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Kinetic Studies of a Coenzyme B_{12} Dependent Reaction Catalyzed by Glutamate Mutase from *Clostridium cochlearium*

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Abstract

The coenzyme B_{12} dependent glutamate mutase is composed of two apoenzyme proteins subunits; S and E_2, which while either fused or separate assemble with coenzyme B_{12} to form an active holoenzyme (E_2S_2-B_{12}) for catalyzing the reversible isomerization between (*S*)-glutamate and (2*S*, 3*S*)-3-methylaspartate. In order to assay the activity of glutamate mutase by UV spectrophotometry, this reaction is often coupled with methylaspartase which deaminates (2*S*, 3*S*)-3-methylaspartate to form mesaconate (λ_{max} = 240 nm, ε_{240} = 3.8 $mM^{-1}\cdot cm^{-1}$). The activities of different reconstitutions of glutamate mutase from separate apoenzyme components S and E in varied amounts of coenzyme B_{12} and adenosylpeptide B_{12} as cofactors were measured by this assay and used to reveal the binding properties of the cofactor by the Michaelis-Menten Method. The values of K_m for coenzyme B_{12} in due to reconstitutions of holoenzyme in 2, 7 and 14 S: E were determined as; 1.12 ± 0.04 μM, 0.7 ± 0.05 μM and 0.52 ± 0.06 μM, respectively, so as those of adenosylpeptide B_{12}; 1.07 ± 0.04 μM and 0.35 ± 0.05 μM as obtained from respective 2 and 14 S: E compositions of holoenzyme. Analysis of these kinetics results curiously associates the increasing affinity of cofactors to apoenzyme with increased amount of component S used in reconstituting holoenzyme from separate apoenzyme components and cofactor. Moreover, in these studies a new method for assaying the activity of glutamate mutase was developed, whereby glutamate mutase activity is measured via depletion of NADH (λ_{max} = 340 nm, ε_{340} = 6.3 $mM^{-1}\cdot cm^{-1}$) as determined by UV spectrophotometry after addition of (2*S*, 3*S*)-3-methylaspartate and pyruvate to a mixture of E_2S_2-B_{12} and two auxiliary holoenzymes system; pyridoxal-5-phosphate dependent glutamate-pyruvate aminotransferase and NADH dependent (*R*)-2-hydroxyglutarate dehydroge-

nase. The activity of glutamate-pyruvate aminotransferase was relatively complete recovered upon the addition of (*S*)-glutamate and pyruvate to the mixtures of hologlutamate-pyruvate aminotransferase and (*R*)-2-hydroxylglutarate dehydrogenase which were incubated with each putative inhibitor of glutamate mutase. Additionally, the new assay was used to determine the kinetic constants of (2*S*, 3*S*)-3-methylaspartate in the reaction of glutamate mutase as K_m= 7 ± 0.07 mM and k_{cat}= 0.54 ± 0.6 s^{-1}. Application of Briggs-Haldane formula allowed the calculation of an equilibrium constant of the reversible isomerization, K_{eq} = [(*S*)-glutamate] × [(2*S*, 3*S*)-3-methylaspartate]$^{-1}$ = 16, where the kinetic constants of (*S*)-glutamate were determined by the standard methylaspartase coupled assay.

Keywords

Coenzyme B_{12}, Adenosylpeptide B_{12}, Glutamate Mutase, (*S*)-Glutamate, (2*S*, 3*S*)-3-Methylaspartate, Methylasparatase

1. Introduction

Glutamate mutase (EC 5.4.99.1) is among the utterly known members of a coenzyme B_{12} (**1**) dependent class of enzymes. This class comprises both mutases and eliminases subtypes of enzymes which use coenzyme B_{12} (**1**) as a cofactor to catalyze vicinal exchange between a hydrogen atom and a carbon skeleton [1] or an electron withdrawing functional group [2] [3] on the molecules of their substrates, where the eliminases achieve further removal of a migrating group as water or NH_3. Glutamate mutase was first described in association with vitamin B_{12} (**2**) by Horace Albert Barker and his co-workers in 1959 owing to the requirement of coenzyme B_{12} (**1**) as a cofactor in the reaction it catalyzes [4] (**Figure 1**). Specifically, glutamate mutase catalyzes the reversible isomerization between (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**) (**Scheme 1**) which form a first metabolic step of (*S*)-glutamate (**4**) fermentation to butyrate, acetate, NH_3, CO_2 and H_2 by *Clostridia*, consequently their anaerobic growth on (*S*)-glutamate (**4**) [5] [6].

The apo-glutamate mutase is composed of two proteins subunits; S, a monomer (σ, 14.8 kDa) and E_2, a homodimer (ε_2, 2 × 53.5 kDa). These proteins domains are encoded by structural genes which are found in operons where they are organized in a reading frame prototype sequence; *glmS*/*glmL*/*glmE* and *mutS*/*mutL*/*mutE* as determined in the genomes of *C. cochlearium* and *C. tetarnomorphum*, respectively. Although a gene encoding a putative chaperone L is detected between those for domains S and E constantly, [7] [8] the component L is not obligatory for the functional expression of the enzyme. As a result, glutamate mutase has been extensively studied by using recombinant components S and E which assemble with coenzyme B_{12} (**1**) *in vitro* to form an active heterotetramer holoenzyme (E_2S_2-B_{12}).

Varieties of study tactics which used different techniques toward understanding

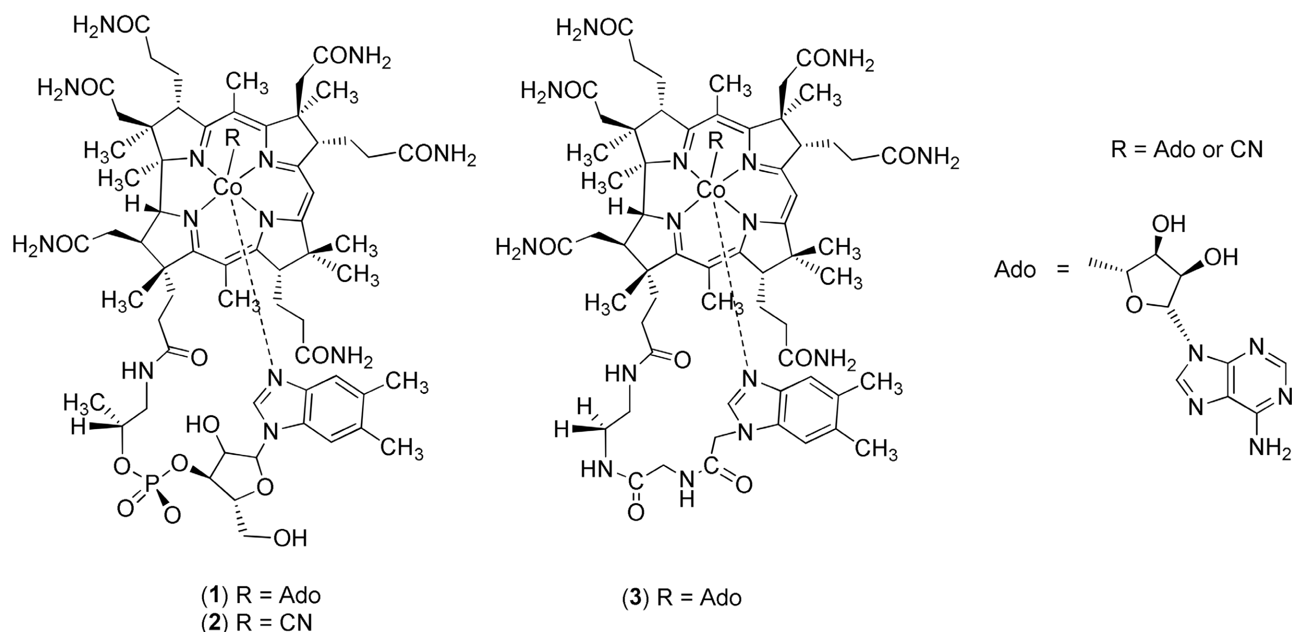
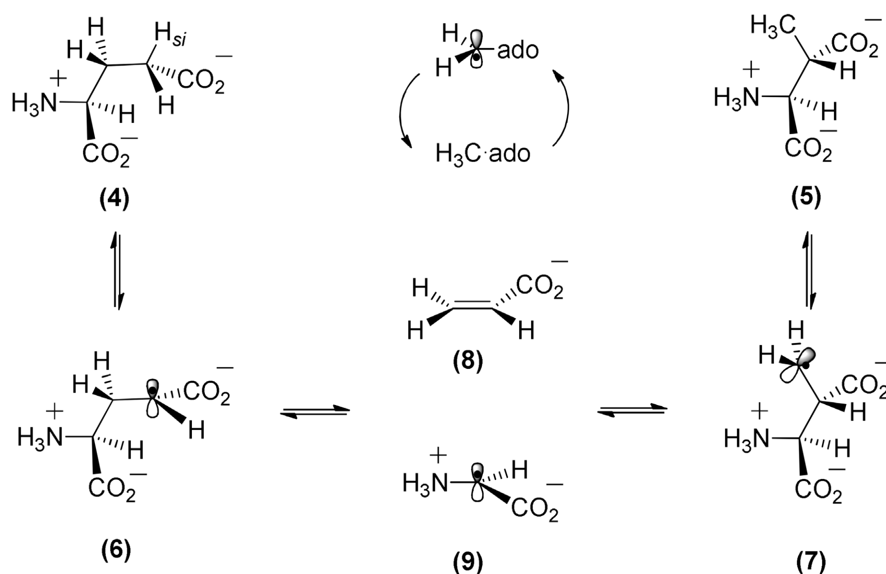


Figure 1. Structures of cobalamins; coenzyme B_{12} (adenosylcobalamin) (**1**), vitamin B_{12} (cynocobalamin) (**2**) and adenosylpeptide B_{12} (**3**).

the mechanism of catalysis by glutamate mutase have mostly produced converging results. Particularly incredible include the determinations of intermediates (*S*)-glutamate (**4**) derived 4-glutamyl radical (**6**) by EPR spectroscopy, so as similar attempts for the apparent (2*S*, 3*S*)-3-methylasparate (**5**) related (2*S*, 3*S*)-3-methyleneaspartate radical (**7**) [9] as well as an acrylate (**8**) which was identified by HPLC [10] [11]. The latter compound is hypothesized to be both; formed along with yet to be identified glycinyl radical (**9**) as a result of fragmentation of either of the former radicals, and allows the reversible isomerization by recombining with the putative glycinyl radical (**9**) to form any of the former radicals [10] [11] [12] (**Scheme 1**). Results from the investigations into the mechanism of glutamate mutase by mutations of specific amino acids residues of the enzyme proteins along with determinations of concomitant impairments in the kinetics have vividly complemented the insights gained from other studies, [13] [14] particularly the enzyme crystal structures which have provided substantial understanding on the mechanism of glutamate mutase. Explicit from the crystal structures include the binding of coenzyme B_{12} (**1**) to apoenzyme by "base off, his on" where the cofactor's DimethylBenzylImidazole (DMBI) coordinate to cobalt is substituted by imidazole of His 16 from domain S as well as binding of substrates; (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**) by hydrogen bonding interactions of carboxylates of the substrates molecules with guanidine bases of Arg 66, Arg 100 and Arg 149 from domain E [12] [15] [16]. While the binding of coenzyme B_{12} (**1**) to S, so as that of (*S*)-glutamate (**4**) to E were demonstrated by mutational studies as revealed in the crystal structures, [14] [17] the conjectured role of displaced lower ligand phospho-*α*-D-ribofuranosyl-5, 6-dimethylbenzylimidazole in enhancing the homolytic cleavage of

Co(III)-C σ bond has also been investigated by several methods which culminated to assenting results [18] [19]. Recently reported decrease of k_{cat} as well as $k_{cat} \cdot K_m^{-1}$ by 10 folds due to reconstitution of hologlutamate mutase with adenosylpeptide B_{12} (**3**) relative to coenzyme B_{12} (**1**) has also implied the conformational changes by the phospho-α-D-ribofuranosyl-5, 6-dimethyl-benzylimida-zole while buried in the protein hydrophobic pocket enhance the cleavage of Co(III)-C σ bond [20]. The enzyme crystal structures also disclose the mechanism of re-orientation of ribose moiety of the cofactor upper ligand by pseudorotation which aid the homolytic cleavage of the Co(III)-C σ bond and direct the primary organic radical, *i.e.* 5'-deoxyadenosyl radical towards the activation of substrate during catalysis [12] [15]. This phenomenon was further studied both computationally by Density Functional Theory (DFT) calculations [21] and various experimental approaches [22]. However, results of the former which implied critical stabilization of substrate activation step by hydrogen bonding interactions between 3'OH (O3RL) of cofactor upper ligand ribose and C_{19}-H of the corrin was challenged by the latter which suggested weak hydrogen bonding interactions by the same atoms based on analysis of both X-ray crystal structures and NMR spectra of diastereoisomers (*R*) and (*S*)-2, 3-dihydroxypropylcobalamins which were used as modals of coenzyme B_{12} (**1**) [22]. The experimental studies went even further to disprove the DFT results on the extent of O3RL role by demonstrating both inability of C_{19}-H to exchange with deuterium when the holoenzyme was incubated with D_2O under all pH conditions as well as cobalt oxidation states and 15 folds decrease in enzyme $k_{cat} \cdot K_m^{-1}$ due to reconstitution of hologlutamate mutase without O3RL [22] [23]. This decrease in enzyme $k_{cat} \cdot K_m^{-1}$ is translated to 7 $kJ \cdot mol^{-1}$, which is the extent of stabilization of substrate activation step contributed by O3RL interactions by hydrogen bonding with C_{19}-H. Nevertheless, both DFT and experimental studies concluded the 2'OH (O2RL) and O3RL of the cofactor upper ligand ribose interact with E330 active site residue and C_{19}-H of corrin by hydrogen bonding to promote the homolytic cleavage of Co(III)-C σ bond and steer 5'-deoxyadenosyl radical to a substrate which is located 6 ˚A away from the cobalt. These results conform to the understandings gathered from the crystal structures which reveal the same hydrogen bonding interactions necessary for the flipping of the ribose moiety that enables substrate activation by 5'-deoxyadenosyl radical after the homolytic cleavage of cofactor Co(III)-C σ bond [12] [15] [22] [23]. The 5'-deoxyadenosyl radical is tight guided by the hydrogen bonding interactions toward the *stereo*-specific abstraction of *pro S* H_{si} on C_4 of (*S*)-glutamate (**4**) or any of the hydrogen atoms on the methyl group of (2*S*, 3*S*)-3-methylaspartate (**5**), thereby switching to the radical mechanism that attain the isomerization [10] [11] [12] (**Scheme 1**). The migration of a hydrogen atom to a vinyl carbon atom on the substrate occurs inter-molecularly via 5'-deoxyadenosine along the intra-molecular migration of acrylate (**8**) to give the isomer product while cob(II)alamin is mechanistically implicated with stabilizations of the intermediate radicals including the primary organic radical before regeneration of the cofactor [24]. The identified 4-glutamyl (**6**) and apparent



Scheme 1. Proposed fragmentation-recombination mechanism of glutamate mutase catalyzed reversible isomerization between (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**). Homolytic cleavage of Co(III)-C σ bond of the cofactor results into cob(II)alamin and the primary organic radical; 5'-deoxyadenosyl radical. Depending on the direction of reaction, 5'-deoxyadenosyl radical *stereo*-specifically abstracts H_{si} on C4 of (*S*)-glutamate (**4**) or any of the hydrogen atom on the methyl group of (2*S*, 3*S*)-3-methylasparate (**5**) to form the respective intermediates 4-glutamly (**6**) and (2*S*, 3*S*)-3-methyleneaspartate (**7**) radicals. These radicals isomerize reversibly; apparently via glycinyl radical (**9**) and acrylate (**8**) before abstracting the hydrogen atom from methyl group of 5'-deoxyladenosine to form the isomer product with the regeneration of coenzyme B_{12} (**1**).

(2*S*, 3*S*)-3-methyleneaspartate (**7**) radicals define the minimal mechanistic scheme which is common to all coenzyme B_{12} (**1**) dependent reactions except those which are catalyzed by Ribonucleotide Reductases class II (RNRs-II) family of coenzyme B_{12} (**1**) dependent enzymes [25] [26].

Likewise any other enzyme, essential for investigating the mechanism of catalysis by glutamate mutase is assaying of its enzymatic activity. The present method of assaying activity of glutamate mutase which was developed in early 1960s uses methylaspartase as an auxiliary enzyme in order to convert (2*S*, 3*S*)-3-methylaspartate (**5**) produced by glutamate mutase to mesaconate. The latter compound is UV active, λ_{max} = 240 nm, ε_{240} = 3.8 $mM^{-1} \cdot cm^{-1}$, hence allows assay of glutamate mutase activity by UV spectrophotometry [27]. This standard methylaspartase coupled assay has been crucial to most mechanistic studies of glutamate mutase, serving from the identifications of fractions containing enzyme proteins during purification of glutamate mutase proteins to kinetic measurements including impaired kinetics in mutational [17] [14] and Structure Activity Relationship (SAR) studies of glutamate mutase [20] [22]. However, the standard methylaspartase coupled assay is limited towards assaying the activity of glutamate mutase in presence of any compound which inhibits glutamate mutase competitively, as such a compound is likely to inhibit the auxiliary methylaspartase too. The standard methylaspartase coupled assay is therefore impotent

in assaying activity of glutamate mutase for purpose of resolving inhibition kinetics. This paper reports the recent development of a new method for assaying activity of glutamate mutase catalyzed conversion of (2*S*, 3*S*)-3-methylaspartate (**5**) to (*S*)-glutamate (**4**) by UV spectrophotometry. The developed assay employs two auxiliary enzymes; the pyridoxal-5-phosphate dependent glutamate-pyruvate aminotransferase and NADH dependent (*R*) 2-hydroxylglutarate dehydrogenase. The paper further describes the suitability of the developed method of activity assay in measurements of glutamate mutase activity in presence of its hypothetical inhibitors; *cis*-glutaconate (**10**), *trans*-glutaconate (**11**), buta-1, 3-diene-2, 3-dicarboxylate (**12**), fumarate (**13**), maleate (**14**) and itaconate (**15**). These compounds (**11** - **15**) were designated to inhibit glutamate mutase competitively, hence appropriate for use in the proposed mechanism based inhibition study of glutamate mutase. Also reported are the kinetic constants of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction catalyzed by glutamate mutase as determined by the new method of the enzyme activity assay and equilibrium constant of the reversible isomerization which is calculated from the kinetic constants of (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase. An illustration of the dependence of affinity of cofactor to apoenzyme on the proportion of proteins components S and E used in reconstituting hologlutamate mutase as gathered from the kinetic constants of the cofactors in the reaction of glutamate mutase in different reconstitutions of holoenzyme from S, E and coenzyme B_{12} (**1**) or adenosylpeptide B_{12} (**3**) is also discussed.

2. Materials and Methods

2.1. General

Partial purified methylaspartase (44 $U \cdot mg^{-1}$, 25% yield, 3.2 enriched) was prepared from the cell-free extracts of *Clostridium tetanomorphum* by following a published procedure [28]. Likewise, as reported in literatures, pOZ3 and pOZ5 vector plasmids with *glmS* and *glmE* inserts, respectively, both from *Clostridium cochlearium* were prepared. Each plasmid construct was used to transform *E. coli DH*5*α* cells. The partial purified recombinant S (66 $U \cdot mg^{-1}$, 65% yield, 51 enriched) apo-enzyme protein component of glutamate mutase was finally prepared after being over-expressed in *E. coli* DH5α cells that carried pOZ3 plasmid construct. Similarly, the partial purified component E (18 $U \cdot mg^{-1}$, 58% yield, 38 enriched) of glutamate mutase was prepared after being over-expressed in *E. coli DH*5 *α* cells that carried pOZ5 plasmid construct [7] [29]. The specific activity of S was measured in excess of E, so as that of E which was measured in excess of S. The PACYCDuet™-1 vector plasmid with (*R*)-2-hydroxylglutarate dehydrogenase encoding *hgdH* insert was obtained from Dr. Ivana Djurdjevic, University of Marburg, and used in the production of recombinant pure (*R*)-2-hydroxylglutarate dehydrogenase (1 $kU \cdot mg^{-1}$, 8 enriched) which was over-expressed in *E. coli* BL21, whereas pure glutamate-pyruvate aminotransferase (81 $U \cdot mg^{-1}$) was

purchased from Sigma-Aldrich. Adenosylpeptide B_{12} (**3**) cofactor was a gift from Prof. Felix Zelder, University of Zurich, while *cis*-glutaconic acid (**10**) and buta-1, 3-diene-2, 3-dicarboxylic acid (**12**) were synthesized by following the literature reported procedures [30]. Other chemicals and biochemical used in these studies were obtained from reliable sources. The light sensitive cofactors; coenzyme B_{12} (**1**), adenosylpeptide B_{12} (**3**) and pyridoxal-5-phosphate were throughout protected from light and all measurements involving them were under red light. The activity of glutamate mutase catalyzed conversion of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) were assayed in varied amount of cofactors; coenzyme B_{12} (**1**) or adenosylpeptide B_{12} (**3**) in order to determine the kinetic constants of each cofactor, so as varied amount of (*S*)-glutamate (**4**) while determining the kinetic constants of (*S*)-glutamate (**4**). Values of initials rates of reaction corresponding to specified amount of cofactor; coenzyme B_{12} (**1**) or adenosylpeptide B_{12} (**3**), as well as (*S*)-glutamate (**4**) were throughout measured by a standard methylaspartase coupled assay. Kinetic constants of (*S*)-glutamate (**4**), coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) in the reaction catalyzed by glutamate mutase were determined by Michaelis-Menten method. A new developed pyridoxal-5-phosphate dependent glutamate-pyruvate aminotransferase and NADH dependent (*R*) 2-hydroxylglutarate dehydrogenase coupled assay of glutamate mutase activity was used to measure the values of initial rates of conversions of (2*S*, 3*S*)-3-methylaspartate(**5**) to (*S*)-glutamate (**4**) by glutamate mutase in various amount of (2*S*, 3*S*)-3-methylaspartate (**5**). The kinetic constants of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reverse reaction of glutamate mutase was also determined by Michaelis-Menten method. In all measurements of glutamate mutase activity, the amount of cofactor; coenzyme B_{12} (**1**) or adenosylpeptide B_{12} (**3**) were not limiting the reaction. The auxiliary enzymes systems; methylaspartase, as well as hologlutamatepyruvate aminotransferase and holo (*R*)-2-hydroxylglutarate dehydrogenase were also not limiting glutamate mutase throughout the measurements. The values of k_{cat} for glutamate mutase were calculated from the amount of component E used in the reconstitution of holoenzyme.

2.2. Assays for Determinations of Kinetic Constants of the Cofactors

Hologlutamate mutase was throughout reconstituted from the partial purified S and E recombinant proteins and cofactor in Tris/HCl (50 mM, pH 8.3) and mercaptoethanol (0.05 mM) at 37˚C. K_M of each cofactor and V_{max} of the reactions in 14, 7 and 2 folds excess of S were obtained from the series of assays which the holoenzyme was reconstituted by S (5 µg): E (2.5 µg), S (2.5 µg): E (2.5 µg) and S (0.15 µg): E (2.5 µg), respectively. The partial purified methylaspartase (36 µg) was also included in all assays mixtures. During the series of assays which the amount of coenzyme B_{12} (**1**) was varied (0.32 µM - 25 µM), each reaction was started by addition of (*S*)-glutamate (**4**) (20 mM) to the assay mixture prior to the determination of glutamate mutase activity at 240 nm by UV spec-

trophotometry. Likewise, the kinetic constants of adenosylpeptide B_{12} (**3**) were measured in 14 and 7 folds excess of S with relative 10 folds increased amount of each apoenzyme component and varied amount of adenosylpeptide B_{12} (**3**) (0.35 µM - 70 µM).

2.3. Assays for Determination of Kinetic Constants of (*S*)-Glutamate (4)

The holo-glutamate mutase with 14 folds excess of component S was throughout reconstituted by S (5 µg), E (2.5 µg) and coenzyme B_{12} (**1**) (0.05 mM) in Tris/HCl (30 mM, pH 8.3) and mercaptoethanol (1 mM) at 37˚C. Partial purified methylaspartase (36 µg) was also included in all assays mixtures. Each reaction was started by addition of a varied amount of (*S*)-glutamate (**4**) (5 mM - 300 mM) to the assay mixture prior to the spectrophotometric determination of glutamate mutase activity at 240 nm.

2.4. Assaying the Interaction of Each Proposed Inhibitor of Glutamate Mutase with Methylaspartase

The partial purified methylaspartase (9 µg) was incubated with each inhibitor compound (**10**)-(**15**) (1 mM or 2 mM) in Tris/HCl (45 mM, pH 8.3) and mercaptoethanol (0.05 mM) at 37˚C for 2 or 6 minutes. In each assay the reaction was started by addition of (2*S*, 3*S*)-3-methylaspartate (**5**) (70 mM) to the assay mixture prior to the spectrophotometric determination of methylaspartase activity at 240 nm. The measured activity of methylaspartase in the presence of each inhibitor for a specified duration was finally reported as a percentage of activity of an inhibitor free methylaspartase which was measured in a similar assay without an inhibitor.

2.5. Assaying the Interactions of Glutamate Mutase with Its Hypothetical Inhibitors

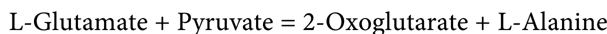
Each inhibitor compound (**10**)-(**15**) (3 mM) was incubated with apoenzyme proteins components; S (10 µg), E (5.2 µg) and coenzyme B_{12}(**1**) (0.05 mM) in Tris/HCl (30 mM, pH 8.3) and mercaptoethanol (1 mM) at 37˚C for 5 minutes. Sephadex G 25 was used to remove excess protein unbound inhibitors and cofactor molecules before methylaspartase (72 µg) was added to each assay mixture. The residual activity of glutamate mutase was finally determined by UV spectrophotometry at 240 nm after addition of (*S*)-glutamate (**4**) (40 mM) to each assay mixture.

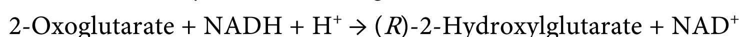
2.6. Assaying the Activity of Glutamate Mutase Catalyzed Conversion of (2*S*, 3*S*)-3-Methylaspartate (5) to (*S*)-Glutamate(4) and Determination of the Kinetic Constants of (2*S*, 3*S*)-3-Methylaspartate (5)

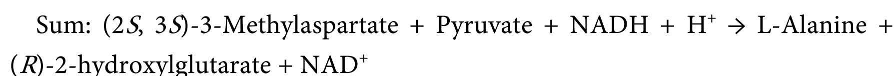
Glutamate mutase activity was determined by UV spectrophotometry at 37˚C via the decreasing absorbance by NADH (λ_{max} = 340 nm, ε_{340} = 6.3 $mM^{-1}\cdot cm^{-1}$)

after each addition of (2*S*, 3*S*)-3 methylaspartate (**5**) (5 - 300 mM) and pyruvate (20 mM) to a mixture of components of hologlutamate mutase; S (10 µg), E (5 µg), coenzyme B_{12} (**1**) (0.025 mM) and two auxiliary holoenzymes; glutamate-pyruvate aminotransferase (40 µg), pyridoxal-5-phosphate (0.02 mM), (*R*)-2-hydroxylglutarate dehydrogenase (20 µg) and NADH (0.2 mM) in Tris-HCl (30 mM, pH 8.3) and mercaptoethanol (0.05 mM).

(2*S*, 3*S*)-3-Methylaspartate = L-Glutamate


L-Glutamate + Pyruvate = 2-Oxoglutarate + L-Alanine


2-Oxoglutarate + NADH + H^+ → (*R*)-2-Hydroxylglutarate + NAD^+


Sum: (2*S*, 3*S*)-3-Methylaspartate + Pyruvate + NADH + H^+ → L-Alanine + (*R*)-2-hydroxylglutarate + NAD^+

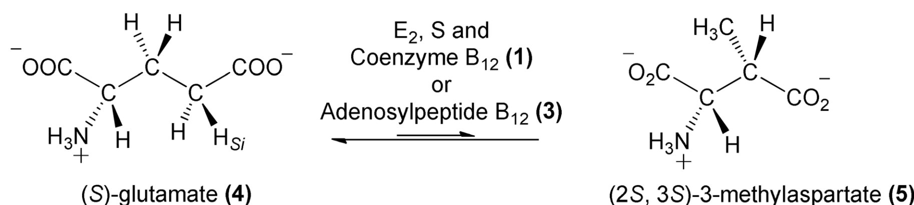
2.7. Assaying the Interactions of the Proposed Inhibitors of Glutamate Mutase with Glutamate-Pyruvate Aminotransferase and (*R*)-2-Hydroxylglutarate Dehydrogenase

Each proposed inhibitor of glutamate mutase (**10**)-(**15**) was assayed separately with hologlutamate-pyruvate aminotransferase and (*R*)-2-hydroxylglutarate dehydrogenase enzymes system. Components of the two holoenzymes; glutamate-pyruvate aminotransferase (40 µg), pyridoxal-5-phosphate (0.02 mM), (*R*)-2-hydroxylglutarate dehydrogenase (20 µg) and NADH (0.2 mM) were incubated with each inhibitor(**10**)-(**15**) (20 mM) in Tris-HCl (30 mM, pH 8.3) and mercaptoethanol (0.05 mM) at 37˚C for 5 minutes. After each incubation the reaction was started by addition of the substrates of glutamate-pyruvate aminotransferase; Pyruvate (20 mM) and (*S*)-glutamate (**4**) (20 mM) to the assay mixture prior to the determination of decreasing amount of NADH (ε_{340} = 6.3 $mM^{-1}\cdot cm^{-1}$) at 340 nm by UV spectrophotometry.

3. Results and Discussion

Similar to many other reported kinetic studies of a coenzyme B_{12} dependent glutamate mutase which used the standard methylaspartase coupled assay to measure activity of the enzyme [14] [17], the recent kinetic investigations into the mechanism of glutamate mutase by coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) cofactors have also relied on the standard methylaspartase coupled assay to measure the activity of glutamate mutase [20]. The kinetic constants of adenosylpeptide B_{12} (**3**) in the glutamate mutase catalyzed transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) were determined by the Michaelis-Menten method in two different relative amount of apoenzyme components S: E 2 and 14, so as those of coenzyme B_{12} (1) which were obtained from the reconstitutions of holoenzyme in 2, 7 and 14 S: E (**Table 1**) [20]. The virtually equal K_m values of coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) in 2 as well as 14 S: E reconstitutions of hologlutamate mutase imply the lower ligand of coenzyme B_{12} (**1**) has no influence in the mechanism of cofactor binding to apoenzyme. Additionally, along with the earlier reported decrease of k_{cat} for the

Table 1. Kinetic constants of cofactors; coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) in the glutamate mutase catalyzed transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) as determined by the Michaelis-Menten method in different relative amount of apoenzyme components S: E with cofactors.



Cofactor	S:E	K_m (μM)	k_{cat} (s^{-1})	$k_{cat} \cdot K_m^{-1}$ (s^{-1}·μM^{-1})
Coenzyme B_{12} (**1**)	2:1	1.12 ± 0.04	1.24 ± 0.36	1.11
	7:1	0.7 ± 0.05	0.9 ± 0.4	1.29
	14:1	0.52 ± 0.06	1.24 ± 0.36	2.39
Adenosylpeptide B_{12} (**3**)	2:1	1.07 ± 0.04	0.12 ± 0.01	0.1
	14:1	0.35 ± 0.05	0.09 ± 0.01	0.26

transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) when the holoenzyme is reconstituted with adenosylpeptide B_{12} (**3**) relative to coenzyme B_{12} (**1**), [20] the same kinetic data have demonstrated the independent of maximum rate of the transformation on the proportions of apoenzyme components. However, intriguingly is the unveiled consistent trends of K_m values of the cofactors during the transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) that correlate with the proportions of apoenzyme components which form the holoenzyme (**Table 1**). The results obtained from the reconstitutions of holoenzyme with both coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) clearly exhibited the decreasing K_m of the cofactors owing to increasing amount of S used in reconstituting holoenzyme from separate apoenzyme components and cofactor. These trends have imply an increase in cofactor binding to apoenzyme due to increased amount of component S used in reconstituting hologlutamate mutase. Congruently, the K_m of coenzyme B_{12} (**1**) in the same transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) by glutamate mutase obtained in another study that used fused SE apoenzyme is 5.5 ± 0.7 μM [17]. This value is 5 folds larger than 1.12 ± 0.04 μM which was obtained in this study as a result of reconstituting holoenzyme from separate apoenzyme components with coenzyme B_{12} (**1**) in S: E 2. The drift of a cofactor (**1**) K_m value as reported by these different studies indicates the binding of coenzyme B_{12} (**1**) to component S of apoenzyme is more efficient if it occurs before the component S has assemble with E than binding to ES. This comparison of the K_m values that led to such conception is based on the fact that the fused enzyme has proportion of apoenzyme proteins components S: E 2, whereas in all reconstitutions both the coenzyme B_{12} (**1**) and adenosylpeptide B_{12} (**3**) lower coordinates are substituted by imidazole of His 16 from subunit S. Likewise, the trends of K_m values of the cofactors obtained in this study resemble those reported from the study which in-

vestigated the binding of coenzyme B_{12} (**1**) to apoenzyme by gel filtration, where the apparent K_m of cofactor and dissociation constant (K_d) of E_2S_2-B_{12} complex decrease from 18 μM to 5.8 μM and 5.4 μM to 1.8 μM, respectively when the reconstitution of holoenzyme was varied from mutS: mutE 1 to 5 [8].

Furthermore, the recently proposed mechanism based inhibitions study of glutamate mutase by using its putative inhibitors compounds (Figure 2) requires the kinetic description of glutamate mutase interaction with each inhibitor compound. Therefore, determinations of the kinetic constants of substrate in the reaction of glutamate mutase in the presence of each inhibitor as well as the inhibition constants are necessary for establishing the nature of glutamate mutase interactions with the inhibitors. Molecules of the putative inhibitors have been designed to resemble 4-glutamly (**6**) or (2*S*, 3*S*)-3-methyleneaspartate radicals (**7**) skeletal structures and have sp2 carbon atoms centers which mimic radical atoms of 4-glutamly (**6**) and (2*S*, 3*S*)-3-methylenespartate radicals (**7**). Based on their structures, the inhibitors are predicted to bind glutamate mutase active site and react by adducting with 5'-deoxyadenosyl radical after the homolytic cleavage of the cofactor (**1**) Co(III)-C bond. Consequently, determination of each putative inhibitor reactivity at glutamate mutase active site by EPR spectroscopy as whether it form radical adduct with the primary organic radical like in the 2-methyleneglutarate mutase [30] were proposed along with determinations of the inhibition kinetics. Since the determination of kinetic constants of (*S*)-glutamate (**4**) in the reaction catalyzed by glutamate mutase requires measurement of glutamate mutase activity by the standard methylaspartase coupled assay, thus, the kinetic characterization of glutamate mutase interaction with each proposed inhibitor is possible only if the auxiliary methylaspartase is not inactivated by an inhibitor compound. However, this study revealed the reduction of methylaspartase specific activity by 10% and 20% after its incubation with 1 mM of *cis*-glutaconate (**10**) for 2 and 6 minutes, respectively (Figure 3). Likewise, the geometrical isomer *trans*-glutaconate (**11**) exhibited similar inhibition potent. Since both *cis*-glutaconate (**10**) and *trans*-glutaconate (**11**) mimic

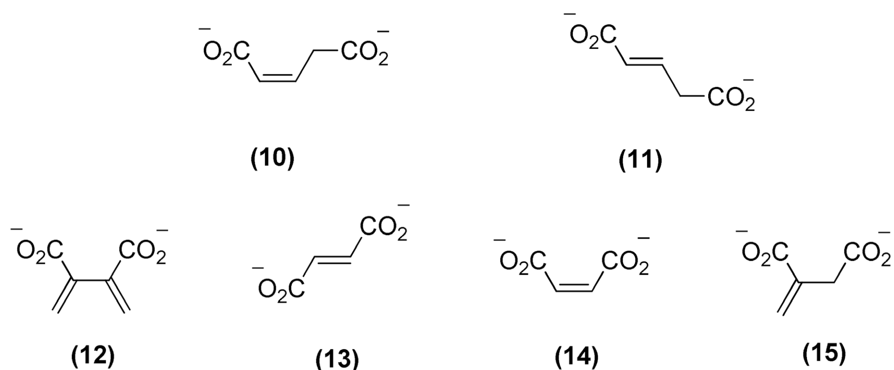


Figure 2. Molecules with structures analogue to the intermediates 4-glutamyl radical (**6**); *cis*-glutaconate (**10**), *trans*-glutaconate (**11**) and (2*S*, 3*S*)-3-methyleneaspartate radical (**7**); buta-1, 3-diene-2, 3-dicarboxylate (**12**), fumarate (**13**), maleate (**14**) and itaconate (**15**).

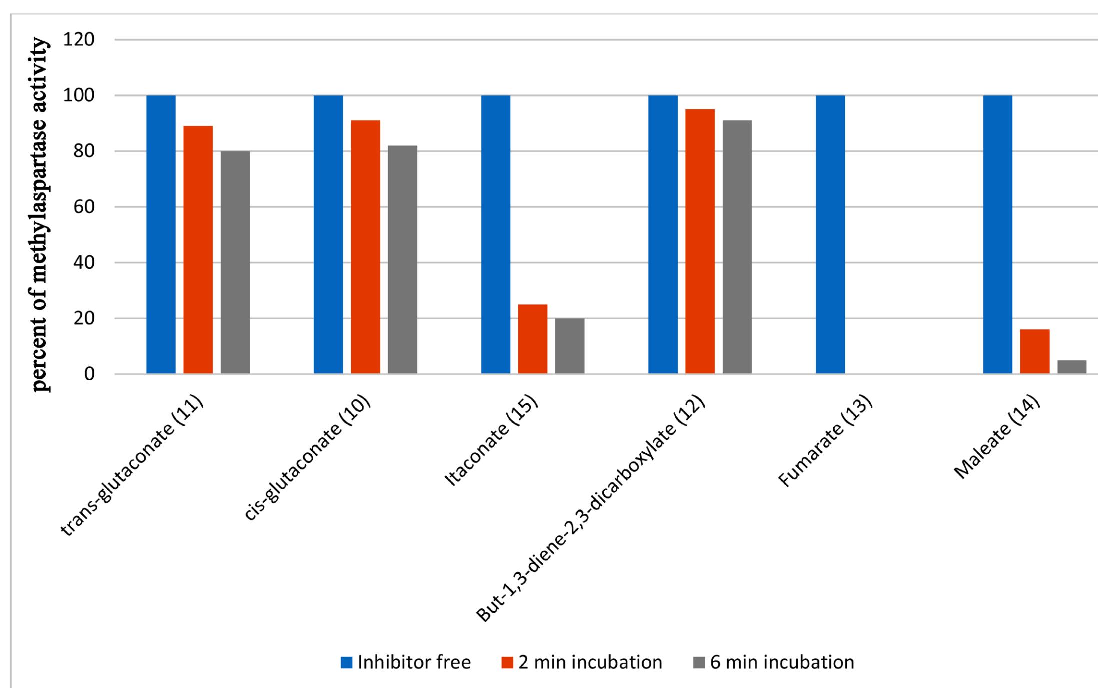


Figure 3. Activity of methylaspartase measured in the presence of each putative inhibitor of glutamate mutase relative to its maximum activity or activity of inhibitor free methylaspartase (44 U·mg^{-1}) (blue bars). Orange and grey bars are for 2 and 6 minutes, respectively incubations of methylaspartase (44 U·mg^{-1}, 9 µg) with 1 mM of each inhibitor.

4-glutamy radical (**6**) in the active site of glutamate mutase, thus the effect of 2 mM of each glutaconate which is equal to K_m of (*S*)-glutamate (**4**) in the reaction of glutamate mutase were determined as a reduction of methylaspartase specific activity to less than 40% within 2 minutes (**Figure 4**). Such considerable reduction of auxiliary enzyme activity by amount of an inhibitor equal to K_m of the substrate that form a mimicked radical in the mechanism of glutamate mutase confirm the inaptness of a methylaspartase coupled assay in measuring the activity of glutamate mutase in the presence of either glutaconates. Similar trends were obtained from the experiments that evaluated the interactions of methylaspartase with but-1, 3-diene-2, 3-dicarboxylate (**12**), fumarate (**13**), maleate (**14**) and itaconate (**15**) which mimic (2*S*, 3*S*)-3-methyleneaspartate radical (**7**) in the mechanism of glutamate mutase. While the most potent inhibition was exhibited by fumarate (**13**) which causes complete inactivation of methylaspartase within 2 minutes upon its 1 mM incubation with the enzyme, the least potent inhibition was by but-1, 3-diene-2, 3-dicarboxylate (**12**) which decrease methylaspartase activity by 50% upon incubation of its 2mM with the enzyme for 6 minutes (**Figure 3** & **Figure 4**). These extent of inhibitions by the amount of inhibitors significant lower than 7 mM which is the K_m of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase are substantial. Therefore, similar to *cis*- and *trans*-glutaconates, the kinetics of glutamate mutase inhibition by any of the latter compounds cannot be determined by the standard methylaspartase coupled assay.

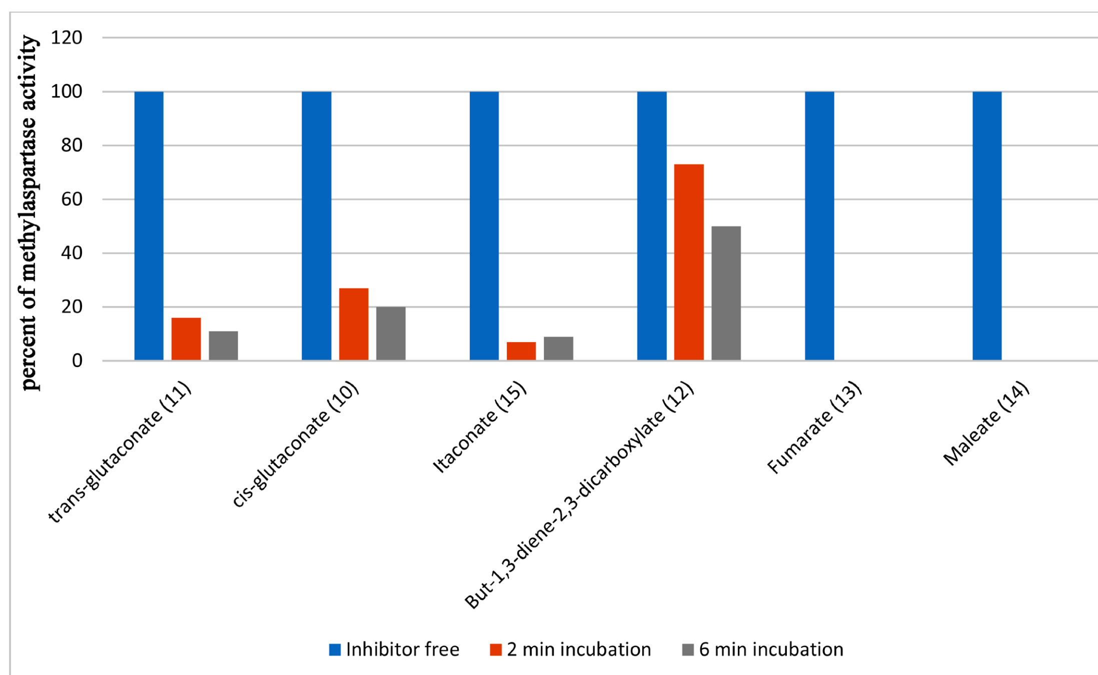
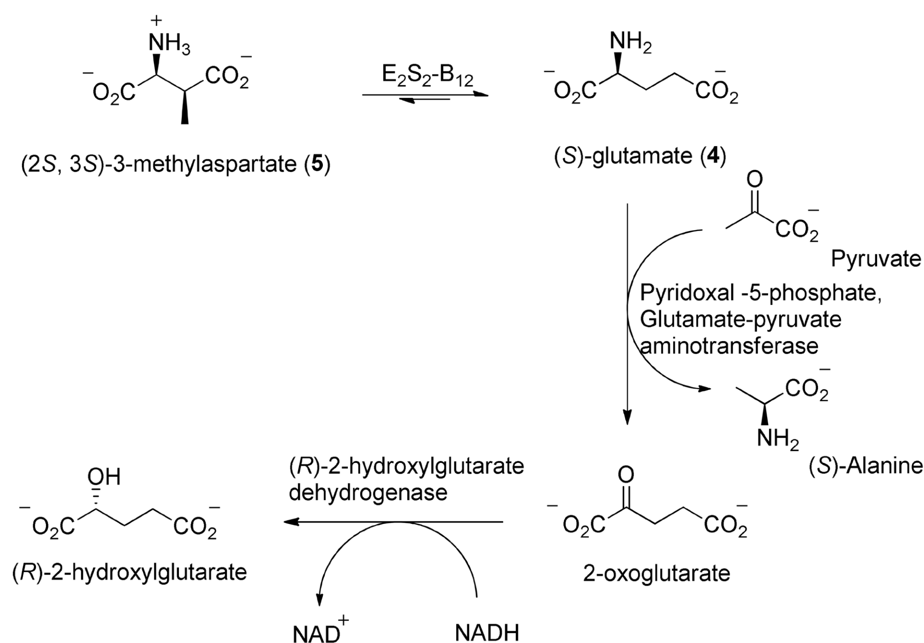


Figure 4. Activity of methylaspartase measured in the presence of each putative inhibitor of glutamate mutase relative to its maximum activity or activity of inhibitor free enzyme (44 $U \cdot mg^{-1}$)(blue bars). Orange and grey bars are for 2 and 6 minutes, respectively incubations of methylaspartase (44 $U \cdot mg^{-1}$, 9 µg) with 2 mM of each inhibitor.

Nevertheless, the residue activities of presumably inhibitor bound hologlutamate mutase were evaluated by the methylaspartase coupled assay. The solution of inhibitor bound holoenzyme that is free from protein unbound inhibitor molecules was prepared by using Sephadex G 25 which remove protein unbound molecules of both inhibitor and cofactor after incubating hologlutamate mutase with each inhibitor, thereby prevent methylaspartase from being inactivated as it is added after the filtration. The Gel filtration has therefore achieve secured inclusion of ancillary methylaspartase and thereafter start of the glutamate mutase reaction by addition of (*S*)-glutamate (**4**). While the residual activity of apparent *cis*-glutaconate (**10**) and itaconate (**15**) bound glutamate mutase were 82% of the activity of inhibitor free glutamate mutase, the apparent *trans*-glutaconate (**11**) bound glutamate mutase exhibited 70% of the activity of inhibitor free enzyme. The activity of inhibitor free glutamate mutase was complete recovered in apparent but-1, 3-diene-2, 3-dicarboxylate (**12**) bound enzyme, whereas, unexpectedly, fumarate (**13**) and maleate (**14**) inactivate glutamate mutase completely. The fact that cob(III)alamin spectrum was recorded throughout upon 1 hour of anaerobic incubations of hologlutamate mutase with fumarate (**13**) and maleate (**14**) (spectra not shown) imply that the inactivation of holoenzyme by (**13**) and (**14**) does not lead to homolysis of Co(III)-C σ bond. These results suggest the allosteric binding of (**13**) and (**14**) on glutamate mutase which change the active site structure and cause the enzyme to lose allegiances to (*S*)-glutamate (**4**).

Deliberately towards gaining insight into whether the molecule of an inhibitor

binds glutamate mutase allosterically or the enzyme active site, a new method of assaying activity for conversion of (2*S*, 3*S*)-3-methylaspartate (**5**) to (*S*)-glutamate (**4**) by glutamate mutase was developed. The assay utilizes (2*S*, 3*S*)-3-methylaspartate (**5**) along with pyruvate as substrates and pyridoxal-5-phosphate dependent glutamate-pyruvate aminotransferase as well as the NADH-dependent (*R*)-2-hydroxyglutarate dehydrogenase as auxiliary enzymes. In this assay (*S*)-glutamate (**4**) produced from (2*S*, 3*S*)-3-methylaspartate (**5**) in the reverse reaction of glutamate mutase is converted by hologlutamate-pyruvate aminotransferase to 2-oxoglutarate in the expense of pyruvate which is dispensed to alanine. Further reduction of 2-oxoglutarate to (*R*)-2-hydroxyglutarate by the dehydrogenase is coupled with the oxidation of NADH to NAD^+. The depletion of NADH (λ_{max} = 340 nm, ε_{340} = 6.3 $mM^{-1}\cdot cm^{-1}$) allows the spectrophotometric assay of glutamate mutase activity at 340 nm (**Scheme 2**). In order to determine the kinetic constants of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase in presence of the putative inhibitor compounds (**10**)-(**15**), the effect of each inhibitor compound against the two auxiliary enzymes system was evaluated. Upon addition of (*S*)-glutamate (**4**) and pyruvate to a mixture of hologlutamate-pyruvate aminotransferase and (*R*)-2-hydroxylglutarate dehydrogenase incubated with each inhibitor, the activity of glutamate-pyruvate aminotransferase were relatively complete recovered. These results demonstrate the suitability of the new pyridoxal-5-phosphate dependent glutamate-pyruvate aminotransferase and NADH-dependent (*R*)-2-hydroxylglutarate dehydrogenase coupled assay in measuring the activity of glutamate mutase in presence of each putative inhibitor. The new assay is therefore recommended for application



Scheme 2. Enzymatic transformations leading to (*R*)-2-hydroxylglutarate from (2*S*, 3*S*)-3-methylaspartate (**5**) in the glutamate-pyruvate aminotransferase and (*R*)-2-hydroxylglutarate dehydrogenase coupled assay of glutamate mutase activity.

in the proposed mechanism based inhibitions study of glutamate mutase, where gaining of insights into the interactions of glutamate mutase with compounds (**10**)-(**15**) is probable.

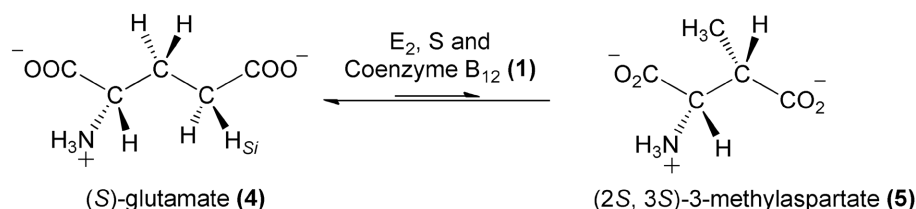
Moreover, the new assay was used to determine the kinetic constants of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase. Together with the kinetic constants of (*S*)-glutamate (**4**) in the reverse reaction as determined by the standard methylaspartase coupled assay (Table 2), an equilibrium constant of the reversible isomerization between (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**), K_{eq} =[(*S*)-glutamate (**4**)] × [(2*S*, 3*S*)-3-methylmethylaspartate (**5**)]$^{-1}$ = $V_{max(\text{forward})} \times V^{-1}_{max(\text{reverse})} \times K_{m(2S,3S)\text{-3-methylaspartate}} \times K^{-1}_{\text{m}(S)\text{-glutamate}} = 16$ was obtained.

This value is comparable to that described in the literature (K_{eq} = 12), [27] which was obtained direct from the concentrations of (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**) at equilibrium under conditions similar to those of the assay mixtures which culminate to the kinetic constants in Table 2.

4. Conclusions

The decreasing K_m of coenzyme B_{12} (**1**), so as adenosylpeptide B_{12} (**3**) in the transformation of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) as a result of increasing amount of S while reconstituting E_2S_2-B_{12} from separate apoenzyme components with cofactor implied the affinity of the cofactor to apoenzyme increases with increasing amount of S. Correspondingly, the K_m values of coenzyme B_{12} (**1**) as obtained in this study where E_2S_2-B_{12} was reconstituted from separate apoenzyme components; S and E and that obtained in another study which used fused apoenzyme components SE have clearly indicated the cofactor binds S more efficient than ES. [17] Therefore the *in vitro*—formation of active E_2S_2-B_{12} occurs via both assemble of S-B_{12} with E_2 when S partners with the cobalamin before it assemble with E_2 and binding of cobalamin to the whole apoenzyme; ES, where data from the kinetic studies have demonstrated the former is more efficient than the latter. Likewise, these findings are corroborated with those reported from a study in which the binding pattern of coenzyme B_{12} (**1**)

Table 2. Kinetic constants of substrates; (*S*)-glutamate (**4**) and (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase reconstituted with coenzyme B_{12} (**1**) in S: E 14 as determined by the Michaelis-Menten method.



Substrates	K_m (mM)	V_{max} (U·mgE^{-1})	k_{cat} (s^{-1})	$k_{cat} \cdot K_m^{-1}$ (s^{-1}·mM^{-1})
(*S*)-glutamate (**4**)	2.25 ± 0.03	3.2 ± 0.5	2.85 ± 0.5	1.3
(2*S*, 3*S*) -3-methylaspartate (**5**)	7 ± 0.07	0.6 ± 0.6	0.54 ± 0.6	7.7 × 10^{-2}

to apoenzyme was evaluated by equilibrium gel filtration. The latter study demonstrated increasing affinity of coenzyme B_{12} (**1**) to apoenzyme with decreasing apparent dissociation constant (K_d) of E_2S-B_{12} complex as a result of increasing amount of mutS while reconstituting the holoenzyme from separate apoenzyme components; mutS and mutE which were prepared from *C. tetarnomorphum* and coenzyme B_{12} (**1**) [8].

Coupling the transformation of (2*S*, 3*S*)-3-methylaspartate (**5**) to (*S*)-glutamate (**4**) by E_2S_2-B_{12} with hologlutamate-pyruvate aminotransferase and holo-(*R*)-2-hydroxylglutarate dehydrogenase in the presence of pyruvate successful lead from (*S*)-glutamate (**4**) to (*R*)-2-hydroxylglutarate via 2-oxoglutarate along with dispense of (*S*)-alanine and oxidation of NADH (λ_{max} = 340 nm, ε_{340} = 6.3 $mM^{-1}\cdot cm^{-1}$). The latter allows measurement of activity of glutamate mutase by UV-spectrophotometry at 340 nm. This new method for assaying activity of glutamate mutase was used to determine the kinetic constants of (2*S*, 3*S*)-3-methylaspartate (**5**) in the reaction of glutamate mutase. Together with the kinetic constants of (*S*)-glutamate (**4**) as determined by the standard methylaspartase coupled assay, an equilibrium constant (K_{eq}) =16 of the glutamate mutase catalyzed reversible transformation was calculated by the Briggs-Haldane equation. This finding reveals an equilibrium towards (*S*)-glutamate (**4**) consistently to the earlier reported K_{eq} =12 which is a constant ratio of the amount of (*S*)-glutamate (**4**) to (2*S*, 3*S*)-3-methylaspartate (**5**) a while after start of reaction [27]. The new assay further demonstrated its usefulness in determination of kinetics of glutamate mutase inhibitions by compounds **10** to **15**, as the activity of hologlutamate-pyruvate aminotransferase were relatively complete recovered after the incubation of each inhibitor compound with hologlutamate-pyruvate aminotransferase and (*R*)-2-hydroxylglutarate dehydrogenase.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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