed by ^{13}C NMR. The methyl transfer occurs in aqueous mildly alkaline (pH 8–12) solution, even in the complete absence of oxygen. ^{31}P NMR and EPR studies demonstrate that cob(II)alamin is the final corrinoid product. However, the pH dependence of the methyl-transfer reaction from methylcobalamin to β-mercaptoethanol is consistent only with a nucleophilic displacement of the methyl group by a thiolate anion, resulting in the heterolytic cleavage of the carbon–cobalt bond. Difference visible spectroscopic measurements of the reaction mixture suggest that cob(I)alamin is formed as an intermediate.

Methionine synthetase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase, EC 2.1.1.13) is a cobalamin-dependent enzyme that catalyzes the methyl transfer from 5-methyltetrahydrofolate (5-methyl-H_4folate) to homocysteine (Taylor, 1982; Matthews, 1984).

$$5\text{-}CH_3\text{-}H_4\text{folate} + \text{homocysteine} \xrightarrow[\text{AdoMet}]{\text{reducing system}} H_4\text{folate} + \text{methionine} \quad (1)$$

Reaction 1 requires a reducing system such as $FMNH_2$ and dithiothreitol and *S*-adenosylmethionine as a cofactor.

Taylor & Weissbach (1967, 1968) demonstrated that the cobalamin–enzyme complex is methylated when [5-^{14}C]-methyl-H_4folate is incubated with a stoichiometric amount of holoenzyme, a reducing system, and AdoMet but without homocysteine. They further showed that the labeled holoenzyme is able to transfer the methyl group to homocysteine. These observations clearly indicate that reaction 1 consists of two half-reactions (1a and 1b).

$$5\text{-}CH_3\text{-}H_4\text{folate} + \text{holoenzyme} \xrightarrow[\text{AdoMet}]{\text{reducing system}} H_4\text{folate} + \text{methylated holoenzyme} \quad (1a)$$

$$\text{methylated holoenzyme} + \text{homocysteine} \rightarrow \text{holoenzyme} + \text{methionine} \quad (1b)$$

The methylated holoenzyme was shown to be a methylcobalamin–protein complex, and thus, the first half-reaction involves the transfer of the methyl group from 5-CH_3-H_4folate to the cobalt atom of the cobalamin. In the second half-reaction the methyl group of methylcobalamin is transferred to the thiol of homocysteine. Indeed, Guest and co-workers (1962) found that preparations of the holoenzyme that catalyze reaction 1 also catalyze the transfer of the methyl group from methylcobalamin to homocysteine. Taylor & Weissbach (1966) demonstrated that methyl iodide is able to replace AdoMet in reactions 1 and 1a and suggested that the holoenzyme is inactive until it is primed by reductive methylation (reducing system and AdoMet).

More than 20 years ago, Guest and co-workers (1962) showed that methyl transfer from methylcobalamin to homocysteine occurs slowly in the absence of methionine synthetase.

Johnson and co-workers (1963) reported that methionine is formed when methylcobalamin is photolyzed in the presence of homocysteine and suggested that a similar enzyme-catalyzed homolysis of the carbon–cobalt bond is not "improbable". Schrauzer (1968) first postulated that the cobalamin-dependent methionine synthesis involves a nucleophilic attack of a thiolate anion on the carbon–cobalt bond to generate the methyl thioether and cob(I)alamin. Schrauzer and co-workers (1967, 1968) demonstrated the formation of methionine from methylcobaloxime and homocysteine in alkaline methanol. A radical mechanism was eliminated because they observed that methyl radicals generated in the presence of thiols gave primarily methane. These conclusions were questioned by several laboratories. Agnes and co-workers (1971) were unable to observe methyl transfer from methylcobalamin to thiols under strictly anaerobic conditions. Methyl transfer was rapid only in the presence of oxidizing agents, and they concluded that the reaction involves a one-electron oxidation attack on methylcobalamin and not a direct reaction with either thiolate anion or thiol. Frick et al. (1976) suggested that the demethylation of methylcobalamin by coenzyme M involves a thiol-promoted homolytic cleavage of the carbon–cobalt bond to cob(II)alamin and the thioether. They also found that the rate of demethylation was very slow under strictly anaerobic conditions, that it was enhanced in the presence of oxygen, and that the reaction rate was not affected by pH in the range 7.0–14.2. In contrast, Brown & Kallen (1972) were unable to detect any methyl thioether or methane as products of the reaction between methyl(aquo)cobaloxime and mercaptoethanol or mercaptoacetate. Their observations indicated that the reaction of these thiols with methyl(aquo)cobaloxime involves only axial ligation and not carbon–cobalt bond cleavage. In a subsequent publication, Schrauzer & Stadlbauer (1974) repeated their earlier experiments in methanol and in aqueous solution and concluded that methylation of mercaptide ions by alkylcobalamins and reactive alkylcobaloximes does occur "provided that the concentration of NaOH is between 0.1 and 1 M and thiol is present in considerable excess to assure high concentrations of thiolate anion".

In an attempt to reconcile these divergent conclusions from well-established laboratories, we have reinvestigated the methyl transfer from methylcobalamin to thiols using several spectroscopic techniques. Our results demonstrate that methyl

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transfer does occur in aqueous buffered solutions under strictly anaerobic conditions and that the rate of the reaction is strongly affected by pH. Our results are consistent only with a heterolytic cleavage of the carbon–cobalt bond of methylcobalamin by thiolate anion as originally postulated by Schrauzer (1968). However, we can find no evidence for the displacement of the lower 5,6-dimethylbenzimidazole ligand by thiol or thiolate.

Experimental Procedures

Materials. Cyanocobalamin was purchased from Rhone-Poulenc Industries, Paris. The cobalamins and their ^{13}C-enriched derivatives were prepared from cyanocobalamin as described before (Hogenkamp et al., 1965). DL-Dithiothreitol (DTT), *trans*-4,5-dihydroxy-1,2-dithiane, DL-homocysteine, and β-mercaptoethanol were obtained from Sigma Chemical Co. Buffer components and inorganic salts were reagent grade and used without further purification. ^2H_2O (99.8%) was obtained from Aldrich Chemical Co.

Methods. Anaerobic conditions were attained by bubbling argon, scrubbed via a heated copper oxide catalyst (Kontes), through solutions for 30 min in NMR tubes or cuvettes sealed with serum stoppers. The vessels were then evacuated (20 mm Hg) for 5 min and filled with argon. This procedure was repeated 3 times, and the vessel was finally filled with argon at slightly positive pressure. All manipulations were done in a glovebox with an argon atmosphere. Pulse Fourier-transform ^1H (250.1-MHz), ^{13}C (62.9-MHz), and ^{31}P (101.3-MHz) NMR spectra were obtained with a Bruker WM 250 spectrometer, locked to the resonance of internal ^2H_2O. For the ^1H spectra the transients resulting from the application of 90° pulses (4 μs) in a spectral width of 4000 Hz were accumulated as 4096 data points in the time domain and transformed into a 2048-point spectrum. The data acquisition time was 512 ms with a 488-ms pulse delay. For the ^{13}C spectra the transients resulting from the application of 90° pulses (38 μs) in a spectral width of 15 000 Hz were accumulated as 8192 data points in the time domain and transformed into a 4086-point spectrum. The data acquisition time was 270 ms with a 200-ms pulse delay. For the ^{31}P spectra the transients resulting from the application of 90° pulses (27 μs) in a spectral width of 2000 Hz were accumulated as 8192 data points and transformed into a 4096-point spectrum. The data acquisition time was 2.048 s without a pulse delay. The ^{13}C and ^{31}P spectra were obtained under conditions of simultaneous broad-band noise decoupling. Peak positions were determined by computer examination of the final Fourier-transformed spectra. Chemical shifts were measured with respect to external neat tetramethylsilane for the ^{13}C NMR spectra, external 85% phosphoric acid for the ^{31}P NMR spectra, and internal ^2H_2O set at 4.90 ppm for the ^1H NMR spectra. The spectrometer temperature was verified with a Cu–constantan thermocouple. Reaction rates were determined by monitoring the decrease of the intensity of the Co–$^{13}CH_3$ resonance. All reaction mixtures for the kinetic experiments were prepared anaerobically in an ice bath, heated to the desired temperature in a heating block (2 min), and inserted into the spectrometer. The spectrometer was then shimmed for 2 min.

Repetitive scan visible spectra were obtained on a Hewlett-Packard (Model 8451A) diode array spectrophotometer equipped for digital data collection and processing. A special cuvette with a 0.127-mm light path was constructed so that solutions of the same concentration needed in the NMR and EPR experiments could be used. The reaction mixtures (0.2–0.4 mL) were injected into the anaerobic cuvette which was placed in a thermostated cell holder set at the desired temperature. Spectra from 300 to 700 nm were taken at various time intervals with a scanning time of 1 s.

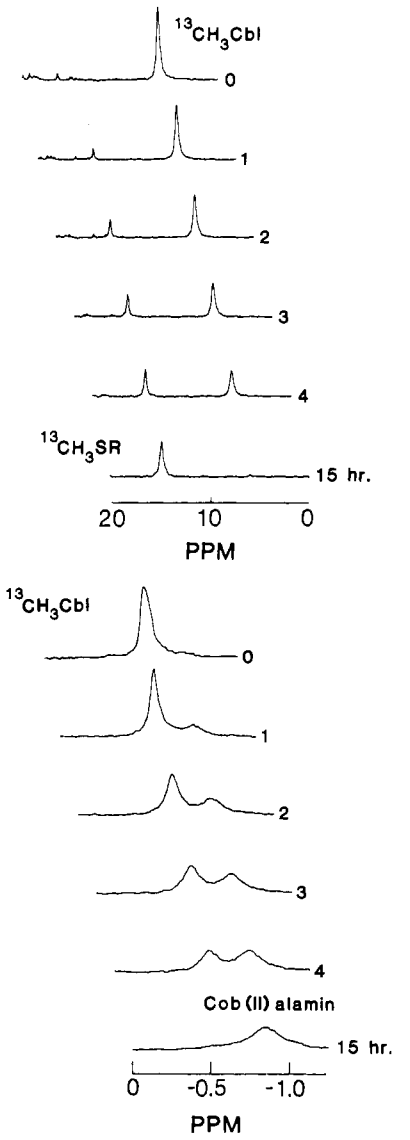


FIGURE 1: (Top) ^{13}C NMR spectra in the methyl region for a reaction mixture containing [^{13}C]methylcobalamin (10 mM), dithiothreitol (100 mM), and glycine buffer (200 mM), pH 9.7, in 99.8% ^2H_2O incubated at 43 °C in the dark. The spectra are obtained 0, 1, 2, 3, 4 and 15 h subsequent to mixing the reagents. Each spectrum represents 3600 transients acquired during the first 30 min of each time interval. (Bottom) ^{31}P NMR spectra in the region from 0 to −1 ppm for the reaction mixture described in the top panel. Each spectrum represents 289 transients acquired during the first 10 min of each time interval.

EPR spectra were recorded at the X band on a Varian E-109 spectrometer with an Oxford Instruments EPR-10 liquid helium cryostat at a temperature of 10 K, a 1-μW microwave power, and a frequency of 9.223 GHz. Spectral data were stored and processed by an interfaced computer.

Results

Nuclear Magnetic Resonance Spectroscopy. The transfer of the methyl group from methylcobalamin to thiols can be readily followed by ^{13}C NMR if methylcobalamin selectively enriched with ^{13}C in the methyl moiety is used. Figure 1 (top) illustrates the ^{13}C NMR spectra in the region of the Co–CH_3 resonance for a 10 mM solution of [^{13}C]methylcobalamin in the presence of a 10-fold excess of DTT–200 mM glycine buffer, pH 9.7, incubated at 43 °C in the dark under strictly *anaerobic conditions*. The spectral changes with time dem-

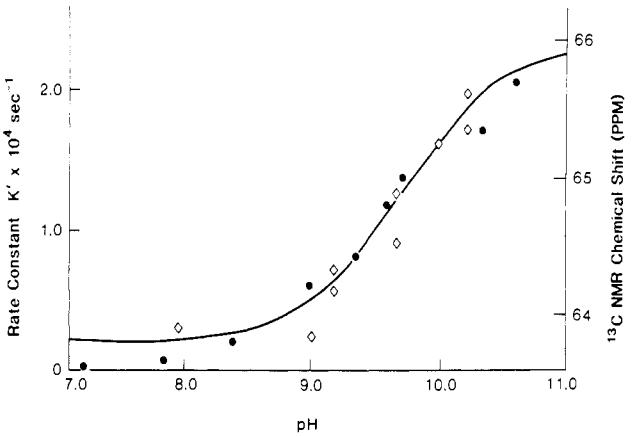
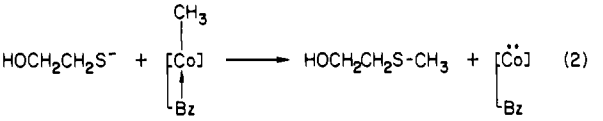


FIGURE 2: pH dependence of the pseudo-first-order rate constant (◊) of the methyl-transfer reaction from [^{13}C]methylcobalamin (10 mM) to β-mercaptoethanol (200 mM) in 200 mM buffer (phosphate and glycine) and 10% ^2H_2O, 43 °C. Titration of β-mercaptoethanol (200 mM) (●). Both sets of data were obtained from ^{13}C NMR measurements made during the time course of the reaction at each pH value. The β-mercaptoethanol titration was verified by independent experiments under the same conditions as the reactions but in the absence of methylcobalamin. The combined data were normalized to the same scale and fitted to the Henderson–Hasselbalch equation by a computer nonlinear least-squares algorithm to determine an "apparent pK" for the methyl transfer. The computed "pK" of 9.7 was used to generate the solid line.

onstrate that the methyl group is transferred from the cobalamin (6.7 ppm) to the thiol (15.4 ppm). Under these reaction conditions the methyl transfer is first order with respect to methylcobalamin. Furthermore, plots of the pseudo-first-order rate constants vs. thiol concentration are linear, indicating that the reactions are also first order with respect to thiol concentration. The methyl-transfer reaction is quite slow ($t_{1/2}$ = 1.5 h; $k = 1.3 \times 10^{-5}$ s^{-1}) at 43 °C. Indeed, an identical reaction mixture incubated at room temperature took more than 5 days to go to completion. It should be pointed out that the ^{13}C resonance of methylcobalamin is not significantly shifted in the presence of the thiol. We have demonstrated before (Needham et al., 1973) that the chemical shift of the $^{13}CH_3$ moiety is markedly affected by the nature of the trans ligand, and thus, the absence of a chemical shift change in the presence of the thiol demonstrates that under these reaction conditions the thiol has not displaced the lower 5,6-dimethylbenzimidazole ligand. In accord with this conclusion the ^{1}H NMR spectrum of the reaction mixture shows resonances for the protons at carbons 4, 2, and 7 of the 5,6-dimethylbenzimidazole moiety at 6.33, 7.01, and 7.19 ppm, respectively. These resonance positions are characteristic for methylcobalamin in the "base-on" form at 43 °C. The methyl transfer can also be followed by ^{31}P NMR. Figure 1 (bottom) shows the ^{31}P NMR spectra of an identical reaction mixture. The spectral changes with time indicate that methylcobalamin (−0.59 ppm) is converted to cob(II)alamin (−0.84 ppm). Indeed, the final reaction mixture also shows the eight-line EPR spectrum characteristic of cob(II)alamin in the base-on form (Hill et al., 1971).

In order to establish the mechanism of the methyl-transfer reaction, the effect of pH on the rate of the reaction was investigated. Instead of DTT, β–mercaptoethanol was used in these experiments because the monothiol would be expected to show a sharper titration curve. The pH dependence of the pseudo-first-order rate constants (Figure 2) shows an inflection point at the pK_a of the thiol (β-mercaptoethanol pK_a = 9.7 at 43 °C), indicating that the thiolate anion is the attacking species.



Visible Spectroscopy. The color of the reaction mixtures changes from red to brown during the course of the methyl-transfer reaction, indicating that cob(II)alamin is one of the final products. However, the nucleophilic displacement reaction outlined in reaction 2 generates a methyl thioether and cob(I)alamin. In order to detect the transient production of this reactive cobalamin, we followed the initial stages of the reaction using a rapid scanning UV–visible spectrophotometer. The UV–visible difference spectra of the reaction mixture

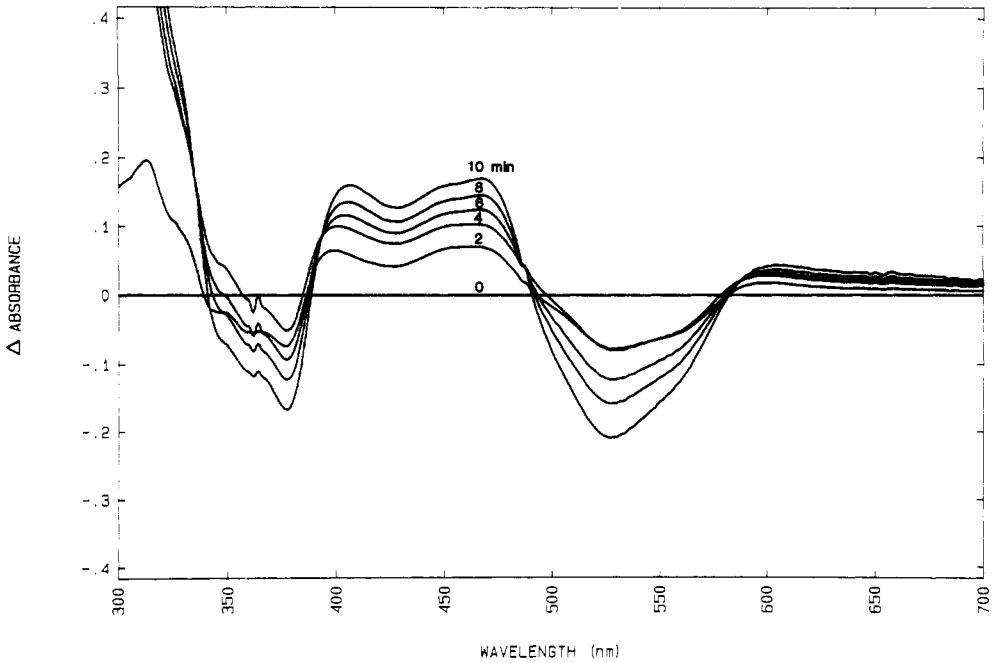


FIGURE 3: Visible difference spectra from 300 to 700 nm for the reaction mixture described in Figure 1 (top) incubated at 70 °C in the dark. The zero time difference spectrum is a 1-s scan immediately after the cell was placed in the spectrophotometer. All difference spectra were generated by mathematically subtracting each spectrum from the zero time spectrum.

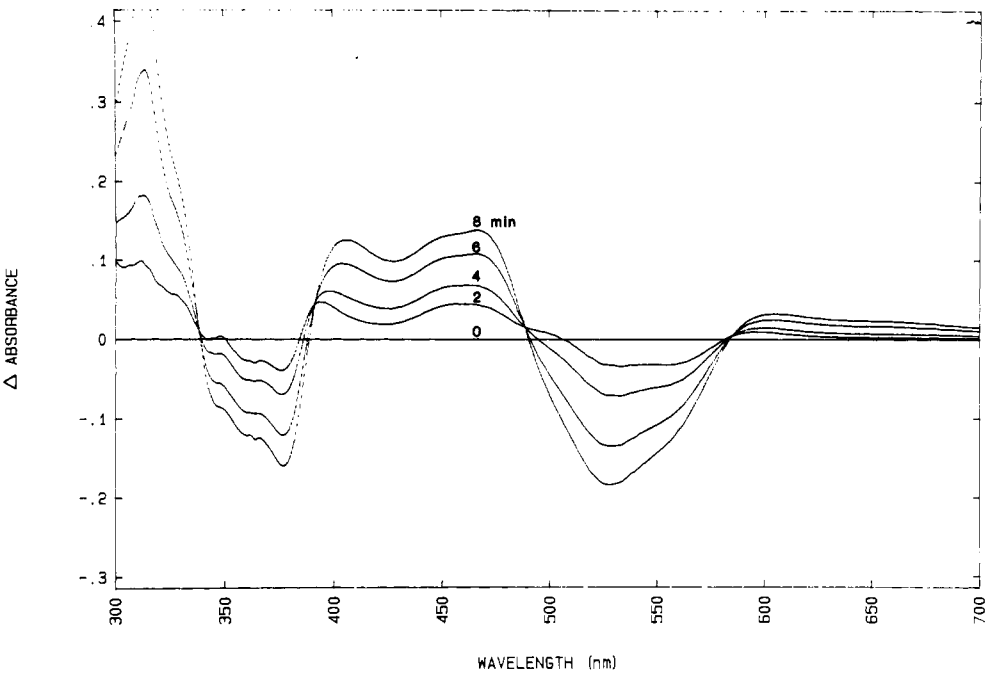
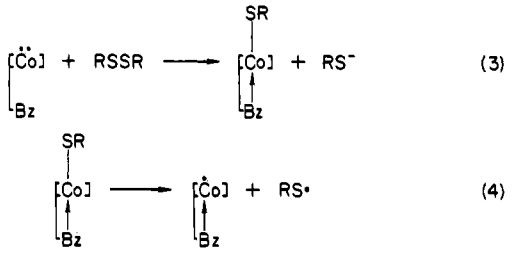


FIGURE 4: Visible difference spectra from 300 to 700 nm for the reaction mixture containing methylcobalamin (10 mM), dithiothreitol (100 mM), *trans*-4,5-dihydroxy-1,2-dithiane (100 mM), and glycine buffer (200 mM), pH 9.7, in ^2H_2O, incubated at 70 °C in the dark. The zero time difference spectrum is a 1-s scan immediately after the cell was placed in the spectrophotometer. All difference spectra were generated by mathematically subtracting each spectrum from the zero time spectrum.

containing 10 mM methylcobalamin and 100 mM DTT in 200 mM glycine buffer, pH 9.7, incubated in the dark, under strictly anaerobic conditions at 70 °C are shown in Figure 3. It is clear that these difference spectra do not show true isosbestic points for the first 10 min of the reaction, indicating that cob(II)alamin is not the initial product. Cob(I)alamin is reasonably stable at pH 9.7 in the absence of oxygen. However, DTT preparations are invariably contaminated by traces of the disulfide, and thus, it is not unreasonable to assume that cob(I)alamin, produced in the initial nucleophilic displacement raction, is oxidized by the disulfide as outlined.



$$2RS\cdot \rightarrow RSSR \quad (5)$$

In this reaction sequence the disulfide is regenerated, and thus, traces can catalyze the oxidation of cob(I)alamin. To test the effect of oxidizd DTT on the transfer reaction, 100 mM *trans*-4,5-dihydroxy-1,2-dithiane was added to an identical reaction mixture. The difference spectra of this reaction mixture, presented in Figure 4, show very sharp isosbestic points at 340, 393, 490, and 586 nm. These results support the hypothesis that the cob(I)alamin formed in the primary reaction is oxidized to cob(II)alamin by the disulfide.

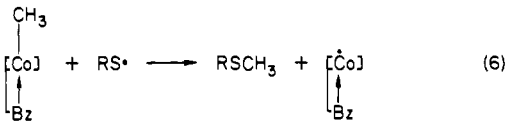
Discussion

The present study demonstrates that methylcobalamin reacts with thiols in aqueous buffered solutions under strictly anaerobic conditions to yield methyl thioethers and cob(II)-alamin. Our results show that this methyl transfer occurs at relatively mild conditions and that the harsh alkaline conditions and the enormous excess of thiol reported by Schrauzer & Stadlbauer (1974) are not necessary. The reaction is quite slow at pH 9.7 and 43 °C if only a 10-fold excess of dithiol is used. Indeed, at room temperature and at pH 9.7 the methyl transfer takes more than 5 days to go to completion. It is, thus, not at all surprising that Brown & Kallen (1972) were unable to detect the formation of methyl thioethers in reaction mixtures containing 10^{-3} M methylaquocobaloxime and a 10-fold excess of β-mercaptoethanol incubated at 25 °C for only 30 min. Our results with methylcobalamin do not provide any evidence for the displacement of the lower 5,6-dimethylbenzimidazole ligand by the thiol. We were unable to detect any shift in the resonance of the ^{13}C-enriched methyl moiety even in the presence of a 100-fold excess of thiol.

Frick et al. (1976) reported that the methyl transfer from methylcobalamin to ethanethiolsulfonate (coenzyme M) was not dependent on the pH of the reaction mixture. In contrast to their observations, we find that the rate of the transfer reaction from methylcobalamin to DTT or β-mercaptoethanol is strongly dependent on the pH of the solution (Figure 2). Indeed, our results show that the pH dependence curve is coincident with the titration curve of β-mercaptoethanol determined from the chemical shift changes of the β-methylene carbon of β-mercaptoethanol with changes in pH at 43 °C. Our results are consistent with a nucleophilic displacement of the methyl moiety of methylcobalamin by a thiolate anion as first postulated by Schrauzer (1968).

Agnes et al. (1971) and Frick and co-workers (1976) reported that they were unable to observe methyl transfer in the absence of oxygen. Our results, presented in Figure 1, clearly demonstrate methyl transfer from [^{13}C]methylcobalamin to DTT under strictly anaerobic conditions. In similar anaerobic reaction mixtures we have also observed methyl transfer to β-mercaptoethanol, cysteine, and homocysteine. The same laboratories also reported that there was a lag period in the reaction even in the presence of oxygen. This lag period could be abolished by increasing the thiol concentration, by increasing the pH, by adding catalytic amounts of cob(II)alamin, or by increasing the oxygen pressure. All these conditions lead

to the oxidation of the thiol to the disulfide. Our results (Figures 3 and 4) suggest that this lag period reflects the production of cob(I)alamin, which under strictly anaerobic conditions is oxidized to cob(II)alamin by traces of disulfide present in the thiol preparations. The conditions reported by Agnes et al. (1971) that abolish the lag period also result in an increase in the disulfide concentration. Increasing the thiol concentration increases the concentration of the contaminating disulfide; increasing the pH, adding cob(II)alamin, and increasing the oxygen pressure also generate more disulfide. Our finding that under strictly anaerobic conditions no true isosbestic points can be observed is entirely consistent with the notion that cob(I)alamin and not cob(II)alamin is the primary product. Our results suggest that the lag periods observed by Agnes et al. (1971) and Frick et al. (1976) are due to the formation of cob(I)alamin, which can be oxidized by a variety of oxidizing agents. Superficially, the overall process outlined in reaction 6 does indeed suggest that the methyl transfer from



(6)

methylcobalamin to a thiol involves the homolytic cleavage of the carbon–cobalt bond by a sulfur radical. However, our mechanistic analysis demonstrates that reaction 6 is the sum of reactions 2–5. Reaction 2 depicts the heterolytic cleavage of the carbon–cobalt bond by a thiolate anion to generate a methyl thioether and cob(I)alamin. Reactions 3–5 show how cob(I)alamin can be oxidized to cob(II)alamin by a disulfide. Since the disulfide is regenerated in reaction 5, the presence of only trace amounts of disulfide will suffice to catalyze this oxidation.

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