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Protection and reactivation of human methylmalonyl-CoA mutase by MMAA protein

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ABSTRACT

Previous studies have reported that some adenosylcobalamin-dependent enzymes suffer inactivation during catalysis due to the oxidation of cobalamin. In addition, the protection or reactivation of their catalytic activities by proteins called "protectases" or reactivases is well known in bacteria. In this study, we examined the influence of human MMAA protein on the kinetics of the reaction catalyzed by methylmalonyl-CoA mutase (MCM) by testing both purified recombinant proteins *in vitro*. Our results showed that MMAA plays dual roles in MCM activity. When it was added at the beginning of the reaction, it prevents inactivation by guarding MCM. After 60 min of reaction, when MCM is inactive, the addition of MMAA increases the enzymatic activity through GTP hydrolysis, indicating reactivation of MCM by exchange of the damaged cofactor. Interaction between MCM and MMAA observed *in vitro* was confirmed *in vivo* by yeast two-hybrid system.

1. Introduction

Methylmalonyl-CoA mutase (MCM; EC 5.4.99.2) catalyzes the reversible isomerization of methylmalonyl-CoA to succinyl-CoA and requires adenosylcobalamin (AdoCbl) as a cofactor [1]. During the reaction catalyzed by MCM, AdoCbl is cleaved homolytically generating cob(II)alamin and a deoxyadenosyl radical, which generates a substrate-centered radical by abstraction of a hydrogen atom. After an intramolecular migration of the thioester moiety, the hydrogen atom is transferred back to generate the product. Finally, a reabstraction of the hydrogen atom from deoxyadenosine occurs, followed by recombination of the cob(II)alamin and deoxyadenosyl radical to regenerate AdoCbl [2]. During catalysis, the MCM cofactor has an unpaired electron state that is very reactive and susceptible to oxidative inactivation, which leads to the formation of OH_2Cbl and the irreversible accumulation of inactive MCM [3,4].

MCM has a broad distribution among living organisms and has been found in both bacterial and animal cells [5]. In higher animals, MCM is involved in the catabolism of the amino acids valine, isoleucine, methionine, and threonine, and it plays an essential role in the conversion of propionyl-CoA to succinyl-CoA, an intermediate of the tricarboxylic acid cycle [6]. Methylmalonic acidemia (MMA) is a rare human disorder caused by mutations in the *mut* gene coding for mitochondrial MCM that decrease the activity and generate the accumulation of methylmalonic acid in the blood and urine of affected individuals. In 2002, Dobson et al., described mutations in another human gene, *MMAA*, which caused vitamin B_{12}-responsive methylmalonic aciduria [7].

Glycerol dehydratase and diol dehydratase, two other adenosylcobalamin-dependent enzymes, also undergo rapid inactivation by glycerol during catalysis although glycerol is a physiologic substrate for these enzymes and for bacterial growth [8]. The solution to this enigma was the discovery of reactivation systems, which are proteins whose respective genes are encoded next to the dehydratases genes in bacteria. The role of reactivating factors is to promote the release of the inactivated cofactor; the resulting apoenzyme can thus be reconstituted into a catalytically active enzyme in the presence of ATP and Mg^{2+} [9]. These enzymes have a low ATPase activity, which is necessary for their function.

To date, the *MMAA* gene has not been expressed, and the protein has not been purified. The protein sequence indicates the presence of motifs that have been associated with the G3E subfamily of P-loop GTPases that includes chaperones [10]. In 2004, studies in *Methylobacterium extorquens* revealed that MeaB, a bacterial ortholog of MMAA, forms a stable complex with MCM that can be seen in native polyacrylamide gels [11,12]. *In vitro* studies suggest that MeaB plays a role in both the assembly of the bacterial mutase and in its subsequent protection against inactivation [13].

In spite of the importance of MMAA protein in humans, nothing is known about the specific role of this protein. In this paper, we demonstrate that MMAA acts as a chaperone of human MCM protein.

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2. Material and methods

2.1. Strain

Yeast strain Y2HGold (*MAT* a, *trp*1–901, *leu*2–3, 112, *ura*3–52, *his*3–200, *gal*4Δ, *gal*80Δ, *LYS*2::*GAL*1$_{UAS}$-*Gal*1$_{TATA}$-*His*3, *GAL2*$_{UAS}$-*Gal2*$_{TATA}$-*Ade*2 *URA*3::*MEL*1$_{UAS}$-*Mel*1$_{TATA}$ *AUR*1-*C MEL*1) was obtained from Clontech. Y2HGold was grown in YPDA medium.

2.2. Cloning of the human mut gene

Full-length human *mut* cDNA from pmMUT (a kind gift from Dr. W. Fenton) [14] was the source of the human *mut* sequence. It was obtained by PCR using the Mut-for and Mut-rev primers (Table 1). The resulting 2154-bp amplicon, which lacked the mitochondrial leader sequence, was cloned into the *Sac*I and *Nco*I sites of the pRSETA expression vector (Invitrogen) to obtain pMCM-2.

2.3. Cloning of the human MMAA gene

Total mRNA was obtained from human leukocytes using the RNeasy Mini Kit (Qiagen). *MMAA* cDNA lacking a hypothetical mitochondrial leader sequence was obtained by RT-PCR using One Step RT-PCR (Qiagen) with the MMAAwl-for and MMAA-rev primers (Table 1). The resulting 1062-bp amplicon was cloned into the *Bam*HI and *Eco*RI sites of the pRSETA vector (Invitrogen) to obtain pMMAAwl.

Both cloned genes were sequenced to confirm that no changes were introduced during PCR by comparison with GenBank sequences Nos. NM_000255 and NM_172250.

2.4. Expression and purification of human MCM and MMAA proteins

pMCM-2 was transformed into the *Escherichia coli* strain BL21(DE3) pLysS and the transformation mixture was used to directly inoculate LB medium with ampicillin (100 μg mL^{-1}) and chloramphenicol (35 μg mL^{-1}). The bacteria were grown overnight at 37 °C. A 1.5 L fermentator containing 1 L of LB medium with ampicillin (100 μg mL^{-1}) was inoculated with this culture and incubated at 22 °C with shaking (200 rpm). When the culture reached an $OD_{600} \sim 1$, the temperature was decreased to 12 °C, the shaking was increased (350 rpm), the culture was fed with 10% glucose (1 mL h^{-1}), and the protein expression was induced with 1 mM IPTG (Sigma) for 41 h (manuscript in preparation). p-MMAAwl was transformed into the *E. coli* strain Rosetta(DE3), and the transformation mixture was used to directly inoculate LB medium with ampicillin (100 μg mL^{-1}) and chloramphenicol (50 μg mL^{-1}). The bacteria were grown overnight at 37 °C. This culture was used to inoculate 400 mL of LB with ampicillin (100 μg mL^{-1}) and chloramphenicol (50 μg mL^{-1}), which was incubated at 37 °C. When the culture reached an $OD_{600} \sim 0.6$, the temperature was decreased to 22 °C and protein expression was induced with 0.5 mM IPTG (Sigma) for 14 h.

The expressed proteins were purified by IMAC according to the manufacturer's instructions (Invitrogen); the buffers used to purify MCM contained 5 μM AdoCbl (Sigma). The protein purity was verified by SDS–PAGE and the concentrations were determined by the Bradford protein assay using BSA as a standard.

2.5. Western blot

His-tagged MCM and His-tagged MMAA were identified in western blots using the anti-HisG-AP antibody (Invitrogen) and detected with the BCIP/NBT substrate kit (Invitrogen) according to the manufacturer's instructions.

2.6. GTPase activity

GTPase activity was measured according to the method reported by Korotkova and Lidstrom [11] with minor modifications.

2.7. MCM activity

Activity of the pure recombinant enzyme activity was determined in 50 μL reaction mixture containing 0.24 mM methylmalonyl-CoA (Sigma), 5 μM AdoCbl (Sigma) in 200 mM Tris/100 mM phosphate buffer pH 7.5, and 3.2–6.2 ng of purified recombinant protein. Activity was stopped with dry ice/ethanol, and 20 μL samples were analyzed by reversed-phase HPLC system as described by Kikuchi et al. [15], with minor modifications. Enzyme activity is expressed as micromole succinyl-CoA min^{-1} mg^{-1} of protein.

2.8. Yeast two-hybrid system plasmid construct

The full-length *mut* cDNA was subcloned into pGBKT7 (Clontech) containing a GAL4 DNA-binding domain to obtain pGBKT7-MCM. *MMAA* cDNA was subcloned into pGADT7 (Clontech) containing GAL4 activation domain to obtain pGADT7-MMAA. Both plasmids were transfected into the yeast strain Y2HGold by using Yeastmaker Yeast Transformation System 2 (Clontech).

2.9. Yeast two-hybrid system

Yeast two-hybrid assay was performed by using the Matchmaker Gold Yeast Two-hybrid system (Clontech) with pGBKT7-MCM as a bait plasmid to screen MMAA protein fused with GAL4 activation domain according to the manufacturer's instructions. Competent Y2HGold cells were co-transformed with both plasmids and mixture was plated on SD/-leu/-trp/X-α-Gal (40 μg mL^{-1}). Control experiments were performed at the same time by co-transformation of different control plasmids as indicated in the manual (positive, negative, and nonspecific interactions). After incubation at 30 °C for 3–5 days, positive colonies were re-streacked on SD/-leu/-trp/X-α-Gal and on SD/-leu/-trp/-his/-ade/X-α-Gal in the presence of aureobasidin A (125 ng mL^{-1}).

Table 1
Primers used in the study.

Designation	Sequence (5′-3′)	Description
Mut-For	tacagcaacgagctcctacaccag	Insertion of *Sac*I restriction site at the beginning of the *mut* gene designed to eliminate the mitochondrial leader sequence
Mut-Rev	ccatggttatacagattgetgcttct	Insertion of *Nco*I restriction site at the end of the *mut* gene
MMAAwl-For	cttaaagagaggatcctgtgtacaaaca	Insertion of *Bam*HI restriction site at the beginning of the *MMAA* gene designed to eliminate the probable mitochondrial leader sequence
MMAA-Rev	tatacaggatgagaattcttagtctctgct	Insertion of *Eco*RI at the end of the *MMAA* gene

Underlined bases indicate restriction sites.

3. Results

3.1. Cloning, expression, and purification of human MCM and MMAA

Both *mut* and *MMAA* cDNAs were cloned and overexpressed in *E. coli* BL21(DE3) cells. For MCM expression, lowering the temperature to 12 °C after induction was necessary to achieve solubility and reach yields of 2.5 mg of protein per L. A temperature of 22 °C was used for MMAA expression. For pure MCM protein a single band with an apparent Mr of 80 KDa was obtained by SDS–PAGE (Fig. 1A), which was also recognized in the western blot (Fig. 1B). MMAA was also purified. Several bands were visualized by SDS–PAGE, although a major band of 40 KDa was observed (Fig. 1C). In the western blot, all the bands were identified as proteins containing histidine tags. These bands were probably degradation products (Fig. 1D) due to the instability of MMAA, which was also reported for *E. coli* YgfD, another MMAA ortholog. Likewise, MMAA precipitated in pure and semipure preparations [16]. The molecular weights for both proteins corresponded to the expected weights taking into account the lack of predicted mitochondrial leader sequences and the addition of His-tags.

3.2. MMAA enzymatic activity

As previously mentioned, amino acid sequence analysis of human MMAA has shown motifs suggesting GTPase activity. To confirm this activity, [α-^{32}P]GTP was used as the substrate of MMAA in the presence of $MgCl_2$; a time-dependent increase in GDP concentration was detected as result of the GTPase activity of MMAA as shown in Fig. 2A. Banerjee et al., reported that the GTPase activity of *M. extorquens* MeaB was enhanced 100-fold in the presence of bacterial MCM [12]. In our case, the addition of human MCM did not increase the GTPase activity of MMAA (Fig. 2B).

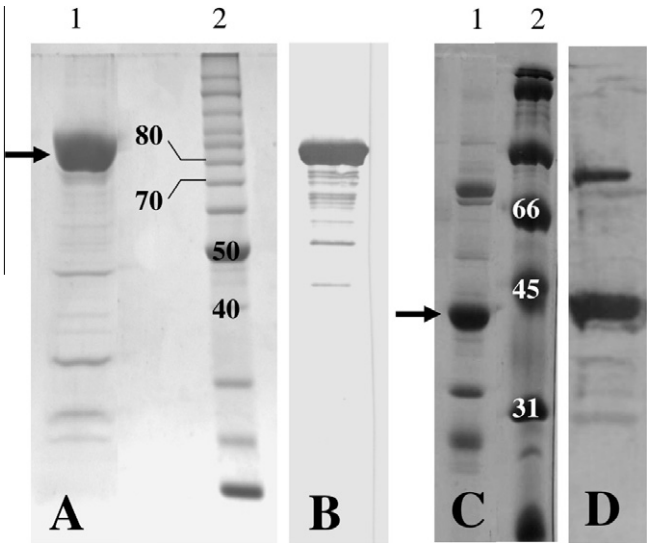

Fig. 1. Expression of human MCM and MMAA proteins. *E. coli* BL21(DE3)pLysS and *E. coli* Rosetta(DE3) cells transformed with pMCM-2 and pMMAAwl, respectively, were grown and induced as described in Section 2. The soluble fraction was prepared and separated on a 10% SDS–polyacrylamide gel, which was stained with Coomassie Brilliant Blue G-250. (A) Lane 1, MCM elution with 150 mM imidazol; lane 2, molecular weight marker. The migration position of MCM is indicated by an arrow. (B) Detection of His-tagged-MCM by western blot. Ten micrograms of enzyme purified by IMAC were electrophoresed, blotted and detected with anti-HisG-AP antibody (Invitrogen). (C) Lane 1, MMAA elution with 200 mM imidazol; lane 2, molecular weight marker. The migration position of MMAA is indicated by an arrow. (D) Detection of His-tagged-MMAA by western blot. Ten micrograms of pure enzyme were electrophoresed, blotted and detected with anti-HisG-AP antibody (Invitrogen).

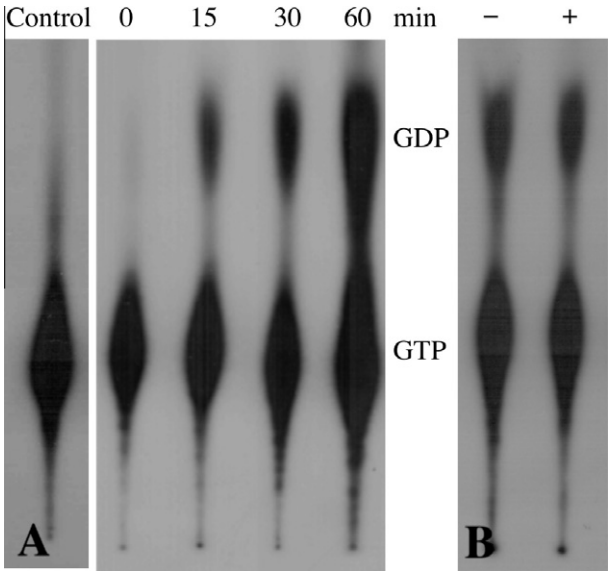

Fig. 2. TLC analysis of the products of GTP hydrolysis mediated by MMAA. (A) The reaction was performed at RT for the indicated periods of time in a reaction mixture containing (in a final volume of 25 μL): 16.5 μg MMAA, 10 μCi [α-^{32}P]GTP, 5 mM $MgCl_2$, 38.5 mM KCl, and 50 mM potassium phosphate buffer pH 7.5. Aliquots were chromatographed on poly(ethyleneimine)-cellulose F plates (Merck) with 1 M KH_2PO_4 pH 4.5 as the solvent. (B) The reaction was performed for 30 min in the presence (+) or absence (–) of MCM (2:1) under the same conditions.

3.3. MCM activity and the effect of MMAA

To determine if the purified recombinant human MCM protein was active, enzymatic activity was determined by quantifying methylmalonyl-CoA after separation from succinyl-CoA by HPLC. The results shown in Fig. 3 (inset) demonstrated that MCM activity with adenosylcobalamin increased linearly during the first 3–4 min. The rate of increase then slows down, probably due to oxidative inactivation of the enzyme. A specific activity of 21 μmoles mg^{-1} min^{-1} was obtained for the recombinant human mutase.

In 2002, Dobson et al. [7], found that the gene responsible for the *cblA* defect, a disorder associated with B_{12}-responsive methylmalonic aciduria, was *MMAA*. However, the role of this protein was not determined. For this purpose, MCM activity was determined in the presence of MMAA protein and GTP or GMPPNP, a non-hydrolyzable analog of GTP, to observe whether MMAA could prevent the inactivation of human MCM.

The addition of MMAA/GTP at the beginning of the reaction had no effect when the activity was measured for short time intervals (Fig. 3A, inset). However, differences were found after 60 min of reaction with MCM activity increasing to 76% with respect to the control without MMAA protein, probably due to interference with oxidative inactivation. The hydrolysis of GTP does not appear to be necessary because the positive effect was also obtained when GMPPNP was used instead of GTP (Fig. 3A). In contrast, when MMAA/GTP was added after 60 min of reaction with a certain level of inactivated MCM, its activity rapidly increased to the identical level obtained when MMAA was supplemented at 0 min. Surprisingly, MMAA alone or MMAA/GMPPNP had no effect on this condition (Fig. 3B).

3.4. Yeast two-hybrid screening of MCM-MMAA interaction

To confirm MCM and MMAA *in vivo* interaction, the full-length *mut* and *MMAA* cDNAs were cloned as a translational fusion of a GAL4 DNA-binding domain and GAL4 activation domain respectively. pGBKT7-MCM and pGADT7-MMAA was con-transformed

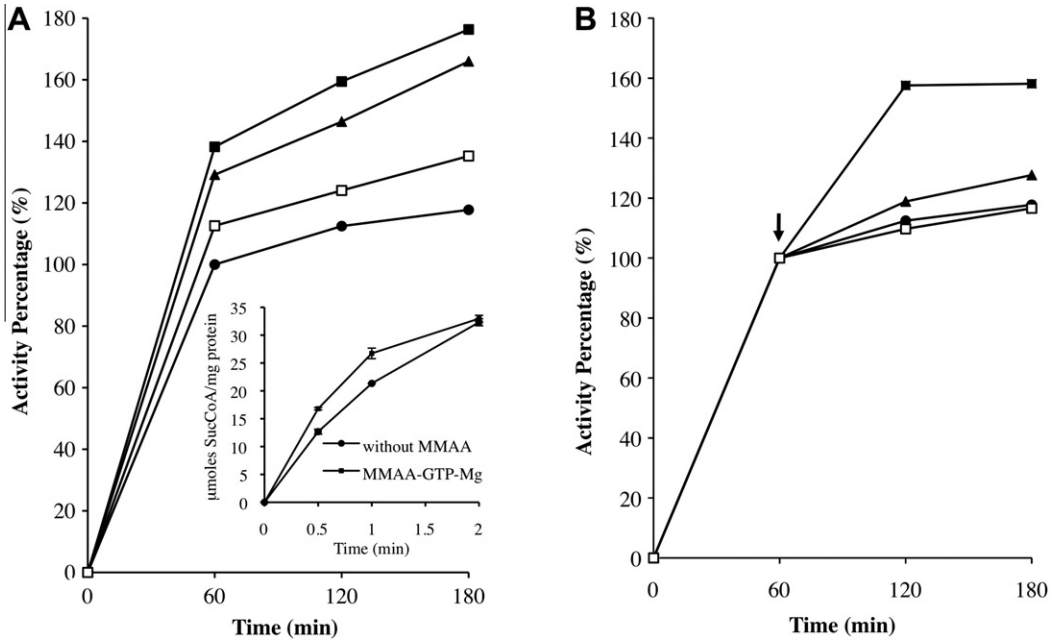

Fig. 3. Effect of addition of MMAA on MCM activity. MCM activity was recorded for long time periods of incubation of 6.5 ng of pure enzyme at 37 °C in a reaction mixture containing 0.24 mM methylmalonyl-CoA and 5 μM AdoCbl in 200 mM Tris/100 mM phosphate buffer pH 7.5 in the absence (●) and presence of 12.5 ng of pure MMAA alone (□) or with GTP (■) or GMPPNP (▲) added at T_0 (Panel A) or T_{60} min (Panel B). Activity was stopped with dry ice/ethanol and 20 μL samples were analyzed by HPLC. Inset: MCM activity at short time intervals in the absence (●) and presence (■) of MMAA-GTP.

into yeast Y2HGold strain and allowed to grow in SD/-leu/-trp/X-α-Gal along with controls. The interaction between these two proteins was measured by the reconstitution of a functional transcriptional activator that triggers the expression of four independent reporter genes (*AUR*1-*C*, *ADE*2, *HIS*3, and *MEL*1). As can be seen in Fig. 4A, colonies were obtained in SD/-trp/-leu/X-α-Gal medium as a result of the presence of both plasmids. As a result of the activation of the reporter gene (*MEL*1) due to interaction between MMAA and MCM proteins, blue colonies were obtained in the presence of X-α-Gal. A very pale blue color yeasts were observed in some controls, however growth in highest stringent conditions by the presence of aureobasidin A and selecting for the four reporter genes (SD/-leu/-trp/X-α-gal/AbA) confirmed the interaction between human MCM and MMAA (Fig. 4B).

4. Discussion

Like other adenosylcobalamin-dependent enzymes, human methylmalonyl-CoA mutase undergoes suicide inactivation during catalysis and the presence of a molecular chaperone that participates in the reactivation of MCM cannot be ruled out. Methylmalonic aciduria can result from mutations in either the *mut* or *MMAA* gene [7], indicating that the MMAA protein also plays an important role in the conversion of methylmalonyl-CoA to succinyl-CoA. To elucidate the function of MMAA, both human *mut* and *MMAA* cDNAs were cloned and expressed in *E. coli* and it was demonstrated that the pure recombinant proteins were enzymatically active. MCM activity was determined by a direct method and was comparable to another reported activity [14].

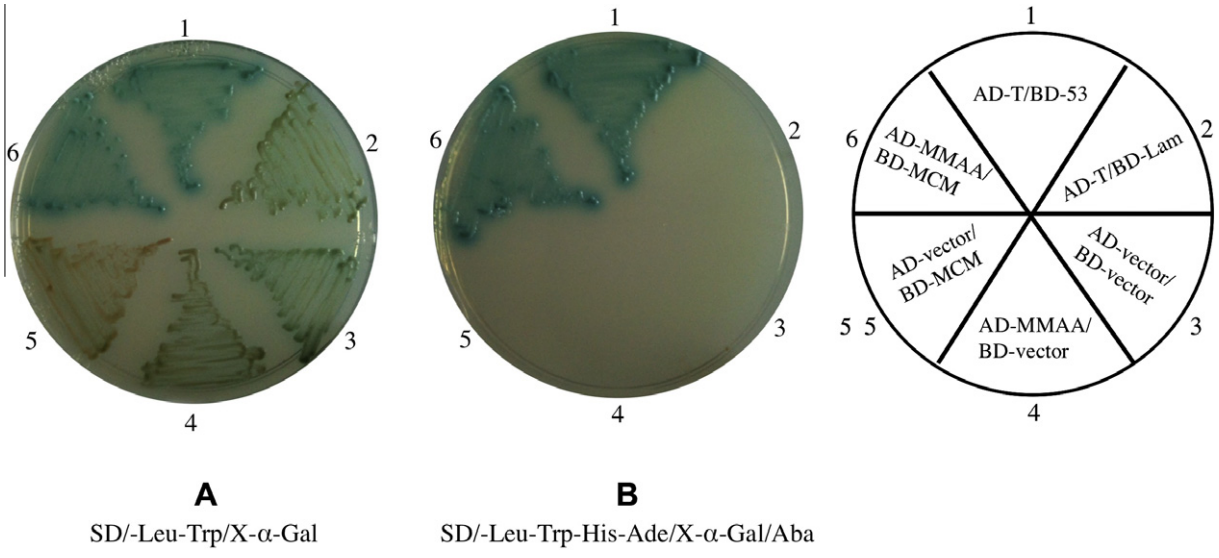

Fig. 4. *In vivo* association between human MCM and MMAA. The yeast strain Y2HGold was transformed with the indicated plasmids and grown on SD medium lacking Trp and Leu for 3–5 days at 30 °C. Each colony was re-streaked on SD medium lacking Trp, Leu, containing X-α-Gal (Panel A) and SD medium lacking Trp, Leu, Ade, His containing X-α-Gal and aureobasidin A (Panel B).

The performance of MMAA was established through GTPase activity; the recombinant protein lacking the leader sequence was active in the presence of GTP and Mg^{2+}. In contrast to the results obtained with *M. extorquens* MeaB, the addition of human MCM did not enhance MMAA activity.

The mutase activity-based data presented herein suggest that human MCM could suffer inactivation because the reaction velocity permanently slows like diol and glycerol dehydratases. Therefore, MMAA could act as a reactivating factor although no amino acid identity was found between this protein and DdR and GdR, the proteins that reactivate diol and glycerol dehydratases, respectively, in bacteria.

Results obtained from the addition of MMAA to the MCM reaction mixture at time zero or 60 min later showed a 70% of increase in activity, which could be due to the promotion of inactive cofactor exchange by MMAA and the restoration of MCM activity. However, MMAA did not require GTP hydrolysis when it was added at the beginning of the reaction, suggesting the protection of MCM instead reactivation. On the other hand, the positive effect of MMAA on mutase activity was not observed when GMPPNP was used and the inactivation was previously carried out. These results are similar to those reported by Korotkova and Lindstrom who found that the addition of MeaB considerably increased MCM activity and was not apparently involved in the release of the cofactor; GTP hydrolysis was not required for this activity [11].

On the contrary, inactivated diol and glycerol dehydratases were shown to be reactivated by their own reactivating factors through the ATP-dependent exchange of an enzyme-bound damaged cofactor for free AdoCbl. The addition of these reactivating factors led to an increase in dehydratase activity involving ATP hydrolysis [17,18], which is similar to the results obtained when MMAA was added at 60 min of the reaction. All these enzyme activity data strongly suggest stable complex formation between MCM and MMAA, however it was necessary the confirmation of the interaction in a physiological context. Using yeast two-hybrid system it was demonstrated interaction between these two proteins. Thus our data strongly suggest that MMAA interacted with MCM and plays two important roles as both the ''protectase'' and reactivase of MCM, which explains the existence of the *cblA* phenotype when its gene contains mutations.

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