The stereochemistry of the formation of the methyl group in the glutamate mutase-catalysed reaction in *Clostridium tetanomorphum*

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The adenosylcobalamin-dependent enzyme glutamate mutase from *Clostridium tetanomorphum* catalyses the reversible rearrangement of (2*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate. In this conversion 6 carbon centers are involved. The stereochemistry of 4 has been elucidated whereas the formation of the methyl group from the methylene group remains to be established. To solve this problem, (2*S*,3*R*)- and (2*S*,3*S*)-[3,3-$^2H_1$,^3H]glutamates were prepared via the 2-oxo[3,3-$^2H_2$ or ^3H]glutarates by incubation with isocitrate dehydrogenase in deuterium oxide or tritiated water. The labelled glutamates were fermented with growing cells of *C. tetanomorphum* to butyrate and acetate. Butyrate was further degraded to acetate in which methyl group over 90% of the tritium of the starting glutamate was retained. The chirality of the acetates was determined with malate synthase and fumarase. In both samples complete racemisation was found. This result confirms the rule that racemisation occurs in all adenosylcobalamin-dependent rearrangements in which methyl groups are formed. A methylene radical as intermediate could explain these observations. In a control experiment inversion of configuration in the formation of the methine group of (2*S*,3*S*)-3-methylaspartate from the methylene group of (2*S*)-glutamate was confirmed. Glutamates stereospecifically labelled at C-4 were synthesized from chiral acetates via citrate.

Adenosylcobalamin *Chiral methyl group* *(2S,3S)-3-Methylaspartate* *(2S)-Glutamate* *Racemisation* *Glutamate mutase*

1. INTRODUCTION

The adenosylcobalamin-dependent enzyme glutamate mutase catalyses the reversible interconversion of (2*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate, the first step of the fermentation pathway leading to NH_3, CO_2, acetate and butyrate (fig.1; review [1,2]). During this reaction the glycine residue (C-1 and C-2) is transferred from C-3 to C-4 whereby the hydrogen H_c migrates in the opposite direction. The abstraction and readdition of H_c is mediated by the coenzyme [3].

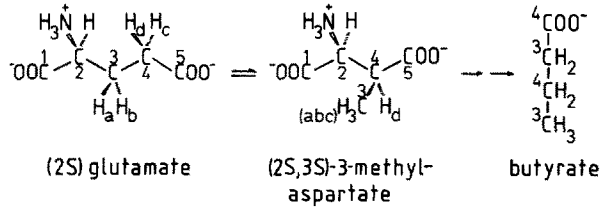

Fig.1. Reversible interconversion of (2*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate.

To obtain an insight into the mechanism of this and related adenosylcobalamin-dependent rearrangements stereochemical approaches were applied successfully (review [4]). In the case of glutamate mutase it was shown that the glycine residue migrates with retention of configuration [5], whereas the formation of the new chiral center at C-4 of methylaspartate occurred with inversion [6]. However, no effort was made to elucidate the stereochemistry at C-3, the formation of the methyl group, although methods for its analysis

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have been available for 15 years [7,8]. This paper tries to fill this gap.

[3-$^2H_1$,3-^3H]Glutamates labelled at H_a and H_b or vice versa are fermented by growing bacteria. Then the chirality of the methyl group of the final product butyrate which is derived from methylaspartate (fig.1) is analysed. In addition the reliability of the use of whole bacteria rather than that of the pure mutase is checked by reconfirming the stereochemistry at C-4 with [4-^3H]glutamates labelled either at H_c or at H_d.

2. MATERIALS AND METHODS

Tritiated water (1 Ci/ml) was from Amersham Buchler (Braunschweig); deuterium oxide (min. 99.75%) was from Merck (Darmstadt).

Isocitrate dehydrogenase (EC 1.1.1.42) and glutamate dehydrogenase (EC 1.4.1.3) were obtained from Boehringer (Mannheim). Glutamates labelled at C-4 were prepared either from [2-^{14}C]acetate (CEA Saclay, France) or from (*R*)- and (*S*)-acetate [2,14]. The latter were gifts from Dr G.T. Phillips (Shell Research Ltd, England). Samples of malate synthase (EC 4.1.3.2) prepared as in [10] were gifts from Dr H. Durchschlag (Universität Regensburg).

Clostridium tetanomorphum H1 was a gift from H.A. Barker (Berkeley, USA) and *Acidaminococcus fermentans* ATCC 25085 was obtained from the American Type Culture Collection. Both organisms were grown on media described in [2,9]. Growth of cultures was conducted in Hungate tubes at 37°C and anaerobic conditions were achieved by treatment with a mixture of nitrogen and hydrogen gas (95:5, v/v) for several time periods; anaerobiosis could be monitored with the indicator resazurin.

Volatile fatty acids were obtained through steam distillation and separated on a BioRex 70 (H^+) 100–200 mesh column (Bio-Rad, Munich) [11]. The labeled butyrates were degraded stepwise to propionate [12] and acetate [13]. The ^3H content of the substance was calculated by difference for positions C-2 (propylamine propionate) and C-3 (ethylamine acetate) or measured directly for C-4 (acetate) in butyrate on the basis of the $^3H/^{14}C$ ratio. Chirality of the labelled acetates was checked with a modification of the established method [7] in which the precipitation of fumarate with barium acetate and chromatography on Dowex 50 W × 8 were omitted. Samples from the fumarase reaction were evaporated to dryness (3 times), dissolved in 1 ml water and counted after addition of 9 ml Quickszint 212 (Zinsser, Frankfurt am Main). Reliability of the procedure was checked with authentic samples of (*R*)- and (*S*)-acetate. The data obtained on the virtually chiral acetates showed a ^3H retention in fumarate of 75.7% for (*R*)- and 24.2% for (*S*)-acetate although being stored as 100 mM potassium salts (pH 7.0) in sterilized and sealed ampoules for about 14 years. Comparison with published results [14] showed very good agreement.

3. RESULTS

3.1. *Design of the experiment*

In principle the substrate stereochemistry of the mutase could be analysed in both directions (fig.1). However, in the back reaction the chemical synthesis of (2*S*,3*S*)-3-methylaspartates with additional chiral methyl groups would have been required. Since no method was available, the second approach, the stereospecific labelling of glutamate at the C-3 methylene group and analysis of the generated chiral methyl group appeared to be much more feasible. 2-Oxoglutarate can be labelled at C-3 by heating the sodium salt in deuterium oxide or tritiated water [15]. On incubation with NADPH and isocitrate dehydrogenase the pro-(3*S*)-hydrogen exchanges with the solvent [16]. Thereby (3*S*)- and (3*R*)-2-oxo[3-$^2H_1$,3-^3H]glutarates can be synthesized, depending on the order in which the isotope is introduced. Finally, the 2-oxoglutarates may be reduced enzymatically to (2*S*)-glutamates [16].

During the conversion of the glutamates obtained to methylaspartates ^1H should migrate from C-4 to C-3 and generate the chiral methyl groups. The other possibility, the labelling at both positions of C-4 with ^2H or ^3H in addition to one position at C-3 also would yield chiral methyl groups. However, this labelling pattern has two disadvantages. Firstly, the migration of the hydrogen via the coenzyme occurs intermolecularly. Thus unlabelled carrier glutamate would interfere significantly and dilute the labelled positions with ^1H, depending on isotope effects. Secondly, in related adenosylcobalamin-dependent rearrange-

ments it was shown that part of the migrating heavy isotope was lost into the solvent, whereas in the opposite direction no exchange was observed. This unexplained effect was termed 'washing-out' process [4] (see section 3.3).

Although the synthesis of the labelled glutamates was a feasible task, their conversion to chiral methyl groups posed a severe problem. Unfortunately, the equilibrium of the glutamate mutase reaction lies over 90% on the side of glutamate with $K_{eq.} = 10.6$ [1]. Thus, using the isolated enzyme, racemisation would occur after only less than 10% conversion. Another possibility would be the use of whole bacteria rather than the pure enzyme since the rate-determining step of this fermentation pathway was shown to be the first enzyme, i.e., glutamate mutase [1]; any methylaspartate should be immediately converted to butyrate (fig.1). Degradation of the latter product [12,13] would yield acetate which could be analysed for chirality.

3.2. *Performance of the experiment*

2-Oxo[3,3-$^2H_2$]glutarate (fig.2b) was synthesized from 2-oxoglutarate (fig.2a) by heating in deuterium oxide. Further incubation with isocitrate dehydrogenase and NADPH in water yielded (3*R*)-2-oxo[3-$^2H_1$]glutarate (fig.2c). The ^{1}H-NMR spectra were consistent with the proposed structures. By repeating the experiment in tritiated water (3*S*)-2-oxo[3-$^2H_1$,3-^{3}H]glutarate was obtained. To measure the kinetics of ^{3}H incorporation a trace of 2-oxo[1-^{14}C]glutarate was included in the incubation mixture (fig.3).

The formation of (3*R*)-2-oxo[3-$^3H_1$,3-^{3}H]glutarate from 2-oxo[3,3-^{3}H]glutarate in deuterium oxide gave almost identical kinetics. The 2-oxoglutarates were reduced enzymatically to the corresponding (2*S*)-glutamates. The chirality at C-3 of

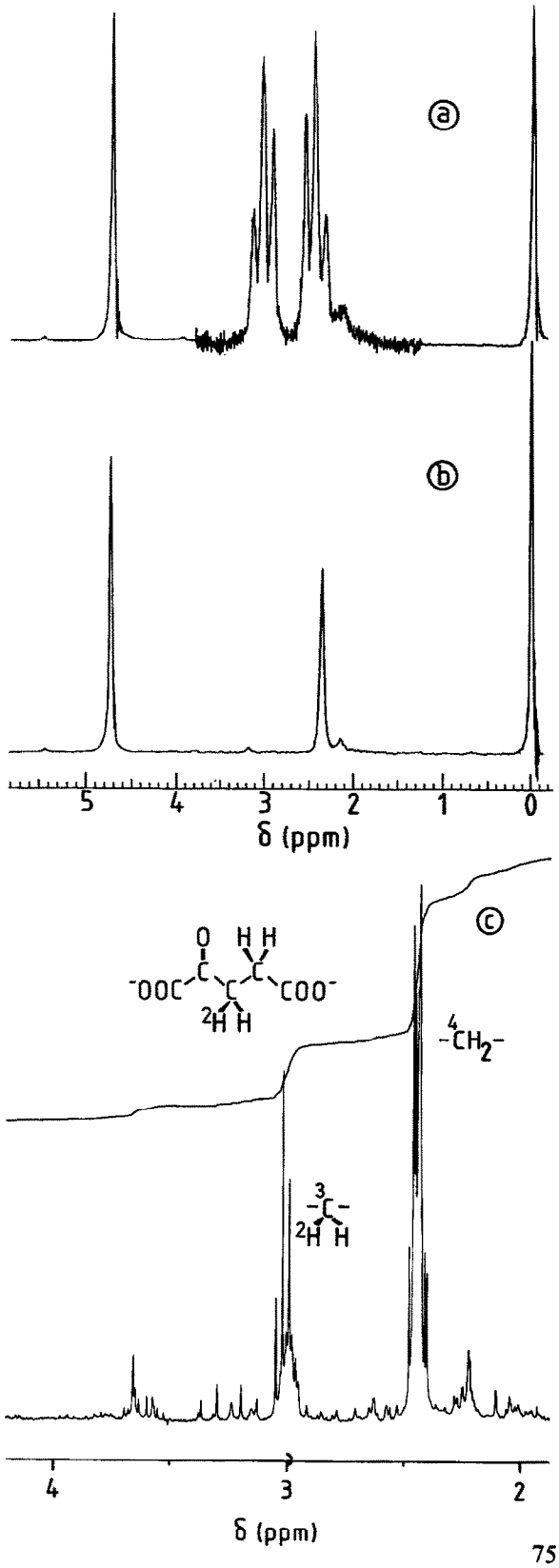

Fig.2. ^{1}H-NMR spectra of 2-oxoglutarates in deuterium oxide at p^{2}H ~ 7 with sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate as internal standard. (a) 2-Oxoglutarate. (b) 2-Oxo[3,3-$^2H_2$]glutarate, the small resonance at 2.15 ppm is due to the hydrated species [19]. Both spectra were recorded on a Varian T60 instrument. (c) (3*R*)-2-Oxo[3-$^2H_1$]glutarate recorded on a Bruker WM 250(FT) instrument; again the hydrated species is visible (around 2.15 ppm).

the ^2H- and ^3H-labelled amino acids was analysed with growing cells of *A. fermentans*. The remaining ^3H in the acetates and butyrates agreed well with earlier observations [9]. The final fermentations were performed with growing cultures rather than with washed cells of *C. tetanomorphum* since an about 10% higher conservation of ^3H in butyrate was observed with the former method.

The ^3H contents of the fermentation products are listed in table 1. No significant difference was observed between (3*R*)-, (3*S*)- and (3*RS*)-glutamates. Degradation of the two butyrates derived from the former two glutamates also gave identical values (table 2). The almost complete (over 90%) conservation of ^3H at the methyl group of butyrate (C-4) was a very important result, since it demonstrated the reliability of the whole experiment. Significant loss of ^3H would have indicated a racemisation of the methyl group during the conversion of the methylaspartate to butyrate.

The acetates obtained by the degradation of the butyrates were analysed for chirality by conversion

Table 1

Fermentation of stereospecifically labelled (2*S*)-[4-^{14}C]glutamates by growing cells of *Clostridium tetanomorphum*

Exp.	(2*S*)-[4-^{14}C]Glutamate labelled at	^3H retention in the fatty acids (%)	
		Acetate	Butyrate[a]
1	(3*RS*)-[3,3-^{3}H]	60.2	103.0
	(3*R*)-[3-$^2H_1$,3-^{3}H]	64.1	113.8
	(3*S*)-[3-$^2H_1$,3-^{3}H]	60.3	106.2
2	(4*RS*)-[4,4-^{3}H]	20.7	44.1
	Sample A[b]	33.0	56.6
	Sample B[b]	10.9	20.0
3	(4*RS*)-[4,4-^{3}H]	n.d.	43.0
4	(4*RS*)-[4,4-^{3}H]	n.d.	26.0

[a] The measured $^3H/^{14}C$ ratio of butyrate was multiplied by 2, since butyrate contains ^{14}C twice (fig.1)
[b] See text

The $^3H/^{14}C$ ratio of glutamate was taken as 100%. The ratios of the purified acetates and butyrates are expressed relative to this value. n.d., not determined

to malate. After treatment with fumarase the following amounts of ^3H remained bound to malate + fumarate (malate before fumarase treatment = 100%): starting with (3*R*)-glutamate the values from two different experiments were 48.6 and 49.9%, whereas (3*S*)-glutamate yielded 49.1 and 49.3%. Thus the formation of the methylene group at C-3 of glutamate catalysed by the adenosylcobalamin-dependent glutamate mutase occurs with complete racemisation.

3.3. *Control experiments*

The stereochemistry of the conversion of the methylene group at C-4 of glutamate to the methine group of methylaspartate should be deducible with glutamates labelled specifically with ^3H either at the pro-(4*R*) or at the pro-(4*S*) hydrogen (H_d or H_c, respectively, fig.1). By inversion of configuration H_d stays on C-4 forming the methine group while H_c migrates to C-3 which becomes the methyl group. In the next step of the fermentation, the β-elimination of NH_3, H_d is removed, whereas H_c remains in the methyl group, a precursor for acetate and butyrate. The required labelling was achieved by synthesizing glutamates from chiral acetates via citrate by the method developed for [4-^{14}C]glutamate [2].

Considering the inversion of configuration during condensation of chiral [$^2H_1$,^{3}H]acetyl-CoA with oxaloacetate and the isotope effect k_H/k_D in this reaction [14], the following (2*S*)-glutamates should be obtained: (*R*)-acetate should yield a mixture of about 75% (4*S*)-[4-$^2H_1$,4-^{3}H]glutamate and 25% (4*R*)-[4-^{3}H]glutamate (sample A) whereas with (*S*)-acetate about 75% (4*R*)-[4-$^2H_1$,4-^{3}H]-glutamate and 25% (4*S*)-[4-^{3}H]glutamate (sample B) should be generated. In addition to samples A and B the reference sample (4*RS*)-[4,4-^{3}H]glutamate was also fermented by growing cells of *C. tetanomorphum* (table 1). Sample A gave fatty acids of which the ^3H contents deviated significantly from those of the reference sample: +11.3% for acetate and +22.5% for butyrate. Almost the same deviations were found for acetate (−9.8%) and butyrate (−24.1%) derived from sample B but with the opposite sign. Thus, the data showed that the compositions of samples A and B were as expected above. Pure (4*R*)- and (4*S*)-[4-^{3}H]glutamates should yield the following deviations from the (4*RS*)-compound: ±20% for acetate and

±44% for butyrate, whereas the observed values lay almost exactly in the middle, indicating a (4*S*):(4*R*) ratio of 3 in sample A and vice versa in sample B. Since H_d, the pro-(4*R*) hydrogen was removed during the fermentation and H_c remained in the methyl group the inversion of configuration was demonstrated in complete agreement with the results in [5]. Furthermore, growing cells as a system for stereochemical studies were found to be reliable.

The data obtained by the degradation of the butyrates could also be checked by the known mechanism of butyrate synthesis from acetyl-CoA. During condensation and elimination of water two out of 6 ^3H were lost into the medium. Thus the ^3H content at C-2, C-3 and C-4 of butyrate should be 25, 0 and 75%, respectively. The slightly higher content at C-2 (30.5%, table 2) was due to the probable isotope effect in the synthesis of acetoacetyl-CoA from two acetyl-CoA. Remarkably, the ^3H contents at C-2 of those butyrates, which were derived from the [3-$^2H_1$,3-^3H]glutamates, were somewhat lower (27.2 and 27.5%), showing a smaller isotope discrimination due to the presence of ^2H in addition to ^1H and ^3H in the methyl groups.

The almost identical ^3H content at C-2 of both butyrates showed that the methyl groups must have been racemized before butyrate synthesis. Otherwise condensation of two chiral acetyl-CoA would generate acetoacetyl-CoA and 3-hydroxybutyryl-CoA with a chiral methylene group at C-2.

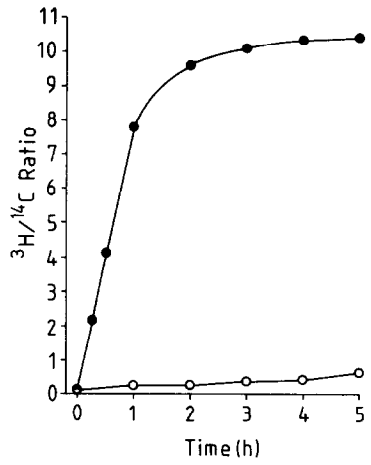

Fig.3. Enzymatic (●) and non-enzymatic (○) replacement of deuterium in 2-oxo[1-^{14}C,3,3-$^2H_2$]glutarate by tritium. The complete incubation mixture (0.1 ml) contained at 37°C: 31 mM 2-oxo[3,3-$^2H_2$]glutarate, 0.04 mM 2-oxo[1-^{14}C]glutarate, 100 mM Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 1.5 mM NADPH and tritiated water (22 mCi/ml). Isocitrate dehydrogenase (0.8 units, 40 μl of a solution in 50% glycerol) was added at zero time. At times indicated 5-μl samples were applied to Dowex 1-X8 (Cl^-) columns (0.5 × 4 cm). After washing with 7 ml water, 2-oxoglutarate was eluted with 8 ml of 1 N HCl and evaporated to dryness. The samples were counted as indicated in section 2. The final $^3H/^{14}C$ ratio of 10.39 corresponds to an incorporation of 0.95 μgatom ^3H/μmol 2-oxoglutarate (corrected by the non-enzymatic replacement. For preparative purposes the concentration of ^3HOH was increased 20-fold and the incubation was terminated after 2 h.

Table 2

Degradation of labelled butyrates obtained by fermentation of $^2H/^3H$-labelled (2*S*)-[4-^{14}C]glutamates

(2*S*)-[4-^{14}C]Glutamate labelled at	^3H content at position of butyrate (%)		
	C-2	C-3	C-4
(3*R*)-[3-$^2H_1$,3-^3H]	24.8	0	91.3
(3*S*)-[3-$^2H_1$,3-^3H]	25.1	0	91.3
(4*RS*)-[4,4-^3H][a]	6.1	0	20.0

[a] Butyrate from table 1, exp.4

The $^3H/^{14}C$ ratio of glutamate was taken as 100%

Discrimination between the two stereoisomers would be readily performed by the subsequent anti-elimination of water [17,18]. This system is therefore comparable to that of the standard procedure for the analysis of chiral acetates.

The remaining problem is the total ^3H content of the butyrates derived from [4,4-^3H]glutamates which varied between 26 and 44%. This was probably due to unknown differences in the fermentation conditions. Since 70% of this ^3H was present in the methyl groups, the ^3H content at this position was considerably lower than expected from the fermentation pathway. Thereby 50% should be lost in the methylaspartase reaction whereas the other 50% should be retained in the methyl group, in which only 20–30% was found. The loss of the

other 20–30% could be caused by the washing-out effect which is only operative if a heavy hydrogen isotope such as ^3H migrates via the coenzyme in the rearrangement [4].

4. DISCUSSION

The fermentation of (2*S*, 3*R* and 3*S*)-[3-$^2H_1$,3-^3H]glutamates to butyrates by *C. tetanomorphum* yielded racemic methyl groups. The data of the main experiment together with those of the controls strongly indicated that the racemisation occurred during the formation of the methyl group of methylaspartate from the methylene group of glutamate catalysed by glutamate mutase.

This observation confirms the rule that every methyl group generated in an adenosylcobalamin-dependent rearrangement becomes racemic [4]. An explanation for this fact could be a methylene radical as intermediate.

Other than a charged species which could be fixed by an opposite charge on the enzyme, the radical species may rotate freely and allow an attack of the third hydrogen from both sides. This proposed radical intermediate and the observed washing-out effect show strong relations to other adenosylcobalamin-dependent enzymes, especially to methylmalonyl-CoA mutase [4]. However, there is still a difference between glutamate and methylmalonyl-CoA mutases. In the conversion of the methylene to the methyl group the former enzyme works with inversion of configuration whereas with the latter retention was observed [4]. Further experiments are required to elucidate whether this difference reflects an important feature of the catalysis.

The fermentation of glutamate via methylaspartate is not the only pathway for the anaerobic degradation of this amino acid. Remarkably, in the other pathway via 2-hydroxyglutarate a radical intermediate has also been proposed [20].

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