Bioorganic Chemistry **28,** 176–189 (2000)
doi:10.1006/bioo.2000.1168, available online at http://www.idealibrary.com on IDEAL®

REVIEW ARTICLE

Coenzyme-B_{12}-Dependent Glutamate Mutase

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Received December 15, 1999

Adenosylcobalamin (coenzyme B_{12})-dependent glutamate mutase catalyzes a most unusual carbon skeleton rearrangement involving the isomerization of L-glutamate to L-*threo*-methylaspartate, a reaction that is without precedent in organic chemistry. This reaction proceeds through a mechanism involving free radical intermediates that are initiated by homolysis of the cobalt–carbon bond of the coenzyme. The enzyme serves as a paradigm for adenosylcobalamin-dependent catalysis and, more generally, provides insights into how enzymes generate and control reactive free radical species. This review describes how recent studies on the mechanism and structure of glutamate mutase have contributed to our understanding of adenosylcobalamin-mediated catalysis.

INTRODUCTION

It has been a little over 40 years since H. A. Barker reported the unusual rearrangement of L-glutamate to L-*threo*-3-methylaspartate (Fig. 1) which occurs as the first step in the fermentation of glutamate by the obligate anaerobe *Clostridium tetanomorphum* (*1*). The enzyme responsible for this transformation, glutamate mutase, was the first vitamin B_{12} enzyme to be discovered. At the time, the structure of vitamin B_{12} (cyanocobalamin, CNCbl) had recently been determined by X-ray crystallography, revealing a remarkably complex macrocycle containing a cobalt atom (*2*). However, its biochemical function remained obscure. In fact, vitamin B_{12} proved to be a precursor of one biologically active form of the coenzyme, adenosylcobalamin (AdoCbl). The X-ray structure of this molecule (Fig. 1) revealed a novel feature of the coenzyme: a covalent bond between the 5′-carbon of adenosine and the central cobalt atom of the cobalamin macrocycle (*3*). The cobalt–carbon bond is key to the coenzyme's biological function as a source of organic free radicals that are "unmasked" by homolysis.

Metal–carbon bonds have proved to be extremely rare in nature, and even today only a handful of examples are known (*4*). One of these is the "sister" compound to AdoCbl, methylcobalamin, in which a methyl group is bonded to cobalt. Despite the structural similarity to AdoCbl, methylcobalamin (MeCbl) fulfills a very different biochemical function, serving as a methyl transfer agent in reactions that proceed through ionic mechanisms (*5*–*7*).

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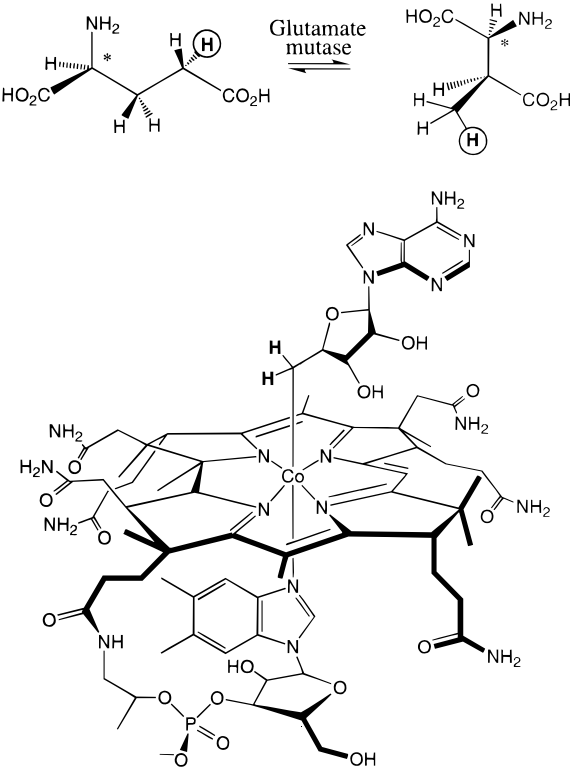

FIG. 1. The structure of adenosylcobalamin and the isomerization catalyzed by glutamate mutase; the migrating hydrogen is circled and the migrating carbon is marked by *.

There are now about a dozen AdoCbl-dependent enzymes known, most of which catalyze unusual isomerization reactions involving the interchange of a hydrogen atom on one carbon with an electron-withdrawing group, *X*, on an adjacent carbon. *X* may be either –OH, $–NH_2$, or a carbon-containing fragment as in the case of glutamate mutase (*8–12*). The minimal mechanistic scheme describing these rearrangements is shown in Fig. 2. It involves homolysis of AdoCbl to generate a 5′-deoxyadenosyl radical that then abstracts the migrating hydrogen to generate 5′-deoxyadenosine and a substrate radical. In a poorly understood step, the migrating group, *X*, undergoes a 1,2 shift to give a product radical, and finally a hydrogen atom is returned from 5′-deoxyadenosine to generate the product and adenosyl radical. The adenosyl radical then recombines with the cobalt to reform AdoCbl. The one apparent exception to this mechanism is AdoCbl-dependent ribonucleotide reductase, which catalyzes the reduction of ribonucleotide triphosphates to 2′-deoxyribonucleotide triphosphates (*13,14*). However, although the details are more complicated, AdoCbl serves the same basic function: a radical is generated on the substrate at the 3′-carbon, adjacent to the site of reduction, and this has the effect of labilizing the 2′-OH toward leaving (*15*).

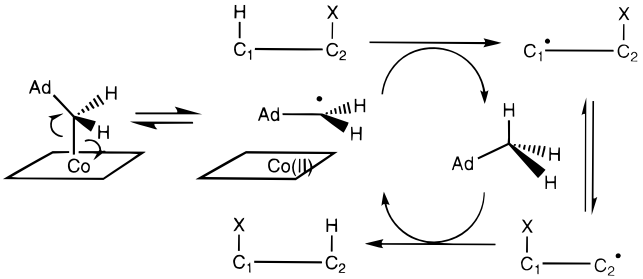

FIG. 2. A minimal mechanistic scheme describing the reactions catalyzed by AdoCbl-dependent isomerases; *X* may be –OH, –NH_2, or, as is the case with glutamate mutase, a carbon-containing fragment.

AdoCbl-dependent enzymes may be viewed as one group in a larger, emerging class of enzymes that use carbon-based radicals to effect a variety of chemically difficult reactions involving the cleavage of carbon–carbon, carbon–oxygen, and carbon–nitrogen bonds (*16–18*). In these reactions an enzyme-generated radical is used to remove a hydrogen atom from an unreactive position in the substrate, thereby activating the substrate toward various chemical transformations that would otherwise be difficult to achieve. Nature has evolved several different mechanisms for generating carbon-based radicals that may be classified as "reductive," "homolytic," and "oxidative" (*17*). In the first two mechanisms, 5′-deoxyadenosyl radical is generated either by a one-electron reduction of S-adenosylmethionine in an iron–sulfur center mediated reaction (*19*) or by homolysis of the carbon–metal bond in AdoCbl. The third mechanism involves the one-electron oxidation of a tyrosine residue to form a radical that is stabilized by a dinuclear iron(III) cluster (*20*).

The different mechanisms may be viewed as evolutionary adaptations that have allowed enzymes to generate radicals in increasingly aerobic environments. The reductive mechanism might be considered the prototypical mechanism, but appears to be confined to organisms living under strictly anaerobic conditions as all the enzymes studied so far are extremely oxygen sensitive. On the other hand, the iron-stabilized tyrosyl radical formed in the oxidative mechanism actually requires molecular oxygen as the oxidizing agent (*21*) and is stable under aerobic conditions. AdoCbl is perhaps the most versatile method of generating radicals as it can function under either anaerobic or aerobic conditions: the reversible homolysis of the cobalt–carbon bond represents an elegant solution to the problem of generating free radicals rapidly at the active site of an enzyme.

The unusual reactions catalyzed by AdoCbl-dependent enzymes have intrigued bioorganic, biophysical, and bioinorganic chemists, and over the past decade our understanding of both mechanism and structure has advanced significantly. This review will focus primarily on glutamate mutase. The "simple" isomerization catalyzed by this enzyme provides a model system for investigating how enzymes harness the intrinsic reactivity of free radicals toward catalysis and exemplifies many structural and mechanistic features common to other AdoCbl-dependent isomerases.

STRUCTURE OF GLUTAMATE MUTASE

Glutamate mutase comprises two subunits, designated E and S. E is a dimer of subunit M_r 54,000 whereas S is a monomer of M_r 14,800 (*22–25*). The subunits are only weakly associated and may readily be separated by gel filtration chromatography. Both subunits are required to bind AdoCbl, and in its active form the enzyme is an E_2S_2 tetramer that binds two molecules of AdoCbl at the interfaces between the E and S subunits (*24*). Crystal structures of the enzyme with the AdoCbl analogs methylcobalamin and cyanocobalamin bound have recently been solved by Kratky's group (*26*).

Structure of the S subunit—A Conserved B_{12}-Binding Domain

The S subunit was initially identified as a conserved cobalamin-binding domain on the basis of sequence similarities with other B_{12} enzymes that are characterized by the "D-x-H-x-x-G" sequence motif (*6,27*). Among the homologous sequences are representatives from both the AdoCbl-dependent carbon-skeleton mutases and the MeCbl-dependent methyltransferases (*5*). Glutamate mutase is unusual in that this domain is present as a separately encoded subunit, whereas in other B_{12} enzymes it is either a C-terminal domain or an internal domain of a larger protein. There appears to be no functional significance to this arrangement, however, as genetic engineering has allowed the construction of a highly active single-subunit version of glutamate mutase in which the S subunit is fused to the C-terminus of the E subunit (*28*).

The X-ray structure of glutamate mutase (Fig. 3) shows the conserved S subunit to possess an α/β structure reminiscent of the Rossmann fold of nucleotide-binding proteins. It consists of a twisted β-sheet of five parallel strands encased by five α-helices that bind the lower, α-face of the corrin macrocycle and the substituents projecting "down" from this face, notably the dimethylbenzimidazole ribofuranosyl nucleotide loop. Upon binding to the enzyme, AdoCbl undergoes a major conformational change whereby the dimethylbenzimidazole moiety dissociates from the cobalt and becomes buried in an elongated hydrophobic pocket deep in the Rossmann domain. Extensive intermolecular contacts are made in this region, with strands β3 and β4 of the S subunit forming one side of the nucleotide-binding pocket and helices α1 and α5 forming the other. The conserved residues of the D-x-H-x-x-G motif lie on a loop between helix α1 and strand β2. The histidine makes intimate contact with the cobalt atom, taking up the vacant Co_α coordination position via the ε-nitrogen of the imidazole ring. The histidine (His 16) is hydrogen-bonded to the carboxylate side-chain of the conserved aspartate residue.

The crystal structures methylmalonyl-CoA mutase and methionine synthase B_{12}-binding domain are also known (*29,30*): these enzymes also possess the conserved domain corresponding to the S subunit, including the D-x-H-x-x-G motif. Not surprisingly the structures are very similar and cobalamin is again bound in the "base-off, histidine-on" conformation. However, not all AdoCbl-dependent enzymes possess this conserved domain, and it is known that for diol dehydrase and AdoCbl-dependent ribonucleotide reductase the dimethylbenzimidazole base remains coordinated to cobalt when B_{12} is bound to the enzyme (*31,32*).

The solution structure of the S subunit apoprotein has also been determined by multidimensional NMR techniques (*33*). It appears that significant changes in the structure of the S subunit accompany cobalamin-binding. In the apoprotein, the five-stranded

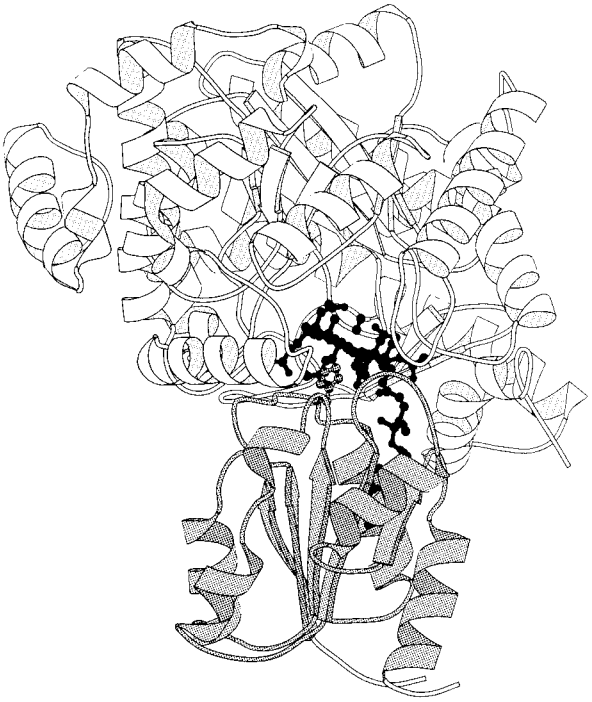

FIG. 3. The crystal structure of glutamate mutase. Only one-half of the E_2S_2 tetramer is shown. The upper domain (E subunit, light shading) adopts a TIM-barrel structure, the lower conserved domain (S subunit, dark shading) is a canonical "Rossmann" nucleotide-binding fold. Cyanocobalamin, in which CN replaces the adenosyl moiety, is bound at the interface of the two domains (ball and stick model). A histidine (shaded ball and stick model) from the S subunit displaces the nucleotide tail of the coenzyme to coordinate cobalt.

β-sheet core is intact but surrounded by just four α-helices (Fig. 4). NMR relaxation measurements revealed considerable dynamic behavior in several parts of the protein, particularly between sheets β1 and β2 that are spanned by a poorly structured loop containing a highly mobile set of residues (Ser13–Phe27) including the D-x-H-x-x-G motif. The hydrogen-bonded network around His16 is also not formed in the apo enzyme. This suggests that, in the absence of coenzyme, only part of the cobalamin-binding site is formed, and that coenzyme binding serves to trigger nucleation of helix α1 to completely bury the nucleotide tail and anchor the coenzyme to the protein.

Structure of the E Subunit—The Catalytic Domain

The E subunit of glutamate mutase contains the active site of the enzyme and takes the form of a $(\alpha/\beta)_8$ TIM-barrel. The C-terminal end of the barrel sits over the upper reactive face of AdoCbl, with most of the protein contacts being made between propionamide side-chains of the coenzyme and the backbone amides of the loops that connect the secondary structure elements of the barrel. Thus the coenzyme is sandwiched between the E and the S subunits. The structure was determined with MeCbl

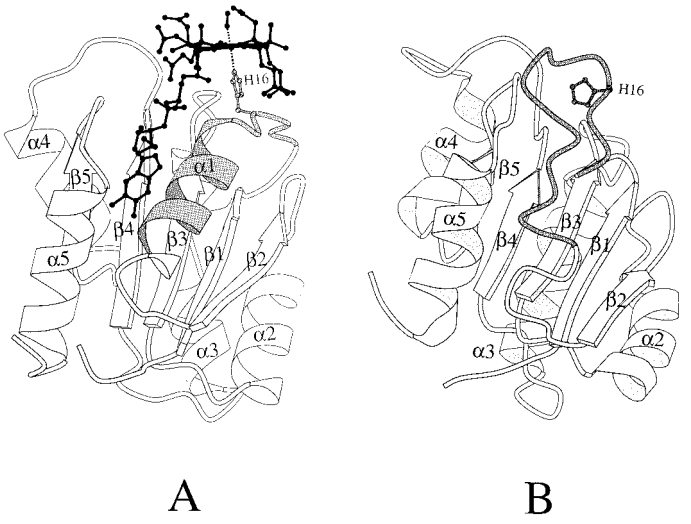

FIG. 4. Conformational changes involving helix α1 that occur in the S subunit when the protein binds the coenzyme. (**A**) The crystal structure of the S subunit with CNCbl bound; (**B**) The solution structure calculated for the S subunit from NMR experiments.

and CNCbl bound, so that the location of the adenosyl moiety is not known. For reasons that are unclear, it appears that the protein:AdoCbl complex is insufficiently stable for the Co–C bond to remain intact during crystallization and X-ray analysis (*34*).

Glutamate mutase was crystallized at low pH from tartrate buffer and contains one tartrate ion (which may be considered a reasonable mimic of methylaspartate) bound in the lumen of the barrel about 5 Å from the coenzyme. A number of residues make hydrogen bonds to the tartrate (Fig. 5): these include Arg66 and Arg149, which bind what would be the α-carboxylate of glutamate, Arg100 and Tyr181, which bind the ω-carboxylate, and Glu171, which binds the α-carboxylate of tartrate but most likely interacts with the positively charged amino group of glutamate. The large number of hydrogen bonds to the substrate analog provides an explanation for the strict substrate specificity observed for the enzyme. Several narrow, solvent-filled channels lead to and from the active site, through which the substrate might gain access; alternatively, (partial) dissociation of the two protein subunits would provide another plausible mechanism for the substrates to enter and leave.

The crystal structures of methylmalonyl-CoA mutase and diol dehydrase have also recently been determined (*29*,*34*,*35*). These enzymes possess different quaternary structures, little sequence similarity (except in the "Rossmann" domain of methylmalonyl-CoA mutase), and, whereas methylmalonyl-CoA mutase and glutamate mutase bind AdoCbl base-off, histidine-on, diol dehydrase binds AdoCbl "base-on." It would seem unlikely that they would share common structures, but all three enzymes adopt a very similar global fold. The coenzyme is bound between a Rossmann-fold domain, which is modified slightly to accommodate base-on AdoCbl in diol dehydrase, and a TIM-barrel domain that binds the substrate. Indeed, the C_α atoms of the barrel domain of methylmalonyl-CoA mutase and glutamate mutase can be superimposed with an rms deviation of only 2 Å (*26*). However, the

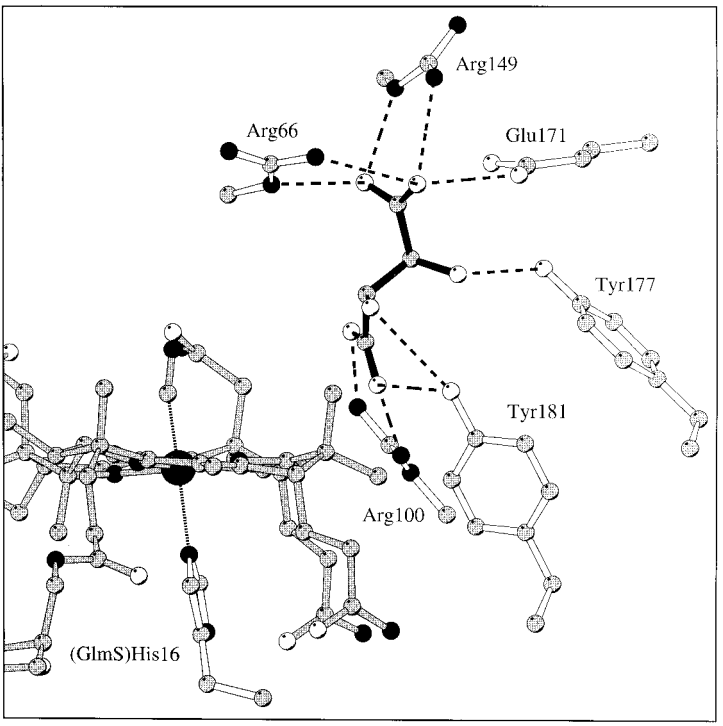

FIG. 5. Detail of the presumed substrate binding site with tartarate bound. Most likely Glu171 interacts with the amino group of the substrates rather than the carboxyl group, as it appears to do in the low-pH crystals of the tartrate complex.

active site residues of these enzymes do not seem to be conserved and the substrates in each case are bound very differently.

MECHANISTIC STUDIES ON GLUTAMATE MUTASE

The isomerization of glutamate to methylaspartate catalyzed by glutamate mutase may usefully be separated into two mechanistic problems. First, how does the enzyme trigger the energetically very unfavorable homolysis of AdoCbl and couple this to generation of the substrate radical? And second, how do the substrate and product radicals rearrange on the enzyme? The first problem is common to all AdoCbl-dependent enzymes and so may have a common solution, whereas the mechanistic details surrounding the interconversion of substrate radicals interconvert are likely to vary, depending on the nature of the migrating group in the substrate.

AdoCbl Homolysis and the Formation of Substrate Radicals

The first step in the mechanism of all AdoCbl-dependent enzymes is homolysis of the cobalt–carbon bond. This reaction can be readily followed by stopped-flow spectroscopy, since the change in the oxidation state and coordination number of cobalt results extensive changes to the UV-visible spectrum of the coenzyme (Fig. 6). For all enzymes examined, homolysis only occurs in the presence of the substrate or substrate analogs.