Reaction of Alkylcobalamins with Thiols[†]

Harry P. C. Hogenkamp,* Gerald T. Bratt, and Ann T. Kotchevar
Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: Carbon-13 NMR spectroscopy and phosphorus-31 NMR spectroscopy have been used to study the reaction of several alkylcobalamins with 2-mercaptoethanol. At alkaline pH, when the thiol is deprotonated, the alkyl-transfer reactions involve a nucleophilic attack of the thiolate anion on the Co–methylene carbon of the cobalamins, yielding alkyl thioethers and cob(II)alamin. In these nucleophilic displacement reactions cob(I)alamin is presumably formed as an intermediate. The higher alkylcobalamins react more slowly than methylcobalamin. The lower reactivity of ethyl- and propylcobalamin is probably the basis of the inhibition of the corrinoid-dependent methyl-transfer systems by propyl iodide. The transfer of the upper nucleoside ligand of adenosylcobalamin to 2-mercaptoethanol is a very slow process; *S*-adenosylmercaptoethanol and cob(II)alamin are the final products of the reaction. The dealkylation of (carboxymethyl)cobalamin is a much more facile reaction. At alkaline pH *S*-(carboxymethyl)mercaptoethanol and cob(II)alamin are produced, while at pH values below 8 the carbon–cobalt bond is cleaved reductively to acetate and cob(II)alamin. The reductive cleavage of the carbon–cobalt bond of (carboxymethyl)cobalamin by 2-mercaptoethanol is extremely fast when the cobalamin is in the "base-off" form. Because we have been unable to detect trans coordination of 2-mercaptoethanol, we favor a mechanism that involves a hydride attack on the Co–methylene carbon of (carboxymethyl)cobalamin rather than a trans attack of the thiol on the cobalt atom.

Methylcorrinoids, such as methylcobalamin, *Coα*-(5-methoxybenzimidazolyl)-*Coβ*-methylcobamide, *Coα*-(5-hydroxybenzimidazolyl)-*Coβ*-methylcobamide, and *Coα*-aquo-*Coβ*-methylcobyric acid, function as coenzymes in the biosynthetic pathways of methionine, acetate, and methane. Methionine synthetase (5-methyltetrahydropteroyl-L-glutamate:L-homocysteine *S*-methyltransferase, EC 2.1.1.13) is a cobalamin-dependent enzyme that catalyzes the transfer of the methyl group from 5-methyltetrahydrofolate to homocysteine (Taylor, 1982; Matthews, 1984). Acetate biosynthesis by acetogenic bacteria involves the reduction of CO_2 to 5-methyltetrahydrofolate, transmethylation to a corrinoid-containing enzyme, and transfer of the Co–methyl moiety to carbon monoxide dehydrogenase (Ljungdahl & Wood, 1982; Ljungdahl, 1984; Wood, 1985; Wood et al., 1986). Some methanogens catabolize acetate to CO_2 and methane via a pathway that involves carbon monoxide dehydrogenase, a corrinoid-containing enzyme, tetrahydromethopterin, and coenzyme M (2-mercaptoethanesulfonate). The methyl moiety of acetate is transferred via these carriers, and the methyl group of methyl coenzyme M is finally reduced to methane (Zeikus et al., 1985; Wood et al., 1986; Fuchs, 1986).

More than two decades ago Ljungdahl et al. (1965) isolated [^{14}C]methylcorrinoids from cells of *Clostridium thermoaceticum*, which were fermenting glucose and had been pulse-labeled with [^{14}C]carbon dioxide. They also isolated a radioactive compound that exhibited properties of a (carboxymethyl)corrinoid. Indeed, cell-free extracts of *C. thermoaceticum* catalyzed the reductive cleavage of the carbon–cobalt bond of (carboxymethyl)cobalamin to acetate. Ljundahl and Irion (1966) showed that (carboxymethyl)cobalamin is reduced to acetate and cob(I)alamin by sodium borohydride. These observations suggested that a (carboxymethyl)corrinoid could function as an intermediate in acetate biosynthesis. However, recent developments have indicated that an acetyl and not a carboxymethyl moiety is generated by carbon monoxide dehydrogenase and not by the corrinoid protein (Wood et al., 1986).

In model studies Schrauzer et al. (1972) reported that the carbon–cobalt bond of alkylcobaloximes and alkylcobalamins can be cleaved to alkanes by thiols and dithiols. They postulated that this cleavage, which occurs optimally at neutral or acidic pH, involves a trans attack on the cobalt atom to yield alkyl carbanions. These carbanions could then react with the solvent to produce the alkane products. They also reported that the methyl carbanions could be trapped by carbon dioxide to generate acetate, albeit in very poor yield. More recently, Kräutler (1984) has demonstrated that acetylcobalamins can be formed from methylcobalamin and carbon monoxide in the light. Evidently, this carbonylation involves the homolytic cleavage of the carbon–cobalt bond, followed by reaction of the methyl radical with carbon monoxide to yield an acetyl radical and finally reaction of the latter with cob(II)alamin.

In an earlier publication we have shown that methyl transfer from methylcobalamin to thiols involves a nucleophilic displacement of the methyl moiety by thiolate anion (Hogenkamp et al., 1985). In the present paper we have extended these studies to include the transfer reactions from several alkylcobalamins and adenosylcobalamin. The aim of these studies is to provide a chemical model for the inhibition of the three corrinoid-dependent transmethylation reactions by alkyl iodides (Taylor, 1982; Wood et al., 1986) and for the facile reductive cleavage of the carbon–cobalt bond of the (carboxymethyl)corrinoids.

EXPERIMENTAL PROCEDURES

Materials. Cyanocobalamin was purchased from Rhone-Poulenc Industries, Paris. DL-Dithiothreitol, 2-mercaptoethanol, and aquocobalamin were obtained from Sigma Chemical Co. $^{2}H_2O$ (99.8%) was obtained from Aldrich

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Chemical Co. Inorganic salts and buffer components were reagent grade and were used without further purification. ^{13}C-enriched alkyl halides were purchased from MSD Isotopes. The cobalamins and their ^{13}C-enriched analogues were prepared as described before (Hogenkamp et al., 1965, 1975; Walker et al., 1974).

Methods. Anaerobic conditions were obtained by bubbling argon, scrubbed via a heated copper catalyst (Kontes), through solutions in closed cuvettes or NMR tubes sealed with serum stoppers for 30 min. The tubes were then evacuated (20 mmHg) for 5 min and filled with argon. This procedure was repeated 3 times, and the tubes were finally filled with argon at slightly positive pressure. In a typical experiment, the cobalamin solution was equilibrated in the spectrometer and a reference spectrum was acquired. The tube was then removed from the spectrometer, the appropriate amount of thiol was injected, and the tube returned to the spectrometer. One minute after the addition of the thiol, data acquisition was started. Pulse Fourier-transform ^{13}C (62.9-MHz) and ^{31}P (101.27-MHz) NMR spectra were obtained with a Bruker WM-250 spectrometer locked to the resonance of internal ^2H_2O. For the ^{13}C NMR spectra, the transients (500) resulting from the application of 90° pulses (36.5 μs) in a spectral width of 5000 Hz were accumulated as 8192 data points in the time domain and transformed into a 4096-point spectrum. The acquisition time for each transient was 819 ms with a 191-ms pulse delay. For the ^{31}P NMR spectra, the transients (1000) resulting from the application of 90° pulses (30 μs) in a spectra width of 2000 Hz were accumulated as 8192 data points and transformed into a 4096-point spectrum. The acquisition time for each transient was 512 ms without an interpulse delay. The ^{13}C and ^{31}P NMR spectra were obtained with simultaneous ^{1}H noise broad-band decoupling of 0.5 W. 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 and 85% phosphoric acid for the ^{31}P NMR spectra.

All experiments were carried out by using automatic program sequences with appropriate time delays between spectra. In most cases, pseudo-first-order reaction rate constants were computed from the decrease of the Co–^{13}C alkyl resonance or of the ^{31}P resonance.

RESULTS

Reactions of Alkylcobalamins with Thiols. Like the methyl-transfer reaction from methylcobalamin to thiols (Hogenkamp et al., 1985), the reaction of the higher alkylcobalamins (ethyl- and propylcobalamin) with thiols involves a nucleophilic attack of the thiolate anion on the Co–methylene carbon. The results of a typical experiment are illustrated in Figure 1. In this experiment [1-^{13}C]propylcobalamin (10 mM) in glycine buffer (200 mM), pH 9.5, was treated with a 20-fold excess of 2-mercaptoethanol (200 mM) at 70 °C in the dark under strictly anaerobic conditions. The ^{13}C NMR spectral changes with time show that the propyl moiety is transferred from the cobalamin (31.9 ppm) to the thiol (34.0 ppm). UV–visible and ^{31}P NMR spectroscopy measurements of identical reaction mixtures demonstrated that cob(II)alamin is the corrinoid product of the transfer reaction. Similar results were obtained with [1,2-^{13}C]ethylcobalamin. Like the methyl-transfer reaction, the reactions of these two alkylcobalamins show a strong pH dependence, indicating that the thiolate anion is the nucleophile. However, the higher alkylcobalamins react more slowy than the methyl derivative (Table I).

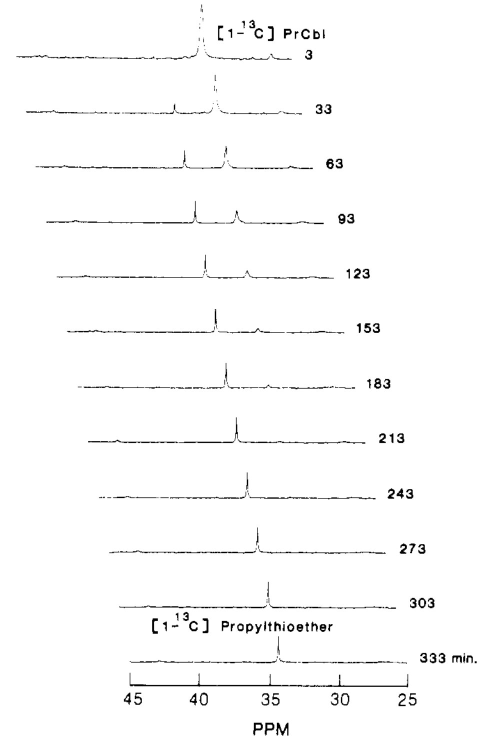


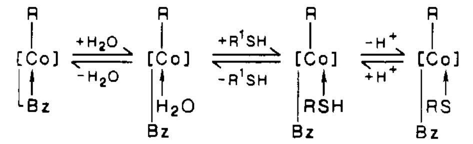
FIGURE 1: ^{13}C NMR spectra in the methylene region of a reaction mixture containing [1-^{13}C]propylcobalamin (10 mM), 2-mercaptoethanol (200 mM), and glycine buffer (200 mM), pH 9.5, in 10% ^2H_2O incubated anaerobically at 70 °C in the dark. The spectra were obtained during the first 10 min of each 30-min interval and represent 500 transients.

Table I: Reaction of Alkylcobalamins with 2-Mercaptoethanol

alkylcobalamin	rate const (k' × 10^4 s^{-1})^a
adenosylcobalamin	0.14^b
propylcobalamin	0.21
ethylcobalamin	0.24
methylcobalamin	0.54
(carboxymethyl)cobalamin	2.6
[(ethoxycarbonyl)methyl]cobalamin	>5.0

^{a} Pseudo-first-order rate constants, pH 9.5, 43 °C. ^{b} 70 °C.

Schrauzer et al. (1972) and more recently Jacobsen et al. (1984) have suggested that alkylcobalamins react with thiols to form trans axial thiol complexes as shown in reaction 1.



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Indeed, Schrauzer and Stadlbauer (1974) have reported that the yield of alkyl thioethers is not quantitative, and they attributed this low yield to the formation of unreactive trans adducts of the alkyl cobalt complexes with the thiolate anion. In an attempt to provide direct evidence for the formation of such trans axial complexes with the alkylcobalamins, we have studied the effect of a large excess of 2-mercaptoethanol on the chemical shift of the Co–methylene carbon of [1-^{13}C]propylcobalamin in the "base-on" and the "base-off" forms. The reaction mixtures in NMR tubes, sealed with serum stoppers, contained 30 mg (20 μmol) of [1-^{13}C]propylcobalamin in 2 mL of 200 mM sodium phosphate buffer, pH 7.0. The solutions were purged with argon, 1 mL of 2-mercaptoethanol (14 mmol) was added, and the reaction mixtures were incubated for 3 h (at this pH no transalkylation is observed).

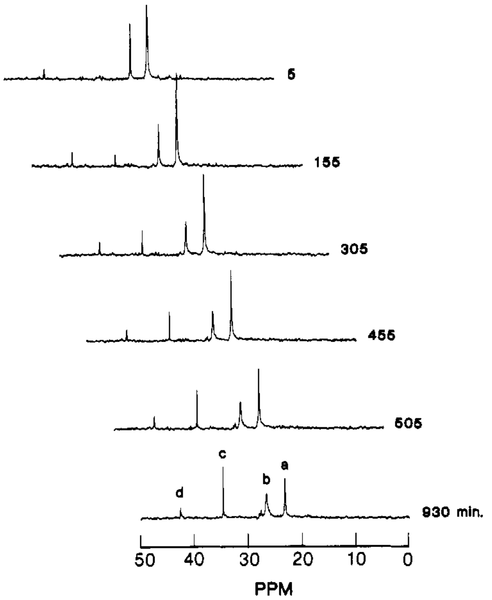

FIGURE 2: ^{13}C NMR spectra in the upfield region of a reaction mixture containing [5′-^{13}C]adenosylcobalamin (10 mM), 2-mercaptoethanol (200 mM), and glycine buffer (200 mM), pH 9.5, in 10% ^2H_2O incubated anaerobically at 70 °C in the dark. Each spectrum represents 500 transients acquired during the first 30 min of each time interval. (a) [5′-^{13}C]adenosylcobalamin; (b) C-2 of 2-mercaptoethanol; (c) [5′-^{13}C]-*S*-adenosylmercaptoethanol; (d) C-1 of glycine.

Even in the presence of a 700-fold excess of thiol, no significant shift of the ^{13}C resonance of propylcobalamin was observed at 43 °C or at 70 °C. These results suggest that trans axial ligation by the thiol does not occur at all or occurs only to a minor extent or that the ligation by the thiol does not cause a significant chemical shift change of the Co–methylene resonance. The latter explanation is unlikely because we have previously demonstrated that the chemical shift of the Co–methylene carbon of the alkylcobalamins is markedly affected by the nature of the trans ligand (Needham et al., 1973). The reaction mixtures were then acidified to pH 1.0 with 5.34 M DCl to protonate the 5,6-dimethylbenzimidazole ligand and convert the cobalamin to the base-off form. The ^{13}C NMR spectrum of such a mixture shows the typical upfield shift of the Co–methylene carbon resonance from 31.3 (base-on) to 28.1 ppm (base-off), indicative of the substitution of a strong-field ligand (5,6-dimethylbenzimidazole) by a weak one (water).

Thioether Formation from Adenosylcobalamin. Preliminary experiments in which the reaction between 2-mercaptoethanol and adenosylcobalamin in glycine buffer, pH 9.5, monitored by ^{31}P NMR, demonstrated that adenosylcobalamin is converted to cob(II)alamin. Compared to the alkyl-transfer reactions, the reaction of adenosylcobalamin with thiolate anion is an extremely slow process (Table I); indeed, it is so slow even at 43 °C that the temperature was adjusted to 70 °C to allow the reaction rate to be measured. If we assume that the reaction rate doubles for every 10 °C for approximately a 7-fold increase in rate and with the difference in the measured rates as shown in Table I, a 27-fold difference in rate between adenosylcobalamin and methylcobalamin would result. In order to identify the product(s) derived from the 5′-deoxyadenosyl moiety, we used [5′-^{13}C]adenosylcobalamin synthesized by Walker et al. (1974) and followed the reaction by ^{13}C NMR. Figure 2 illustrates the ^{13}C NMR spectra in the region of the Co–methylene resonance of a reaction mixture containing 10 mM [5′-^{13}C]adenosylcobalamin, 200 mM 2-mercaptoethanol, and 200 mM glycine buffer, pH 9.5, incubated at 70 °C in the dark under strictly anaerobic conditions. The spectral changes with time show that the carbon–cobalt bond is cleaved and that only one labeled product is formed. The nucleoside product was identified from a reaction mixture containing adenosylcobalamin (100 μmol) and 2-mercaptoethanol (11 mmol) in 200 mM glycine buffer, pH 9.5, incubated at 70 °C for 3 days. The mixture was then exposed to air and acidified to pH 3.0 with 6 M HCl, and the reaction products were separated by chromatography on Dowex 50X2, 200–400 mesh, pH 3.0, as described before (Hogenkamp & Gleason, 1971). The pass-through and water washes contained oxidized mercaptoethanol, and the nucleoside was eluted with 0.1 M sodium acetate buffer, pH 6.0. The tubes containing the nucleoside were pooled, acidified to pH 3.0 with 6 M HCl, and desalted on a Dowex 50X2 [H$^+$] column. The column was washed with water, and the nucleoside was eluted with 0.1 M NH_4OH. The eluate was evaporated to dryness and the residue dissolved in 2 mL of ^2H_2O. A ^{13}C NMR spectrum of the nucleoside showed seven lines in the upfield region (<100 ppm): 33.74 (C-5′), 34.31 (-SCH_2–), 60.34 ($HOCH_2$–), 72.45 (C-3′), 73.34 (C-2′), 83.61 (C-4′), and 87.52 ppm (C-1′), identifying the nucleoside as *S*-adenosylmercaptoethanol.

Table II: Reaction of (Carboxymethyl)cobalamin with 2-Mercaptoethanol

	rate const ($k' \times 10^4$ s^{-1})			rate const ($k' \times 10^4$ s^{-1})	
pH	43 °C 10 mM	29 °C 5 mM	pH	43 °C 10 mM	29 °C 5 mM
1.0	>200[a]		6.9	2.5[e]	
2.2	~100[b]	8.7	8.3	1.1[f]	
3.0	37.9[b]	3.0	9.5	2.3[b]	
4.0	3.6[c]	0.6	10.5	5.2[b]	
4.9	1.6[c]		12.5	10.4[g]	
6.9	5.4[d]				

[a] 200 mM HCl. [b] 200 mM glycine. [c] 200 mM sodium succinate. [d] 200 mM dimethyl glutarate. [e] 200 mM PIPES.[1] [f] 200 mM Tris. [g] 200 mM NaOH.

Reaction of (Carboxymethyl)cobalamin with Thiols. The reaction of (carboxymethyl)cobalamin with 2-mercaptoethanol shows a strong pH dependence (Table II). At low pH where the (carboxymethyl)cobalamin is in the base-off form, the reaction is extremely fast; acetate and cob(II)alamin are the products. On the other hand, at alkaline pH where the thiol is ionized, the reaction yields *S*-(carboxymethyl)mercaptoethanol and cob(II)alamin. These results suggest that at alkaline pH the reaction involves primarily a nucleophilic attack of the thiolate ion on the Co–methylene carbon, while below pH 8 the reaction involves a reductive cleavage of the carbon–cobalt bond. These results also demonstrate that (carboxymethyl)cobalamin in the base-off form is more susceptible to reductive cleavage. Indeed, (carboxymethyl)aquo(3,5,6-trimethylbenzimidazolyl)cobamide, which is in the base-off form at all pH values due to the methylation of N-3, is converted to acetate at all pH values at rates similar to that of (carboxymethyl)cobalamin at pH 1.0. Furthermore, the rate of the reductive cleavage of the carbon–cobalt bond of the cobamide by mercaptoethanol does not show strong pH dependence (Table III). The dealkylation of [(ethoxycarbonyl)methyl]cobalamin by mercaptoethanol at pH 9.5 is much faster than the dealkylation of (carboxymethyl)cobalamin (Table I). When the reductive cleavage of (carboxy-

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

Table III: Reaction of (Carboxymethyl)aquo(3,5,6-trimethylbenzimidazolyl)cobamide with 2-Mercaptoethanol

pH	rate const ($k' \times 10^4$ s^{-1})	pH	rate const ($k' \times 10^4$ s^{-1})
2.1	15.1[a]	7.0	23.0[c]
3.1	11.6[a]	7.1	24.0[d]
4.0	13.5[b]	9.3	10.2, 12.3[a,e]
4.9	10.1[b]	10.2	13.6[a]

[a] Glycine. [b] Sodium succinate. [c] HEPES. [d] Dimethyl glutarate. [e] Two independent experiments.

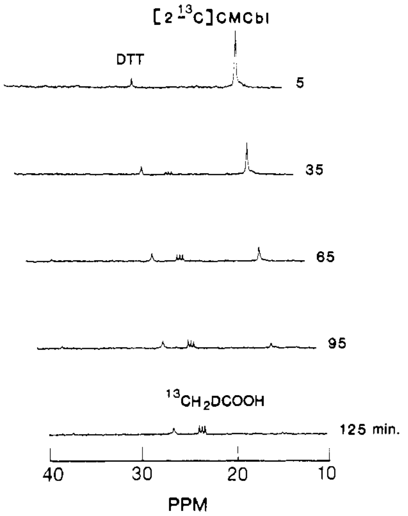


FIGURE 3: ^{13}C NMR spectra in the methylene region of a reaction mixture containing [2-^{13}C](carboxymethyl)cobalamin (10 mM), DL-dithiothreitol (100 mM), and phosphate buffer (200 mM), pH 7.0, in 99.8% ^2H_2O incubated anaerobically at 43 °C in the dark. The spectra were obtained during the first 10 min of each 30-min interval and represent 500 transients.

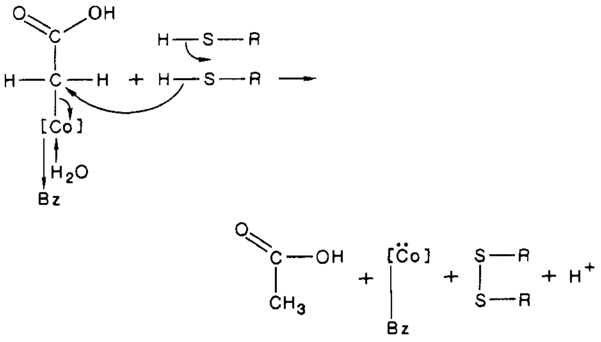
methyl)cobalamin is run in ^2H_2O, one deuterium atom is incorporated at the methyl carbon of acetate which now appears as a triplet in the ^{13}C NMR spectrum (Figure 3).

DISCUSSION

The results presented here demonstrate that alkylcobalamins and adenosylcobalamin react with thiols at alkaline pH under anaerobic conditions to yield the corresponding thioethers and cob(II)alamin. Like the methyl-transfer reaction from methylcobalamin to thiols, the reactions involve a nucleophilic attack of the thiolate anion on the Co–methylene carbon of the upper ligand, and thus, cob(I)alamin is formed as a transitory intermediate. The higher alkylcobalamins react more slowly than methylcobalamin (Table I). The lower reactivity of the higher alkylcobalamins on these displacement reactions is probably the basis for the inhibitory effect of the alkyl halides on the corrinoid-dependent transmethylation reactions. For instance, Brot and Weissbach (1965) and Taylor and Weissbach (1967) showed that the normal C_2 to C_8 alkyl iodides are able to alkylate the corrinoid coenzyme of N^5-methyltetrahydrofolate–homocysteine transmethylase and thus inhibit the enzyme. Our observations suggest that such alkylation leads to inactivation because the alkyl groups cannot readily be transferred to homocysteine. It should be noted that iodoacetic acid does not inhibit the transmethylase reaction, suggesting that the (carboxymethyl)corrinoid is unstable under the assay conditions (vide infra).

The transfer of the upper nucleoside ligand of adenosylcobalamin to 2-mercaptoethanol is almost 25-fold slower than the corresponding methyl-transfer reaction. At alkaline pH *S*-adenosylmercaptoethanol and cob(II)alamin are the only products, indicating that this reaction also involves a nucleophilic attack of the thiolate anion on the 5′-methylene carbon of the upper ligand. Schrauzer et al. (1972) reported that at neutral pH the carbon–cobalt bond of adenosylcobalamin is cleaved reductively by mercaptoethanol. However, this reductive cleavage is a much slower process; with a 1500-fold excess of 2-mercaptoethanol a 3-month incubation at 65 °C was required to give 5′-deoxyadenosine in 60% yield.

The reaction of (carboxymethyl)cobalamin and 2-mercaptoethanol involves both a nucleophilic attack of the thiolate anion and a reductive cleavage of the carbon–cobalt bond. At alkaline pH, where the thiol is completely ionized, *S*-(carboxymethyl)mercaptoethanol and cob(II)alamin are the products, indicating a nucleophilic attack of the thiolate anion on the Co–methylene carbon. As expected, the rate of the nucleophilic displacement reaction is increased when the carboxymethyl group is esterified, as in [(ethoxycarbonyl)methyl]cobalamin (Table I). As the pH is lowered, both thioether and acetate are formed, demonstrating that in the presence of the thiol the carbon–cobalt bond is cleaved reductively. Indeed, at pH values below pH 8, acetate is the only product. At pH values where the cobalamin is in the base-on form, the reductive cleavage of (carboxymethyl)cobalamin is a relatively slow process. However, at acidic pH, where the cobalamin is in the base-off form (pK_a = 2.2), or when N-3 of the 5,6-dimethylbenzimidazole nucleotide is methylated, preventing its coordination to cobalt, the reductive cleavage is an extremely facile process. The rates of the dealkylation at 43 °C of (carboxymethyl)cobalamin at low pH values (Table II) and (carboxymethyl)aquo(3,5,6-trimethylbenzimidazolyl)cobamide at any pH value (Table III) are too fast to be measured with ^{13}C NMR, and thus these rates were detemined at 29 °C and at lower concentrations of the corrinoid. Similar results were obtained by Schrauzer et al. (1972), who reported that the alkylcobinamides are more susceptible to reductive cleavage by thiols than the corresponding alkylcobalamins. They suggested that the reaction is initiated by a trans attack of the thiol on the cobalt atom, followed by the heterolytic cleavage of the carbon–cobalt bond to yield carbanionic species which react with solvent protons to form the alkanes. Such a mechanism seems to be unlikely because we have been unable to obtain evidence for trans coordination of thiols using ^{13}C-enriched alkylcobalamins. It has been demonstrated by Ljungdahl and Irion (1966) that (carboxymethyl)cobalamin is reduced to acetate and cob(I)alamin by sodium borohydride. Analogous to this reductive cleavage of the carbon–cobalt bond by sodium borohydride, we propose that the reductive cleavage of (carboxymethyl)cobalamin by thiols involves a hydride attack on the Co–methylene carbon to yield acetate and cob(I)alamin (reaction 2). (Carboxymethyl)cobalamin in the base-on form is less



(2)

susceptible to nucleophilic attack because the strong ligand in the lower coordination position forces the cobalt into the plane of the corrin ring and thus sterically hinders the approach of the nucleophile. In contrast, at acidic pH where the cobalamin is in the base-off form the weak lower ligand (H_2O) allows the cobalt to remain above the plane of the ring, and thus the approach is less hindered. Furthermore, in the base-off form the carbon–cobalt bond is polarized toward the cobalt atom and thus the Co–methylene carbon is more susceptible to nucleophilic attack.

The inability of iodoacetic acid to inhibit N^5-methyltetrahydrofolate–homocysteine transmethylase (Taylor & Weissbach, 1967) is undoubtedly due to the fact that the carboxymethylated coenzyme is readily reduced to acetate and cob(I)alamin by the thiols included in the reaction mixtures.

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