# Glutamate and 2-Methyleneglutarate Mutase: From Microbial Curiosities to Paradigms for Coenzyme $B_{12}$-dependent Enzymes

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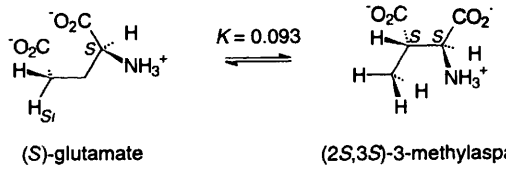
*Dedicated to H. A. Barker in his ninetieth year*

## 1 Introduction

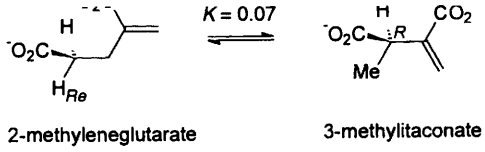
In the late 1950s H. Albert Barker discovered a light-sensitive, yellow–orange cofactor for the carbon-skeleton rearrangement of glutamate to 3-methylaspartate in *Clostridium tetanomorphum*, a strict anaerobic bacterium fermenting glutamate to ammonia, $CO_2$, acetate, butyrate and $H_2$.[1] The enzyme catalysing this process was isolated, named glutamate mutase (EC 5.4.99.1), and shown to act specifically on (*S*)-glutamate, which equilibrated with (2*S*,3*S*)-3-methylaspartate (Scheme 1).[2] Barker proved that the cofactor was




**Scheme 1** Interconversion of (*S*)-glutamate with (2*S*,3*S*)-3-methylaspartate catalysed by coenzyme $B_{12}$-dependent glutamate mutase

related to vitamin $B_{12}$, actually pseudo-vitamin $B_{12}$, by showing that treatment with an excess of cyanide gave the characteristic reddish purple dicyanocobalamin (see Box concerning cobamide nomenclature). Subsequently, dark-red crystals of the coenzyme form of vitamin $B_{12}$ were isolated from *Propionibacterium shermanii* and subjected to X-ray analysis, which yielded the structure shown in the Box.[3] The presence of a cobalt–carbon $\sigma$-bond in coenzyme $B_{12}$ (adenosylcobalamin) was an unexpected feature that would ultimately prove to be of crucial importance for the biological action of the coenzyme.

A decade later Thressa C. Stadtman identified 2-methyleneglutarate mutase (EC 5.4.99.4) in *Clostridium barkeri*, which ferments nicotinate *via* 2-methyleneglutarate to ammonia, $CO_2$, acetate and propionate.[4] During this degradation the enzyme catalyses the interconversion of 2-methyleneglutarate with (*R*)-3-methylitaconate (Scheme 2) and requires coenzyme $B_{12}$ as




**Scheme 2** Interconversion of 2-methyleneglutarate and (*R*)-3-methylitaconate catalysed by coenzyme $B_{12}$-dependent 2-methylenegulatarate mutase

cofactor. Hence, the reaction catalysed by 2-methyleneglutarate mutase is very similar to that of glutamate mutase. Both enzymes remained as microbial curiosities until cloning and over-expression of their genes in *Escherichia coli* enabled the production of relatively large amounts of homogeneous apo-proteins.[5–9] Today, there are three distinct classes of molecular rearrangements known to be catalysed by an enzyme in partnership with coenzyme $B_{12}$ (see Table 1). In these reactions a migrating group X and a hydrogen atom exchange places on adjacent carbon atoms. The reactions differ according to the nature of the migrating group X and the substituent Y (H or OH) at the adjacent carbon from which the hydrogen atom is abstracted. In this review, we focus on the mechanisms of the reactions catalysed by glutamate and 2-methyleneglutarate mutase. We aim to stimulate the reader to learn more about the fascinating chemistry of the radicals postulated as intermediates in these arrangements.

## 2 Mechanism of Action – Initial Conclusions

Early studies of coenzyme $B_{12}$-dependent enzymatic reactions demonstrated that the essence of coenzyme function was to be found in the 5′-methylene group of the 5′-deoxyadenosyl residue bound to cobalt. It was shown by isotopic labelling that the migrating hydrogen became attached to this group, leading to the supposition that 5′-deoxyadenosine is an intermediate.

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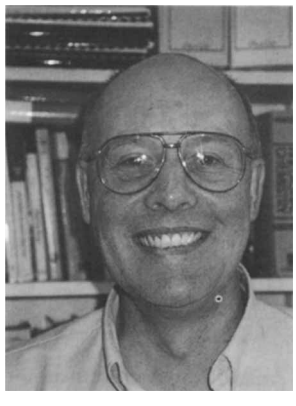
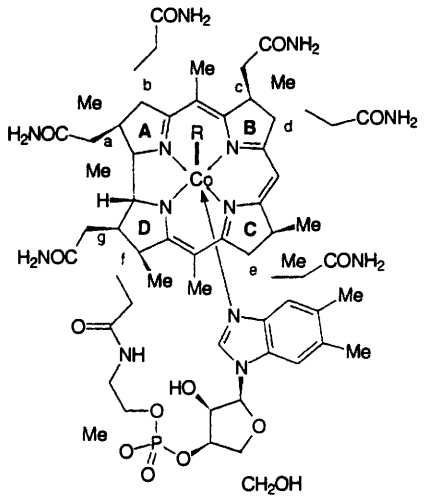
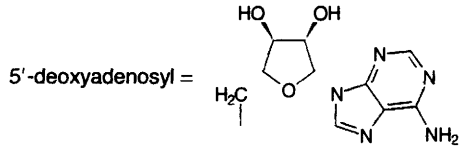
*Bernard Golding studied chemistry at Manchester, where he worked with Rod Rickards as a PhD student. Postdoctoral work in the mid-sixties with Albert Eschenmoser on the synthesis of vitamin $B_{12}$ led to investigations of the mode of action of $B_{12}$ coenzymes. He is professor of organic chemistry and currently head of department at Newcastle, where he also has research groups studying carcinogenesis and anticancer drug design, and a bicycle to evade the city's traffic.*



**Box $B_{12}$ Nomenclature**



In this review coenzyme $B_{12}$ refers to the substance adenosylcobalamin (AdoCbl) in which R = 5′-deoxyadenosyl in the above structure By definition all cobalamins contain 5,6-dimethylbenzimidazole Coenzyme $B_{12}$ is also a cobamide (*i e* any $B_{12}$ derivative as shown with a heterocyclic base connected to the lower ribose) In pseudo-vitamin $B_{12}$ the base is adenine, connected to ribose at N-7 and ligated to cobalt at N-9 In dicyanocobalamin, the 5,6-dimethylbenzimidazole has been displaced by cyano, but remains connected to ring *D* through the ribose–phosphate–propanolamine, however, the adenosyl has been replaced by a cyano group



Homolytic cleavage of the Co–C $\sigma$-bond generates cob(II)alamin and the 5′-deoxyadenosyl radical, which should be reactive enough to abstract a hydrogen atom even from an unactivated position (*e g* methyl group) of a substrate molecule SH (see Scheme 3) The substrate-derived radical S· rearranges to a product-related radical P·, which is quenched by 5′-deoxyadenosine with regeneration of the 5′-deoxyadenosyl radical and formation of product PH [10] The mode of conversion of S· into P· has been the subject of controversy At one extreme, it has been postulated that the rearrangement proceeds *via* organic radicals with cob(II)alamin as a mere spectator [10] At the other extreme, the cob(II)alamin may conduct the rearrangement through organocorrinoid intermediates [11] In Sections 3—7 experimental evidence pertaining to the mechanistic questions is presented leading to decisions about the mode of action of glutamate and 2-methyleneglutarate mutase It is concluded that the most likely mechanisms involve fragmentation of S· to the alkene acrylate and a carbon-centred radical (X·, *i e* 2-glycinyl radical for glutamate mutase and 2-acrylylyl radical for 2-methyleneglutarate mutase), which recombine to give P·, perhaps with the assistance of cob(II)alamin In the conclusion to this review the extension of this mechanism to other coenzyme $B_{12}$-dependent reactions (see Table 1) is considered Are glutamate and 2-methyleneglutarate mutase paradigms for coenzyme $B_{12}$-dependent enzymes?

## 3 Enzymology

Glutamate mutase was first isolated from *C tetanomorphum*[2] and more recently from the related *C cochlearium* [12] The enzymes are very similar in their properties, the only significant difference being the higher stability of the enzyme from the latter organism Glutamate mutase is composed of two components E, a dimer ($\epsilon_2$, $m$ = 107 600) and S, a monomer ($\sigma$, 14 700) [2 12] The genes coding for the polypeptide chains $\sigma$ and $\epsilon$ have been cloned from both organisms in *E coli*, they were designated as *mut* genes in *C tetanomorphum*[5–7] and as *glm* genes in *C cochlearium* [9] In both organisms the genes are clustered in the same order *mutS-mutL-mutE-bma*[6 7] and *glmS-glmL-glmE-bma*,[9] respectively The *S*- and *E*-genes code for the corresponding glutamate mutase components, whereas the *L*-genes possibly code for proteins that act as molecular chaperones, but are not required for functional expression of the *E*- and *S*-genes in *E coli* The fourth gene of both clusters, *bma*, codes for the consecutive enzyme in the glutamate fermentation pathway, $\beta$-methylaspartase [(2*S*,3*S*)-3-methylaspartate ammonia lyase, EC 4 3 1 2] [13] The deduced amino acid sequences of MutE and GlmE show 90% identity to each other but no significant similarity to any other known protein In contrast, MutS and GlmS, which are only 82% identical to each other, share significant amino acid sequence similarities to domains of the cobalamin-dependent enzymes 2-methyleneglutarate mutase (see below), methylmalonyl-CoA mutases from several microorganisms and mammals including humans (EC 5 4 99 2), as well as methionine synthase from *E coli* (MetH, EC 2 1 1 13) [5 8]

The separate overexpression of the *glmE*- and *glmS*-genes in *E coli*, followed by simple two-step purifications, led to homogenous components E and S containing not a trace of a cobamide [9] Addition of an excess of coenzyme $B_{12}$ to a mixture of both components immediately led to an active enzyme Activity was not only observed with mixtures of the components from the same organism, but also with MutE + GlmS and GlmE + MutS, which were active in the presence of the coenzyme (U Leutbecher and W Buckel, unpublished) Upon gel filtration, the active enzyme, composed of GlmE and S, eluted as a complex, $\epsilon_2\sigma_2$, which contained 1 0 coenzyme Hence, the coenzyme glues both components together [9] This is not the case, however, with MutE and S, which, even in the presence of the coenzyme, separate on a gel filtration column [6] Interestingly, GlmS is able to bind the coenzyme alone, albeit in sub-stoichiometric amounts (0 5 mol/mol), whereas GlmE contains no trace of coenzyme after incubation with an excess of adenosylcobalamin followed by gel filtration The binding of the coenzyme to GlmS alone is consistent with the amino acid sequence similarities of MutS and GlmS with the cobalamin binding domains of other enzymes (*n b* the binding of the coenzyme to MutS is probably too weak to be observable by gel filtration) [6] It should be mentioned that all these binding experiments were performed with the commercially available adenosylcobalamin (coenzyme $B_{12}$) rather than with the natural coenzyme, which, in the case of *C tetanomorphum*, was identified as the corresponding derivative of pseudo-vitamin $B_{12}$ [1] Before the recombinant mutases became available, it was erroneously assumed by all workers in this field that the coenzyme binds to component E rather than to S Now it is obvious that the former purifications of component E from the original clostridia gave a mixture of the colourless apoprotein, as well as an inactive $\epsilon_2\sigma_2$ complex containing aquocobamide and cob(II)amide, most probably derivatives of pseudo-vitamin $B_{12}$ Only GlmE was recently obtained from its native organism *C cochlearium* as a homogeneous, highly active (specific activity 11 s $^{-1}$), colourless apoprotein devoid of any GlmS [14]

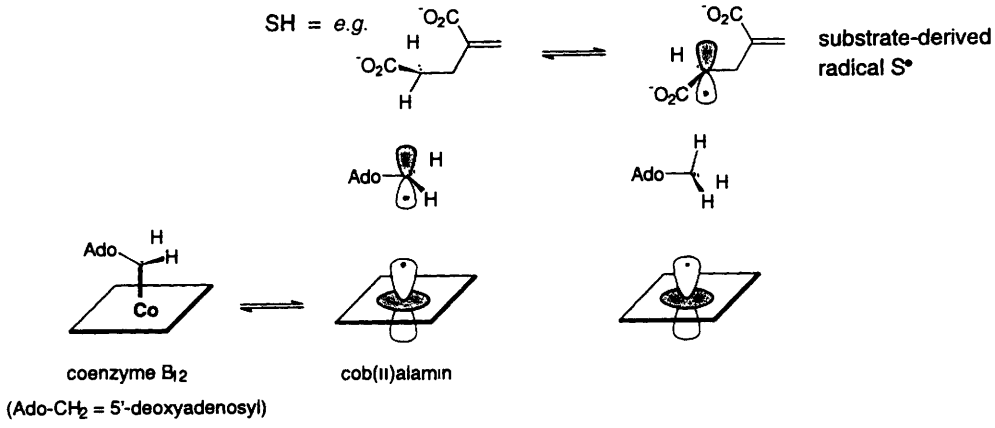
2-Methyleneglutarate mutase was purified from *C barkeri* in the dark (*n b* the Co–C bond of adenosylcobalamin is sensitive towards light) as an apparent homotetramer ($\alpha_4$, 267 000) containing up to two adenosylcobalamin and 0 1—0 2 cob(II)alamin [15 16] In contrast to glutamate mutase, the enzyme required no additional adenosylcobalamin for activity, owing to its content of this coenzyme Upon treatment with 8 mol dm $^{-3}$ urea followed by dialysis against buffer, the enzyme was completely inactivated but had lost only about half of the cobalamins Addition of adenosylcobalamin restored the activity almost completely Hence, the enzyme contains at least four different cobalamin species, which are distinguished by their content of 5′-deoxyadenine and by their binding to the enzyme It can be concluded that only those coenzyme $B_{12}$ molecules that are reversibly attached to the enzyme yield activity

**Table 1** Coenzyme $B_{12}$-dependent enzymes (a, b and Y are variable substituents; X is the migrating group – OH, $NH_3^+$ or a carbon-centred group)

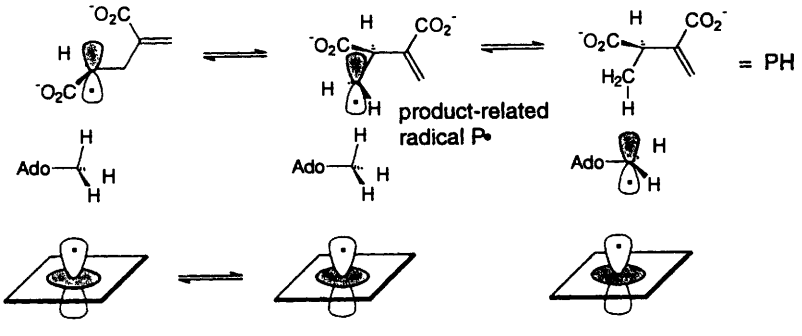
*n.b.* For class II enzymes the species on the right-hand side of the above equilibrium loses HX with the formation of $bCH_2CYa$ With ribonucleotide reductase the product is the species on the rhs of the equilibrium with X = H

| Substituent | a | b | X | Y | Ref |
|---|---|---|---|---|---|
| *Class I carbon skeleton mutases* | | | | | |
| 1 Glutamate mutase, EC 5 4 99 1 | $CO_2$- | H | 2-Glycinyl | H | 2,9,21 |
| 2 2 Methyleneglutarate mutase, EC 5 4 99 4 | $CO_2$ | H | 2-Acrylate | H | 8,15,20 |
| 3 Methylmalonyl-CoA mutase, EC 5 4 99 2 | $CO_2$ | H | Formyl-CoA | H | 32 |
| 4 Isobutyryl-CoA mutase, EC 5 4 99 | Me | H | Formyl-CoA | H | 39 |
| *Class II eliminases* | | | | | |
| 5 Propanediol dehydratase, EC 4 2 1 28 | H | H, $CH_3$, $CF_3$ | OH | OH | 42 |
| 6 Glycerol dehydratase, EC 4 2 1 30 | H | $CH_2OH$ | OH | OH | 42 |
| 7 Ethanolamine ammonia-lyase, EC 4 3 1 7 | H | H, $CH_3$ | $NH_3^+$ | OH | 43 |
| 8 Ribonucleotide-triphosphate reductase, EC 1 17 4 2 | C 4′ of ribonucleotide | C 1′ of ribonucleotide | OH | OH | 28 |
| *Class III aminomutases* | | | | | |
| 9 β-Lysine-5,6-aminomutase, EC 5 4 3 3 | 4-(3-Aminobutyrate) | H | $NH_3^+$ | H | 44 |
| 10 D-Ornithine-4,5 aminomutase, EC 5 4 3 4 | 3-D-Alanine, 4-(D-2-Aminobutyrate) | H | $NH_3^+$ | H | 44 |

(*a*)




(*b*)




**Scheme 3** Pathway for coenzyme $B_{12}$-dependent enzymic reactions illustrated with 2-methyleneglutarate as substrate SH and (*R*)-3-methylitaconate as product PH Part (*a*) shows conversion of SH to the substrate derived radical S·, part (*b*) shows conversion of S· into the product-related radical P· and hence product PH (Ado-$CH_2$ = 5′-deoxyadenosyl)

The gene *mgm* encoding the single polypeptide $\alpha$, of which 2-methyleneglutarate mutase is composed, has been cloned, sequenced and overexpressed in *E coli* [8] By comparison with other cobalamin-dependent enzymes the C-terminus (*ca* 100 amino acids) of the deduced amino acid sequence was identified as the coenzyme-binding domain (see above) In a manner analogous to the genes coding for glutamate mutase, *mgm* is followed by the gene *mii* coding for the consecutive enzyme in the pathway of nicotinate fermentation, 3-methylitaconate $\Delta$-isomerase (EC 5 3 3 6) Like $\beta$-methylaspartase, this enzyme eliminates the methine hydrogen from its substrate The homogenous overproduced apo-2-methyleneglutarate mutase contained no trace of a cobalamin, consistent with the inability of *E coli* to synthesise these compounds But on addition of adenosylcobalamin, holo-2-methyleneglutarate mutase was immediately obtained with a specific activity twice as high as that of the enzyme purified from *C barkeri* (B Beatrix, O Zelder, F Kroll and W Buckel, unpublished) Furthermore, the reconstituted enzyme also contained no cob(II)alamin (see above), which was erroneously suggested to be required for activity [15] The apoenzyme was shown to bind one coenzyme $B_{12}$ per two polypeptides suggesting a similar structure to glutamate mutase from *C cochlearium*, since the size of the $\alpha$-polypeptide (66 800) equals about that of $\epsilon + \sigma$ (68 500) Hence, in 2-methyleneglutarate mutase the part of the enzyme containing the active site, corresponding to subunit $\epsilon$ of glutamate mutase, apparently is fused together with the coenzyme-binding domain

## 4 Cryptic Substrate Stereochemistry

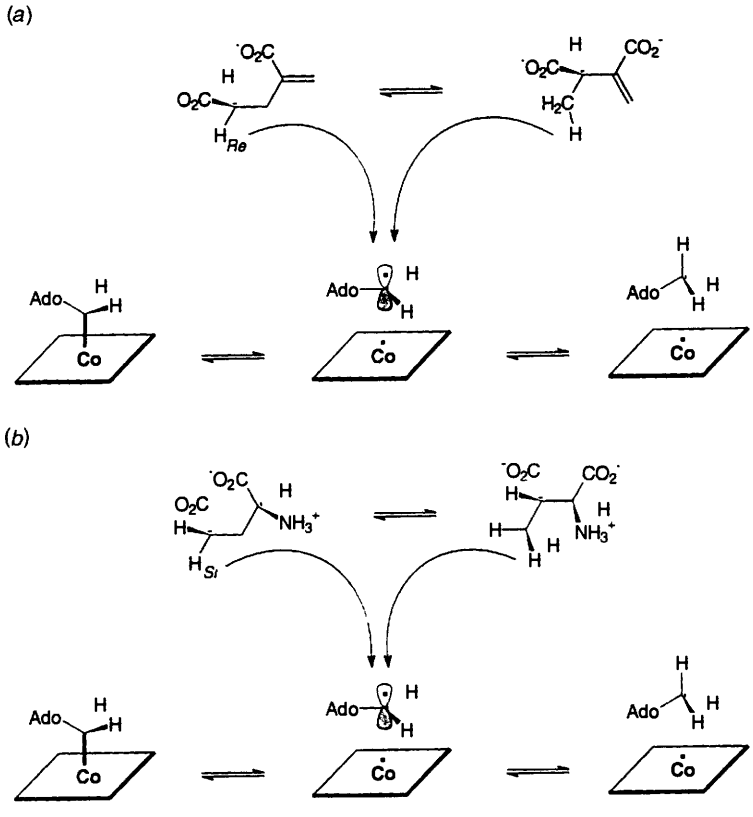
The diastereotopic methylene protons at C-4 of glutamate and the enantiotopic methylene protons at C-4 of 2-methyleneglutarate are expected to be distinguished by the respective enzymes Isotopic labelling has revealed that $H_{Si}$ is removed from glutamate,[17] whilst $H_{Re}$ is removed from 2-methyleneglutarate [18] Considering the absolute configurations of the substrate and product molecules, it was deduced that both enzymes cause an inversion of configuration at C-4 during the sequence of hydrogen abstraction and group migration The stereochemical data described are summarised in Scheme 4

To determine the stereochemistry of formation of the methyl groups of (2*S*,3*S*)-3-methylaspartate and (*R*)-3-methylitaconate from their respective precursors, all three hydrogen isotopes have been applied (*R*)-2-Oxo[3-$^2H_1$,3-$^3$H]glutarate was prepared by heating 2-oxoglutarate in $D_2O$ and incubating the resulting 2-oxo[3-$^2H_2$]glutarate with isocitrate dehydrogenase in tritiated water After conversion into (2*S*,3*S*)-[3-$^2H_1$,3-$^3$H]glutamate, the labelled amino acid was fermented with *C tetanomorphum*, to give labelled butyrate from which chiral acetate was obtained applying two consecutive Schmidt degradations The acetate contained *ca* 90% of the original tritium but was racemic (2*S*,3*S*)-[3-$^2H_1$,3-$^3$H]Glutamate, prepared by introducing the hydrogen isotopes in the reverse order, also gave racemic acetate [19] This result supports the postulated intermediacy (see Section 2) of a methylene radical corresponding in structure to 3-methylaspartate (*cf* Scheme 5) However, the experiment should be repeated with purified glutamate mutase to exclude the possibility that the racemisation is caused by another enzyme in the multistep fermentation pathway A similar approach to that described for glutamate was used with 2-methyleneglutarate and again, there was an apparent racemisation (G Hartrampf, P Sanchez, J W Cornforth, and W Buckel, unpublished results)

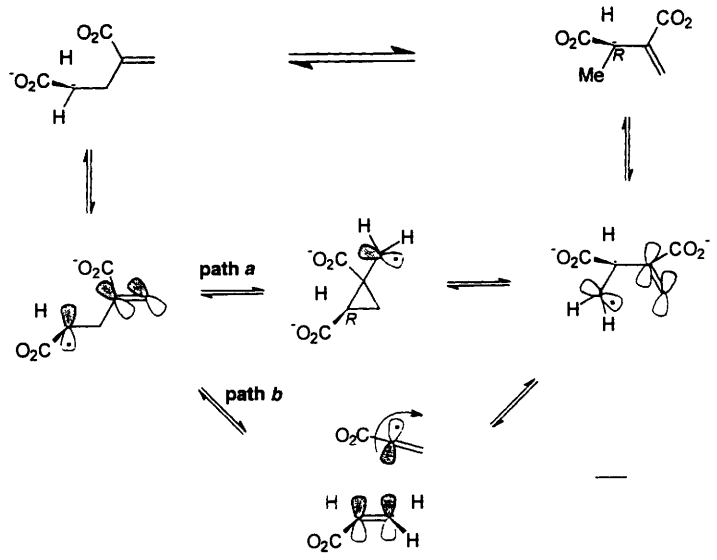
In an approach intended to probe for the intermediacy of a cyclopropylcarbinyl radical in the 2-methyleneglutarate mutase reaction, (*E*)-2-(*methylene*-$^2H_1$)methyleneglutarate was synthesised and shown to equilibrate with its (*Z*)-isomer on exposure to the enzyme [20] The significance of this result is discussed in Section 8

## 5 Kinetic Properties

Both glutamate and 2-methyleneglutarate mutase are highly specific for their respective substrates Despite a wide ranging search no other substrates have been discovered [2 15 23] The reason for this




**Scheme 4** Stereochemistry of the 2 methyleneglutarate reaction (*a*) and glutamate mutase reaction (*b*) (*a*) $H_{Re}$ is abstracted from C 4 of 2 methyleneglutarate, the abstracted H mixes with the 5′ methylene hydrogens of adenosylcobalamin, the acrylate residue migrates to this C-4 with inversion of configuration (*b*) $H_{Si}$ is abstracted from C 4 of (*S*) glutamate, the abstracted H mixes with the 5′ methylene hydrogens of adenosylcobalamin, the glycinyl residue migrates to this C 4 with inversion of configuration




**Scheme 5** Equilibration of 2 methyleneglutarate and (*R*) 3 methylitaconate and their corresponding radicals, either *via* a cyclopropylcarbinyl radical (path *a*), or by fragmentation to acrylate and the 2 acrylate radical (path *b*)

unusual specificity could lie in the unique mechanism whereby three carbon centres are implicated in the molecular rearrangement

Not even fluoro- or methyl-substitution of glutamate permits substrate behaviour, although some such derivatives are inhibitory (see Table 2) [12 16 21–23] Of the diastereoisomeric 4-fluoroglutamates only the (2*S*,4*S*)-isomer is inhibitory, presumably because the fluoro substituent replaces the hydrogen that is normally abstracted, the (2*S*,4*R*)-isomer does not interact with the enzyme *rac*-2-Methylglutarate does not inhibit glutamate mutase, whereas 2-methyleneglutarate is an active inhibitor, presumably by occupying the glutamate binding site in a manner that cannot be matched by either enantiomer of 2-methylglutarate The inhibition of glutamate mutase by 2-methyleneglutarate was taken as an additional argument for the intermediacy of an imino derivative in the glutamate mutase reaction [7 12] However, the recent discovery (see Section 8) that glutamate mutase is inhibited synergistically by acrylate and glycine[21] may explain this result, if the key structural feature of 2-methyleneglutarate is the presence of an acrylate moiety that occupies the acrylate binding site Both enantiomers of 3-methylitaconate are inactive with glutamate mutase It was originally reported[12] that (*S*)-3-methylitaconate inhibits glutamate mutase, but this is now known to be due to inhibition of the auxiliary enzyme β-methylaspartase, a component of an assay system for glutamate mutase [2]

Using (*S*)-[2,3,3,4,4-$^2H_5$]glutamate as a substrate,[9] a kinetic isotope effect, $V_H/V_D = 7$, was observed, whereas $K_m = 2\ 4$ mmol dm$^{-3}$ remained similar to that observed with the unlabelled amino acid (1 5 mmol dm$^{-3}$) [2] By applying regiospecifically labelled glutamates, it was shown that only a hydrogen at C-4, most likely $H_{5'}$, which is abstracted by the 5′-deoxyadenosyl radical, contributes much to the rate-limiting step [23] The isotope effect of the transfer of a tritium from the coenzyme to the substrate, *i e* from 5′-deoxyadenosine to the product-related radical, was estimated as 13 5—18, from which a deuterium isotope effect of 6—7 4 was calculated In this experiment it was shown that the hydrogen was removed from the coenzyme at a rate comparable to that of its appearance in the product 3-methylaspartate This important observation excludes the intermediacy of a protein-based radical in the catalytic turnover, with the coenzyme acting merely as a radical initiator [24]

Already 25 years ago Kung and Stadtman reported a series of inhibitors for 2-methyleneglutarate mutase,[22] among which itaconate proved to be the most effective [16] An intriguing result of the earlier work was the purported inhibitory action of *trans*-1-methylcyclopropane-1,2-dicarboxylate with the corresponding *cis*-isomer being less active, these compounds were regarded as analogues of a presumed intermediate Using a continuous optical assay with homogenous 3-methylitaconate Δ-isomerase as an auxiliary enzyme, it was recently shown that none of the four stereoisomers

**Table 2** Substrates and inhibitors of glutamate and 2-methyleneglutarate mutase [a,b]

| Enzyme | Compound | Effect | $K_m$ or $K_i$/mmol dm$^{-3}$ | Ref |
|---|---|---|---|---|
| Glutamate mutase | (*S*) Glutamate | Substrate | 1 5 | 12 |
| | (2*S*,3*S*) 3 Methylaspartate | Substrate | 0 5 | 12 |
| | (2*R*,3*RS*) 3 Fluoroglutamate | Competitive inhibition | 0 6 | 12 |
| | (2*S*,4*S*) 4-Fluoroglutamate | Competitive inhibition | 0 07 | 12 |
| | 2 Methyleneglutarate | Competitive inhibition | 0 4 | 12 |
| | Glycine + acrylate | Inhibition | *ca* 5 each | 21 |
| 2 Methyleneglutarate mutase | 2 Methyleneglutarate | Substrate | 3 7 | 16 |
| | (*R*)-3 Methylitaconate | Substrate | [c] | |
| | Itaconate | Competitive inhibition | 0 7 | 16 |
| | Mesaconate (methylfumarate) | Competitive inhibition | > 1 | 22 |
| | Succinate | Competitive inhibition | > 1 | 22 |
| | Acrylate | Inhibition | *ca* 1—10 (see text) | 21 |

Glutamate mutase was not inhibited by (*R*) glutamate (*R*) or (*S*) 3 methylitaconate 4 mmol dm$^{-3}$ (2*S* 4*R*) 4 fluoroglutamate 2 methyl 3 methyl [23] 4 methyl [23] *N* methylglutamate (10 mmol dm$^{-3}$ each) 20 mmol dm$^{-3}$ glycine 20 mmol dm$^{-3}$ acrylate, 10 mmol dm$^{-3}$ (*S*) aspartate [c] 2 Methyleneglutarate mutase was not inhibited by 10 mmol dm$^{-3}$ (*S*) glutamate 15 mmol dm$^{-3}$ (*R*,*S*) 2 methylglutarate, 20 mmol dm$^{-3}$ of all four stereoisomers of 1 methylcyclopropane 1 2 dicarboxylate Not determined

of 1-methylcyclopropane-1,2-dicarboxylate was able to inhibit significantly 2-methyleneglutarate mutase [21] The very recent discovery of the inhibitory power of the simple compound acrylate leads to an entirely new mechanistic proposal, which will be discussed below The plot of the reciprocal initial velocity as a function of the acrylate concentration (Dixon plot) fitted better to a quadratic equation than to a linear one This agrees well with the requirement that two acrylate molecules mimic intermediates in the 2-methyleneglutarate mutase reaction [21]

A remarkable feature of coenzyme $B_{12}$-dependent enzymes is their apparent ability to handle safely free radicals which would be highly reactive if detached from the protein [25] The enzymes are not perfect, however, because they are slowly destroyed in the presence of substrate, a process which is accelerated by exposure to air[9] Prolonged anaerobic incubation in the dark (15 h, 37 °C) yields the 'inactive complexes' of glutamate and 2 methyleneglutarate mutase, in which the enzyme-bound coenzyme $B_{12}$ has been converted into aquocobalamin and cob(II)alamin (Section 6) The presence of these inactive complexes in the native clostridia shows that this also happens *in vivo* [12 14–16] It would be of interest to see whether these complexes are repaired, degraded or simply washed out by the growing bacteria Remarkably, the sensitivity of the coenzyme in 2-methyleneglutarate mutase towards light *decreases* during catalysis This experiment shows that the Co–C bond of the coenzyme, the final target of light with $\lambda < 600$ nm, has already been cleaved during substrate turnover [26]

## 6 Electron Paramagnetic Resonance Studies

Electron paramagnetic resonance spectroscopy (EPR) has proved to be a powerful tool for gaining insights into the structure and function of glutamate and 2-methyleneglutarate mutase According to the mechanism described in Section 2, EPR signals of cob(II)alamin ($g_{xy}$ *ca* 2 3) and of an organic radical ($g$ 2 00) should be observable during catalysis The first EPR spectra of the three carbon-skeleton rearranging mutases, which were published in 1992,[14 15 27] clearly showed, however, only one 'catalytic' signal around $g$ *ca* 2 1 This was generated by addition of the corresponding substrate to the EPR-silent mixture of enzyme and coenzyme Owing to the lack of sufficient amounts of enzymes and to the presence of inactive cob(II)alamin in some preparations (see Section 3), the signals were of low resolution and therefore difficult to interpret The subsequent introduction of molecular biology into coenzyme $B_{12}$ research led to the availability of large amounts of enzymes yielding intense EPR spectra with excellent resolution [9 21] Thus, addition of glutamate to a mixture of component E with a twofold molar excess of component S (10—20 mg protein/sample) and a tenfold molar excess of coenzyme $B_{12}$ gave the expected signal in the $g_{xy}$ *ca* 2 1 region with an eightfold hyperfine splitting of the $g$ line centred at 1 985 Comparison of this 'catalytic' spectrum with that of cob(II)alamin revealed similarities and differences The signal of cob(II)alamin with $g_{xy}$ *ca* 2 3 was shifted to $g$ *ca* 2 1, whereas the coupling constant ($A$ = 106 5 G) of the eightfold hyperfine splitting of the $g$ line was reduced to 50 G The characteristic threefold superhyperfine splittings of each of the eight $g$ lines due to coupling with the $^{14}N$ nucleus of the axial base ($I$ = 1) were not resolved The spectrum of the catalytic species was interpreted in terms of a tight coupling of the unpaired electron of cob(II)alamin with that of a carbon-centred radical [9] Recently, the spectrum has been almost perfectly simulated by using parameters similar to those applied for the interaction of a thiyl radical with cob(II)alamin in ribonucleoside triphosphate reductase from *Lactobacillus leichmanni* [28] The simulation of the spectrum of glutamate mutase revealed that $Co^{II}$ and an organic radical are coupled together by an isotropic exchange coupling which is at least 10 GHz This means that the electrons are interacting either directly *via* orbital overlap or indirectly *via* a super-exchange† mechanism In addition, the electrons are interacting *via* a zero field splitting term of about 300 MHz A rough estimate of the distance between the two species gives ~6 Å (G Gerfen, personal communication) The very similar and highly resolved spectrum obtained with 2-methyleneglutarate mutase[21] can be explained in the same way Interestingly, the inhibitors of glutamate mutase, (2*S*,4*S*)-fluoroglutamate and 2-methyleneglutarate, induced spectra similar but not identical to those induced by glutamate Double integration yielded spin concentrations up to 150%, as compared to the concentration of component E These high values support the idea of a biradical being responsible for the EPR spectrum In contrast, (*S*)-glutamate induced a spectrum with only 50% spin concentration [9] Freeze-stopped experiments showed that glutamate induced the full EPR spectrum within less than 25 ms, whereas (2*S*,4*S*)-fluoroglutamate required more than 5 s [12] Interestingly, the combination of glycine plus acrylate, but not the single compounds, induced an EPR spectrum with glutamate mutase showing the characteristic signal at $g$ *ca* 2 1 This is consistent with the synergistic inhibition of the enzyme by both substances [21] Likewise, addition of acrylate to 2-methyleneglutarate mutase afforded an EPR spectrum similar to that obtained with 2-methyleneglutarate[21] or the competitive inhibitor itaconate [16] In summary, the EPR spectra suggest that during catalysis the substrate-derived radical closely interacts with $Co^{II}$ Although a direct coordination of the radical by $Co^{II}$ should result in radical pairing and give rise to an EPR-inactive species, acrylate itself could coordinate to $Co^{II}$, with the 2-glycinyl or 2-acrylate radical located nearby (see Figure 1)

The nature of the axial base coordinated to the cobalt of coenzyme $B_{12}$ was also revealed by EPR spectroscopy *p*-Cresolylcobamide in the $Co^{II}$ state does not show the characteristic threefold superhyperfine splitting of the $g$ lines due to the absence of an axial nitrogen base (base off) Upon binding to a methyltransferase, however, this splitting occurs indicating coordination to a $^{14}N$-containing ligand The latter was identified as histidine by incorporation of (*imidazole*-$^{15}N$)histidine into the protein Now a twofold splitting was observed due the spin $I$ = 1/2 of the $^{15}N$ nucleus [29] X-Ray crystallography of the coenzyme $B_{12}$ binding domain of the methionine synthase (MetH) from *E coli* directly showed the coordination of the conserved histidine residue (see section 3) to the cobalt atom [30] Mixing of unlabelled component E and glutamate with the completely $^{15}N$-labelled component S yielded an EPR spectrum of the catalytic species with sharper $g$ lines By using a histidine-requiring mutant of *E coli*, a $^{15}N$-labelled component S was prepared in which only the histidines remained unlabelled Its EPR spectrum could not be distinguished from that of the completely unlabelled component S, indicating the coordination of a histidine to the cobalt Upon formation of cob(II)alamin by prolonged incubation of the completely labelled component S with unlabelled component E and glutamate, the typical twofold superhyperfine splitting of the $g$ lines was observed, demonstrating that a histidine also coordinates to the inactive species [31] The conserved histidine residue 359 of 2-methyleneglutarate mutase, which was identified by sequence alignment with methionine synthase,[5 8 30] was converted into a glutamine residue by site-directed mutagenesis The resulting mutant was completely inactive, since it was not able to bind coenzyme $B_{12}$ Furthermore, wild type 2-methyleneglutarate mutase is also active when combined with adenosyl-*p*-cresolylcobamide, demonstrating the low importance of the axial base of the coenzyme for biological activity (E Stupperich, F Kroll and W Buckel, unpublished)

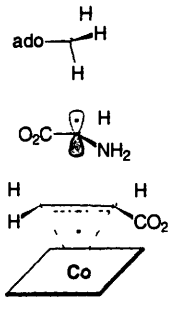
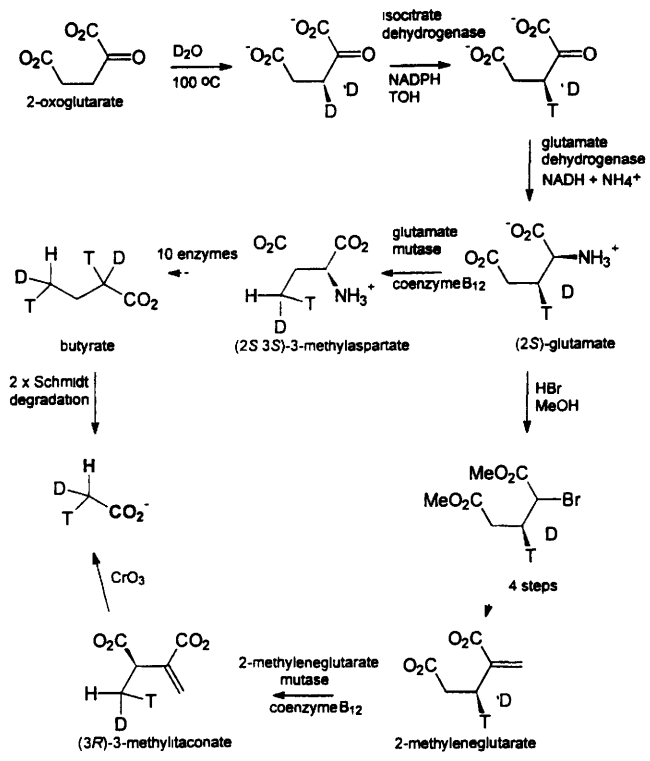



**Figure 1** Postulated intermediate state in the glutamate mutase reaction showing an acrylate cob(II)alamin complex

† Mediated by orbitals of amino acid residues or the solvent




**Scheme 6** Syntheses of (2*S*,3*S*) [3 $^{2}$H,3 $^{3}$H]glutamate and (3*S*) 2 methylene[3 $^{2}$H,3 $^{3}$H]glutarate, their conversion to chiral methyl labelled (2*S*, 3*S*) 3 methylaspartate and (3*R*) 3 methylitaconate, respectively, and the degradation to chiral acetates The conversion of glutamate to butyrate was performed with growing cells of *C tetanomorphum*

Recently, the coordination of a histidine nitrogen to cobalt within the enzymes catalysing carbon-skeleton rearrangements has been confirmed by the crystal structure of methylmalonyl-CoA mutase from *P shermanii* revealing an extraordinary long Co–N distance of 2 53 Å The observed 0 32 Å difference from the corresponding Co-dimethylbenzimidazole bond length in free adenosylcobalamin (2 21 Å) is thought to facilitate homolysis of the Co–C bond [32] It would be of interest to measure the Co–N distance in methionine synthase, which catalyses heterolytic cleavage of the Co–methyl bond

## 7 Model Studies

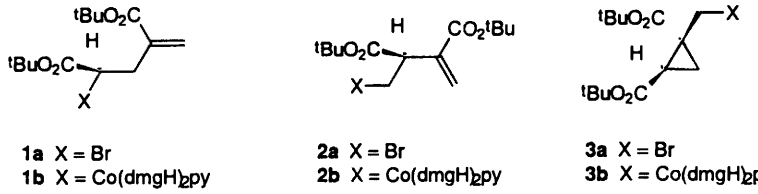
Possible mechanisms for the equilibration of 2-methyleneglutarate with 3-methylitaconate catalysed by 2-methyleneglutarate mutase are shown in Scheme 6 [33] The key intermediate in path *a* is a cyclopropylcarbinyl radical, which by cleavage of its C(1)–C(2) bond connects with the substrate-derived radical, whilst cleavage of the C(1)–C(3) bond leads to the product-related radical (*cf* Scheme 3) There is ample precedent for these processes in non-enzymatic chemistry Thus, conversion of the cyclopropylcarbinyl radical to the but-3-enyl radical is one of the fastest unimolecular reactions known ($k$ = $10^{8}$ s $^{-1}$ at 298 K), whilst the reverse reaction is also relatively fast ($k$ = $10^{3}$ s $^{-1}$ at 298 K) Many examples of these types of interconversion have been described in which the butenyl or cyclopropylcarbinyl system bears alkyl, aryl and/or ester substituents [34] It has been shown[33] that treatment with triphenyltin hydride of each of the bromides (**1a**—**3a**) matching in structure the species proposed as intermediates in the 2-methyleneglutarate reaction (see Scheme 6), gave primarily di-*tert*-butyl 2-methyleneglutarate, presumably *via* the corresponding free radicals The bromides were also reacted with cobaloxime(I), which gave the alkylcobaloxime **1b** from bromides **1a** and **3a**, and the alkylcobaloxime **2b** from bromide **2a** Alkylcobaloxime **2b** did not readily rearrange into alkylcobaloxime **1b** It was therefore proposed that this lack of reactivity of organocobalt species **2b** compared to the corresponding free radicals supports the mechanism of Scheme 6, path *a* (see also Scheme 3 and Section 8)

Murakami and his coworkers have described attempts to model glutamate mutase by preparing an organocorrinoid bearing an alkyl group derived from 3-methylaspartate, and photolysing this material in a micellar matrix to give glutamate in low yield [35] This is the only model system to achieve the conversion of 3-methylaspartate into glutamate, but further studies are needed to elucidate the reaction pathway

## 8 Mechanism of Action - Decision

For 2-methyleneglutarate mutase, model studies (see Section 7) supported a mechanism in which the substrate-derived and product-related radicals are interconverted *via* an intermediate cyclopropylcarbinyl radical (see Scheme 6, path *a*) A similar mechanism was, however, impossible for glutamate mutase because of the lack of suitable $\pi$-bond with which the radical centres in S· and P· could interact The proposal[7] that such a $\pi$-bond could be generated by formation of an imine from the amino group of glutamate and a carbonyl function within the protein cannot be sustained (see Section 3) Furthermore, a mechanism whereby the 2-glycinyl moiety migrates *via* a bridged transition state can also be excluded because of the predicted high energy of such a species [36] However, the discovery of synergistic inhibition of glutamate mutase by glycine and acrylate gave the first experimental support for a fragmentation–recombination mechanism for this enzyme (Scheme 7*a*), already proposed many years ago [36,37] The similarities between 2 methyleneglutarate and glutamate mutase with respect to the reactions catalysed, enzymes, cofactor and EPR data point to a commonality of mechanism We have, therefore, proposed fragmentation–recombination mechanisms for both of these enzymes in which acrylate is a common intermediate [21] Such a mechanism for 2-methyleneglutarate mutase arose during our studies of the four isomers of 1-methylcyclopropane-1,2-dicarboxylates as potential inhibitors of 2-methyleneglutarate mutase The surprising failure of any of these compounds to inhibit 2-methyleneglutarate mutase, and especially the inactivity of the (*R*,*R*)-isomer, led us to re-evaluate the long-held mechanistic hypothesis of Scheme 6, path *a* The startling discovery that 2-methyleneglutarate mutase is inhibited by acrylate,[21] with a square dependence on acrylate concentration, supports the mechanism of Scheme 6, path *b* for this enzyme (see also Scheme 7*b*)

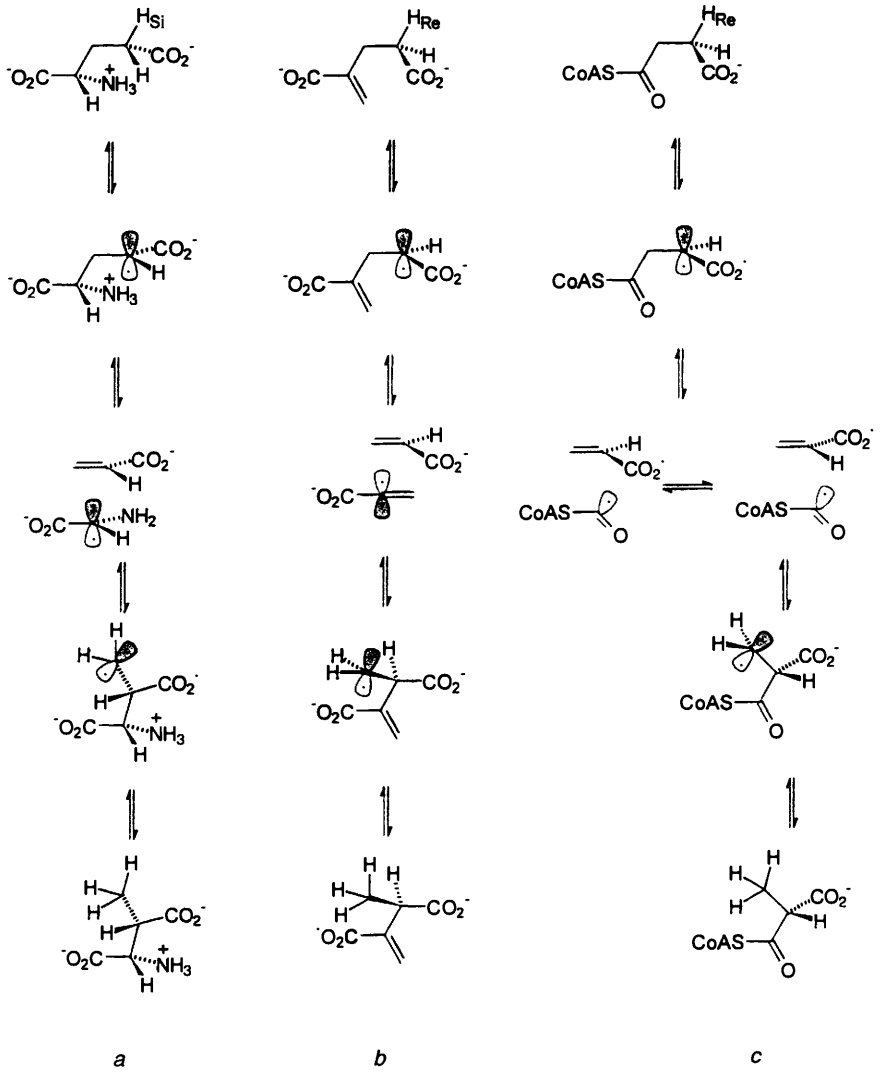
The stereochemical features of the glutamate and 2-methyleneglutarate mutase reactions (see Section 4) are fully explicable by the mechanisms of Scheme 6, path *b* and Scheme 7 The stereochemistry of the initial hydrogen abstraction will be governed by the precise positioning of the adenosyl radical with respect to the protein-bound substrate The fact that $H_{Si}$ is removed from glutamate whilst $H_{Re}$ is removed from 2-methyleneglutarate is not surprising even though the active sites of the enzymes may be similar Thus, a 120° rotation about the C(3)–C(4) bond causes a lateral movement of the carboxylate of only *ca* 2 Å and serves to present either $H_{Si}$ or $H_{Re}$ to the adenosyl radical Small differences in




**1a** X = Br
**1b** X = Co(dmgH)$_2$py

**2a** X = Br
**2b** X = Co(dmgH)$_2$py

**3a** X = Br
**3b** X = Co(dmgH)$_2$py




**Scheme 7** Proposed reaction pathways for coenzyme $B_{12}$-dependent reactions [(*a*) glutamate mutase; (*b*) 2-methyleneglutarate mutase; (*c*) methylmalonyl-CoA mutase] (from ref. 21).

protein structure, especially with respect to the positions of carboxylate-binding functions, could suffice to bring about this alteration.

Fragmentation of the substrate-derived radical requires a specific conformation in which its C(2)–C(3) bond is nearly parallel to the p orbital at the radical centre (see Scheme 7). This leads to acrylate and a 2-glycinyl radical from glutamate, and acrylate and the 2-acrylate radical from 2-methyleneglutarate. Addition of the 2-glycinyl radical to the *Re* face at C-2 of acrylate leads to a product-related radical of correct stereochemistry [*i.e.* that corresponding to (2*S*,3*S*)-3-methylaspartate]. Provided that addition of the 2-acrylate radical occurs to the *Si* face of acrylate, a product-related radical of correct stereochemistry is also generated [*i.e.* that corresponding to (*R*)-3-methylitaconate]. Both processes lead to the observed inversion of configuration at the centre from which hydrogen is abstracted and to which a group migrates. Throughout the processes described, the migrating group remains in contact with a particular face of the acrylate molecule. The observed equilibration of (*E*)-(*methylene*-$^2H_1$)2-methyleneglutarate with its (*Z*)-isomer catalysed by 2-methyleneglutarate mutase[20] (see Section 4 and Scheme 6) can be explained by noting the linearity of the 2-acrylate radical and postulating a time dependent rotation of this radical, either about the C(1)–C(2) bond or the C(1)–C(2)–C(3) axis, that is slower than substrate turnover (see Scheme 6, path *b*). This result, however, is also consistent with the intermediacy of a cyclopropylcarbinyl radical (Scheme 6, path *a*). The fragmentation–recombination route discussed for the Class I enzymes glutamate and 2-methyleneglutarate mutase (see Table 1) can be immediately applied to methylmalonyl-CoA mutase (Scheme 7*c*), with acrylate and the 2-formyl-CoA radical as intermediates. In support of this proposal, recent studies in Karlsruhe have shown that methylmalonyl-CoA mutase is synergistically inhibited by acrylate and formyl-CoA (A. Abend and J. Rétey, personal communication). The mechanism shown in Scheme 7*c* contains a stereochemical subtlety. In contrast to the glutamate and 2-methyleneglutarate reactions, which both proceed with inversion at C-4 of substrate, the transformation of methylmalonyl-CoA to succinyl-CoA takes place with retention of configuration at the corresponding carbon centre. This observation can be explained if the acrylate exists in two conformations (see Scheme 7*c*), which interconvert by rotation about their C(1)–C(2)-bond. This enables 'the error in the cryptic stereochemistry of methylmalonyl-CoA mutase'[17,38] to be understood. In addition to the expected migration of $H_{Re}$ at C-3 of succinyl CoA to the methyl group of methylmalonyl-CoA, the 'exchangeable hydrogen' at C-2 of methylmalonyl-CoA migrated to C-3 of succinyl-CoA. To elucidate this result, a 1,2-hydrogen shift in the intermediate succinyl-CoA radical was invoked, but it can be better explained if there is an occasional removal of $H_{Si}$ from C-3, leading *directly* to the correct acrylate conformation for further elaboration to (*R*)-methylmalonyl-CoA.[21] Recently, it has been shown that isobutyryl-CoA mutase,[39] to which the fragmentation mechanism may also be applied, proceeds with retention of configuration.[39] The stereochemical 'error' made by this enzyme, which has to handle propene according to the fragmentation–recombination mechanism, is even more pronounced than that observed with methylmalonyl-CoA mutase.

## 9 Concluding Comments

Remarkably, the coenzyme $B_{12}$-dependent eliminases and aminomutases (Table 1) have coenzyme $B_{12}$-independent counterparts, which catalyse essentially identical reactions The existence of several coenzyme $B_{12}$-independent ribonucleotide reductases is well established [28] In addition, a coenzyme $B_{12}$-independent, but iron-containing diol dehydratase, has been reported [40] The iron–sulfur dependent lysine-2,3-aminomutase also uses the 5′-deoxyadenosinyl radical, but this reagent is derived from *S*-adenosylmethionine (SAM, the "poor man's coenzyme $B_{12}$") [41] On the other hand, no coenzyme $B_{12}$-independent counterpart to the carbon-skeleton mutases (Class I, Table 1) has been discovered yet This may due to the fact that only the Class I enzymes require $Co^{II}$ for coordination of the acrylate, in order to enable the addition of the radical fragment to the $\alpha$-carbon of this intermediate, leading to the branched products Hence, in these enzymes, $Co^{II}$ might act not as a mere spectator, but as a conductor of the catalysis In contrast, the Class II and III enzymes apparently require coenzyme $B_{12}$ only as a generator of 5′-deoxyadenosyl radicals The intermediacy of such a hypothetical $Co^{II}$-acrylate $\pi$-complex (Figure 1) in the catalysis of the carbon-skeleton mutases causing the enhancement of the reactivity of the $\alpha$-carbon of acrylate, remains, however, to be established

Soon after his discovery of glutamate mutase H A Barker wrote 'the precise role of the coenzyme in the interconversion of glutamate and $\beta$-methylaspartate is not yet known' [1] Nearly 40 years later one may begin to understand

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