Structural and Electronic Similarity but Functional Difference in Methylmalonyl-CoA Mutase between Coenzyme B_{12} and the Analog 2′,5′-Dideoxyadenosylcobalamin†

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ABSTRACT: The cofactor analog 2′,5′-dideoxyadenosylcobalamin (ddAdoCbl) differs from the natural cofactor coenzyme B_{12} [5′-deoxyadenosylcobalamin (dAdoCbl)] by lacking only one oxygen atom. The ^1H and ^{13}C NMR spectra of ddAdoCbl have been assigned unambiguously by homonuclear and heteronuclear 2D NMR techniques. The ^1H, ^{13}C, and ^{31}P chemical shift values for ddAdoCbl were compared with those of another organocobalamin, namely dAdoCbl. This assessment shows that the analog is very similar both electronically and structurally to the natural cofactor. The effectiveness of ddAdoCbl as a cofactor for both the human and *Propionibacterium shermanii* methylmalonyl-CoA mutases was compared with that of the natural cofactor. ddAdoCbl was found to be a competitive inhibitor with respect to dAdoCbl. Similar binding affinities to both enzymes were found for both the ddAdoCbl analog and the natural cofactor. However, in the presence of ddAdoCbl, the rate of conversion of methylmalonyl-CoA to succinyl-CoA was only 1−2% of that seen with the natural cofactor. There were no changes with time in the visible absorption spectrum of the bound cofactor analog in the presence of substrate, suggesting that the Co−C bond was not cleaved. The CD (circular dichroism) spectra of dAdoCbl and ddAdoCbl are very similar, consistent with the NMR results. The CD spectral changes upon binding to *P. shermanii* methylmalonyl-CoA mutase are large compared to those reported on the binding of dAdoCbl to ethanolamine ammonia lyase. Furthermore, the CD spectra of both enzyme-bound cobalamins are very similar, suggesting that similar changes in the conformation or structure in these cobalamins occur on binding to the enzyme. Since the natural cofactor binds with the 5,6-dimethylbenzimidazole displaced by a nitrogenous ligand, probably a His residue, the analog must bind in this same way. The CD spectral changes are thus a potential signature for such displacement. The inactivity of the cofactor most probably does not lie in the nature of the interaction of the Co(III) cofactor with the protein. We hypothesize that the 2′-OH group participates in the formation of the Co(II) form of the cofactor, most likely by stabilization of the initially formed radicals.

Methylmalonyl-CoA mutase (EC 5.4.99.2) catalyzes the intramolecular rearrangement of the carbonyl-CoA group of methylmalonyl-CoA to succinyl-CoA and is dependent on the cofactor 5′-deoxyadenosylcobalamin (coenzyme B_{12}, dAdoCbl) for activity. The role of this organometallic cofactor is generally considered to be that of a free radical initiator which undergoes homolysis of the Co−C bond, a step that is believed to involve enzyme-induced distortion of dAdoCbl (Hill et al., 1969; Pratt, 1982; Bresciani-Pahor et al., 1985; Halpern, 1985; Randaccio et al., 1989; Marzilli, 1993). The rate of homolytic cleavage of the Co−C bond in the enzyme-bound cofactor is estimated to be accelerated by a factor of $\sim 10^{11}$ (Hay & Finke, 1987). The mechanisms by which this labilization is achieved and the structural attributes of the cofactor that are important for effecting this remain obscure. Thus, it is of interest to characterize, both structurally and kinetically, dAdoCbl analogs to better understand the binding and the mechanism of Co−C bond cleavage in this interesting class of enzymes.

Either large or small changes in the cofactor could be informative. In this study, we have examined the cofactor analog, 2′,5′-dideoxyadenosylcobalamin (ddAdoCbl), in which a single atom, the oxygen atom in the hydroxyl group at the 2′-position of the deoxyadenosyl moiety, has been deleted. We report the complete assignment by 2D NMR techniques of the ^1H and ^{13}C spectra of ddAdoCbl. Such spectra afford insight into both the structural and electronic properties of the organocobalamins. From this analysis, the structural and electronic properties of dAdoCbl and ddAdoCbl appear to be very similar. The more accurate ^1H NMR assignments reported here disagree in several assignments with the recent

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partial assignment of ddAdoCbl by homonuclear 2D NMR methods (Yan et al., 1993, 1994). We also report on the kinetic characterization of the analog with both the human and a bacterial methylmalonyl-CoA mutase from *Propionibacterium shermanii*. The ddAdoCbl analog exhibits competitive kinetics with respect to dAdoCbl with K_i values that are the same as the K_m for the natural cofactor within the experimental error of our measurements. In addition, similar circular dichroism (CD) spectra were obtained for both enzyme-bound dAdoCbl and ddAdoCbl. However, despite similarity in the binding affinity, the activity of the enzymes in the presence of ddAdoCbl is only 1−2% of that in the presence of the natural cofactor, dAdoCbl. This indicates the importance of the 2′-OH of the ribofuranose ring to the cofactor function of dAdoCbl.

MATERIALS AND METHODS

Reagents. ddAdoCbl was synthesized according to published methods (Chen et al., 1993). Samples for NMR analysis were prepared by dissolving 18 mg of lyophilized ddAdoCbl in 0.5 mL of 99.96% D_2O (MSD Isotopes). No pD adjustment was carried out. (*R,S*)-Methylmalonyl coenzyme A, dAdoCbl, dithiobis(nitrobenzene), thiokinase, and guanosine diphosphate were purchased from Sigma.

Physical Measurements. UV−visible spectra were recorded at 5 °C with a Cary 3 spectrophotometer. CD spectra were recorded at 5 °C on a Jasco 600 spectropolarimeter.

NMR Spectroscopy. The 1D NMR spectral measurements were made with Omega GN-600 (599.64 MHz, ^1H) and GE GN-500 (202.44 MHz, ^{31}P) spectrometers. ^1H and ^{13}C chemical shifts were referenced externally to TSP (sodium 3-(trimethylsilyl)tetradeuteriopropionate) and ^{31}P to TMP (trimethyl phosphate). The 2D NMR experiments were performed on an Omega GN-600 spectrometer at 25 °C without sample spinning and processed with the Felix program (Biosym Technologies Inc.). The NMR tubes were wrapped in aluminum foil to minimize exposure to light.

Phase-Sensitive NOE Spectroscopy (Jeener et al., 1979; States et al., 1982). The phase-sensitive NOE spectrum of ddAdoCbl resulted from a 2048 × 2048 data matrix size, after zero filling, with 32 scans per t_1 value. The mixing time was 300 ms. Presaturation of the residual HOD peak was used. A 2 Hz exponential line-broadening function followed by a cubic spline base line correction routine and a 90°-shifted sine bell squared filter were used prior to Fourier transformation in the t_2 and t_1 dimensions, respectively.

^1H-Detected Heteronuclear Multiple Quantum Coherence Spectroscopy (HMQC) (Muller, 1979; Bax & Subramanian, 1986). The one-bond $^1H-^{13}C$ shift correlation spectrum of ddAdoCbl resulted from a 128 × 2048 data matrix size with 304 scans (preceded by 4 dummy scans) per t_1 value. Predelay was 1.6 s. A 27 μs 90° ^{13}C pulse width and 71 dB of ^{13}C rf power were used. A 90°-phase-shifted sine bell filter was used prior to Fourier transformation in both the t_2 and t_1 dimensions.

^1H-Detected Multiple-Bond Heteronuclear Multiple Quantum Coherence Spectroscopy (HMBC) (Bax & Summers, 1986). The multiple-bond $^1H-^{13}C$ shift correlation spectrum of ddAdoCbl resulted from a 256 × 2048 data matrix size with 304 scans (preceded by 4 dummy scans) per t_1 value. Predelay was 1.6 s. A 27 μs 90° ^{13}C pulse width and 71 dB of ^{13}C rf power were used. The delay between the first 90° ^1H pulse and the first 90° ^{13}C pulse was 3.3 ms. The delay between the first and the second 90° ^{13}C pulses was 53.3 ms. The 45°- and 30°-phase-shifted sine bell filters were used prior to Fourier transformation in the t_2 and t_1 dimensions, respectively.

Strains. The recombinant expression vector (pMEX2/pGP1-2) harboring the *P. shermanii* genes in *Escherichia coli* strain K 38 was a gift from Dr. Ian Scott (Texas A&M University) and was constructed by Leadlay and co-workers (McKie et al., 1990). The yeast expression system (yEPV/hMCM) (Andrews et al., 1993) for the recombinant human mutase gene was generously provided by Dr. Fred Ledley (GeneMedicine, Houston).

Protein Purification. The human and bacterial mutases were purified as described previously (McKie et al., 1990; Taoka et al., 1994; Padmakumar & Banerjee, 1995).

Inhibition Kinetics. A continuous spectrophotometric assay described previously (Taoka et al., 1994) was employed to monitor the effects of the ddAdoCbl analog. The assay mixture contained phosphate-Tris buffer (pH 7.5) (50 mM in phosphate), $MgCl_2$ (10 mM), guanosine diphosphate (1 mM), thiokinase (0.1 unit), dithiobis(nitrobenzene) (50 μM), dAdoCbl (0.1−6.4 μM), (*R,S*)-methylmalonyl-CoA (800 μM), and apomethylmalonyl-CoA mutase (59 or 118 ng) in a total volume of 300 μL. The concentration of ddAdoCbl varied from 0 to 4 μM. All components except for the mutase were mixed in a 0.3 mL cuvette and temperature equilibrated by incubation in a water-jacketed cuvette holder maintained at 30 °C. The background absorption was recorded over 5 min. The reaction was initiated by addition of enzyme, and the increase in absorption at 412 nm was recorded for an additional 5 min. The reported rates were corrected for the background rate recorded in the absence of enzyme, which is typically at least 10-fold lower. Concentrations of dAdoCbl and ddAdoCbl were determined spectrophotometrically prior to use [$\epsilon_{522} = 8\ mM^{-1}\ cm^{-1}$ and $\epsilon_{524} = 7.8\ mM^{-1}\ cm^{-1}$ for dAdoCbl and ddAdoCbl, respectively (Chen et al., 1993)]. Cofactor activity of ddAdoCbl was monitored in the standard assay described above with the following modifications: dAdoCbl was substituted by an equivalent concentration of ddAdoCbl and a 5−10-fold higher concentration of enzyme was employed.

The data were analyzed by nonlinear regression analysis with Sigma Plot (Jandel Scientific, Corte Madera, CA) using eq 1:

$$v = \frac{V_{max}[\text{AdoCbl}]}{K_m\left(1 + \frac{[\text{ddAdoCbl}]}{K_i}\right) + [\text{AdoCbl}]} \quad (1)$$

Protein concentration was determined by the method of Bradford (Bradford, 1976) (using reagents purchased from Bio-Rad), with bovine serum albumin as a standard.

Spectroscopic changes, if any, of enzyme-bound ddAdoCbl were monitored following addition of substrate ((*R,S*)-methylmalonyl-CoA, 175 μM) to the bacterial mutase (8 μM) reconstituted with ddAdoCbl (6 μM in 50 mM potassium phosphate buffer, pH 7.5). The visible range was scanned from 350 to 650 nm.

RESULTS

Assignment of the NMR Spectra. Most of the NMR signals of ddAdoCbl (supporting information) were assigned by

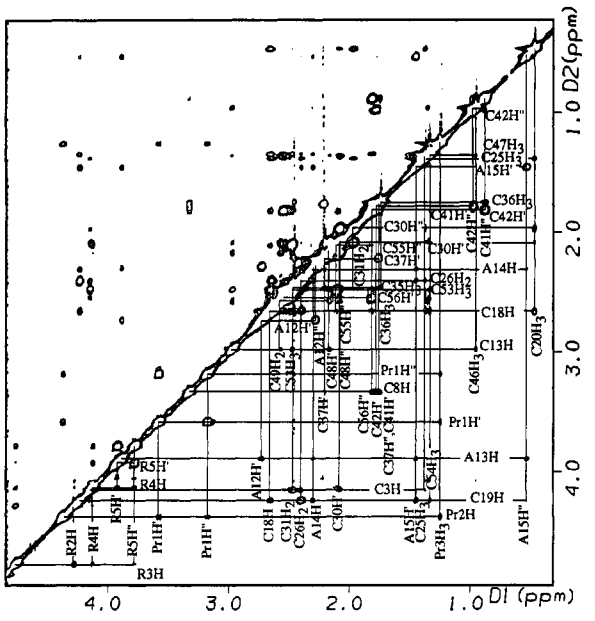

FIGURE 1: Upfield region of the NOESY spectrum of ddAdoCbl.

using the strategy of combining NOESY (Figure 1 and supporting information) and $^1H-^{13}C$ reverse detection methods (supporting information) described previously (Summers et al., 1986; Bax et al., 1987; Pagano & Marzilli, 1989; Pagano et al., 1989, 1991; Alelyunas et al., 1991). The HMBC and NOESY connectivities for ddAdoCbl, used for ^1H and ^{13}C assignments, are given in the supporting information. The ^1H nuclei are designated by the carbon atom to which they are attached. For nonequivalent geminal signals, H′ and H″ refer to the downfield and upfield signals, respectively. The ^{13}C nuclei are designated in Scheme 1 with numbers, following B for carbons of the DMBz moiety, C for carbons of the corrin ring and side chains, A for carbons of the 2′,5′-dideoxyadenosyl residue, and Pr for carbons of the propanolamine group.

The most downfield ^1H NMR signals must correspond to B7H, B4H, B2H, C10H, R1H, A2H, and A8H (supporting information). The signal at 7.18 ppm, with NOEs to R1H, R2H, and $B11H_3$, was assigned to B7H. The resonance at 6.25 ppm, which correlated to $B10H_3$, corresponded to B4H. A signal showing NOEs to C8H, $C46H_3$, and $C47H_3$ must be C10H. The singlet at 6.97 ppm with correlations to R1H and R2H was assigned to B2H. The most downfield signal, with no NOEs, was assigned to A2H. The remaining signal at 8.03 ppm, with NOEs to A11H, A12H″, and A13H, was assigned to A8H. From C10H, most of the remaining corrin and methylene signals were assigned as described previously (Summers et al., 1986; Pagano & Marzilli, 1989; Pagano et al., 1989, 1991).

All the methyl and protonated corrin carbon signals of ddAdoCbl were assigned from the HMQC spectrum (supporting information), which gives one-bond $^1H-^{13}C$ correlations. Assignment of the methylene carbons was difficult using HMQC, since the signals overlap in the ^1H dimension and are sometimes in a crowded region in the ^{13}C dimension as well. The HMBC spectrum, which displays two- and three-bond $^1H-^{13}C$ connectivities, was used to confirm the HMQC-based assignments and to assign the non-protonated carbons (supporting information).

Kinetic Studies with Methylmalonyl-CoA Mutase. ddAdoCbl is not an effective cofactor for either the human or the bacterial methylmalonyl-CoA mutase. When ddAdoCbl

Scheme 1. Structure and Numbering Scheme for ddAdoCbl

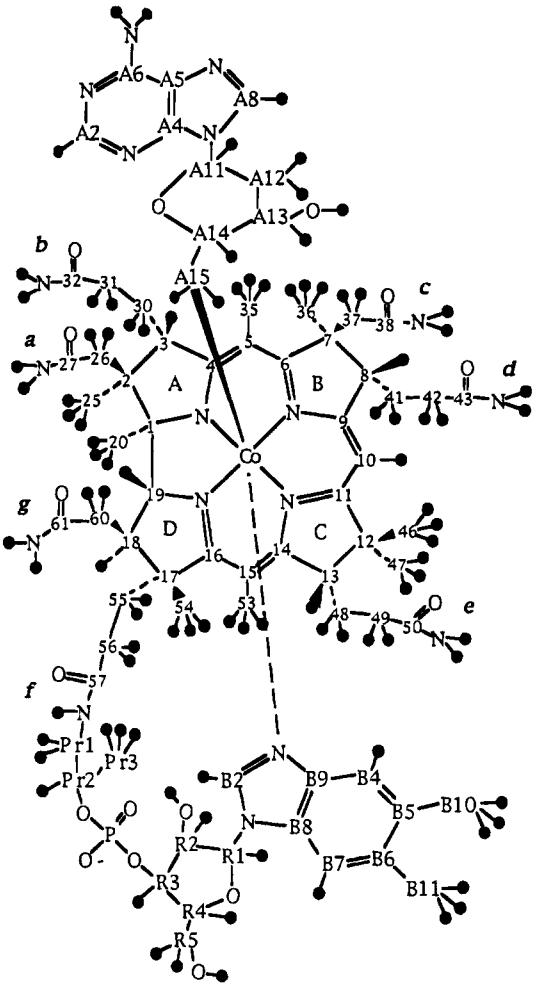

Table 1: Kinetic Parameters of dAdoCbl and ddAdoCbl with the Two Mutases

	human mutase	bacterial mutase
(a) $K_{m[dAdoCbl]}$	$0.22 \pm 0.02\ \mu M$	$0.24 \pm 0.02\ \mu M$
(b) $K_{i[ddAdoCbl]}$	$0.17 \pm 0.01\ \mu M$	$0.28 \pm 0.02\ \mu M$
(a) % activity with dAdoCbl	100	100
(b) % activity with ddAdoCbl	1.1	1.4

(10 μM) was substituted for dAdoCbl in the spectrophotometric assay, only ~1−2% of the activity was observed (Table 1). To determine whether the observed failure to support catalysis was due to lack of binding, the kinetics of inhibition of the ddAdoCbl analog were studied. Our data (supporting information) show that ddAdoCbl functions as a competitive inhibitor with respect to the natural cofactor, dAdoCbl. For both the bacterial and human mutases, K_i for ddAdoCbl (Table 1) is the same as K_m for dAdoCbl, within the experimental error of our measurements. This indicates that the cofactor analog is able to bind as tightly as the natural cofactor to the active site of the mutases.

No changes were observed in the visible absorption spectrum over 2 h following addition of substrate to the bacterial enzyme reconstituted with ddAdoCbl (data not shown). This result indicates that the failure to catalyze the isomerization reaction was not due to escape of the dideoxyadenosyl radical out of the catalytic cycle, as has been seen with deoxyadenosylcobinamide phosphate methyl ester (Ishida & Toraya, 1993). Thus, ddAdoCbl is not an inefficient cofactor because it undergoes Co−C bond homolysis but not recombination.

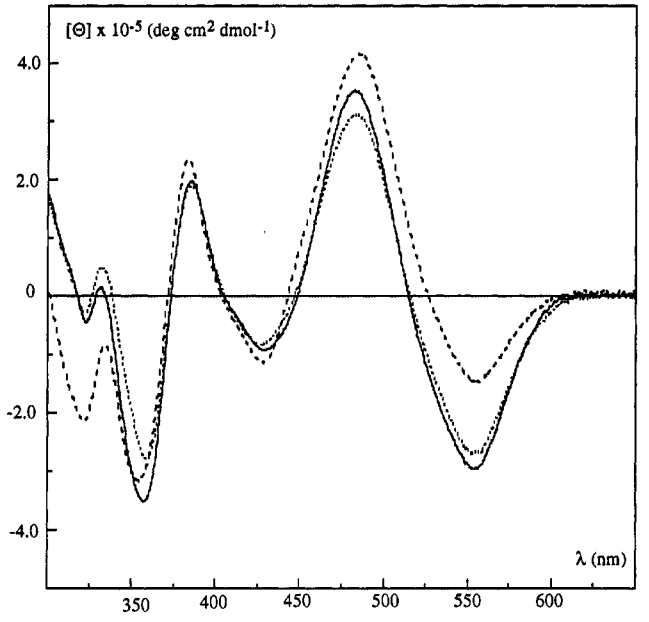

FIGURE 2: Circular dichroism spectra of dAdoCbl (—), ddAdoCbl (• • •), and MeCbl (- - -).

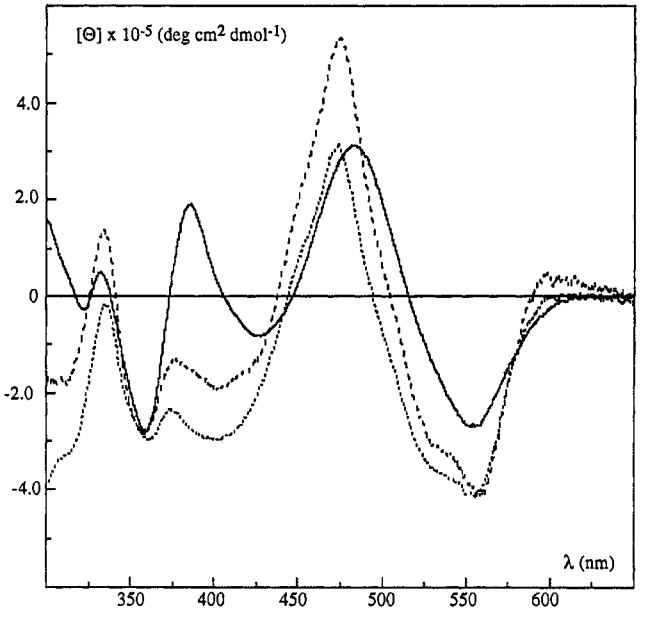

FIGURE 3: Circular dichroism spectra of ddAdoCbl (—), enzyme-bound ddAdoCbl (• • •), and enzyme-bound dAdoCbl (- - -).

Circular Dichroism. The CD spectra of dAdoCbl and ddAdoCbl are very similar, with positive bands at ~332, 385, and 482 nm and negative bands at ~322, 358, 428, and 553 nm (Figure 2). In the figure, we also show the CD spectrum of the other principal B_{12} cofactor, methylcobalamin (MeCbl). The spectrum has distinct differences from those of dAdoCbl and ddAdoCbl. Although there are somewhat larger differences in ^{13}C, ^{31}P, and ^{1}H NMR spectral properties between MeCbl and dAdoCbl than between dAdoCbl and ddAdoCbl (Calafat & Marzilli, 1993), the structures of the two natural cofactors are rather similar (Rossi et al., 1985). Therefore, the CD spectra appear to be sensitive even to small structural and electronic differences in these cobalamins.

On cofactor binding to the bacterial mutase, the resulting CD spectra are similar to each other and different from those of the free cobalamins (Figure 3). In particular, upon binding to the enzyme, the dAdoCbl positive peaks at 332, 385, and 482 nm shifted to 334, 377, and 475 nm, respectively, and the negative peaks at 357, 430, and 554 nm shifted to 358, 405, and 558 nm, respectively; a shoulder at 535 nm on the 558 nm band was also observed (Figure 3). Likewise, on binding to the mutase, the ddAdoCbl positive peaks at 332, 386, and 483 nm shifted to 335, 373, and 474 nm, respectively, while the negative bands at 359, 428, and 552 nm shifted to 360, 402, and 555 nm, respectively (Figure 3). A shoulder at 542 nm appeared. In both cases, upon binding to the enzyme, there was an increase in the intensity of the negative bands at $\lambda > 400$ nm and a significant change at ~380 nm (an intense positive band at ~385 nm in spectra of the free cofactors vs a negative band at ~375 nm for bound cofactors).

The visible spectra of the solutions used for the CD measurements were not changed by the measurement. However, there were changes on binding. For example, the unbound dAdoCbl had a band in the 450−600 nm region ($\lambda_{max} \sim 525$ nm) with a clear shoulder at 490 nm and a slight shoulder at ~550 nm. The bound cofactor had $\lambda_{max} \sim 525$ nm, but the shoulders changed. The shoulder at 490 nm had blue-shifted to ~480 nm and was relatively less intense, whereas the ~550 nm shoulder had red-shifted to ~560 nm and was relatively more prominent.

DISCUSSION

The chemical shifts of some of the corrin ring protons and the NOE correlations can provide some information on the orientation in solution of the adenine moiety. In dAdoCbl (Summers et al., 1986; Bax et al., 1987) and AdePrCbl (Pagano et al., 1991), several NOEs between corrin and ribose protons were observed which are also consistent with the crystal structures. In addition, there was an NOE between A8H and $C46H_3$ (Bax et al., 1987), which suggested a different orientation of the adenine ring and led to the proposal for a fluxional character of the adenosyl moiety (Bax et al., 1987; Pagano et al., 1991). In dAdoCbl, C19H showed NOEs to A14H and A15H′; $C54H_3$ had a cross-peak to A14H and $C26H_2$ to the A15 protons (Summers et al., 1986; Bax et al., 1987). For ddAdoCbl, there are NOE cross-peaks between A14H and both C19H and $C54H_3$ and between the A15 protons and C19H and $C26H_2$. In addition, no NOEs between the adenine and the corrin protons were detected. These results suggest that, in all significant solution conformers of ddAdoCbl, the ddAdo group probably has a location similar to that observed for the adenosyl moiety in the crystal structure of dAdoCbl (Glusker, 1982; Savage et al., 1987; Bouquiere et al., 1993).

Most of the ^{1}H NMR shifts of ddAdoCbl are quite similar to those of dAdoCbl. Except for the ribose moiety of the ddAdo group, the biggest differences (~0.08 ppm) correspond to C ring protons $C46H_3$, C13H, and C48H″ and B ring protons C42H″ and C41H″, which are shifted downfield in ddAdoCbl relative to dAdoCbl. A minor change in the average relative position of the ddAdo and dAdo moieties in ddAdoCbl and dAdoCbl, respectively, could be responsible for these shift changes. Previously, the significant downfield shift (~0.2−0.3 ppm) for C13H and $C46H_3$ in AdePrCbl relative to dAdoCbl was attributed to a change in position of the adenine ring (Pagano et al., 1991).

Most of the ^{13}C NMR shifts of ddAdoCbl are close to those of dAdoCbl. The significant difference in the A11 ^{13}C shift (~35 ppm) in the ribose moiety of the ddAdo group is due to the absence of the hydroxyl group in ddAdo relative

to dAdo. It has been shown that for cobalamins the ^{13}C NMR shifts of most of the 5,6-dimethylbenzimidazole (DMBz) and ribose carbons and some of the corrin ring carbons are primarily influenced by inductive electronic effects (Calafat & Marzilli, 1993). The data also suggest modulations in the shifts of the DMBz carbons, reflecting steric interactions of the dAdo group with the corrin. In turn, these effects are reflected in the Co−N(DMBz) bond lengths (Rossi et al., 1985; Pagano et al., 1991; Calafat & Marzilli, 1993; Kratky et al., 1995).

The ^{13}C NMR shifts of the respective corrin ring and DMBz carbons in ddAdoCbl and dAdoCbl (supporting information) exhibit no significant differences. This result suggests that the 5′-deoxyadenosyl and 2′,5′-dideoxyadenosyl groups have quite similar electron donor abilities. Furthermore, ^{31}P shifts in cobalamins have been reported to respond to the trans influence of the axial ligand and reflect changes in the phosphodiester conformation as the axial ligand changes (Brown et al., 1984; Rossi et al., 1985). The similar ^{31}P shifts for dAdoCbl (−4.34 ppm) and ddAdoCbl (−4.27 ppm) suggest comparable electron donor abilities and steric effects for the 5′-deoxyadenosyl and 2′,5′-dideoxyadenosyl groups.

The reactivity of ddAdoCbl has been examined with other dAdoCbl-dependent enzymes (Zagalak & Pawelkiewicz, 1965; Sando et al., 1975). The cofactor activity of ddAdoCbl in these systems varied from 5% in ribonucleotide reductase (Sando et al., 1975) to between 20 and 30% with diol dehydrase (Zagalak & Pawelkiewicz, 1965). Like methylmalonyl-CoA mutase, these enzymes did not appear to discriminate between the two cobalamins, with the K_m (Zagalak & Pawelkiewicz, 1965) or K_i (Sando et al., 1975) for the analog being the same as the K_m for the cofactor. These results point to the importance of the 2′-hydroxyl group as a determinant of reactivity but not of binding of the cofactor to the active sites in all three enzymes examined so far. Diol dehydrase appears to be relatively less affected by changes in the ribose moiety; e.g., several analogs have >35% relative coenzyme activity (Toraya et al., 1977).

The similarity in the enzyme binding of dAdoCbl and ddAdoCbl for the bacterial methylmalonyl-CoA mutase was further assessed with CD data. CD studies on the interaction between (adenylalkyl)cobalamins and ethanolamine ammonia lyase (EAL) associated the changes between 350 and 600 nm of the CD spectra of dAdoCbl analogs with changes in the conformation of the corrin ring (Krouwer et al., 1980). Relatively little change was observed in the CD spectrum of dAdoCbl on binding to ethanolamine ammonia lyase. In contrast, the CD spectra of dAdoCbl and ddAdoCbl showed significant changes in the region between 300 and 600 nm on binding to the enzyme, particularly at ~400 nm. Therefore, our data suggest that, for both dAdoCbl and ddAdoCbl, some relatively large changes in the conformation of the cofactor (and/or the ligating groups) occur on binding to the enzyme. In addition, there is a remarkable resemblance in the CD spectra between enzyme-bound dAdoCbl and ddAdoCbl (Figure 3). This suggests that the interaction of bacterial methylmalonyl-CoA mutase with dAdoCbl and ddAdoCbl is substantially the same.

Recent unambiguous EPR evidence indicates that the benzimidazole group is displaced by a nitrogenous ligand (probably the imidazole of a histidine) on the binding of dAdoCbl to methylmalonyl-CoA mutase (Padmakumar et al., 1995). The similarity of the CD spectral changes and in binding affinity establishes that the cofactor analog binds in

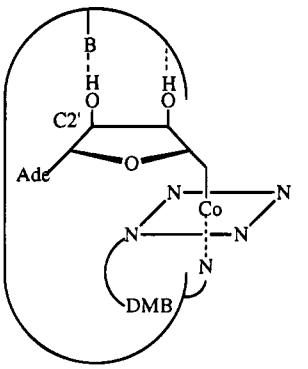

FIGURE 4: Hypothetical model showing the interaction between the 2′-hydroxyl group of the deoxyribose moiety and a general base at the active site.

essentially the same way. The enzyme, methionine synthase, also binds cofactor by displacing the benzimidazole group; in this case, it is known that the displacing ligand is an imidazole side chain (Drennan et al., 1994). The relatively small CD spectral changes found for Cbl binding to EAL (Krouwer et al., 1980) leads us to predict that the conformation of dAdoCbl bound to EAL is base-on, i.e., that the bottom axial ligand is dimethylbenzimidazole.

It is not surprising that the difference of only one atom between ddAdoCbl and dAdoCbl causes little difference in overall chemical and spectroscopic ground state properties. In contrast, this apparently subtle change at a position four bonds removed from the reactive carbon−cobalt bond of the cofactor has a profound effect on methylmalonyl-CoA mutase holoenzyme reactivity. The effect could be manifest primarily either in the organocobalt(III) form or in the radical, cobalt(II) form. We can rule out the possibility that the radical self-destructs since the spectrum of the ddAdoCbl-reconstituted enzyme is unaffected in the presence of substrate.

For the organocobalt(III) form, one possibility is that the 2′-hydroxyl group is involved in a hydrogen-bonding interaction at the active site (Figure 4) that contributes to the steric enzyme-induced distortion of dAdoCbl proposed to be important for labilizing the Co−C bond (Hill et al., 1969; Pratt, 1982; Bresciani-Pahor et al., 1985; Halpern, 1985; Randaccio et al., 1989; Marzilli, 1993). An alternative possibility is that the absence of a hydroxyl group at the 2′-position results in an altered conformation of the ribofuranose ring of the enzyme-bound cofactor, with a consequent impact on the Co−C bond. To this end, it would be of interest to determine the lengths of the Co−C and trans Co−N bonds in enzyme-bound ddAdoCbl for comparison with that in the bound normal cofactor. However, although no relevant X-ray structures of dAdoCbl-dependent enzymes have been reported, it is unlikely that such bond length changes would be precise enough to allow assessment of the structural changes, given the large size of the enzymes and similarity in the bound cofactors suggested by the CD spectra. An X-ray structure might reveal that the 2′-hydroxyl is involved in H bonding or has a particular ribose conformation in the organocobalt(III) form.

We believe the explanation for the different activities more likely lies in the nature of the radical, cobalt(II) form. While the enthalpy of activation of bond dissociation of free dAdoCbl has been estimated to be 26 ± 2 kcal/mol (Halpern et al., 1984) and 30 ± 2 kcal/mol (Finke & Hay, 1984; Hay & Finke, 1986), a value for ddAdoCbl has not, to our knowledge, been reported. Preliminary data (Kim et al.,

1988; Jensen et al., 1995; H. Chen, unpublished results) indicate that such minor variations in the alkyl group do not lead to large changes in the ease of Co−C bond homolysis. Furthermore, it is a reasonable assumption that the dimethylbenzimidazole-displaced cofactor undergoes Co−C bond cleavage. Since the displacement of the nucleotide loop is a recent discovery (Drennan et al., 1994; Padmakumar et al., 1995), the consequences on Co−C bond cleavage have not been probed experimentally. However, the effect on bond homolysis of the dimethylbenzimidazole displacement by the nitrogenous ligand should be similar for both ddAdoCbl and dAdoCbl. In the enzyme-bound forms, the Co−C bond dissociation energy of ddAdoCbl would be larger than that of dAdoCbl if, during the later phases of Co−C bond cleavage, the 2′-hydroxyl oxygen of this natural cofactor forms a favorable H bond with the protein.

In conclusion, we have shown that ddAdoCbl is very similar to both free dAdoCbl, and the similarity extends to the form bound to methylmalonyl-CoA mutase. The ddAdoCbl analog, like the natural cofactor, appears to bind by displacement of the benzimidazole. This conclusion is supported by both the similar binding affinity and the similar large changes in CD spectra. When dAdoCbl binds to ethanolamine ammonia lyase, there are only relatively small changes in CD spectra. Therefore, CD spectroscopy appears to be a useful tool in assessing dAdoCbl binding modes. The relatively low activity of the ddAdoCbl analog is best understood as arising from interactions of the 2′-OH group with the protein during or after Co−C bond cleavage. Further structural and functional characterization of dAdoCbl analogs is clearly warranted in elucidating the mechanisms by which this interesting class of enzymes enhances the reactivity of the organometallic cofactor.

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SUPPORTING INFORMATION AVAILABLE

One table of the ^{1}H and ^{13}C NMR chemical shifts and signal assignments for ddAdoCbl and dAdoCbl; one table of the observed HMBC and NOESY connectivities for ddAdoCbl; HMBC, NOESY, and upfield region of the HMQC spectra of ddAdoCbl; and a figure showing the effect of ddAdoCbl on the reaction catalyzed by human methylmalonyl-CoA mutase (14 pages). Ordering information is given on any current masthead page.

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