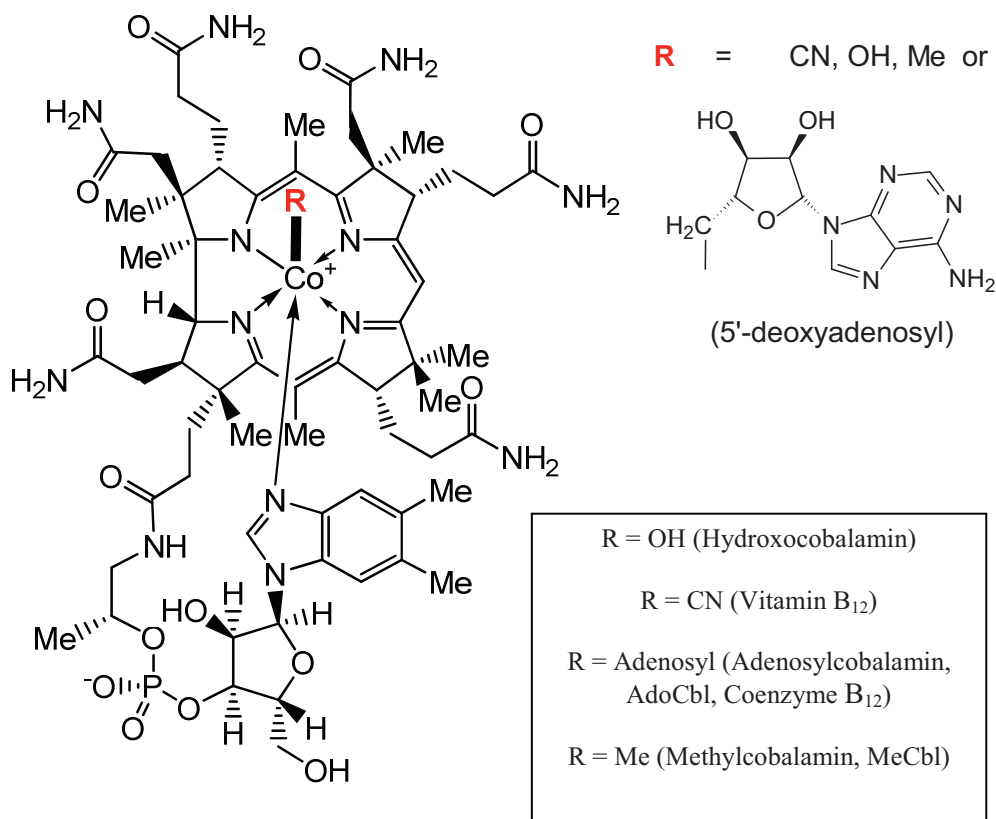


## 1.1 INTRODUCTION TO VITAMIN B<sub>12</sub>

Vitamin B<sub>12</sub> was discovered because of its relationship to the disease pernicious anemia (PA). PA was a fatal illness before the 1920s. But this changed after Whipple suggested raw liver as a treatment. He found that ingesting large amounts of liver seemed to cure anemia from blood loss.<sup>1</sup> Minot and Murphy<sup>2</sup> then described the dramatic recovery of 45 patients suffering from PA after they consumed a special diet of lightly cooked liver. The three men shared the 1934 Nobel Prize in Medicine for the discovery of the cure of a previously fatal disease of unknown origin.

The active anti-PA factor was a mystery until 1948, when Folkers<sup>3</sup> and Smith<sup>4</sup> independently isolated a small quantity of a red crystalline compound from liver. After the substance was shown to lead directly to the recovery of PA patients it was named vitamin B<sub>12</sub>.

Vitamin B<sub>12</sub> has the most complicated structure; the unique corrin ligand was only revealed by X-ray crystallography in full 10 years later its discovery. The structures of vitamin B<sub>12</sub> and coenzyme B<sub>12</sub> were established by X-ray crystallography in the laboratory of D.C. Hodgkin.<sup>5</sup> The structure of vitamin B<sub>12</sub> is based on a corrin ring, which is a near planar, macrocyclic ring like the porphyrin system found in hemes, chlorophylls, and cytochromes. A cobalt atom lies at the center of corrin ring. The cobalt is in oxidation state +3,<sup>6</sup> four of the six coordination sites are provided by the corrin ring, and a fifth ( $\alpha$  face, bottom site of the corrin ring) by a dimethylbenzimidazole group. The sixth coordination site ( $\beta$  face), the biological activity centre of vitamin B<sub>12</sub>, is variable (**Figure 1**).



**Figure 1:** The chemical structure of vitamin B<sub>12</sub> and derivatives

Vitamin B<sub>12</sub> and its analogues are often called corrinoids while the forms of vitamin which contain the ribonucleotide  $\alpha$ -D-ribofuranosyl-5, 6-dimethylbenzimidazole are also named cobalamins.

Vitamin B<sub>12</sub> occurs naturally in several forms. Though the vitamin was isolated in the form of cyanocobalamin (CNCbl), vitamin B<sub>12</sub> is biologically active in only three forms, adenosylcobalamin (AdoCbl), hydroxocobalamin (HOCbl) and methylcobalamin (MeCbl). They can be found in the serum and tissues of man and higher animals. MeCbl is the preferred form for oral absorption because of its immediate activity. CNCbl and HOCbl must lose the cyanide or hydroxide moiety and add either a methyl or adenosyl group in order to be converted into either MeCbl or AdoCbl in

*vivo*. MeCbl or AdoCbl can be effective to cure pernicious anaemia. The structures of the most important cobalamins are shown in **Figure 1**.

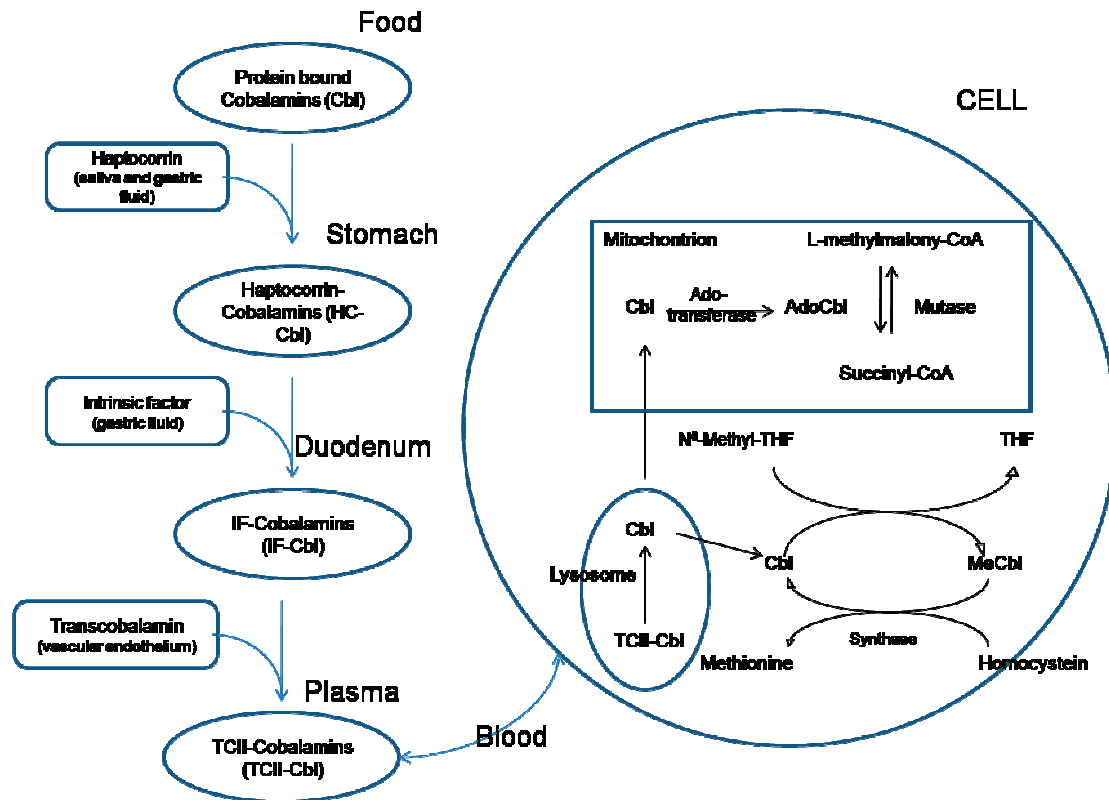
## 1.2 VITAMIN B<sub>12</sub> NUTRITION AND MEDICINAL USES

Humans and higher animals require vitamin B<sub>12</sub> for only two enzymes: (*R*)-methylmalonyl-CoA mutase (MCM; E.C.5.4.99.2) and methionine synthase (5-methyltetrahydrofolate, E.C.2.1.1.13). MCM catalyses the conversion of (*R*)-methylmalonyl-CoA to succinyl-CoA using adenosylcobalamin (AdoCbl) as a cofactor. In the cytosol, methylcobalamin (MeCbl) is synthesised through reduction and methylation of Cbl and is employed as a cofactor for methionine synthase in the conversion of homocysteine to methionine. These two enzymes have impact on DNA synthesis and regulation particularly, and on fatty acid synthesis and energy production as well.

Vitamin B<sub>12</sub> is essential for rapid DNA production during cell division. This is especially important in tissues such as the bone marrow, where cells are dividing rapidly. Where there is a lack of B<sub>12</sub>, the synthesis of DNA can be disrupted and abnormal cells called megaloblasts are produced during red blood cell formation. This can result in megaloblastic anaemia.<sup>7,8</sup> B<sub>12</sub> deficiency could also lead to damage in the nervous system by interrupting the maintenance of myelin. Despite the extensive animal-model research of B<sub>12</sub> deficiency for more than 30 years, the specific role of vitamin B<sub>12</sub> in maintaining normal myelination remains poorly understood.<sup>9</sup> Failure to convert methylmalonyl CoA into succinyl CoA results in a high level of methylmalonic acid, which is a destabiliser of myelin.<sup>9,10</sup>

Mammals are not able to synthesis cobalamins, so they must be supplied from the diet. B<sub>12</sub> can be found primarily in meat, fish, eggs and dairy products. The daily requirement of vitamin B<sub>12</sub> for the human body is low 1-2 µg.<sup>7</sup> Consequently, there is an elaborate mechanism for absorption, blood transportation and cellular uptake of dietary B<sub>12</sub>. The absorption of vitamin B<sub>12</sub> begins in the mouth where a small amount of unbound Cbl is absorbed through the mucosa membrane. Most of the vitamin B<sub>12</sub> which is protein bound in food is digested in the stomach by proteolytic gastric enzymes, which require an acidic pH. Meanwhile hapotocorrin is secreted to bind to free Cbl and forms a hapotocorrin-bound Cbl. This can prevent free Cbl from breakdown in the low-pH environment of stomach. In the duodenum hapotocorrin-bound Cbl is digested by protease and Cbl becomes bound to intrinsic factor (IF), which is a protein synthesised by gastric parietal cells. The Cbl-IF complex is taken into the circulation in a complex with transcobalamin II (TC-II).<sup>11</sup>

After the complex TCII-Cbl has been transferred into the cell, Cbl is released into the cytoplasm. Subsequently, it is reduced from the 3+ to the 1+ oxidation state and converted to AdoCbl or MeCbl in the mitochondrion and cytoplasm, respectively. In cytoplasm, methionine synthase (synthase) catalyses the reaction, which converts homocysteine to methionine with 5-methyltetrahydrofolate as methyl group donor and MeCbl as a cofactor. AdoCbl is required for the activity of methylmalonyl-CoA mutase (mutase), which converts (*R*)-methylmalonyl CoA to succinyl CoA in the mitochondrion<sup>12</sup> (**Figure. 2**).



**Figure 2:** Cobalamin absorption and metabolic pathway. Cbl: cobalamin; HC: haptocorrin(transcobalamin I/III); IF: intrinsic factor; HC-Cbl: haptocorrin-bound cobalamin; IF-Cbl: intrinsic factor-bound cobalamin; TCII-Cbl: cobalamin bound to transcobalamin II.<sup>13</sup>

Vitamin B<sub>12</sub> deficiency can be caused by failure in any of the steps of the elaborate mechanism of absorption, transport and synthesis of cobalamins. The total amount of vitamin B<sub>12</sub> stored in body is about 2-5 mg in adults, which is normally maintained from the diet. Vitamin B<sub>12</sub> deficiency can be treated with intramuscular injections or less well by oral intake of vitamin B<sub>12</sub>.<sup>14</sup>

Hydroxocobalamin is not only used for B<sub>12</sub> deficiency but also used as an acute treatment for cyanide poisoning in both Europe and United States. The mechanism of

action is that the hydroxide ligand of hydroxycobalamin is displaced by the toxic cyanide ion, resulting in the very stable harmless cyanocobalamin.

Cobalamins can be selectively modified at several functional sites due to its structural complexity without affecting its metabolic pathway in the human body. Therefore, these modified cobalamin analogues can be used in diagnosis and chemotherapy.<sup>15,16</sup>

### **1.3 B<sub>12</sub> DEPENDENT ENZYMES**

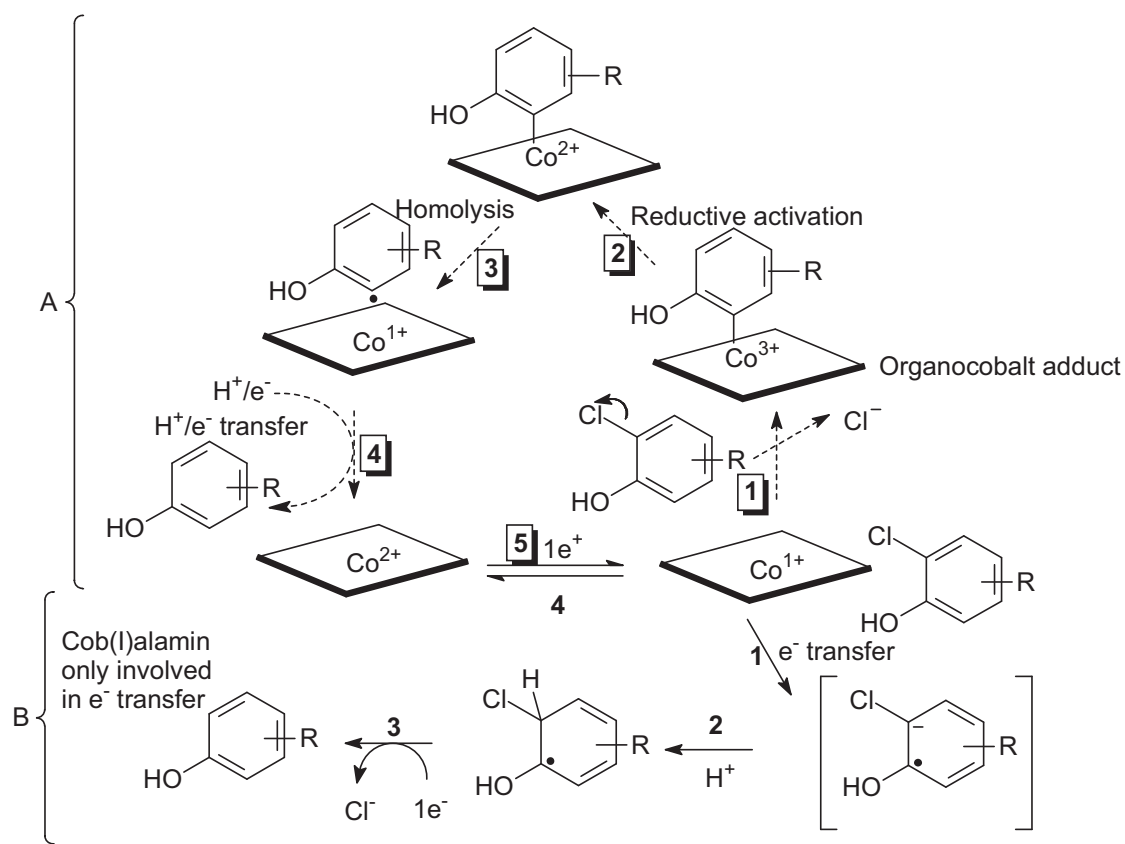
B<sub>12</sub> dependent enzymes have been divided into three large classes on the basis of different types of biological reactions that they catalyse: isomerases, methyltransferases, and reductive dehalogenases.<sup>17</sup>

The isomerases are the largest subfamily of B<sub>12</sub>-dependent enzymes found in bacteria. The only exception is methylmalonyl-CoA mutase, which is found in both bacteria and animals. The isomerases catalyse carbon skeleton rearrangements, that is 1,2 interchange between a variable substituent and a hydrogen atom on vicinal carbons. This type of enzyme is an AdoCbl-dependent enzyme. These enzymatic reactions are initiated by homolysis of the Co-C bond in AdoCbl.

The B<sub>12</sub>-dependent methyltransferases catalyse the transfer of a methyl group from a methyl donor to a nucleophilic acceptor. This type of enzyme plays an important role in the anaerobic acetogenesis, methanogenesis and catabolism of acetic acid to methane and carbon dioxide.<sup>18</sup>

The B<sub>12</sub>-dependent dehalogenases remove chloride ion from aliphatic or aromatic chlorinated organic compounds; such as chlorinated ethenes, chlorinated phenols, and polychlorinated biphenyls (PCBs), which are all in the 12 priority pollutant list of the USA Environmental Protection Agency.<sup>19 20</sup> The reductive dehalogenation was observed in a variety of anaerobes, including methanogenic and homoacetogenic bacteria; i.e. 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei*<sup>21</sup> and *O*-chlorophenol reductive dehalogenases of *Desulfitobacteria*.<sup>22</sup> The role of B<sub>12</sub> in the reductive dehalogenation is different from that of B<sub>12</sub>-dependent isomerisation and methyltransfer.

Two mechanistic pathways have been proposed for B<sub>12</sub>-dependent reductive dehalogenation (**Figure 3**). In Path A, an organo-cobalt adduct is formed, which undergoes subsequent  $\beta$ -elimination of the chlorine substituent. Then, homolysis of the Co-C bond gives an aryl radical, which is converted to aryl product *via* abstraction of a hydrogen atom. In Path B, the cobalamin serves as an electron donor. A radical anion intermediate is formed by a one-electron transfer from the cob(I)alamin. Then a chloro-aryl radical is produced as a result of protonation. Another electron transfer causes the cleavage of the chlorine anion and formation of product. Many questions regarding the dehalogenation mechanism remain unanswered as the B<sub>12</sub> dependent dehalogenases have only been discovered in recent years.

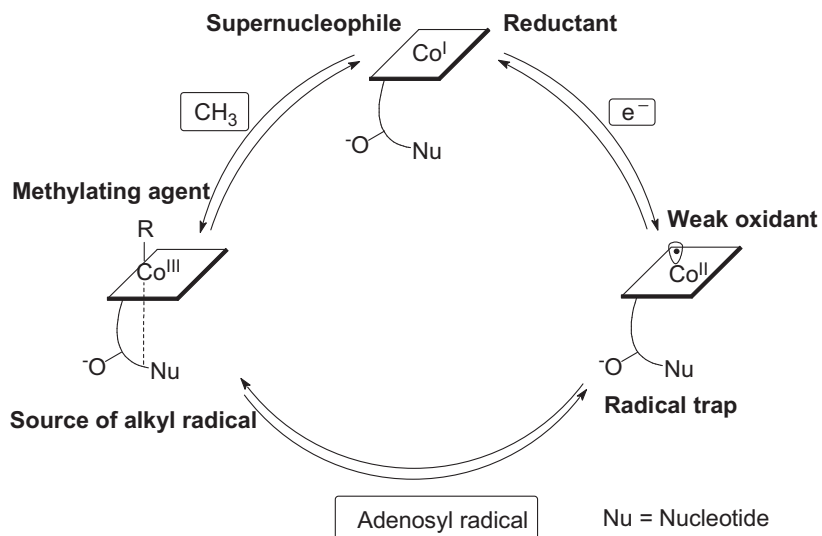


**Figure 3:** Proposed mechanistic pathways for  $\text{B}_{12}$ -dependent reductive dehalogenation.<sup>23</sup> The two pathways, A (dashed arrows) and B (solid arrows), are different in the roles of cobalamin.

The majority of methyltransferases have been found in bacteria, though *N*-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) and methylmalonyl-CoA mutase have been found in mammals.  $\text{B}_{12}$  dependent enzymes have been classified into two major sets, either methylcobalamin (MeCbl) or adenosylcobalamin (AdoCbl) dependent enzymes, according to the coenzyme involved.

Recent progress in the structural analysis of the enzymes involved in both coenzyme systems has led to a clearer understanding of both the chemical environment at the active site and the mode of B<sub>12</sub> binding. (See section 1.6 and 1.7)

Natural cobalamins are known to exist in three different oxidation states: +3, +2 or +1<sup>24, 25</sup> (**Figure 4**). The cob(I)alamin is highly reactive, known as a ‘supernucleophile’. It has been used in the synthesis of alkylcobalamins.<sup>26</sup> The catalytic activity of B<sub>12</sub> dependent enzymes can be traced back to the reactivity of the B<sub>12</sub>-derivatives with different oxidation states. In MeCbl dependent enzymatic reactions, the heterolytic formation/cleavage of the Co-C bond triggers the oxidation/reduction of cob(III)alamin and cob(I)alamin (formally a two electron reduction/oxidation of cobalt). It is represented by the reaction of cob(I)alamin with alkylating agents and by the nucleophile-induced demethylation of methyl-cob(III)alamin. This is particularly important in enzymatic methyl-transfer reactions. The coordination of Co-nucleotide (‘base-on’ mode) has a notable thermodynamic effect on the heterolytic reactions of methylcobalamin.<sup>27</sup> Occasionally, cob(I)alamin is oxidatively inactivated to cob(II)alamin, which requires reductive activation.



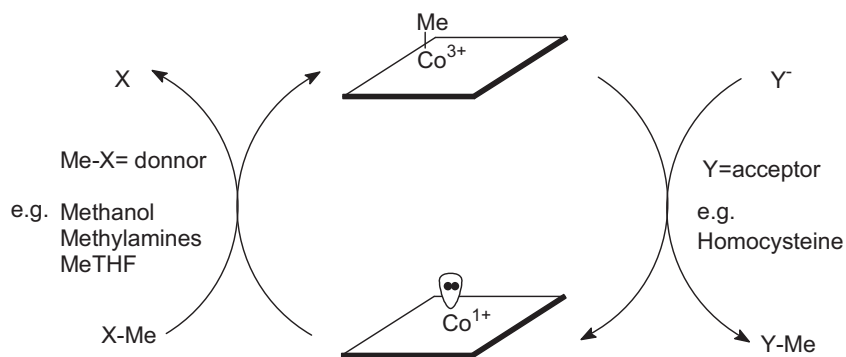
**Figure 4:** Reaction steps and reactivity relating to the oxidation state of cobalamins in  $B_{12}$ -dependent enzymes.<sup>18</sup>

The oxidative/reductive interconversion of cob(III)alamin and cob(II)alamin (formally a one-electron oxidation/reduction of the cobalt) is involved in AdoCbl-dependent enzymatic reactions. Homolysis of the Co-C bond generates the alkyl radical (i.e. 5'-adenosyl radical) and cob(II)alamin. Therefore one role of cobalamin is to act as a carrier of the highly reactive adenosyl radical, which is released after the binding of a substrate molecule to the enzyme, whereas cob(II)alamin acts as a radical trap, which will bind to the adenosyl radical after the catalytic cycle. Thus in AdoCbl-dependent enzymatic reactions the enzyme acts as a reversible free radical reservoir.

## 1.4 METHYLCOBALAMIN DEPENDENT ENZYMES

Methyltransferases catalyse a methyl transfer reaction using either vitamin B<sub>12</sub> or the CFeSP (corrinoid iron-sulfur protein)<sup>28</sup> as a cofactor. Methylcobalamin is the vitamin B<sub>12</sub> derivative involved in the reaction. Methylcobalamin-dependent enzymes exist in many bacteria and animals. Anaerobic acetogenesis,<sup>29</sup> methanogenesis<sup>30</sup> and catabolism of acetic acid to methane and carbon dioxide all depend on B<sub>12</sub>-dependent methyl transfer reactions.

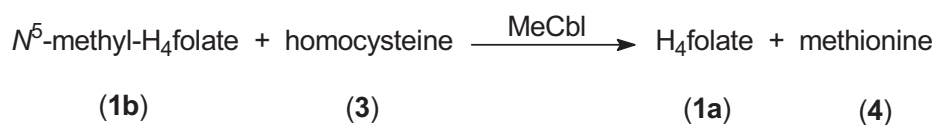
Methylcobalamin transfers its methyl group from donor to acceptor. Thiols are the methyl group acceptors in most cases of methylcobalamin dependent enzymatic reactions. The substrates are the methyl group donors, which are variable in different organisms, i.e. methanol, aromatic methyl esters, methylamines and methyltetrahydropterins (such as *N*<sup>5</sup>-methyltetrahydrofolate). (**Figure 5**)



**Figure 5:** Catalytic cycle of methylcobalamin-dependent enzymatic reactions

### 1.4.1 Methionine Synthase

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13) catalyses the transfer of a methyl group from  $N^5$ -methyltetrahydrofolate (**1b**) to homocysteine (**3**), forming tetrahydrofolate (**1a**) and methionine (**4**).<sup>31, 32</sup> (**Figure 6**) Low activity of methionine synthase in humans could result in megaloblastic anemia, and eventually cause the subacute combined degeneration of the spinal cord.<sup>33</sup>

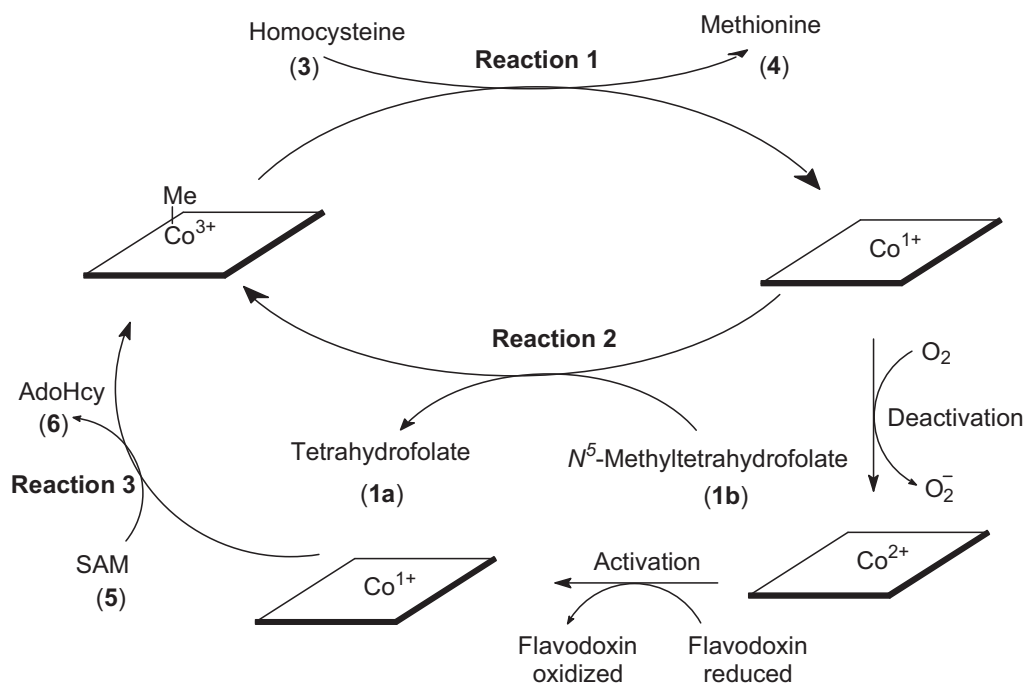


**Scheme 1:** Methylcobalamin-dependent methionine synthase reaction.

The overall catalytic mechanism involves two successive methyl transfer reactions from methyltetrahydrofolate to cob(I)alamin and from MeCbl to homocysteine. In the methylcobalamin-dependent methionine synthase reaction, cobalt-carbon  $\sigma$ -bond heterolytic cleavage and formation are important and the ‘supernucleophilic’ cob(I)alamin is an intermediate that can remove a methyl group from a substrate of relatively low reactivity, such as  $N^5$ -methyltetrahydrofolate<sup>34</sup> (**1b**) and  $N^5$ -methyltetrahydromethanopterin<sup>35</sup> (**2b**). (**Scheme 1**)



and the methylcobalamin form with the homocysteine binding domain, which correspond to the three reactions in **Figure 7**.



**Figure 7:** The complete catalytic cycle and the deactivation/activation of the coenzyme in methionine synthase

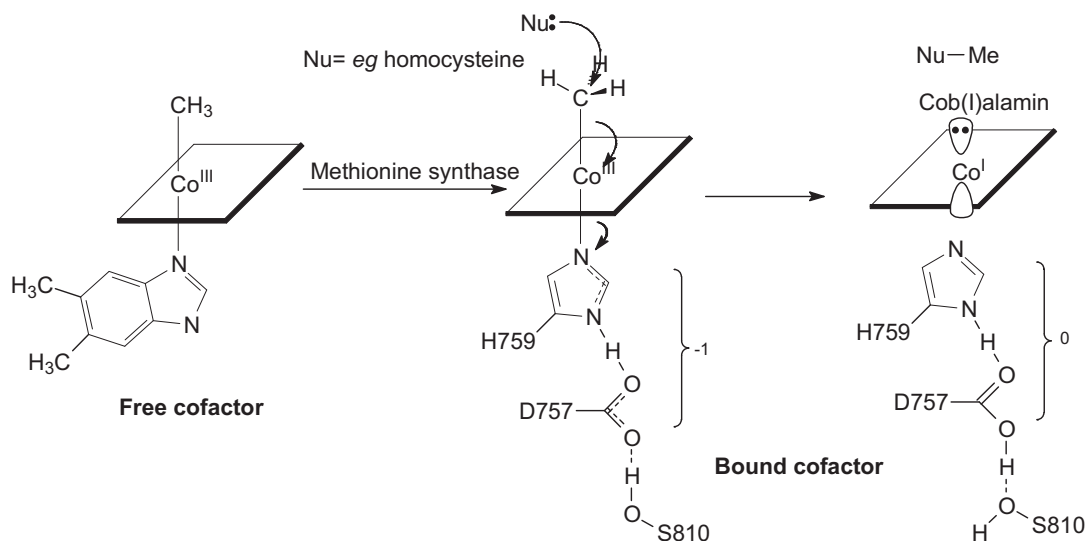
In the catalytic cycle (**Figure 7**), a methyl group is transferred from the methylcobalamin to homocysteine (3), producing methionine (4) and cob(I)alamin. The cob(I)alamin is methylated by *N*<sup>5</sup>-methyltetrahydrofolate (1a).<sup>37</sup> During the catalytic cycle, the cob(I)alamin form is occasionally converted to cob(II)alamin in the presence of oxygen, which occurs once in every 100-2000 turnovers.<sup>38</sup> The cob(II)alamin is catalytically inactive in the reaction. So, the enzyme is reactivated by reductive methylation of the cob(II)alamin form where SAM (5) binds to the C-

terminal domain as the methyl donor. In the bacterial systems, the reductant (reduced flavodoxin) is comprised of two flavoproteins, NADPH-flavodoxin (or ferredoxin) oxidoreductase<sup>39</sup> and flavodoxin,<sup>32</sup> which transfer the electron from NADPH to methionine synthase. Recent investigation of the reactivation reaction showed that the inactive cob(II)alamin form is firstly reduced to cob(I)alamin by an electron from flavodoxin and its then methylated by SAM. Flavodoxin is lacking in mammals and the physiological reducing mechanism was completely unknown until recently. A dual-flavoprotein family member, methionine synthase reductase, is the reductant for the reactivation of cob(II)alamin.<sup>40</sup>

Methionine synthase from *Escherichia coli* is the most extensively studied B<sub>12</sub>-dependent methyltransferase. The X-ray crystal structure of the cobalamin binding domain led to the discovery of the 'base-off/His-on' binding mode.<sup>34</sup> It revealed that the dimethylbenzimidazole (Dmb) side chain of the methylcobalamin is displaced from the cobalt by a histidine residue from the protein as the axial ligand in the cobalt complex. As a result of this displacement, the stability and reactivity of cobalamin derivatives is directly controlled by the protein rather than by a substituent of the cofactor. Model studies have shown that in free alkyl cobalamins, the cobalt-carbon bond is stabilised by the basic lower ligand, which increases the electron density on the cobalt. This ligand acts the homolytic cleavage to form cob(II)alamin and heterolytic cleavage to form cob(I)alamin. So the 'base off/His on' mode is believed to modulate the reactivity and stability of the cobalamins and facilitate the heterolytic cleavage of the cobalt-carbon bond.

In the ‘base off/His on’ mode, the key His759 residue is found in the consensus motif (DX**H**XXG), where **H** represents the lower axial histidine ligand. The structure study<sup>34</sup> revealed that it is a set of hydrogen-bonded residues, His759-Asp757-Ser810, named ‘ligand triad’ that transfers a proton from solvent to His759 and ensures that His759 is positioned to the corrin ring. Therefore, a catalytic quartet is formed by the His759-Asp757-Ser810 of the enzyme and the cobalt of cobalamin. It facilitates the methylation and demethylation by transferring protons in and out of the said region. In this mechanism, Asp757 and His759 share a single proton, with one negative charge delocalised across the two residues. Ser810 provides an essential connection between bulk solvent and the two residues. **(Figure. 8)**

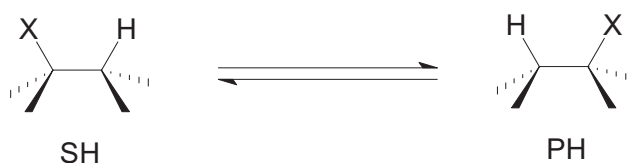
This ‘base-off/His-on’ mode is also conserved in all AdoCbl-dependent mutases (e.g. methylmalonyl-CoA mutase and glutamate mutase), which also features the consensus motif (DX**H**XXG).<sup>18,41</sup>



**Figure 8:** Displacement of dimethylbenzimidazole by His759-Asp757-Ser810, named ‘ligand triad’, in MeCbl on binding to methionine synthase and heterolysis of protein-bound cofactor leading to cob(I)alamin. Charges have been assigned assuming a partial imidazolate character for H759.<sup>42</sup>

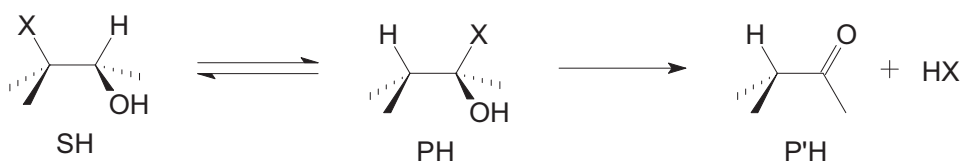
## 1.5 ADENOSYLCOBALAMIN DEPENDENT ENZYMES

At least ten biological reactions are catalysed by AdoCbl dependent enzymes, using a variety of substrates (**Table 1**). A general rearrangement of the type shown in **Figure 9** can be seen in all cases, where X is an electron withdrawing group.



**Figure 9:** General type of rearrangement catalysed by AdoCbl dependent enzymes, where X is an electron withdrawing group.

AdoCbl-dependent enzymes catalyse the stereospecific 1, 2-shift of the X group in the substrate molecule SH, exchanging places with an adjacent hydrogen atom, to form the product PH. In most cases this step is reversible and leads to an equilibration between SH and PH. Some AdoCbl dependent ‘eliminase’ enzymes promote the loss of water or ammonia from the rearranged product: an irreversible process shown schematically in **Figure 10**.



**Figure 10:** AdoCbl catalysed rearrangement of a substrate molecule (SH) into a product molecule (PH) followed by the subsequent dehydration to give P'H (X=OH or NH<sub>2</sub>)

CLASS	ENZYME	REACTION	GROUP X
Class I enzymes	Glutamate mutase		-CH(NH <sub>2</sub> )CO <sub>2</sub> H
	2-Methyleneglutarate mutase		-C(=CH <sub>2</sub> )CO <sub>2</sub> H
	Methylmalonyl-CoA mutase		-COSCoA
	Isobutyryl-CoA mutase		-COSCoA
Class II enzymes	Ribonucleotide reductase		None
	Ethanolamine ammonia lyase		-NH <sub>2</sub>
	1,2-Diol dehydrase		-OH
	Glycerol dehydrase		-OH
Class III enzymes	D-α-Ornithine mutase		-NH <sub>2</sub>
	D-α-Lysine mutase		-NH <sub>2</sub>

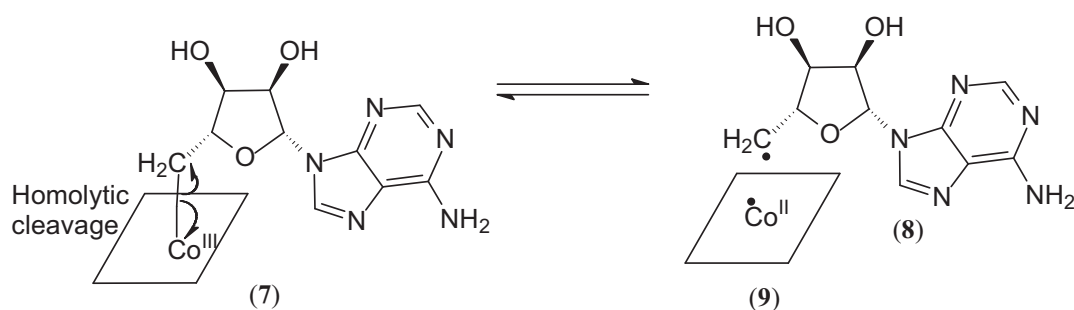
**Table 1:** Enzymatic reactions catalysed by AdoCbl

These AdoCbl-dependent reactions can be divided into three classes on the basis of the specifics of the migrating group and the receiving carbon.<sup>43, 44</sup> Class I enzymes are carbon skeleton mutases, which are reversible reactions; Class II enzymes are eliminases including lyases (such as dehydratases and deaminases) and ribonucleotide reductase. This type of enzyme catalyses the 1,2 shift of the H atom with an OH or NH<sub>2</sub> group resulting elimination of water or ammonia and the irreversible formation of the corresponding aldehyde. Class III enzymes are amino mutases which catalyse the reversible 1,2 shift of a H atom with a NH<sub>2</sub> group.

Depending on the binding structure of enzymes and the AdoCbl form, these three classes can be grouped into two categories.<sup>11</sup> The cobalamin exists in the base-on (Dmb-on) or base-off (Dmb-off) conformations with different B<sub>12</sub> dependent enzymes. The Class I and Class III enzymes (mutases) bind AdoCbl in base-off form, with the dimethylbenzimidazol(Dmb) replaced by the imidazole of a histidine residue, known as 'base-off/His-on'. All enzymes feature the consensus sequence (DXHXXG). The 'ligand triad' in the sequence of His-Asp-Ser was first discovered for methylcobalamin in the B<sub>12</sub> dependent methionine synthase. The Class II enzymes (eliminases) all bind AdoCbl in base-on form with the Dmb group coordinated to cobalt in the lower axial position.

AdoCbl (**7**) has been associated with free radical biochemistry for more than 30 years. Unlike the methylcobalamin dependent enzymes, it is now widely accepted that AdoCbl dependent enzymes operate *via* homolytic rather than heterolytic cleavage of the Co-C  $\sigma$ -bond. Homolytic cleavage of this relatively weak, long bond (130 kJ mol<sup>-1</sup>,

$\sim 2 \text{ \AA}$ ),<sup>45</sup> leads to formation of cob(II)alamin (**9**) and a highly activated organic radical, which is localised on the methylene group of 5'-deoxyadenosine (**8**). (Figure 11)

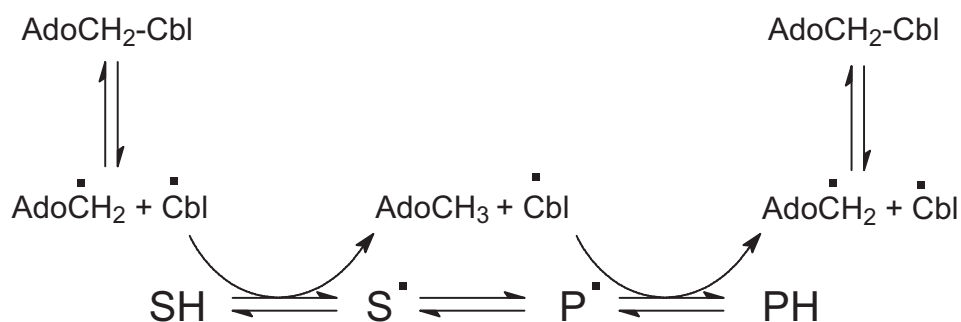


**Figure 11:** Homolytic cleavage of the cobalt-carbon  $\sigma$ -bond of AdoCbl (**7**) leading to cob(II)alamin (**9**) and 5'-deoxyadenosyl radical (**8**).

The pressure for Co-C  $\sigma$  bond homolysis must derive primarily from the protein, by specific interactions with the periphery amide side chains of the corrin ring and from the influence of the imidazole *trans* to the adenosyl group.<sup>46, 47</sup>

Therefore, activation of the AdoCbl is known as the first step in the catalytic cycle of the adenosylcobalamin dependent reactions. The Co-C  $\sigma$ -bond is cleaved, forming a 5'-deoxyadenosyl radical and cob(II)alamin. The adenosyl radical subsequently abstracts a specific hydrogen atom from a substrate molecule to give a substrate-derived radical (S') and 5'-deoxyadenosine. The next step is the rearrangement between the substrate-derived radical (S') and product-related radical (P'). But how does this isomerisation occur? This is the key question that many groups in many countries are trying to probe. The product related radical (P') abstracts the hydrogen atom from the methyl group of 5'-deoxyadenosine forming a product molecule and the adenosyl radical. Product release occurs where the coenzyme B<sub>12</sub> is regenerated

by the combination of the 5'-deoxyadenosyl radical and the cob(II)alamin species, (Figure 12). It is considered to be the final step of one catalytic cycle.



**Figure 12:** ‘Minimal mechanism’ for coenzyme B<sub>12</sub>-dependent enzymatic rearrangements (AdoCH<sub>2</sub>-Cbl = adenosylcobalamin (coenzyme B<sub>12</sub>), AdoCH<sub>2</sub>• = 5'-deoxyadenosyl radical, AdoCH<sub>3</sub> = 5'-deoxyadenosine, Cbl• = cob(II)alamin; SH = substrate, PH = product, S• = substrate-derived radical, P• = product-related radical).

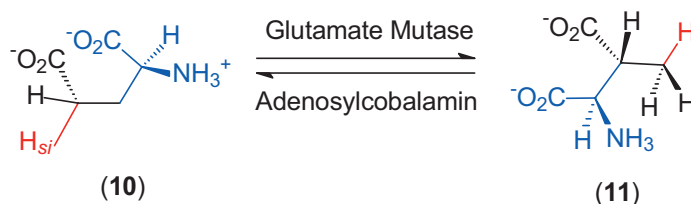
Experiments with tritium-labelling substrate in the AdoCbl-dependent diol dehydratase carried out by Frey *et al.*<sup>48</sup> indicated the intermolecular transfer of the migrating hydrogen atom between substrate/product and cofactor. Furthermore, the cofactor serves as a hydrogen carrier which transfers the hydrogen atom from substrate to product. Experiments carried out by Zagalak *et al.*<sup>49</sup> proved that a product radical will acquire either the hydrogen from its substrate precursor, or one hydrogen atom of the cofactor from another substrate precursor.

X-ray crystal structures showed that in the Class II enzymes (eliminases, e.g. diol dehydratase), intermediate radicals are generated about 10 Å away from the cobalt atom in cob(II)alamin. Therefore, cob(II) alamin is proposed to act as a ‘spectator’ in

the catalysis. The fact that some Class II enzymes use a radical generator other than adenosylcobalmin (i.e. *S*-adenosylmethionine) supported this premise. In the crystal structure of glutamate mutase the 5'-deoxyadenosyl radical remains within 3-4 Å of the cobalt atom with the intermediate radicals approximately 6 Å away. It is suggested that cob(II)alamin acts as a 'conductor' which stabilises the highly reactive methylene radical. This distance also avoids the possibility of the covalent bond formation between intermediate and cob(II)alamin.<sup>50</sup> Recently it has been proposed that the cob(II)alamin reduce the transition-state energy leading to the intermediate radical.<sup>51</sup>

## 1.6 GLUTAMATE MUTASE

The coenzyme-B<sub>12</sub> dependent glutamate mutase catalyses the reversible isomerisation of (*S*)-glutamate (**10**) to (2*S*,3*S*)-3-methylaspartate (**11**).<sup>52</sup> (**Scheme 2**) In this reaction, the H<sub>si</sub> at C-4 and the glycyl moiety of glutamate are interchanged. The glycyl group migrates with retention of configuration at C-2, whereas C-4 undergoes inversion of configuration. The resulting methyl group of 3-methylaspartate becomes ‘racemic’. This enzyme is extremely specific for (*S*)-glutamate (**10**) and (2*S*,3*S*)-3-methylaspartate (**11**), it did not show any activity with other substrates.<sup>53</sup>

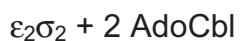
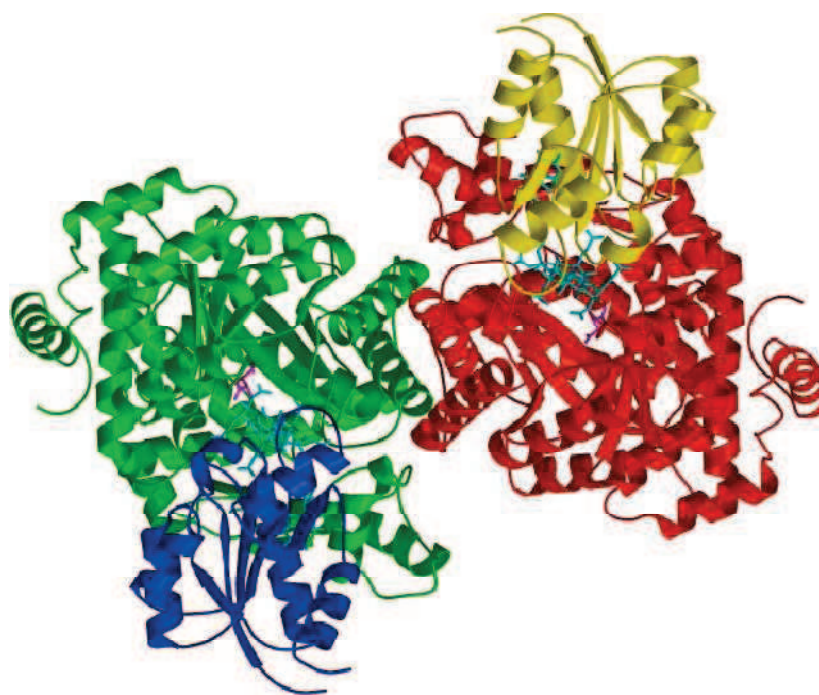


**Scheme 2:** Reaction catalysed by glutamate mutase

Glutamate mutase (EC 5.4.99.1) was first discovered in *Clostridium tetanomorphum* by Barker.<sup>54</sup> It was the first discovered coenzyme B<sub>12</sub> dependent mutase. More recently, glutamate mutase was purified from the closely related organism *Clostridium cochlearium*. It can be found in various clostridia and many other anaerobic bacteria. In these microorganisms, the mutase is involved in the first step of fermentation of glutamate to ammonia, CO<sub>2</sub>, acetate, butyrate and molecular hydrogen. (**Scheme 3**)<sup>55</sup>

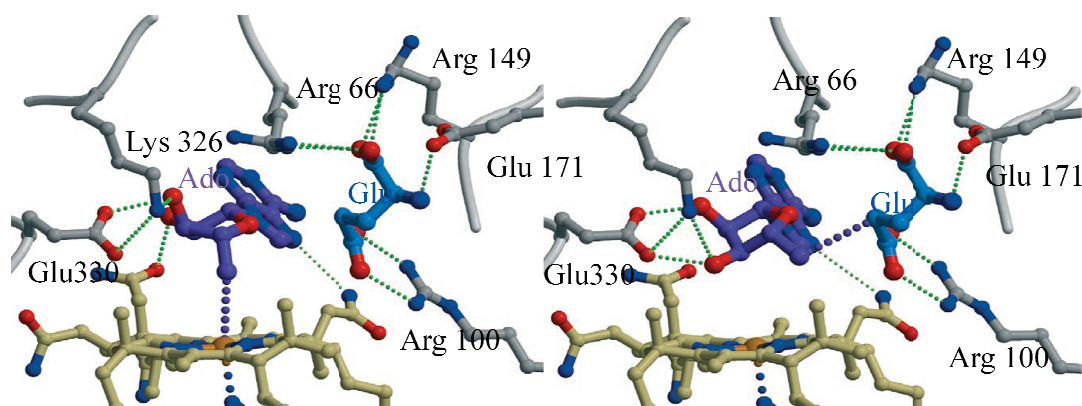


two protein components; E, a homodimer ( $\epsilon_2$ ,  $2 \times 53.5$  Kda) and S, a monomer ( $\sigma$ , 14.8 Kda). Incubation of the purified recombinant components E and 2S with AdoCbl results in the formation of the active enzyme ( $\epsilon_2$ ,  $2\sigma$ ).<sup>57</sup> The  $\sigma$  subunit acts as the B<sub>12</sub> binding unit and  $\epsilon$  as the catalytic unit. The cofactor is bound at the interface of the two subunits. (**Figure 13**) The  $\sigma$  subunit recognises the upper face of AdoCbl and contains the substrate-binding site, and the  $\epsilon$  subunit is a conserved cobalamin-binding domain that interacts with the lower face of the coenzyme, called ‘base off’ mode.

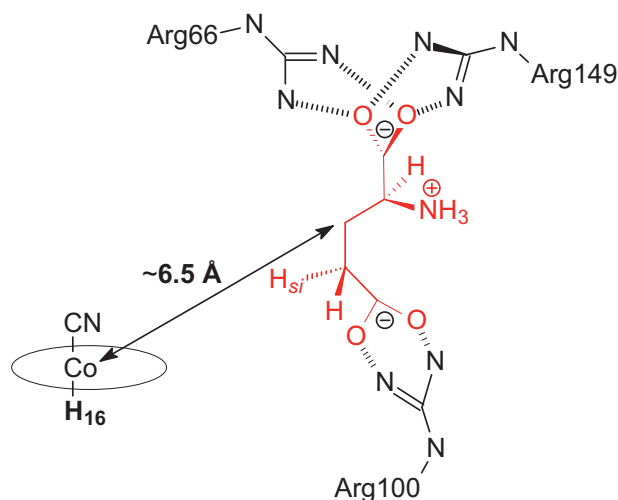


**Figure 13:** Crystal structure of glutamate mutase from *Clostridium Cochlearium*.  $\epsilon$  subunits are shown in green and red,  $\sigma$  subunits are shown in blue and yellow. The cofactor and substrate are shown in light blue and purple, respectively.

According to the research of Kratky's group, the substrate was held in the active site by three arginines and one glutamate of the glutamate mutase. (**Figure 14**) Marsh's experiments<sup>58</sup> indicate that the Glu171 acts as a general base which deprotonates the amino group of the substrate during the catalysis. It also shows no effect on the stability of the substrate/product radical themselves. So the active site of glutamate mutase contains the interaction of three arginines (R66, R100 and R149) from the enzyme and two carboxylic acid groups of substrate, the so called 'arginine claw'. The crystal structure also confirmed the distance ca. 6.5 Å derived from the simulation of the EPR spectra of Co(II)alamin and the substrate radical<sup>59</sup>(**Figure 15**).



**Figure 14:** Stereoview of the protein's arginine claw; the illustration alongside shows the adenosyl radical released from coenzyme B<sub>12</sub> attacking a substrate molecule.<sup>57</sup>



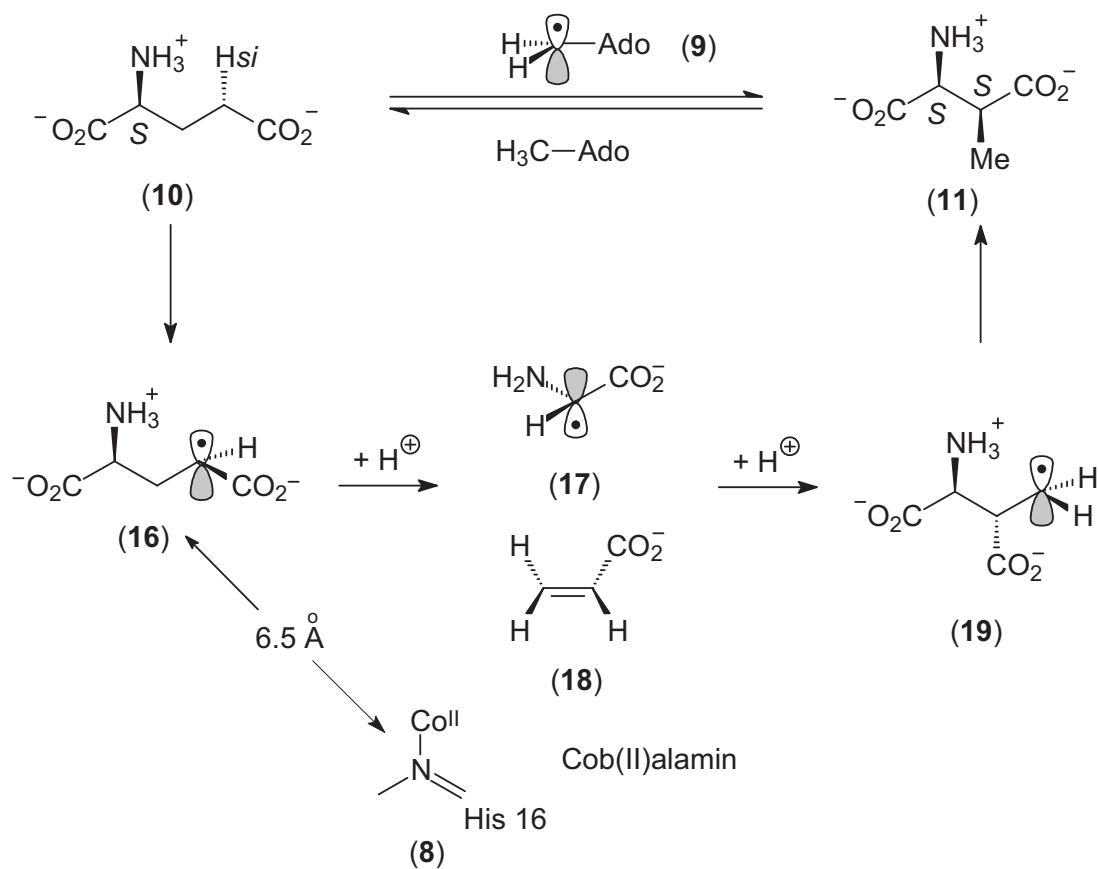
**Figure 15:** Schematic representation of the active site portion of glutamate mutase with cyanocobalamin as cofactor and glutamate as substrate showing the ‘arginine claw’ and the ‘base off’ mode of the B<sub>12</sub>.

### 1.6.2 Mechanism of action of glutamate mutase

According to the generally accepted mechanism, the initial step is the homolysis of the carbon-cobalt bond of the coenzyme B<sub>12</sub>, due to the enzyme-coenzyme binding the substrate as occurs in all other AdoCbl-dependent enzymatic reactions, by which the 5'-deoxyadenosyl radical (**9**) and cob(II)alamin (**8**) is formed. The 5'-deoxyadenosyl radical abstracts a specific hydrogen atom from a substrate molecule to form a substrate-derived radical, which rearranges to a product-related radical.

A cyclic intramolecular pathway is chemically implausible for this reaction, while a fragmentation/recombination route is feasible.<sup>60</sup> During the catalysis of glutamate mutase the initially formed 4-glutamyl radical (**16**), which was identified by EPR spectra,<sup>59</sup> fragments into acrylate (**18**) and 2-glycinyl radical (**17**), which recombine to 3-methylaspartate radical (**19**). Final redonation of hydrogen from 5'-methyl group of

5'-deoxyadenosine yields the product and recycles the initial radical. (**Scheme 4**) This idea is supported by the synergistic inhibition of this enzyme by glycine and acrylate. Furthermore, the characteristic EPR signal at  $g = 2.1$  is only induced by both glycine and acrylate.<sup>60</sup> Recently, rapid quench flow studies have provided evidence for the formation of a glycy radical and acrylate at kinetically competent rates, although the amount of these species detected was very low.<sup>61</sup> This experiment supports the 'fragmentation-recombination' mechanism.



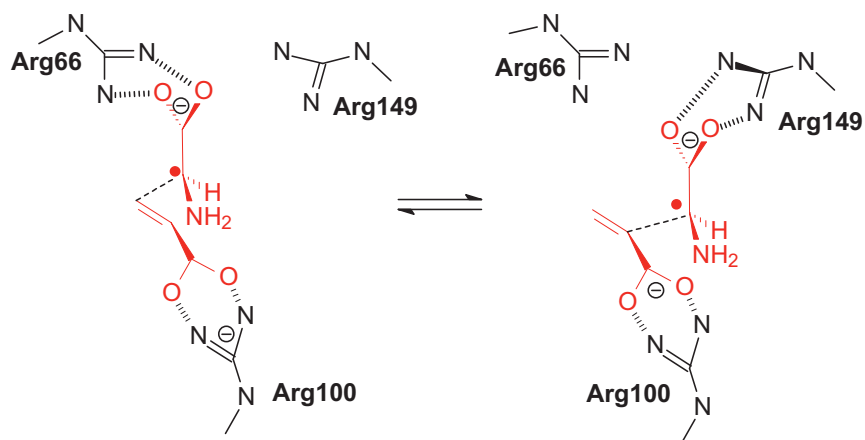
**Scheme 4:** Fragmentation mechanism proposed for glutamate mutase reaction.

### 1.6.3 Active site and radical rearrangement.

*Ab initio* molecular orbital calculations predict that the fragmentation barrier for a substrate with neutral substate (carboxylate protonated and amino group unprotonated) is significantly lower than for one in which the glutamyl radical is either deprotonated or protonated.<sup>62</sup> The calculations also indicate that the fragmentation-recombination mechanism is carried out by glutamate mutase controlling the protonation state of the migrating glycine through appropriate proton transfer in the active site, i.e., by (partially) deprotonating the  $\text{NH}_3^+$  group and (partially) protonating the  $\text{COO}^-$  substituent. This proved that the arginine claw (interaction between three arginine residues and the two carboxylic acid groups) and the hydrogen bond between E171 and amino group of the substrate play an important role in the fragmentation-recombination mechanism.

In the proposed mechanism, a key question is how glutamate mutase stabilises the highly reactive radical substrates and intermediates and directs the fragmentation of the substrate radical and the combination of the intermediates towards the product radical. As discussed above, the relative positions of the two carboxylate groups must change during the rearrangement of glutamate to 3-methylaspartate. It is proposed that E171 in the active site of glutamate mutase is a general base which deprotonates the amino group to stabilise the radical intermediate, and the arginine claw adjusts the structural modification of substrates. Deprotonation of the amino group by E171 is expected to facilitate the formation of glycine radical intermediate during the proposed mechanism of rearrangement.<sup>58</sup> Protonation of the amino group might be

expected to initiate the homolysis of the Co-C bond and the formation of glutamyl radical.



**Figure 16:** Using the ‘arginine claw’ to ‘handover’ glyceryl radical from C-3 to C-2 of acrylate.

One arginine (R100) in the active site anchors the C-5 carboxylate of glutamate (or C-4 carboxylate of 3-methylaspartate) to hold the acrylate intermediate. The handover of migrating glycine radical by the other two arginines (R66 and R149) enables the shift of glycinyl moiety from C-3 to C-2 of the acrylate. (**Figure 16**) After formation of the 3-methylaspartyl radical, protonation of the amino group by E171 stabilises the product radical. The arginine claw holds the product radical to abstract a hydrogen atom from 5'-deoxyadenosine intermediate.

## 1.7 GLYCEROL AND DIOL DEHYDRATASE

Diol dehydratase and glycerol dehydratase have been identified as exclusively coenzyme B<sub>12</sub> dependent enzymes until recently. The only exception for diol dehydratase has not been well characterised. EPR study of the B<sub>12</sub> independent diol dehydratase from membranes of *Clostridium glycolicum* with propanediol indicated that a stable organic radical formed.<sup>63</sup> Raynaud *et al.*<sup>64</sup> reported the molecular characterisation of B<sub>12</sub>-independent glycerol dehydratase from *Clostridium butyricum*. It is a glyceryl radical enzyme activated by *S*-adenosylmethionine (SAM). The B<sub>12</sub>-independent glycerol and diol dehydratase will not be discussed any further in this work.

The coenzyme B<sub>12</sub>-dependent glycerol and diol dehydratases are involved in the anaerobic utilisation of small molecules of 1,2-diol (**Scheme 5**), e.g., propane-1,2-diol (**20**) and glycerol (**24**). They catalyse the intermolecular rearrangements to generate aldehyde in the first step of the fermentation of small molecules. 3-Hydroxypropionaldehyde (**25**) can be reduced to 1,3-propanediol by consuming 50-66 % of glycerol (**24**) into acetate, ethanol, lactate, H<sub>2</sub> and CO<sub>2</sub>, which is the major product in glycerol fermentation pathways.<sup>65</sup> In propane-1,2-diol (**20**) fermentation pathways, the propionaldehyde (**21**) is converted to equal amounts of propanol and propionic acid. The propanol was oxidised to propionic acid eventually. The propionic acid was degraded into propionate and CO<sub>2</sub>.<sup>66</sup> In the bacterial strains which lack of glycerol dehydratase, diol dehydratase instead of glycerol dehydratase is involved in the conversion of 3-hydroxypropionaldehyde (**25**).

