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Review:

Role of vitamin B_{12} on methylmalonyl-CoA mutase activity*

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Abstract: Vitamin B_{12} is an organometallic compound with important metabolic derivatives that act as cofactors of certain enzymes, which have been grouped into three subfamilies depending on their cofactors. Among them, methylmalonyl-CoA mutase (MCM) has been extensively studied. This enzyme catalyzes the reversible isomerization of L-methylmalonyl-CoA to succinyl-CoA using adenosylcobalamin (AdoCbl) as a cofactor participating in the generation of radicals that allow isomerization of the substrate. The crystal structure of MCM determined in *Propionibacterium freudenreichii* var. *shermanii* has helped to elucidate the role of this cofactor AdoCbl in the reaction to specify the mechanism by which radicals are generated from the coenzyme and to clarify the interactions between the enzyme, coenzyme, and substrate. The existence of human methylmalonic acidemia (MMA) due to the presence of mutations in MCM shows the importance of its role in metabolism. The recent crystallization of the human MCM has shown that despite being similar to the bacterial protein, there are significant differences in the structural organization of the two proteins. Recent studies have identified the involvement of an accessory protein called MMAA, which interacts with MCM to prevent MCM's inactivation or acts as a chaperone to promote regeneration of inactivated enzyme. The interdisciplinary studies using this protein as a model in different organisms have helped to elucidate the mechanism of action of this isomerase, the impact of mutations at a functional level and their repercussion in the development and progression of MMA in humans. It is still necessary to study the mechanisms involved in more detail using new methods.

Key words: Vitamin B_{12}, Methylmalonyl-CoA mutase (MCM), MMAA, MeaB, Methylmalonic academia (MMA), Protectase, Reactivase

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1 Introduction

Vitamin B_{12} was discovered by Minot and Murphy (1926) when they were studying pernicious anemia. They supplemented the diet of 45 anemic patients with liver and meat and reverted their anemia in 1–6 months. Rickes *et al.* (1948) isolated the crystalline compound responsible for this effect from the liver, and Smith and Parker (1948) obtained the same compound at a high concentration and purity. Hodgkin *et al*. (1956) determined the structure of vitamin B_{12} by X-ray crystallography, and Lenhert and Hodgkin (1961) determined the organometallic nature of the cofactor and the corrin ligand.

Vitamin B_{12} is made up by four pyrrole rings (tetrapyrrole) with a central cobalt (Co) atom coordinated by four equatorial nitrogen ligands donated by the pyrroles forming a corrin ring. The molecule bound to the Co atom on the upper part of the corrin ring is called the upper axial ligand (β-axial ligand), and the molecule bound to the Co atom on the lower part of the ring is called the lower axial ligand (α-axial

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ligand). Vitamin B_{12} that contains a cyanide group in the upper axial ligand (cyanocobalamin) is a relatively inert Co(III) corrinoid and does not participate as a cofactor. Derivatives of vitamin B_{12} of physiological importance are coenzyme B_{12} or adenosylcobalamin (AdoCbl; Fig. 1) and methylcobalamin, which have a 5′-deoxy-5′-adenosine or methyl group as the upper axial ligand, respectively. The most common corrinoids have 5,6-dimethylbenzimidazole (DMB, green in Fig. 1) as a nucleotide base, and are known as cobamides. In some derivatives, such as pseudovitamin B_{12}, the lower axial ligand is adenine (Kraütler *et al*., 2003).

The biological role of vitamin B_{12} was discovered when Barker *et al.* (1958) described a derivative of vitamin B_{12}, AdoCbl, which is the cofactor of the enzyme glutamate mutase. Four years later, Guest *et al.* (1962) described methylcobalamin (MeCbl) as a cofactor of the bacterial enzyme methionine synthase.

Under physiological conditions, Co can present three possible oxidation states in B_{12} derivatives: Co(III), Co(II), and Co(I). The oxidation state determines which coordinate axial ligands will be present in the molecule. In derivatives with both α- and β-axial ligands, the Co to which they are bound is Co(III); after homolysis of the cofactor in which only one ligand remains, the Co is Co(II); and without ligands, the Co is Co(I). This particular feature determines the functions of B_{12} derivatives in their role as enzyme cofactors (Kräutler, 2005).

2 Adenosylcobalamin-dependent enzymes

Vitamin B_{12}-dependent enzymes form a family that can be divided into three subfamilies: (1) AdoCbl-dependent isomerases, (2) MeCbl-dependent methyltransferases, and (3) B_{12}-dependent reductive dehalogenases.

The subfamily of isomerases performs radical enzyme catalysis, by which they catalyze chemically difficult reactions using highly reactive free radicals originated from the cofactor AdoCbl (Toraya, 2000). The general mechanism of action of these enzymes starts with the homolytic cleavage of the coenzyme C-Co bond generating a 5′-deoxy-5′-adenosyl radical in the active site. This radical generation allows a hydrogen atom to migrate from a substrate carbon to an adjacent carbon in exchange for a group X, which moves in the opposite direction; this mechanism is also known as ‘radical roulette’ (Fig. 2).

The subfamily of isomerases is formed by three classes, which depend on the type of reaction. Class I includes the enzymes that rearrange the carbon skeleton. The enzymes that eliminate heteroatoms belong to Class II. Class III includes the enzymes that catalyze intramolecular migration of amino groups (Fig. 3) (Toraya, 2000).

Members of the isomerase subfamily can in turn be grouped into two classes according to the type of cofactor to which they bind. Class II includes the enzymes that bind to the coenzyme in the conformation known as ‘DMB-on’ (or ‘base-on’), in which the α-axial ligand of the coenzyme (DMB) remains bound to the Co (Abend *et al*., 1999; Sintchak *et al*., 2002; Yamanishi *et al*., 2002). On the other hand, in Class I or ‘His-on’ conformation enzymes, DMB is protonated in the coenzyme-binding domain of the protein, generating a ‘DMB-off’ (or ‘base-off’) conformation. Generation of this conformation is followed by the binding of a histidine residue of the protein to the Co, which occupies the place of DMB, thus generating the ‘His-on’ conformation. This histidine is embedded in a highly conserved sequence, DXHXXG (Reitzer *et al*., 1999; Mohamed *et al*., 2005).

Four members of Class I isomerases are currently known, methylmalonyl-CoA mutase (MCM), glutamate mutase, methyleneglutarate mutase, and isobutyryl-CoA mutase. These enzymes play an important role in bacterial metabolic processes. The only enzyme of this class found in mammals is the MCM, which participates in the catabolism of odd-chain fatty acids, some branched amino acids and cholesterol to generate the metabolite succinyl-CoA. Most isomerases are heterodimeric, and only one of the two subunits binds the cofactor. Exceptions to this are methyleneglutarate mutase and methylmalonyl-CoA mutase. The latter is heterodimeric in almost all bacteria except in *Escherichia coli*, which is homodimeric like in mammals.

Glutamate mutase catalyzes the conversion of glutamate to methylaspartate. This enzyme is isolated from *Clostridium tetanomorphum* and *Clostridium cochlearium*. The enzyme is a heterotetramer composed of two ε subunits and two σ subunits. The heterologous expression of these genes in *E. coli* allows

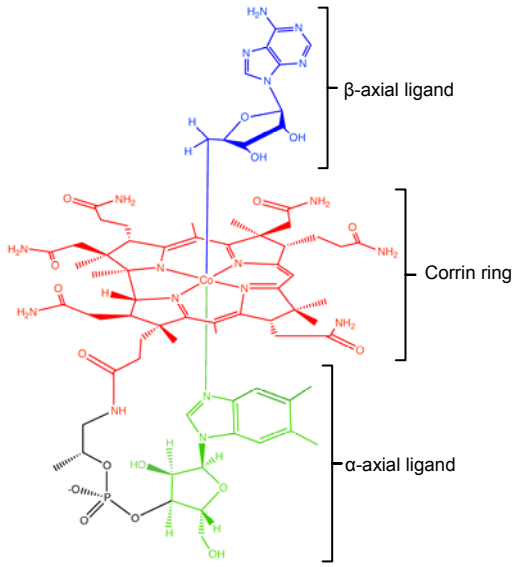

Fig. 1 Chemical formula of adenosylcobalamin (AdoCbl)

its production and molecular characterization. The crystal structure was obtained from recombinant protein of *C. cochlearium* (Buckel and Golding, 1996; Reitzer *et al*., 1999).

Methyleneglutarate mutase converts 2-methyleneglutarate to 3-methylitaconate, and is purified from the strict anaerobic *Clostridium barkeri*. The active enzyme is a homotetramer (α_4) and the gene encoding the protein has been cloned and overexpressed in *E. coli* (Buckel and Golding, 1996).

MCM isomerizes methylmalonyl-CoA to succinyl-CoA; details of this enzyme will be discussed later. Isobutyryl-CoA mutase converts isobutyryl-CoA to butyryl-CoA (Banerjee, 2001) and has been widely studied in *Streptomyces cinnamonensis* (Zerbe-Burkhardt *et al*., 1998). This protein is an $\alpha_2\beta_2$ heterotetramer and recent studies have shown

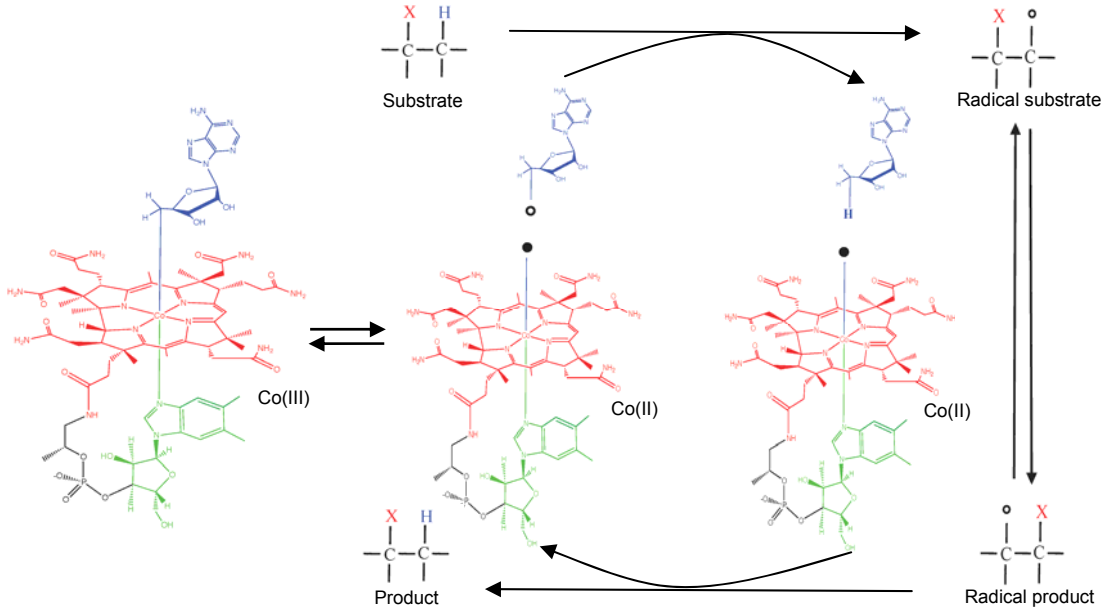

Fig. 2 General mechanism of reaction of adenosylcobalamin (AdoCbl)-dependent enzymes
Homolytic cleavage of C-Co bond triggers the 'radical roulette' allowing X (red) and H (blue) to switch positions

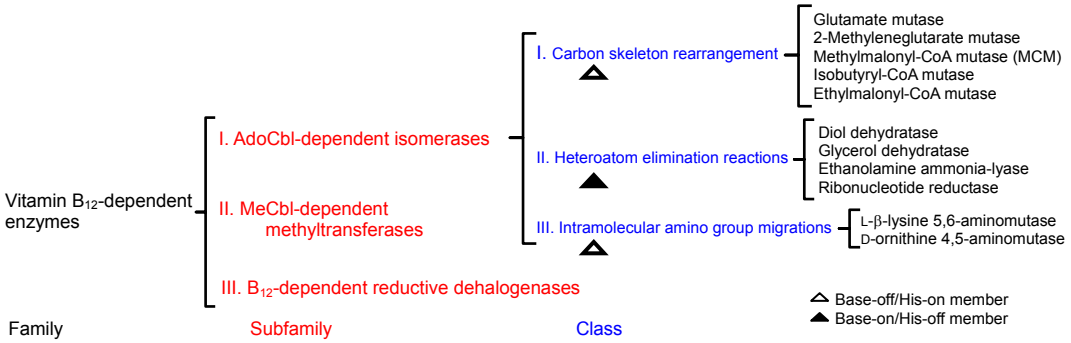

Fig. 3 Classification of adenosylcobalamin (AdoCbl)-dependent enzymes

its fusion with a P-loop guanosine 5′-triphosphatases (GTPases) in some phyla (Cracan *et al*., 2010). Cracan and Banerjee (2012) reported that this enzyme is also responsible for catalyzing the conversion of isovaleryl-CoA to pivalyl-CoA.

Ethylmalonyl-CoA mutase catalyzes the carbon rearrangement of ethylmalonyl-CoA to methylsuccinyl-CoA. This enzyme has been studied in *Rhodobacter sphaeroides* and its cloning and expression in *E. coli* allows its purification, molecular mass determination (75 kDa), and biochemical characterization (Erb *et al*., 2008).

Class II isomerases include diol dehydratase, glycerol dehydratase, ethanolamine ammonia-lyase, and ribonucleotide reductase. The enzyme diol dehydratase, also known as propanediol dehydratase, converts (*R*) or (*S*) 1,2-propanediol and other 1,2-diols into aldehydes and requires the presence of monovalent ions such as K^+ to perform the reactions. It is made up of three subunits and has been isolated from *Klebsiella oxytoca* and *Lactobacillus brevis*. The enzyme synthesis is induced by 1,2-propanediol in some enterobacteria during anaerobic growth and its metabolic role is to produce an electron acceptor and a metabolic intermediary for the incomplete oxidation of 1,2-diols (Lee and Abeles, 1963; Tobimatsu *et al*., 1997).

The enzyme glycerol dehydratase, also called glycerol hydrolyase, catalyzes the conversion of glycerol and 1,2-diols to their corresponding aldehydes. It is produced by some enterobacteria when they grow anaerobically on glycerol to produce the electron acceptor β-hydroxypropionaldehyde. The enzyme consists of three subunits and has been isolated from *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Clostridium pasteurianium* (Toraya and Fukui, 1977; Forage and Foster, 1979; Toraya *et al*., 1980).

The enzyme ethanolamine ammonia-lyase, also known as ethanolamine deaminase, catalyzes the conversion of ethanolamine to its acetaldehyde in the presence of AdoCbl. Its synthesis is induced in some bacteria when they grow on ethanolamine in the presence of exogenous vitamin B_{12}. It is made up of six α-subunits and six β-subunits and is involved in the acetaldehyde production pathway as an acetate mediator and/or electron acceptor in anaerobic fermentation of ethanolamine. The resulting ammonium ion may be employed as a nitrogen source (Bradbeer, 1965; Faust and Babior, 1992).

The enzyme ribonucleotide reductase catalyzes the conversion of ribonucleotides to 2-deoxyribonucleotides, important compounds for DNA replication and repair. The first studies of this enzyme were conducted in *Lactobacillus leichmannii* and in *E. coli*, and established the importance of certain amino acids using directed mutagenesis to study the interaction of this enzyme with the substrate under different conditions (Booker *et al*., 1994).

Class III enzymes include L-β-lysine (D-lysine) 5,6-amino mutase and D-ornithine 4,5-amino mutase, both of which catalyze intramolecular migrations of amino groups. L-β-lysine (D-lysine) 5,6-amino mutase rearranges the 1,2-terminal amino group of DL-lysine and L-β-lysine and depends on pyridoxal-5′-phosphate (Berkovitch *et al*., 2004). The enzyme D-ornithine 4,5-amino mutase catalyzes the reversible conversion of ornithine to 2,4-diaminovaleric acid. It can be purified from *Clostridium sticklandii* and characterized as homodimeric protein with a molecular weight of 180 kDa. It plays an important metabolic role in the oxidative metabolism of ornithine (Somack and Costilow, 1973).

3 Methylmalonyl-CoA mutase in bacteria

MCM is widely distributed in all living organisms except plants. It has been studied and crystallized from the Gram-positive bacteria, *Propionibacterium freudenreichii* var. *shermanii*, in which it plays an important role in the fermentative route of pyruvate to propionate. The enzyme was described as a heterodimer formed by a large subunit (α) and a small subunit (β), forming a 150-kDa protein with one domain binding to acyl-CoA and another one to coenzyme B_{12} per dimer. The crystal obtained by Mancia *et al.* (1996) generated much progress in the study of the architecture of the enzyme's domains, its interactions with the substrate and cofactor, and also contributed to the study of the amino acids that control the radicals generated during catalysis, which thus protect the enzyme from undesirable collateral reactions.

MCM has also been intensely studied in *Saccharopolyspora erythrae*, due to the biotechnological

importance in the production of the antibiotic erythromycin in the fermentative metabolism of carbon compounds. It is a heterodimer formed by a small subunit (α) and a large subunit (β). Reeves *et al*. (2006) studied how deletions in MCM genes affect erythromycin production and their association to the production of this antibiotic with the culture medium (based either on oil or on carbohydrates) that affects the direction of the reaction performed by MCM. This enzyme has also been studied in the facultative methylotrophic bacterium, *Methylobacterium extorquens*, in which the enzyme participates in the cycle of glyoxylate, an essential element in methylotrophic metabolism (Korotkova *et al*., 2002).

4 Methylmalonyl-CoA mutase in mammals

As mentioned earlier, the importance of MCM in mammals is related to its key role in the degradation of the amino acids valine, isoleucine, methionine, and threonine, odd-chain fatty acids, and cholesterol. Their carbon skeletons are broken down to produce succinyl-CoA, an intermediate product of the tricarboxylic acid (TCA) cycle. Methionine donates its methyl group to an acceptor through *S*-adenosylmethionine, and three of its four carbon atoms are converted to propionate as propionyl-CoA. Isoleucine undergoes transamination reactions followed by oxidative decarboxylations to produce one molecule of acetyl-CoA and one molecule of propionyl-CoA as end products. In human tissues, threonine is converted to propionyl-CoA. The propionyl-CoA formed from these amino acids is carboxylated to methylmalonyl-CoA, epimerized and finally converted to succinyl-CoA by MCM. Valine follows a similar route; after transaminations and decarboxylations, a series of oxidations converts the remaining carbon skeleton into methylmalonyl-CoA.

Odd-chain fatty acids are oxidized from the carboxyl end, and the remaining product from the β-oxidation is a 5-C fatty acid, which is broken down to acetyl-CoA and propionyl-CoA. Acetyl-CoA enters directly into the TCA cycle. Propionyl-CoA undergoes carboxylation, producing the isomer *D* methylmalonyl-CoA via propionyl-CoA carboxylase, on which epimerase then converts to its *L* form and is subsequently isomerized to succinyl-CoA via MCM (Lehninger *et al*., 1993).

Flavin *et al*. (1955) and Katz and Chaikoff (1955) firstly studied the mammalian MCM, and they determined the function of this enzyme extracted from sheep kidney and rat liver. Cannata *et al*. (1965) purified it from sheep kidney to homogeneity, and determined the enzyme properties, including its molecular weight of 78 kDa, its K_m, optimal pH and specific activities under different conditions, which revealed the instability of the apoenzyme. They also found that the enzyme binds one mole of its cofactor per mole of subunit.

Fenton *et al.* (1982) purified MCM from human liver to homogeneity obtaining its enzyme molecular weight (77.5 kDa), the K_m for methylmalonyl-CoA (1.5 mmol/L), the specific activity (12–14 μmol succinyl-CoA/(min·mg)) and the optimum pH (approximately 7.5). They also determined that the enzyme activity is stimulated by polyvalent anions and that high concentrations of divalent cations inhibit its activity. Fenton *et al*. (1984) reported that the enzyme is synthesized in the cell cytoplasm as a larger precursor and is subsequently exported to the mitochondrial matrix where it is processed, losing approximately 3–4 kDa, generating the mature enzyme. Ledley *et al*. (1988) obtained a cDNA library of human liver and detected the clone with the gene that codes for MCM, using antibodies raised in chicken. Later, Jansen *et al*. (1989) described the cloning and sequencing of the gene that encodes this enzyme, confirming the authenticity of the sequence and predicting the amino acid sequence for the first time. They found an open reading frame coding for a protein of 750 amino acids. The leader sequence consisted of 32 amino acids whose content is characteristic of mitochondrial matrix enzymes. Using hybridization techniques, Nham *et al*. (1990) determined that the enzyme is encoded by a single gene located on chromosome 6p12-21.1. This gene, named *mut*, consists of 13 exons contained in a DNA region of 35 kb; the promoter region is localized in exon 1. Heterologous expressions of the enzyme were achieved in *Saccharomyces cerevisiae* (Andrews *et al*., 1993) and *E. coli*, and the enzyme was purified from the latter by affinity chromatography (Janata *et al*., 1997).

Mutations in the human *mut* gene generate a recessive autosomic metabolic disease called methylmalonic acidemia (MMA) due to a partial or total deficiency of the enzyme activity. This disease may

also appear in children with insufficient consumption or deficient metabolism of vitamin B_{12}. MMA usually appears during the first year of life, and patients display vomiting, lethargy, dehydration, muscular hypotonia, metabolic acidosis, mental retardation, and, sometimes, death. Accumulated methylmalonic acid in patients may be detected in blood and urine, and concentrations may range from 2.1 to 49 mmol, equivalent to 240 to 5 700 mg/d, which represents approximate concentrations of 50 to 1 000 times higher than normal (Rosenblatt and Fenton, 2001). Disease incidence has been difficult to establish because its early appearance in life makes timely diagnosis complicated. Wilcken *et al*. (2003) reported an incidence of 1 in 140 000 in a screening of newborns realized in Australia. Treatment generally consists of a restrictive diet and administration of vitamin B_{12} to control methylmalonate and propionate production (Rosenblatt and Fenton, 2001); however, this treatment has not reduced the high mortality index of the disease. Murine models have been proposed to investigate the pathophysiology, specifically the neurological and developmental complications of MMA (Peters *et al*., 2003).

5 Interactions of methylmalonyl-CoA mutase with adenosylcobalamin and substrate

MCM-mediated isomerization was initially studied using radioactively labeled substrates, such as methyl-^{14}C-malonyl-CoA and methyl-^{3}H-malonyl-CoA. Labeled atoms were found to remain in the succinyl-CoA formed in the presence of the enzyme (Erfle *et al*., 1964). As previously mentioned, the study of MCM crystal from *P. freudenreichii* var. *shermanii* has contributed considerably to the understanding of the enzyme-substrate interaction. Mancia *et al*. (1996) obtained a crystal with 0.2-nm resolution and described the two following primary domains of the enzyme: one substrate-binding domain with a triosephosphate isomerase (TIM) barrel fold $(\alpha/\beta)_8$ at the N-terminal end and another domain showing a Rossman-type fold $(\alpha/\beta)_5$ at the C-terminal end. The linker region encloses the barrel domain and connects it to the C-terminal domain. The active-site cavity is formed by the loops of the β strand of the barrel. Modeling the substrate in to the active site allowed determining the amino acids that interact with it, such as Hisα244, which binds to the carbonyl oxygen in the rearrangement reaction, and Argα207 and Tyrα89, which are also involved in binding with the substrate. Likewise, the MCM form that binds to the cofactor is 'base-off/His-on'. The displaced DMB is located in a deep hydrophobic cavity between the β strand and the C-terminus helix. The amino acid that substitutes DMB is Hisα610, which is bound to Co through its ε nitrogen (Nε2). In addition, a chain of H-bonds to Aspα608 and Lysα604 begins at the nitrogen atom (Nδ1) of this histidine. The bond between Hisα610 (Nε2) and Co is very long, about 0.25 nm, which is characteristic of cobalamins in which the upper axial ligand is an alkyl group (Mancia *et al*., 1996). Mancia and Evans (1998) completed the substrate-free MCM crystallography from *P. freudenreichii* var. *shermanii*, and Mancia *et al*. (1999) obtained the crystal with the substrate and product and thus determined the enzyme-substrate interaction in greater detail. Argα207 attaches to the carboxyl group of the substrate, Hisα244 contributes to the catalysis and stabilizes the intermediate radicals, and Tyrα89 guides the stereochemistry of the reaction by placing the adenosyl radical at a certain distance from Co. In their studies, they explained how the binding enzyme and substrate close the active site and how this conformational change separates the adenosyl group from Co by steric interference of Tyr89. The development of quantum mechanical methods like quantum mechanical/molecular mechanical (QM/MM) methods for the analysis of biological systems enabled a theoretical study that confirmed the importance of the amino acids Tyr89 and His244 in H-bond formation with the substrate and the fact that they contribute energetically to the isomerization reaction (Loferer *et al*., 2003).

The importance of the ligand formed by Co of the coenzyme and histidine (base-off/His-on) was analyzed by Vlasie and Banerjee (2002), using MCM from *P. freudenreichii* var. *shermanii* mutated at His610 (H610A and H610N) and the properties of their kinetic constants K_m, k_{cat}, and K_d were determined and compared with the wild-type enzyme. For H610A, the K_m for the substrate (364 μmol/L) is about three-fold higher compared with the wild-type (133 μmol/L); K_m for the cofactor (0.45 μmol/L) is twice that for the wild-type (0.24 μmol/L); K_d for the

cofactor (300 μmol/L) is much higher than that for the wild-type (0.17 μmol/L); and, the k_{cat} value is 40 000 times lower (0.024/s). The H610N mutant shows a K_m value (102 μmol/L) similar to that of the wild-type; K_m for the cofactor is almost double in value (0.52 μmol/L); K_d is the same as that for H610A; and, the k_{cat} value is 5 000 times lower (0.003/s). The ultraviolet (UV)-visible absorbance spectrum of the H610A mutant bound to AdoCbl shows a peak at 460 nm, characteristic of the base-off conformation, as well as two peaks at 358 and 535 nm, typical of OH-Cbl, reflecting an increase in lability of the Co-C bond. Comparison of the two spectra of the mutant H610N obtained at different concentrations of the analogous cofactor AdoCbi (adenosylcobinamide methyl phosphate, analog of AdoCbl lacking the nucleotide loop moiety) shows an increase in absorption at 360, 448, 507, and 535 nm, which indicates the cleavage of AdoCbi and hydroxycobinamide (OHCbi). These results show that a change in coordination of histidine 610 with the cofactor increases the lability of the Co-C bond (Vlasie *et al*., 2002).

Thomä and Leadlay (1996) made a model of the human homodimeric MCM based on the *P. freudenreichii* var. *shermanii* MCM crystal structure because human MCM shares 65% identity with the large subunit in these bacteria. In this work, they established certain structural bases to justify MMA-causing mutations. The two principal domains present in the structure are the TIM barrel $(\alpha/\beta)_8$-binding domain at the amino-terminal end and the $(\alpha/\beta)_5$ cofactor-binding domain at the carboxyl-terminal end. The cobalamin DMB is situated in a deep cavity adjacent to strands IIβ3 and IIβ4 on one flank and to strands IIα1 and IIα5 on the other flank. The hairpin structure formed by strands IIβ1 and IIα1 contains His627, which constitutes the ligand with Co of the coenzyme (base-off/His-on) and is equivalent to Hisα610 of *P. freudenreichii* var. *shermanii*. Amino acids Asp625 and Lys621 are also important in H-bond formation, which presumably controls the reactivity of Co, possibly by acting as a proton relay or by positioning the histidine ligand at an unusually long distance from the Co atom. All glycine residues in this loop are conserved and essential for human MCM activity. G262 is particularly important because its NH group forms an H-bond with the propionamide chain of the corrin ring C-8. G630 and G703 are part of the deep cavity that contains DMB. Arg103 and Arg108 bind the ADP-ribosyl molecule of the CoA ester of the substrate (Thomä and Leadlay, 1996).

Recently, Froese *et al*. (2010) determined the structure of human apoenzyme MCM and in complex with AdoCbl or with an analog substrate (malonyl-CoA). They confirmed the presence of the substrate domain (TIM barrel) connected to a small AdoCbl-binding domain via a 100-amino acid inter-domain per subunit. Despite the good model made from the bacterial crystal structure, the human MCM revealed important differences mainly due to the presence of two catalytic subunits instead of one in bacteria. The comparison of the apoMCM and holoMCM structures allowed for observations of rearrangements upon cofactor and substrate binding. The cofactor binding causes significant outward displacement in several C-domain helices, inducing the formation of a pocket at the domain interface for binding AdoCbl, positioning the His627 imidazole to coordinate with the Co atom, as previously predicted by the model, that enables the 'base-off/His-on' cofactor configuration. The N-domain remains largely stationary in the apoenzyme and MCM complex with the cofactor; however, in the substrate-bound enzyme, the TIM barrel domain changes from an open substrate channel to a tightened and constricted barrel, protecting it from the solvent exterior.

6 Role of the lower axial ligand in the methylmalonyl-CoA mutase reaction

As mentioned earlier, MCM binds to the cofactor by the base-off/His-on configuration. This binding conformation raises the question about the exact role of the DMB molecule in the reaction. Chowndhury and Banerjee (1999) evaluated the impact of a modification of the cofactor's lower axial ligand in the catalysis reaction, affinity for the ligand and His-on formation. They used homologous cofactors modified at the α-axial ligand (AdoCbi and AdoCbi-PMe) and the intrinsic fluorescence emission of the MCM tryptophan molecules (there are 11 tryptophan molecules located in the cofactor-binding domain) whose emission diminishes as MCM binds to AdoCbl. To determine the dissociation equilibrium constants, they recorded the increase in fluorescence emission at

340 nm by titration with the cofactor AdoCbl. The K_d obtained for the native cofactor was 0.17 μmol/L, and the values for AdoCbi and AdoCbi-PMe were 0.62 and 3.33 μmol/L, respectively, which led to the conclusion that modifications at the analog DMB region do not impede the binding of the cofactors to the enzyme but considerably increase their dissociation. The authors also compared the UV-visible absorption spectra obtained from AdoCbl or analogs without enzyme and AdoCbl or analogs bound to MCM. Spectra obtained using the native cofactor showed an increase in absorbance in the range of 300–600 nm as a result of the binding of the cofactor to the enzyme, whereas this increase was not observed in the spectra obtained with cofactor analogs. The increase in absorbance in the spectra using the wild-type cofactor results in emission at 525 nm, reflecting the bond formed between the MCM His610 and the Co in the cofactor, which generates the His-on conformation. This maximum is found at 460 nm in the spectra obtained for cofactor analogs. Thus, Chowdhury and Banerjee (1999) concluded that no bond forms between Co and the MCM His610. On the contrary, they remain in the DMB-off conformation at the active site of MCM. The absence of DMB probably results in a faulty organization of the binding site to the cofactor, impeding the Co-His610 bond. These results, together with the null activity of the MCM enzyme bound to analogs, led to the conclusion that the main contribution of DMB is in organizing the binding domain to cobalamin, thus enabling the catalysis reaction.

7 Activation of Co-C bond in methylmalonyl-CoA mutase

A surprising fact in the study of MCM-mediated radical catalysis is the increase in the rate of homolytic Co-C cleavage. Under non-catalyzed conditions, the Co-C cleavage rate is 3.8×10^{-9}/s, which increases 10^{12} times in the presence of the enzyme. This enzymatic activation involves a decrease in the dissociation enthalpy of the Co-C from 31.4±1.5 to ~14 kcal/mol, thus contributing to a ~17 kcal/mol decrease.

Brooks *et al*. (2005) used the magnetic circular dichroism (MCD) to study the spectra of the MCM enzyme with cob(II)alamin at the binding domain to the coenzyme and in the presence and absence of analog substrates. The spectra show differences between the enzyme bound to cob(II)alamin and the enzyme without the cofactor, indicating a conformational change caused by the bond with the cofactor, which could labilize or activate the Co-C bond. Contribution of the bond between the enzyme and cofactor to the Co-C cleavage is not significant, reducing the bond dissociation enthalpy by only about 0.5 kcal/mol. Results also indicate that the protein bound to its cofactor in the presence of the substrate shows an important change in the spectrum, compared to the spectrum of the protein in the absence of the substrate, which suggests that the presence of the substrate is a crucial activator of the Co-C bond. The amino acid Tyr89 undergoes great changes at the active site, which suggests its major role in labilizing the Co-C bond. The entrance of the substrate into the TIM barrel shuts the barrel. Once the substrate is in its position, Tyr89 occupies the area previously dominated by the 5′-deoxyadenosine of the cofactor, causing steric labilization of the Co-C bond. MCM mutants in this residue were obtained, and the proteins were purified to determine the K_m, k_{cat}, and K_d parameters. Mutants show a modified k_{cat} value and an unchanged K_m value for the substrate. Mutants at the His224 amino acid, which binds to the substrate via an H-bond, generate an unstable, highly oxygen-sensitive protein due to the increase in the intermediate cob(II)alamin (Thomä *et al*., 2000). Mutants were obtained for Y89 (Y89F and Y89A), and they show a significant change in K_m (357 and 926 μmol/L for Y89F and Y89A, respectively) compared with that of the wild-type (133 μmol/L). The UV-visible absorbance spectrum of the Y89F-AdoCbl mutant compared with the spectrum of the wild-type shows no increase in absorbance at 470 nm, which is characteristic of the formation of cob(II)alamin, generating an enzyme less susceptible to collateral oxidative reactions (Vlasie and Banerjee, 2003).

Brooks *et al*. (2004) obtained the spectra of MCM bound to its native cofactor (AdoCbl) and to an analogue (MeCbl) using the MCD and electronic absorption spectroscopy (ABS) techniques combined with computer methods based on density functional theory (DFT) and time-dependent DFT (TD-DFT). The AdoCbl spectra show a broad and prominent feature at low energy identified as the α/β region, in

addition to several bands of greater intensity at higher energy identified as the γ region. Comparing these spectra with those obtained for MCM-AdoCbl, some modifications may be observed due to the bond between the enzyme and the cofactor, which increases the peaks of the spectra, because the cofactor adopts a well-defined form at the active site once it stabilizes in one of the various possible isoenergetic orientations that the adenosine molecule may adopt after MCM has bound to the cofactor. Brooks *et al*. (2005) continued using the MCD and ABS techniques to analyze the spectra of cob(II)alamin bound to MCM. Much more pronounced modifications were observed in these spectra, which led them to conclude that distortions of the cofactor in its Co(II) form are a significant source of activation of the Co-C bond. Thus, the binding of the enzyme to the coenzyme and later to the substrate generates distortions in the electronic structure of the cofactor, which contribute to homolysis. However, the dominant contribution to the enzymatic activation of the Co-C bond is caused by the stabilization of the products cob(II)alamine/Ado generated post-homolysis, more so than by destabilization of AdoCbl.

8 Protection of collateral reactions in methylmalonyl-CoA mutase

The MCM isomerization reactions normally occur in the presence of oxygen; therefore, the enzyme must be protected from oxygen interception while catalysis is carried out, which would inactivate the enzyme. The crystal structure reveals that the active site of the enzyme is protected within a deep and inaccessible cavity, isolated from the surroundings. Once the substrate penetrates and binds to the active site, MCM undergoes conformational changes that lead to a filled cavity of the CoA end of the substrate along the TIM barrel.

Wild-type MCM has been shown to become inactive by the action of oxygen. This inactivation occurs at a slower rate (inactivation 1.5×10^5 times slower) than the one observed for MCM mutant His244Ala (once every 22 reactions). Another characteristic of the His244Ala mutant is to accumulate the intermediate product cob(III)alamin in the form of hydroxycob(III)alamin. Consequently, these characteristics confer the function of protecting the enzyme from inactivation by oxygen to this specific amino acid because the imidazole ring of His244 obstructs the passage of oxygen to the intermediate radical products at the active site (Thomä *et al*., 2000; Banerjee and Vlasie, 2002).

Vlasie and Banerjee (2004) performed a study with the R207Q mutant and showed that the enzyme's kinetic parameters are different from the wild-type protein's parameters: k_{cat} (diminished from 7200 to 0.702/min) and K_m (increased from 133 to above 4000 μmol/L). Two spectra of UV-visible absorbance were obtained using this mutant and wild-type MCM bound to AdoCbl. Comparison of the spectra reveals changes in the mutant spectrum caused by the formation of inactive cofactor OHCbl. The formation of this time-dependent inactive cofactor was studied by UV-visible spectroscopy using the mutant R207Q. The changes induced in the mutant spectrum show a time-dependent increase in zone α/β at 351 nm in the presence of oxygen equivalent to the appearance of the inactive cofactor OHCbl without accumulation of the intermediate cob(II)alamin. Other studies have been performed with mutant amino acids at the active site (e.g., Y89F and Y243A), showing that they are important for controlling the reactivity of the radicals generated during substrate isomerization and to avoid collateral reactions that cause inactivation of the enzyme (Padovani and Banerjee, 2006).

Evidence has also been found that nitric oxide (NO) and nitrous oxide are potent inactivators of MCM. A 50% reduction of its activity has been reported in the presence of NO (Kambo *et al*., 2005). This is an important fact because the enzyme, which works at the mitochondrial matrix, is exposed to NO in considerable amounts due to the passage of this gas from the cytoplasm into the mitochondrion by passive diffusion. Kambo *et al*. (2005) studied this inactivation and showed that it is produced by reaction with Co(II), deoxyadenosyl radical, or both. Crystallography studies, which reveal the inaccessibility of the active site to other molecules for its protection, have not examined nonpolar molecules such as O_2 and NO. In addition, the protein has been modeled from bacterial MCM and this model may therefore be somewhat imprecise, and thus, does not reveal the protein differences, which could increase the sensitivity of the enzyme to NO and O_2.

9 MeaB, a protective protein for methylmalonyl-CoA mutase

We previously mentioned that the intervention of radicals during the reaction conducted by AdoCbl-dependent isomerases requires adequate management of the intermediate radicals reactivity formed in order to prevent undesirable collateral reactions, which could lead to inactivation of MCM. Recently, a protein that contributes to the prevention of MCM inactivation has been studied.

Bobik and Rasche (2001) described two genes located in a cluster with MCM in bacterial arrangements. One of them was considered an accessory protein to the function of MCM. Korotkova and Lindstrom (2004) studied one of the genes present in this arrangement of *Methylobacterium extorquens* AM1, and named it *meaB*. This gene codes for a protein that belongs to the family of P-loop GTPases, whose members are auxiliary proteins in the assembly of enzymes with metallic cofactors and may act as chaperones. The precise function of these proteins has not been elucidated, but they are known to show a low GTPase activity.

The same authors found that the bacterial mutant with interruption in *meaB* loses MCM activity. They purified the protein and showed the formation of MeaB-MCM complexes in nondenaturing polyacrylamide gel electrophoresis. They determined that the MeaB protein is capable of increasing MCM specific activity from 1.4 to 3.9 U/mg and depends on GTP and $MgCl_2$ (Korotkova and Lidstrom, 2004).

Research to determine the role of MeaB protein continued, and Padovani and Banerjee (2006) studied the interaction of MeaB with MCM in *M. extorquens*, as well as that of MeaB with GTP and guanosine diphosphate (GDP). They obtained the thermodynamic parameters (K_d, $\Delta H°$, $T\Delta S°$, and $\Delta G°$) associated with the binding of MeaB to nucleotides by using isothermal titration calorimetry (ITC). MeaB binds GDP and GTP (they used a non-hydrolyzable analogue) with similar affinity (GDP: K_d=(6.2±0.7) μmol/L and GTP analogue: K_d=(7.3±0.6) μmol/L), which confirms the characteristic low affinity of this family of GTPases to nucleotides. The MeaB-GDP bond is enthalpically driven, while MeaB-GTP generates changes in $\Delta H°$ (negative) and $T\Delta S°$ (positive) that favor the formation of the complex. The thermodynamic parameters to describe the formation of the complex apoMCM-MeaB-GDP or -GTP and holoMCM-MeaB-GDP or -GTP were also obtained by ITC. The binding parameters for apoMCM-MeaB-GDP or -GTP are similar, and both are enthalpically driven ($\Delta H°$=(−22.4±0.5) and (−22.1±0.6) kcal/mol, respectively) with unfavorable association entropy ($T\Delta S°$=(−13.0±0.7) and (−15.0±0.8) kcal/mol, respectively). In the case of holoMCM-MeaB-GDP or -GTP, binding is more favorable for the latter. The K_d of holoMCM-MeaB-GDP is much higher ((524±66) nmol/L) than that of holoMCM-MeaB-GTP ((34±4) nmol/L), thus favoring the formation of the former by a factor of 15. The binding of holoMCM-MeaB-GTP is 4.6 times stronger than that of apoMCM-MeaB-GTP and is driven by a decrease in the enthalpy of association. The K_d of MeaB bound to GTP or GDP diminishes in the presence of MCM, i.e., MCM increases MeaB affinity for nucleotides. This result is compatible with the increase in MeaB GTPase activity in the presence of MCM. This work also suggests that the MeaB protein forms a complex with MCM because it functions as an auxiliary protein, transferring AdoCbl with GTP dependence (increase in GTPase activity) (Padovani *et al*., 2006). To corroborate the role of MeaB as a mediator of AdoCbl turnover, the influence of this protein in the formation of the complex MCM-AdoCbl was analyzed by ITC. The $\Delta G°$ of MCM-AdoCbl in the absence of MeaB ((−8.6±0.1) kcal/mol) and in the presence of MeaB ((−8.9±0.2) kcal/mol) is similar and, in both cases, entropically driven. The presence of MeaB-GDP increases the K_d of MCM-AdoCbl compared with that obtained without MeaB-GDP by a factor of 5. Titration of the formation of MCM-AdoCbl in the presence of MeaB-GTP does not release heat; thus, these experiments conclude that hydrolysis of GTP is necessary to allow the binding of AdoCbl with MCM. The UV-visible absorbance spectra of MCM incubated with substrate (methylmalonyl-CoA) in the presence of AdoCbl shows the changes in the spectrum at different incubation times (5–190 min). These changes consist of larger peaks as time passes, which reflect the pattern obtained by oxidation of the 5-deoxycob(II)alamin to cob(III)alamin, leading to enzyme inactivation. In the presence of MeaB-GTP, this oxidation rate, monitored by an increase in 351 nm absorbance, diminishes by a factor of 15.

Thus, Padovani and Banerjee (2006) propose a dual function for MeaB, which involves a role in GTP-dependent binding of AdoCbl to apoMCM as well as in the protection of intermediate radical products during MCM catalysis, independent of GTP.

Hubbard and Drennan (2007) crystallized the MeaB protein of *M. extorquens* in its free and GDP-bound forms. They confirmed that the protein is a homodimer, formed by two monomers with the following three main regions: (1) one central G domain, which includes a 7-stranded β-parallel sheet; (2) one C-terminal extension composed of 70 amino acids, critical for dimer formation; and, (3) one N-terminal extension, composed of 50 amino acids, which forms three α-helices. They also confirmed that MeaB is a member of the G3E family of GTPases (metallochaperones) because it contains the four following characteristic motifs: the P-loop motif (Walker A: GXXGXGK) [S/T] in the form GVPGVGKS, the function of which is to coordinate the phosphate molecules of the guanine nucleotide with the nitrogen atoms of the skeleton by hydrogen bonds; one specificity loop of the guanine base (NKXD) as NKAD; and, the switch I and switch II regions, which were not directly determined from the structure but were inferred by structural alignment with HypB, a member of the same subfamily of GTPases (metallochaperones). These switch regions interact with the nucleotide and Mg^{2+} at the active site, positioning one water molecule for GTP hydrolysis and signaling by conformational modification that hydrolysis has occurred (Hubbard *et al*., 2007).

Studies using *E. coli* demonstrated that a similar mechanism to the one previously described in *M. extorquens*, could be present in *E. coli*. Froese *et al*. (2009) reported the expression of Sbm and YgfD proteins, contiguous members of an operon comprised of *sbm-ygfD-ygfG-ygfH*. Sbm is MCM, and the name comes from the acronym 'sleeping beauty mutase' because of the belief of the lack of a functional promoter. YgfD has GTPase activity and shares 48% sequence identity with MeaB. Both proteins form a complex in vitro and in vivo.

Padovani and Banerjee (2009) demonstrated that the MeaB protein uses its GTP hydrolysis activity and GTP binding energy in a gating/editing mechanism. GTP hydrolysis is used for cofactor gating, which refers to the delivery of the cofactor from holo-ATR (adenosyltransferase loaded with AdoCbl) to MCM. The editing process exercised by MeaB occurs prior to the delivery of the cofactor and during turnover conditions. In the first case, MeaB prevents transference of the inactive cob(II)alamin form of the cofactor from ATR to MCM, protecting it from being loaded with the wrong cofactor and shielding MCM from irreversible inactivation. In the second case, the process of editing occurs during catalytic turnover when occasional loss of the deoxyadenosine moiety occurs, and then, MeaB triggers the release of the cofactor. In both editing processes, GTP binding energy is what is required.

A protein orthologous to MeaB, MMAA, is present in humans. Dobson *et al*. (2002) identified the gene that codes for this human protein using bioinformatic tools to analyze bacterial gene arrays that include the enzyme MCM. They obtained the sequence of a gene that appears adjacent to MCM in the arrays and looked for this sequence in the human genome and named it MMAA. These authors also sequenced the DNA from patients with methylmalonic acidemia of the *cblA* complementation group and showed that the disease is caused by mutations in this gene. They also determined a probable mitochondrial leader sequence of approximately 65 amino acids in length. MeaB and MMAA show 46% identity and 67% similarity excluding the N-terminal sequence. Hubbard *et al*. (2007) used this high-identity percentage to map mutations, employing the crystal structure of MeaB to infer the structural basis of the pathology. Mutations R57Q and R272Q of MeaB (R145Q and R359Q in MMAA) result in instability of the protein, reflected as the formation of inclusion bodies. Mutation F119C in MeaB (Y209C in MMAA) generates the formation of a disulfide bond, the structural effect of which has not yet been elucidated. Mutations L11P, G59E, and G130E (L89P, G147E, and G218E, respectively, in MMAA) lead to protein disruption by the interruption of an α-helix, a steric and electrostatic modification and a change in conformational flexibility. Recently, the structure of human MMAA has been determined and has revealed a central 7-stranded G-domain harboring the four GTPase signature motifs. In addition, human MMAA dimerizes in a different manner compared to bacterial MeaB; while MeaB uses the dimerization arms and G-domain from both subunits resulting in a more

closed configuration than MMAA that only uses dimerization arms (Froese *et al*., 2010).

Two groups demonstrated the human MCM-MMAA interaction with different results. Froese *et al*. (2010) observed the formation of the complex between MCM and MMAA only if MCM was in the apoenzyme form and MMAA was bound to GTP. This result led them to infer that MMAA is implicated in the assembly of MCM cofactors such as MeaB, but with some significant differences. They speculate that AdoCbl binding releases human MCM from its complex with MMAA in a GTP-dependent manner and found no evidence of the editing process. More recently, Takahashi-Iñiguez *et al*. (2011) not only demonstrated the interaction of both human proteins in vivo and in vitro but also identified a probable dual role of MMAA. First, they demonstrated that the addition of MMAA to the MCM reaction mixture containing AdoCbl at time zero protects MCM from progressive inactivation by oxidation. This effect is nucleotide-dependent and then MMAA acts as a 'protectase'. Additionally, in a reaction with inactivated MCM, the addition of MMAA generates a 70% increase in activity operating as a 'reactivase', which requires hydrolysis of GTP. In this 'reactivase' function, it is proposed that MMAA may promote exchange of the inactive cofactor, restoring MCM activity.

10 Conclusions

AdoCbl-dependent enzymes perform isomerization reactions using radical catalysis. The mechanism of radical generation at the active site and the role of the cofactor in substrate isomerization have been intensely studied with tools such as ITC, MCD, spectroscopic techniques, and directed mutagenesis. Together with crystal structure and kinetic studies, these techniques have been used to elucidate details of the isomerization process, which is now known to depend on the labilization of the Co-C bond, and to identify important amino acids at the active site that stabilize intermediate radicals which prevent considerable enzyme inactivation. ITC studies have revealed the thermodynamic parameters of MCM binding to the cofactor and have allowed researchers to infer the function of MeaB, a chaperone protein involved in the transference of AdoCbl to the enzyme and in protection from inactivation. The crystal structure of this accessory protein has been studied to determine the distinctive characteristics of the G3E family of GTPases and also revealed that despite being similar in sequence to bacterial counterparts, the structure and mode of action show significant differences. The main difference is the involvement of MMAA not only as a protectase but also as a reactivase, preventing enzyme inactivation and recovering the inactive mutase's activity. Despite the discovery of the interaction between MMAA and MCM and the disclosure of the role of the complex, further studies are required to solve the intricate molecular trafficking of the cofactor to the target enzymes and to determine the physiological causes of the generation of MMA in patients with mutations in metallochaperones.

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