Coenzyme B_{12} Is Coordinated by Histidine and Not Dimethylbenzimidazole on Methylmalonyl-CoA Mutase

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Derivatives of vitamin B_{12} or cobalamin participate in two major classes of reactions, both of which involve manipulation of the reactive organometallic bond of the cofactor. The methylcobalamin-dependent methyltransferases utilize S_N2 chemistry to cleave the $Co-CH_3$ bond heterolytically. The adenosylcobalamin (AdoCbl)[1]-dependent isomerases, on the other hand, cleave the Co–C bond homolytically. How these two classes of proteins modulate the reactivity of the Co–C bond and thus effect such radically different chemical choices is as yet an open question. Considerable debate has focused on the influence of the lower axial (α) ligand in dictating the propensity of the upper axial (β) bond for cleavage *via* the radical *versus* the ionic pathway.[2–8] This position is occupied in solution by the bulky intramolecular base, dimethylbenzimidazole, which is appended from the corrin ring. The recent structure elucidation of the cobalamin-binding domain of methionine synthase,[9] a member of the methyltransferase family, revealed that the conformation of the bound cofactor is "base-off" and that a histidine ligand donated by the protein occupies the sixth coordination position. Based on the presence of a conserved histidine embedded in a homologous sequence (*DXHXXG*-41–42 amino acids-*SXL*-21–22 amino acids-*GG*), it was proposed that methylmalonyl-CoA mutase, a member of the isomerase family, also utilizes a similar binding motif.[9,10] In addition, a histidine ligand to the cobamide bound to a corrinoid protein from *Sporomusa ovata* has been reported.[11] We present evidence from EPR and UV–visible absorption spectroscopy and from diethyl pyrocarbonate (DEPC) inhibition studies for the donation of a histidine ligand from methylmalonyl-CoA mutase to the bound cobalamin.

Methylmalonyl-CoA mutase catalyzes the reversible isomerization of methylmalonyl-CoA to succinyl-CoA. The *Propionibacterium shermanii* enzyme was purified in the apoenzyme form as previously described[12] and reconstituted[13] with a cofactor analog, adenosylcobinamide (AdoCbi), which lacks the nucleotide loop. The unbound cofactor was separated from the holoenzyme by FPLC on a Superose 12 (Pharmacia) gel filtration column.[12] AdoCbi binds tightly to both the human and the bacterial mutases, with K_i values of 8.4 ± 0.8 and 7.4 ± 0.5 μM, respectively.[14] Since AdoCbi lacks the dimethylbenzimidazole moiety, it has a UV–visible absorption spectrum (λ_{max} = 458 nm) that is typical of base-off[15] cobalamin (Figure 1). On binding of AdoCbi to the mutase, the spectrum undergoes a dramatic red shift, and the absorption maximum changes to 525 nm, characteristic of "base-on" cobalamin. Since the absorption spectrum of AdoCbl, the natural cofactor, is the same in solution and in the active site of the mutase, it is reasonable to conclude that the observed red shift with AdoCbi is due to a change in ligation. The magnitude of the shift is consistent with ligation by nitrogen,[16] which in this case would have to be provided by a protein residue (Scheme 1).

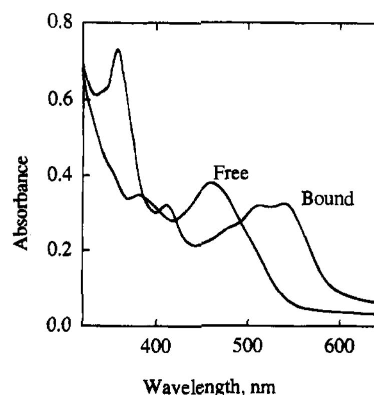

Figure 1. UV–visible absorption spectrum of free and enzyme-bound AdoCbi.

Based on the sequence similarity between the cobalamin-binding domain of methionine synthase and a segment of the mutase, it was expected that the coordinating ligand is a histidine. We have examined the sensitivity of apo- and holomutase to DEPC, which reacts with histidyl residues to yield an *N*-carbethoxyhistidyl derivative[17] (Table 1). The apomutase is very sensitive to DEPC and loses >90% activity in 10 min when exposed to 200 μM concentration of the inhibitor. On the other hand, the holomutase is unaffected by the presence of the inhibitor. Alkylation of histidine and tyrosine, but not other susceptible amino acids (viz. lysine and cysteine), is reversed by hydroxylamine. Hydroxylamine partially alleviates DEPC inhibition of the apomutase. Thus, the presence of the cofactor affords protection to a histidine (or a tyrosine) residue which is essential for catalysis by the mutase (Scheme 1).

The EPR spectrum of enzyme-bound cob(II)alamin generated either by anaerobic photolysis of bound AdoCbi (data not shown) or of AdoCbl (Figure 2, upper trace) shows an axial spectrum with $g_{\parallel}$ = 2.0 and $g_{\perp}$ = 2.3. Interaction of the unpaired electron with the cobalt nucleus ($I = {}^7/_2$) results in hyperfine splittings into an octet which is visible as eight lines, with a spacing of 105 G in the $g_{\parallel}$ component of the spectrum.

Superhyperfine coupling to the α nitrogen ligand (I = 1) further splits each of the eight lines into triplets which are clearly resolved (coupling constant, 19 G). To unambiguously establish the protein origin of the nitrogen ligand, we have examined the EPR spectrum of the mutase purified from cells grown in

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(1) Abbreviations used: AdoCbl, deoxyadenosylcobalamin; DEPC, diethyl pyrocarbonate; AdoCbi, deoxyadenosylcobinamide; MeCbl, methylcobalamin.

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(13) The enzyme was reconstituted by mixing 10 mg of protein (~66 nmol) with 500 nmol of AdoCbi in 1 mL of 50 mM potassium phosphate buffer, pH 7.5, on ice for 2 h. The unbound cofactor was separated by gel filtration chromatography using conditions described in ref 12.

(14) AdoCbi is not an effective cofactor for the mutases. The activity of the bacterial mutase in the presence of 40 μM AdoCbi is ~3% that of the normal cofactor.

(15) Base-on and base-off conformations of cobalamin refer specifically to the presence or absence of dimethylbenzimidazole as the lower axial ligand.

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Scheme 1. Mechanism of Conversion of AdoCbi from the 458 to the 525 nm Species

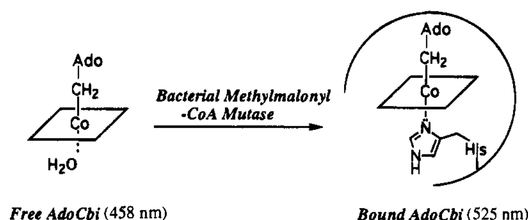

Table 1. Effect of DEPC on the Activity of the Bacterial Methylmalonyl-CoA Mutase

assay conditions	specific activity[a]	activity remaining (%)
standard assay[b]	24	100
apoenzyme preincubated with 200 μM DEPC[c]	1.4	5.8
holoenzyme preincubated with 200 μM DEPC	24	100
DEPC-inhibited apoenzyme + 500 μM hydroxylamine + 500 μM histidine	6.2	26
standard assay + 500 μM hydroxylamine	21	87

[a] Specific activity is measured in units of μmol of succinyl-CoA formed min^{-1} mg^{-1} of protein at 37 °C. [b] The standard radiolabel assay described previously[18] was employed. [c] The enzyme was preincubated with DEPC for 10 min on ice before being added to the assay mixture.

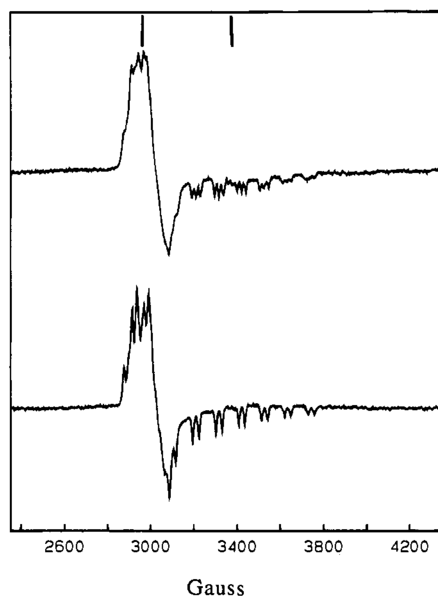

Figure 2. X-band EPR spectra of cob(II)alamin bound to ^{14}N- or ^{15}N-labeled methylmalonyl-CoA mutase. The EPR spectra were recorded on a Bruker ESP 300 instrument with the following settings: sweep width, 2000 G; modulation frequency, 100 kHz; modulation amplitude, 10 G; power, 40 mW; temperature, 100 K. The resonator frequency was 9.443 GHz. Both spectra were obtained by addition of five scans. The line markers indicate g values of 2.0 and 2.27.

minimal medium supplemented with $^{15}NH_4Cl$.[18] The ^{15}N-labeled apoenzyme was reconstituted with [^{14}N]AdoCbl and purified as described above. The enzyme-bound cofactor (120 μM) was converted to its paramagnetic cob(II)alamin form by irradiation of an anaerobic sample with a 60 W tungsten lamp. The observed spectrum (Figure 2, lower trace) confirms the presence of an ^{15}N-labeled lower axial ligand which is derived from the protein rather than the nucleotide loop of the cofactor. Seven of the eight lines in the $g_{\parallel}$ region are clearly resolved, with a spacing of 108 G. Superhyperfine coupling to the ^{15}N-nucleus ($I = {}^1/_2$) results in the appearance of doublets rather than triplets, with a coupling constant of 28 G. In addition, the resolution of fine structure in the $g_{\perp}$ region of the spectrum is considerably higher than that observed with ^{14}N-labeled enzyme. This rules out tyrosine coordination and provides evidence for a protein-derived histidine ligating to the bound cofactor.

It is confounding that the mutase and methionine synthase have a similar physical mechanism for tethering the cofactor but distinct chemical routes for manipulating it. The apparently similar "cobalamin pockets" in the two families have probably come to exist by conservation of an architectural motif that may or may not confer chemical advantages to both groups of enzymes. For instance, histidine coordination is expected to facilitate formation of square planar cob(I)alamin, which is an intermediate for methyltransferases and is postulated for the isomerases.[4,7,9] It will be of interest to determine whether noncatalytic proteins such as intrinsic factor have a similar binding pocket for cobalamin. Based on model studies, it has been proposed that a key role in labilization of the Co–C bond to homolysis is played by the lower axial base, which until recently had been presumed to be dimethylbenzimidazole. The Co–N bond to DMB is unusually long, at 2.24 Å.[19] The bulkiness of the DMB ligand contributes to the upward folding of the corrin ring,[20,21] resulting in steric crowding on the upper face with consequent weakening of the Co–C bond. However, recent studies from Kräutler's group on imidazolylcobamide[22] argue against both of these factors being influential in promoting homolysis on the mutase. Thus, the presence of an imidazole ligand shortens the Co–N bond by ~0.04 Å and diminishes the upward bending of the corrin ring. Similarities in the binding motifs on the lower face of the corrin ring in the two different families of cobalamin-dependent enzymes suggest that perhaps key factors governing the choice between heterolysis and homolysis may lie in the upper face of the corrin, where the substrate binding sites are also located. To this end, determination and comparison of complete structures of both AdoCbl- and MeCbl-binding enzymes will be critical in understanding how these proteins modulate the reactivity of the organometallic bond.

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(18) The recombinant *Escherichia coli* strain K38, harboring the pMEX2 and pGP1-2 plasmids, was grown in 6 × 1 L of M9 minimal medium containing 9.5 mM $^{15}NH_4Cl$. The enzyme was purified in the apoenzyme form as described in ref 12. The specific activity of the ^{15}N-labeled mutase was 26 units/mg of protein at 37 °C in the standard radiolabel assay described before: Taoka, S.; Padmakumar, R.; Lai, M-t.; Liu, H-w.; Banerjee, R. *J. Biol. Chem.* **1994**, *269*, 31630–31634.

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