PROBING THE COB(II)ALAMIN CONDUCTOR HYPOTHESIS WITH GLUTAMATE MUTASE FROM *CLOSTRIDIUM COCHLEARIUM*

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ABSTRACT

It had been proposed that during reversible coenzyme B_{12} dependent rearrangements, cob(II)alamin is not merely present as a spectator but also acts as a conductor by stabilizing the methylene radical intermediates. Density functional theory (DFT) calculations suggested a hydrogen bond between C_{19}-H of the corrin ring and the 3'-OH moiety (O3RL) of the 5'-deoxyadenosyl radical resulting in a decrease of the activation energy by about 30 kJ mol^{-1}. We tested this hypothesis with glutamate mutase and artificial coenzyme B_{12} derivatives. The assembly of coenzyme B_{12} (adenosylcobalamin) with recombinant components GlmS and GlmE of glutamate mutase from Clostridium cochlearium reconstitutes an active holoenzyme that catalyses the reversible rearrangement between (S)-glutamate and (2S,3S)-3-methylaspartate. Glutamate mutase activity was also demonstrated upon incubation of GlmS and E with 3',5'-dideoxyadenosylcobalamin, but not with 2',5'-dideoxyadenosylcobalamin and peptidoadenylcobalamin. In the latter cobalamin, the ribose unit of the upper ligand was replaced with a peptide mimic that contains the same number of atoms between Co(III) and the adenosine base. Measurements of the kinetic constants of glutamate mutase with coenzyme B_{12} and 3',5'-dideoxyadenosylcobalamin suggested similar binding properties of the cofactors to the apo-enzyme. However, the catalytic efficiency (k_{cat}/K_m) of glutamate mutase was 15 times reduced with 3',5'-dideoxyadenosylcobalamin compared to catalysis with coenzyme B_{12} as cofactor. This translates into a stabilization of only 7 kJ mol^{-1} in the substrate activation step. We attribute this effect to weak interactions of O3' of the riboise moiety with either C_{19}-H of the corrin ring or with the glutamate residue 330 of component E (Glu330). The catalytic inactivity of 2',5'-dideoxyadenosylcobalamin and peptidoadenylcobalamin reveals critical interactions of the 2'-OH moiety (O2') during the catalytic cycle. Evidence for H-bonding between O2' and Glu330 is obtained from the crystal structure analysis of glutamate mutase's active site.

Keywords: Methylene radical, carbon skeleton rearrangement, cob(II)alamin, glutamate mutase, dideoxyadenosylcobalamin

INTRODUCTION

All coenzyme B_{12} dependent enzymes, mutases as well as eliminases, catalyze an 1,2-exchange between hydrogen and an adjacent electron withdrawing group on the substrate molecule. In mutases the migrating moiety is a carbon fragment or an amino group, whereas in the eliminases either an amino or hydroxyl group exchanges with the vicinal hydrogen and is further irreversibly eliminated as ammonia or water. Glutamate mutase (EC 5.4.99.1) from various clostridium species catalyzes the first step of glutamate fermentation, in which (*S*)-glutamate (**1**, Scheme 1) reversibly rearranges to (2*S*,3*S*)-3-methylaspartate (**2**) (Buckel 2001). Nature uses this rearrangement to enable the removal of the amino group catalyzed by the subsequent enzyme methylaspartase to yield

mesaconate. Further hydration and cleavage affords acetate and pyruvate that is oxidized to CO_2 and acetyl-CoA by ferredoxin, which is regenerated by H_2 and butyrate synthesis.

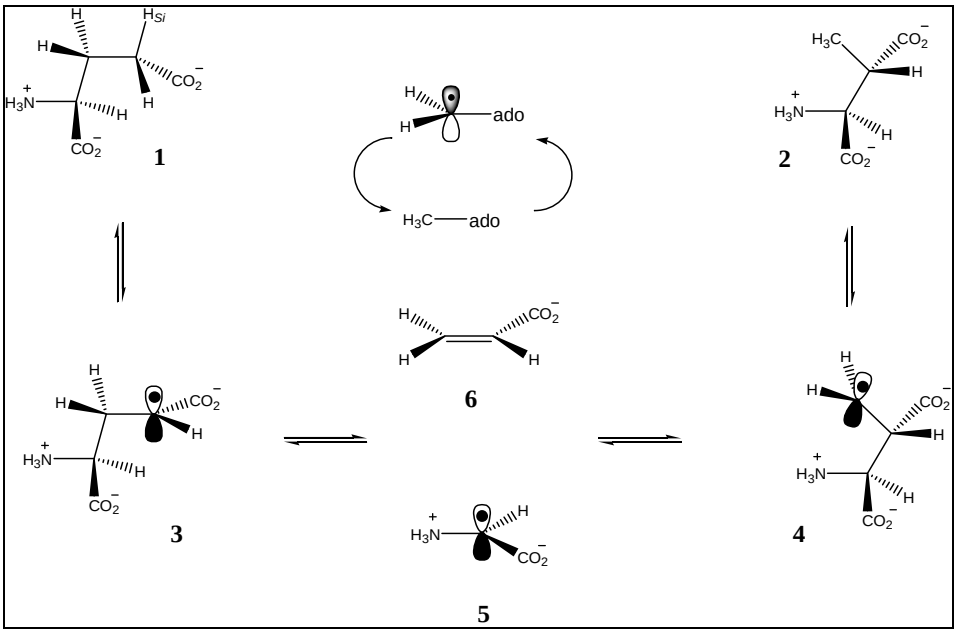
The enzyme from *Clostridium cochlearium* consists of two different protein components; E, a homodimer (ε_2, 2 x 53.5 kDa) and S, a monomer (σ, 14.8 kDa). Between the genes coding for E and S, an open reading frame had been detected, which codes for a putative chaperon L (λ, 50.2 kDa). While component L is not necessary for functional gene expression, glutamate mutase has been extensively studied by using components S and E overproduced in *E.coli* (Huhta *et al.* 2001, Huhta *et al.* 2002, Cheng and Marsh 2005, Patwardhan and Marsh 2007). Incubation of both components with two molecules of coenzyme B_{12} leads to the formation of an active holoenzyme ($\varepsilon_2\,\sigma_2$) (Reitzer *et al.* 1999). Like other mutases, the coenzyme is bound to the protein by cobalt coordination to a conserved histidine residue from component S which replaces dimethylbenzimidazole (base off, his on). Binding of substrate to hologlutamate mutase triggers homolysis of the coenzyme Co-C bond to yield the 5'-deoxyadenosyl radical (5'-DAR) and cob(II)alamin. 5'-DAR selectively abstracts the hydrogen from the inert C_4-H_{Si} bond on the substrate molecule to generate the substrate derived 4-glutamyl radical **3** which rearranges to the product related methylaspartate radical **4** by fragmentation-recombination (Buckel and Golding 1996). The proposed intermediates glycine radical **5** and acrylate (**6**) have been experimentally detected (Beatrix *et al.* 1995, Chih and Marsh 1999). The re-abstraction of hydrogen from 5'-deoxyadenosine by the product related radical **4** leads to the formation of the reaction product with regeneration of 5'-DAR that recombines with cob(II)alamin to coenzyme B_{12} (Scheme 1).

In the generally accepted mechanism of coenzyme B_{12}-depentent rearrangements, cob(II)alamin is thought to act just as a spectator that does not participate in the reaction. Recently, however, it has been proposed that cob(II)alamin acts as a conductor by stabilizing the intermediate methylene radicals during the rearrangement (Buckel *et al.* 2005). EPR studies of glutamate mutase reaction indicated that cob(II)alamin interacted with the substrate derived 4-glutamyl radical **3** at a distance of 6 Å (Bothe *et al.* 1998).

Furthermore, crystal structures of glutamate mutase showed that the 5'-deoxyadenosyl radical remained within 3-4 Å from Co^{2+} with substrate and product radicals approximately 3 Å further away. The crystal structures also reveal that after Co-C cleavage the ribose moiety of 5'-DAR suffers pseudorotation by which the radical bearing 5'-carbon moves towards the substrate to enable hydrogen abstraction. Thereby O3' approaches C_{19}-H of cob(II)alamin within hydrogen bonding distance, whereas O2' interacts with a conserved glutamate of component E (Glu330) in both conformations (Fig. 1) (Buckel *et al.* 2005, Gruber and Kratky 2002). The importance of ribose orientation for substrate activation in glutamate mutase reaction has also been studied by functional density theory which reported a stabilization of ca. 30 kJ mol^{-1} by a hydrogen bond between C_{19}-H and O3' (Kozlowski *et al.* 2007, Durbeej *et al.* 2009).

To better understand the role of cob(II)alamin in substrate activation by hydrogen abstraction in the glutamate mutase mechanism, we studied the reaction by using three structural derivatives of coenzyme B_{12} (**7**): 2',5'-dideoxyadenosylcobalamin (**8**), 3',5'-dideoxyadenosylcobalamin (**9**) and peptidoadenylcobalamin (**10**). In the latter

cobalamin, the ribose unit of the upper ligand was replaced with a peptide mimic that contains the same number of atoms between Co(III) and the adenosine base. In these cofactor derivatives no interactions of the enzyme-coenzyme complex with either O2' or O3' of ribose or with both oxygens are possible. In this paper we report on the impairments of the enzyme kinetics resulting from the replacement of coenzyme B_{12} by **8**, **9**, and **10**.



Scheme1: Proposed reversible fragmentation-recombination rearrangement mechanism for coenzyme B_{12} dependent glutamate mutase reaction. Homolysis of the Co-C bond of the coenzyme yields cob(II)alamin and the 5'-deoxyadenosine radical that abstracts $4H_{Si}$ from the substrate glutamate. The formed 4-glutamyl radical **(3)** fragments into the glycine radical **(5)** and acrylate (**6**) which recombine to the product related methylene aspartate radical **(4)**. This radical removes a hydrogen from adenosine to give methylaspartate (**2**) with regeneration of the 5'-deoxyadenosine radical that together with cob(II)alamin recycles coenzyme B_{12}

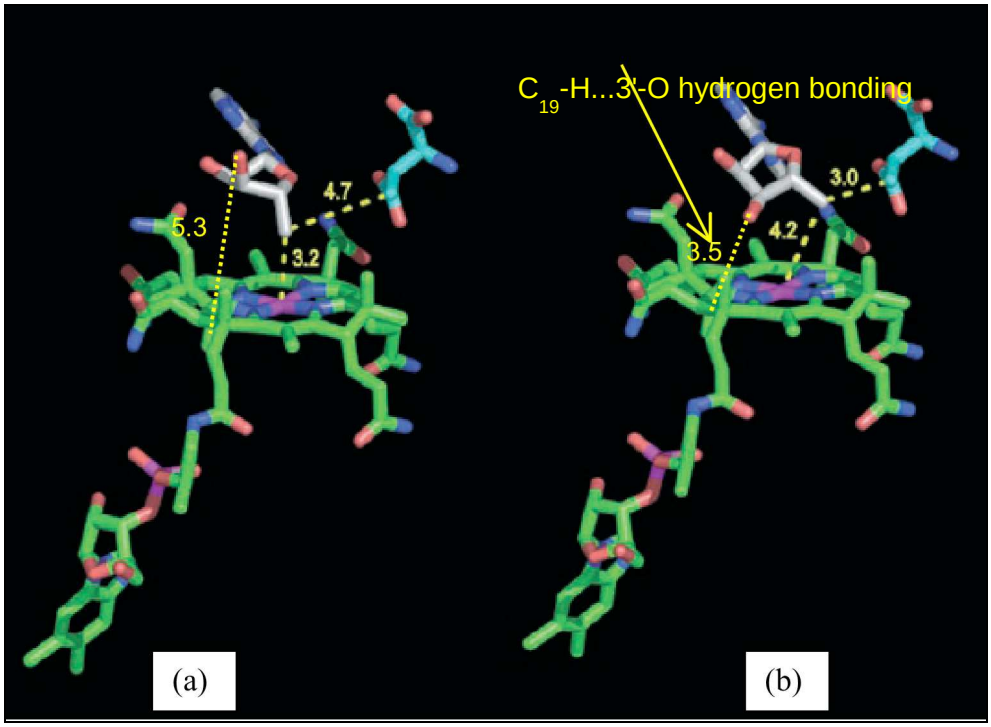


Figure 1: Crystal structure of coenzyme B_{12} bound to glutamate mutase after homolytic cleavage of the Co-C bond. (a) Ribose O3' points away from the corrin ring and (b) after pseudorotation of the ribose, which brings O3' and C_{19}-H from cob(II)alamin into hydrogen bonding distance.

RESULTS AND DISCUSSION

Synthesis of coenzyme B_{12} derivatives

In these studies we used derivatives **8**-**10** of coenzyme B_{12} (**7**). Compounds **8** and **9** were synthesized by procedures slightly modified from those described in the literature (Zagalak and Pawelkiewicz 1965, Jacobsen *et al.* 1979, Brown *et al.* 1998, Jensen and Halpern 1999, Gschösser *et al.* 2005). Compound **10** was obtained in a similar way from B_{12} and a chloroethane amine modified adenosine building block under reductive conditions. Detailed syntheses and characterizations of compounds **8**-**10** will be described elsewhere.

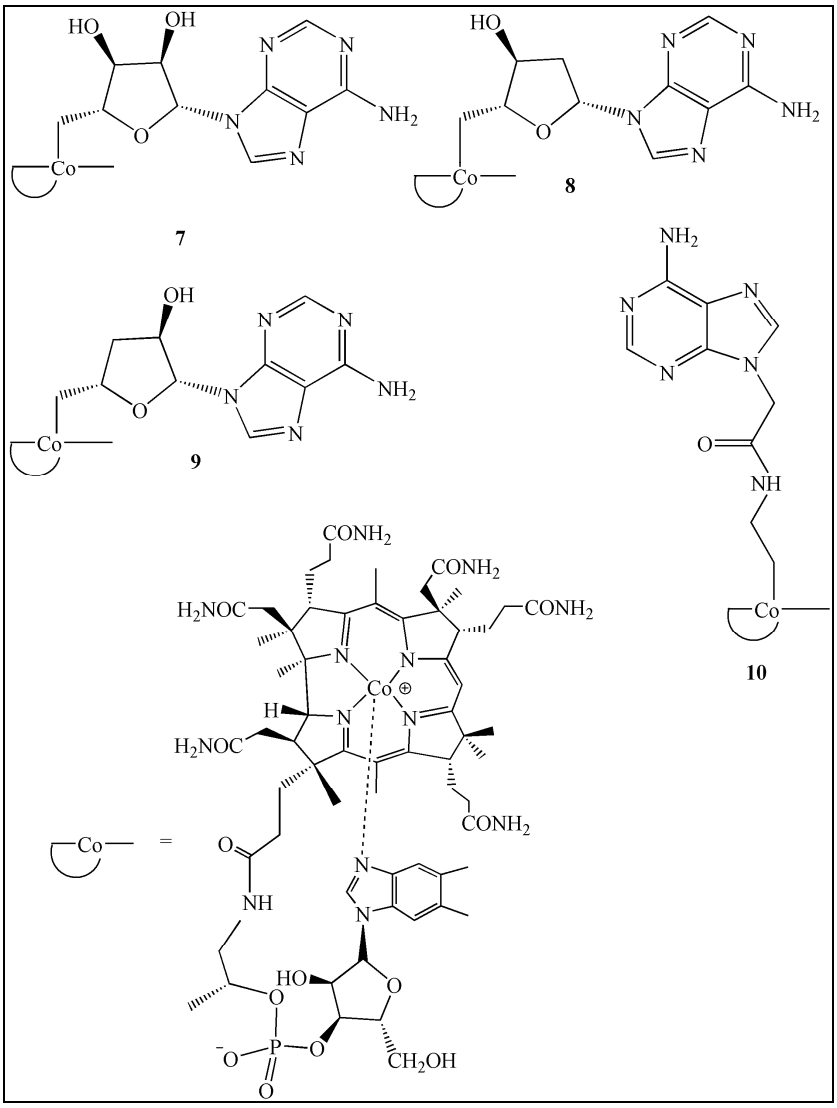


Figure 2: Coenzyme B_{12} (**7**), the natural cofactor of glutamate mutase and structural derivatives thereof; 2',5'-dideoxyadenosylcobalamin (**8**), 3',5'-dideoxyadenosylcobalamin (**9**) and peptidoadenylcobalamin (**10**).

Kinetic probing of the cofactor derivatives

The activity of glutamate mutase was determined in a standard coupled assay using the auxiliary enzyme methylaspartase for further conversion of the produced methylaspartate to mesaconate which absorbs at 240 nm (Barker *et al.* 1964). With coenzyme B_{12} as a cofactor being the control, we measured a 15 times reduced catalytic efficiency with 3',5'-dideoxyadenosylcobalamin (**9**) using a 14-

fold excess of component S over E (GlmS: GlmE 14) (Table 1). Because of the similar K_m values for coenzyme B_{12} (**7**) and **9** which suggested an equal affinity of the cofactors to the enzyme, the reduction of the rate has been attributed to the ribose O3' interactions. These results have primarily indicated that the 3'OH of ribose is a useful functional group for rate enhancement in coenzyme B_{12} dependent glutamate mutase. Furthermore, the difference of the catalytic efficiencies between the two cofactor reactions has revealed that the 3'OH interactions stabilize the 5' DAR during substrate activation by only 7 kJ mol^{-1} rather than by 30 kJ mol^{-1} as derived from density functional theory calculations.

Table 1: Kinetic constants of coenzyme B_{12} (**7**) and 3',5'-dideoxyadenosylcobalamin (**9**) in the reaction catalyzed by glutamate mutase from *Clostridium cochlearium*. All measurements were taken in a 14-fold excess of component S over E.

	Coenzyme B_{12} (5'-deoxyadenosylcobalamin)	**3',5'-Dideoxyadenosylcobalamin (9)**
K_m	0.52 ± 0.06 μM	0.56 ± 0.02 μM
k_{cat}	1.24 ± 0.36 s^{-1}	0.089 ± 0.01 s^{-1}
$k_{cat} K_m^{-1}$	2.38×10^6 s^{-1} M^{-1}	1.59 x 10^5 s^{-1} M^{-1}

Based on the analysis of the enzyme active site structure, we suggest a stabilization of the substrate activation step by a weak hydrogen bond between O3' with either C_{19}-H of cob(II)alamin or the active site glutamate 330 (Fig. 3). These hydrogen bonding interactions may facilitate ribose pseudorotation which brings the 5'-DAR closer to the hydrogen to be abstracted from the substrate molecule.

Glutamate mutase activity was not detected when the enzyme was reconstituted with 2',5'-dideoxyadenosylcobalamin (**8**) or peptidoadenylcobalamin (**10**) in separate assays. The consecutive addition of coenzyme B_{12} to these inactive glutamate mutase complexes could not recover the enzyme activity. Similarly to these cobalamin derivatives, also hydroxocobalamin formed an inactive glutamate mutase complex, which led to the suggestion that the adenosyl group has a minor role in binding of the cofactor to the enzyme. The catalytic inactivity of **8** and **10** demonstrates a critical stabilization of the 5'-DAR due to ribose O2' interactions. This view is supported by analysis of the enzyme's active site structure that predicts 2'-OH hydrogen bonding with glutamate 330 before and after Co-C cleavage. In agreement with these observations, the activity of methylmalonyl-CoA mutase in complex with 2',5'-dideoxyadenosylcobalamin (**8**) was reduced to 1% and no substrate-induced homolysis of the cofactor Co-C bond was observed by UV-vis spectroscopy (Calafat *et al.* 1995). (Notably, by this method 1% homolysis cannot be detected.) This result indicates that homolysis may be driven by the substrate-induced pseudorotation, whereby the 2'-OH acts as hinge.

From these kinetic probings of the glutamate mutase reaction by using coenzyme B_{12} derivatives, we conclude that Co-C homolysis and hydrogen abstraction from glutamate or methylaspartate are stabilized by O2' and O3' interactions. C_{19}-H of cob(II)alamin may contribute to a weak stabilization of the 5'-DAR through hydrogen bonding interaction with ribose O3'.

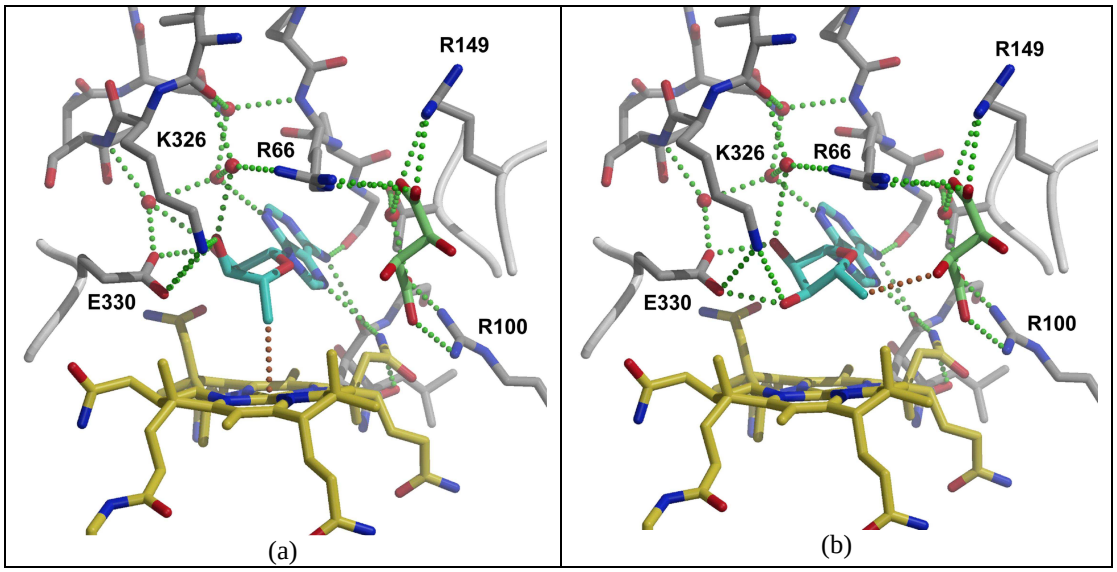


Figure 3: Crystal structures of the active site of glutamate mutase after Co-C-bond homolysis. (a) Before and (b) after pseudorotation, whereby the 5' DAR gets close to the 3-methylaspartate analogue tartrate fixed by three arginines (red dotted lines). Note the ribose pseudorotates to the orientation in which O3' fits into hydrogen bonding distance to both C_{19}-H and E330. O2' interacts within hydrogen bonding distance with E330 before and after pseudorotation. Adapted from Gruber and Kratky (2002).

EXPERIMENTAL SECTION

Material and Methods

Coenzyme B_{12} (**7**) and 2-Mercaptoethanol were purchased from Sigma-Aldrich. Methylaspartase and both recombinant glutamate mutase components S and E were prepared as previously described (Barker *et al.* 1959, Zerdel *et al.* 1994).[22, 23]

Assays for glutamate mutase kinetic studies

Hologlutamate mutase was prepared from glutamate mutase components S, E and coenzyme B_{12}. All kinetic constants were measured at 37 °C by using standard methylaspartase coupled glutamate mutase assay in 50 mM Tris/HCl pH 8.3 and 0.05 mM mercaptoethanol. Coenzyme B_{12} and its structural derivatives were throughout protected from light and measurements were taken under red light. Enzymes were partially purified to the indicated specific activities: methylaspartase (43.5 U/mg), GlmS (66 U/mg), and GlmE (18.1 U/mg). K_m and V_{max} for the natural cofactor were determined in the presence of 5 µg GlmS, 2.6 µg GlmE, 36 µg methylaspartase, 20 mM glutamic acid and 0.32 – 25 µM coenzyme B_{12}.

K_m and V_{max} for the cobalamin derivative were determined with 10-times increased amounts of components S and E. The concentration of 3',5'-dideoxyadenosylcobalamin was varied between 0.35 µM and 70 µM. K_m and V_{max} were obtained from the Michaelis-Menten equation. The calculations of k_{cat} for both coenzyme B_{12} and 3',5'-dideoxyadenosylcobalamin were based on component E.

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