Stereochemical Course of the Decarboxylation of 2-Amino-2-methylmalonic Acid by Serine Hydroxymethyltransferase[1]

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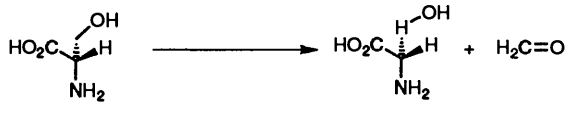
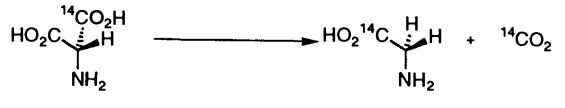
2-Amino-2-methylmalonic acid has been shown to be a slow decarboxylation substrate for the enzyme serine hydroxymethyltransferase from both rabbit liver cytosol and *E. coli*. For both enzymes the amino acid product was (2*R*)-alanine. The enantiomers of 2-amino-2-methyl[1-^{13}C]malonic acid have been synthesized and used to probe the stereochemical course and mechanism of the reaction. The *pro-R* carboxy group of 2-amino-2-methylmalonic acid was removed during the decarboxylation reaction catalysed by each enzyme and was replaced by a proton with retention of configuration at C-2. Similar results were also obtained with the H228N mutant of the *E. coli* enzyme. These results contrast earlier findings where it was suggested that 2-aminomalonic acid, a fast substrate for serine hydroxymethyltransferase, was decarboxylated non-stereospecifically.

Serine hydroxymethyltransferase (SHMT) is a ubiquitous pyridoxal 5'-phosphate (PLP)-dependent enzyme which catalyses the retro-aldol cleavage of L-serine to give glycine and formaldehyde (Scheme 1).[2] The enzyme exhibits a low regard for reaction-type specificity with α-amino acid substrates and is able to catalyse retro-aldol cleavage/aldol condensation, transamination, racemisation and decarboxylation reactions with the appropriate amino acid substrates.[2,3]



Scheme 1 *Reagents:* SHMT, PLP

In 1973 Palekar, Tate and Meister showed that the decarboxylation of aminomalonic acid by SHMT, in incubations conducted in tritiated water, gave equal amounts of (2*R*)- and (2*S*)-tritiated glycine.[4] Further studies in which the *pro-R* carboxy group of the substrate was ^{14}C-labelled gave labelled glycine and labelled carbon dioxide which each contained half of the original radiolabel, apparently confirming that the enzymic decarboxylation occurred non-stereospecifically, Scheme 2.



Scheme 2 *Reagents:* SHMT, PLP

The result was of importance because it was the first and remains the only reported example of a non-stereospecific decarboxylation catalysed by a PLP-dependent enzyme. Furthermore, the result was particularly difficult to rationalise in the light of the emerging stereochemical imperative for reactions catalysed by PLP-dependent enzymes in which it had been suggested that a diverse range of such systems, including transaminases, decarboxylases and SHMT, had originated from a common ancestor (for reviews see refs. 5–7) and, hence, shared common stereochemical features.

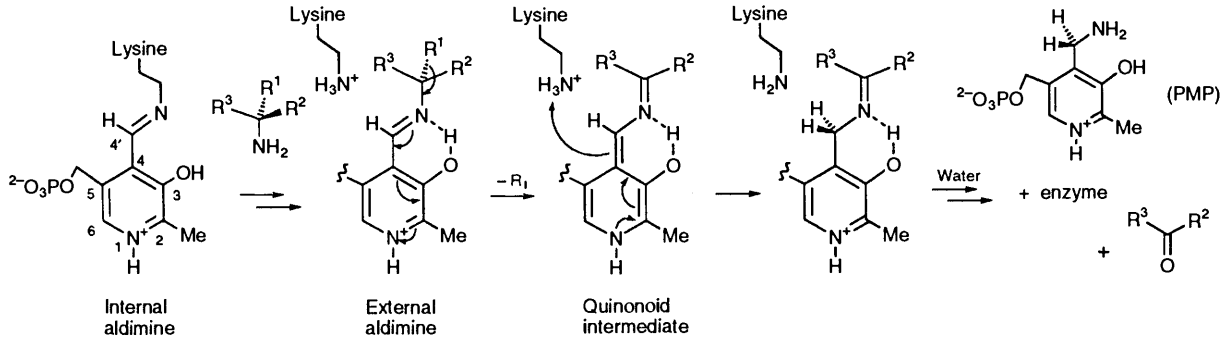
The original proposals stemmed from observations that indicated that proton-transfers to C-4' of quinonoid intermediates, formed during the normal catalytic cycle for transaminases, during the transamination of (2*R*)-alanine for SHMT, and during an abortive decarboxylation–transamination, in the case for glutamate decarboxylase (Scheme 3, reactions A, B, and C respectively), occur only from the C-4'-*si*-face of the coenzyme.

Recent work has reinforced this view for an extended number of decarboxylase systems,[8,9] and it has become apparent that the imperative also accounts for the stereospecificity of the decarboxylation reaction catalysed by α,ω-*meso*-diaminopimelate [(2*R*,6*S*)-diaminopimelate] decarboxylase.[10,11] In contrast to other PLP-dependent decarboxylases which operate on L-amino acids, both the bacterial and plant forms of this enzyme catalyse the decarboxylation of the 'D-amino acid centre' of the substrate, with inversion of configuration at C^{α}, to give L-lysine. It is believed that the C^{α}–CO_2 bond is cleaved on the C-4'-*re*-face of the coenzyme and that protonation of the quinonoid intermediate occurs from the C-4'-*si*-face.

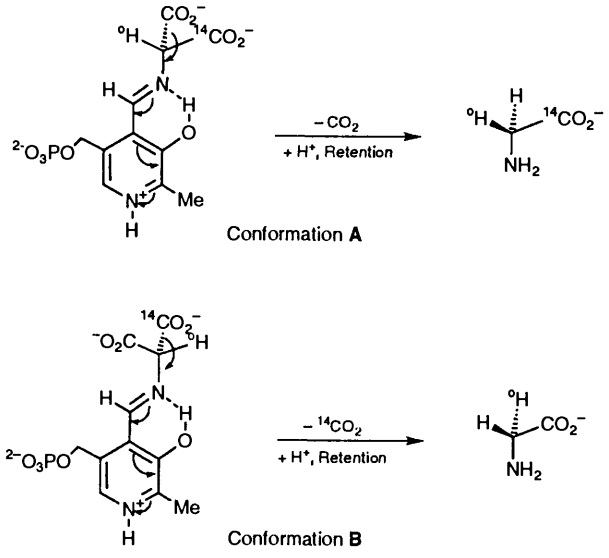
In order to explain the apparent lack of stereospecificity observed for the decarboxylation of aminomalonic acid by serine hydroxymethyltransferase within the confines of the stereochemical imperative, Palekar *et al.*[4] envisaged that the prochiral aminomalonic acid substrate could bind in two distinct conformations (**A** and **B**) at the active site of the enzyme, Scheme 4. Thus, each of the two carboxy groups could be positioned perpendicular to the plane of the coenzyme and could maximise orbital overlap between the nascent negative charge, formed during C^{α}–CO_2 bond cleavage, and the pyridinium π-system, in accord with the Dunathan postulate.[12]

In order to account for the fact that the labelled decarboxylation products were completely racemic, Palakar *et al.* suggested that each of the two distinct binding conformations would be equally populated and that the subsequent protonation steps, in each case, would occur stereospecifically.[4] In support of this mechanism, which requires a somewhat symmetrical active site possessing two carboxy group-binding pockets, it was noted that both the (2*R*)- and (2*S*)-antipodes of several amino acid substrates bind to and are processed by the enzyme.[13]

An alternative mechanism, racemisation of the substrate prior to stereospecific decarboxylation, was not considered as a likely explanation. Indeed, Palekar *et al.* had shown earlier that another PLP-dependent enzyme, aspartate β-decarboxylase,[14] catalysed the decarboxylation of the same substrate,



Scheme 3 Stereospecificity of protonation at C-4′ of the quinonoid intermediate. Reaction A, the transaminase system, R^1 = H, R^2 = CO_2^-, R^3 = amino acid side-chain. Reaction B, the SHMT system with (2*R*)-alanine, R^1 = H, R^2 = Me, R^3 = CO_2^-. Reaction C, the glutamate decarboxylase system with (2*S*)-2-methylglutamic acid, R^1 = CO_2^-, R^2 = $CH_2CH_2CO_2^-$, R^3 = Me.



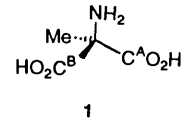
Scheme 4 Proposed explanation for non-stereospecific decarboxylation

aminomalonic acid, in a stereospecific manner under similar conditions to those used for SHMT. By analogy, this suggested that racemisation had not occurred.

The explanation offered by Palekar *et al.*, while consistent with the experimental data, invoked a rather unlikely situation in which a substrate could bind to an enzyme in two distinct but equally populated conformations, **A** and **B** in Scheme 4.*

In order to gain further information on the system, the use of the substrate analogue 2-amino-2-methylmalonic acid **1** was explored. It was reasoned that if the compound was a substrate for the enzyme, there would be no possibility for racemisation *via* C^{α}-deprotonation/protonation or for the occurrence of a well documented transamination reaction[15] between aminomalonic acid and non-enzyme-bound PLP that is promoted by the acidity of C^{α}–H.

Thanassi and Fruton had reported[16] that 2-amino-2-methylmalonic acid was a competitive inhibitor of the decarboxylation of aminomalonic acid by the cytosolic rat liver enzyme. These researchers did not observe any enzyme-catalysed decarboxylation when 2-amino-2-methylmalonic acid was incubated as a substrate, but reported that some non-enzymic decarboxylation occurred in the presence of PLP under the conditions of the assay. Since L-α-methylserine[17] was known to be a slow retro-aldol substrate for the enzyme, and



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differs from the 2-amino-2-methylmalonate only in that the carboxy group is replaced by a hydroxymethyl group, it seemed as though there was sufficient room at the active site for the extra methyl group to be accommodated. Hence, it appeared that Thanassi and Fruton's decarboxylation assay might have been too insensitive to detect the enzymic decarboxylation and that the reaction should be re-examined.

Accordingly, 2-amino-2-methylmalonic acid was prepared by the method of Bailey *et al.*[18] and was tested as a substrate for cytosolic and mitochondrial rabbit liver SHMT, and for the enzyme from *E. coli*. 2-Aminomalonic acid readily undergoes non-enzymic decarboxylation below pH 7 even in the absence of PLP, whereas, at high pH, SHMT is not an effective catalyst. Therefore, it was necessary to perform a number of trial experiments in order to optimise the conditions for the enzymic decarboxylation. At pH 7.5 decarboxylation was catalysed by each enzyme and occurred at a rate significantly faster than for control incubations containing PLP but no enzyme. Thus, 2-amino-2-methylmalonic acid was a substrate for SHMT. The expected product, alanine, could be detected by TLC analysis on cellulose, after developing the chromatogram with ninhydrin, and by conducting incubations on a larger scale it was possible to isolate sufficient quantities of the TLC-purified products to allow characterisation by ^{1}H NMR spectroscopy. The rates of the enzymic decarboxylations were, nevertheless, very slow for each system, 10^2–10^3-times slower than the rates for the retro-aldol cleavage of L-*allo*-threonine, a standard activity assay for SHMT[19] (see Experimental section). Owing to these low rates and other complications including racemisation of the product and abortive transamination to give pyruvate (see below) the kinetic parameters V_{max} and K_m (the Michaelis constant) were not determined. However, it was evident that the rates for each system were different, that the *E. coli* enzyme was the most active (see Fig. 1, below) and that the mitochondrial liver enzyme was the least active. In view of the low decarboxylase activity displayed by the mitochondrial enzyme, the system was not subjected to stereochemical scrutiny. However, an *E. coli* mutant SHMT (H228N), in which the histidine residue adjacent to the internal aldimine-forming lysine residue (see Scheme 3) had been altered to an asparagine residue,[20] was available to us and did process 2-amino-2-methylmalonic acid at a rate similar to that for the wild-type enzyme.

The absolute stereochemistry of the alanine product was determined by two different methods. In the first, aliquots of the decarboxylation reaction solution were taken at various time

* Note that this explanation includes the possibility that one conformation in Scheme 4 is less highly populated than the other, but reacts faster.

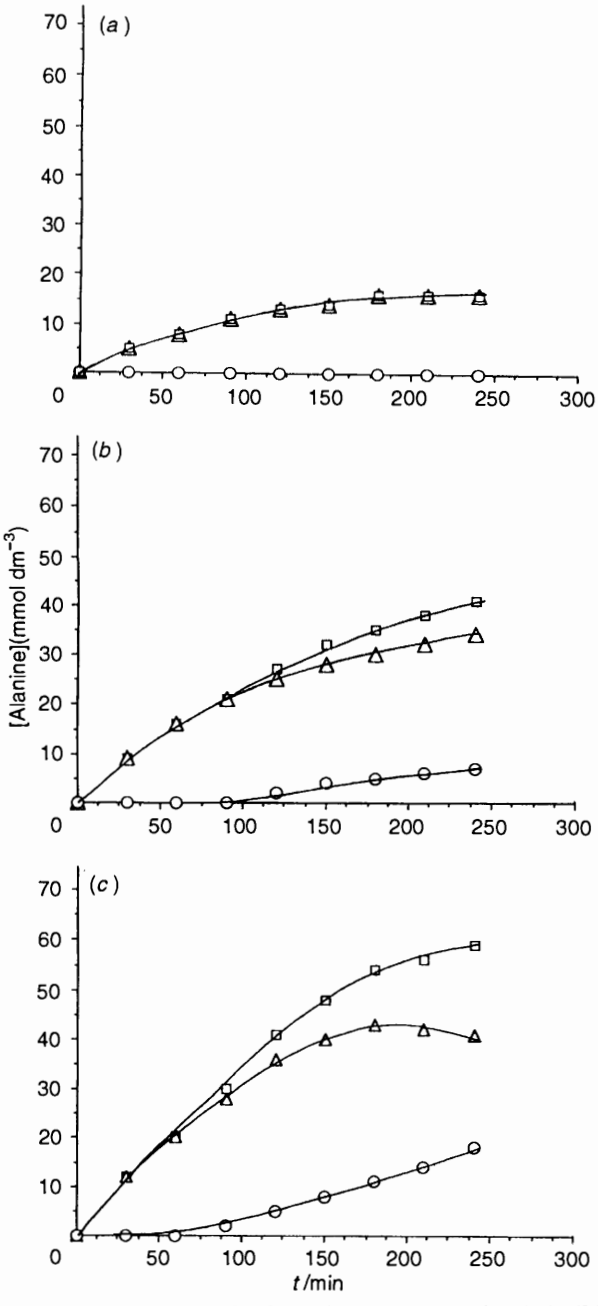
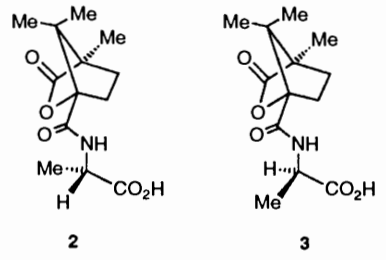


Fig. 1 Graphs showing the formation of (2*R*)-alanine (△), (2*S*)-alanine (○) and total alanine (□) in incubations of 2-amino-2-methylmalonic acid in the presence of PLP and various forms of SHMT. (*a*) Cytosolic SHMT; (*b*) *E. coli* SHMT; (*c*) *E. coli* SHMT H228N mutant. The starting concentration of the substrate was 75 mmol dm^{-3} in each experiment. See Experimental section for details.

intervals and were added to each of two 'enzyme cocktails' to estimate for (2*R*)- and (2*S*)-alanine, respectively, following the method of Walsh.[21] The cocktail for estimating (2*R*)-alanine contained D-amino acid oxidase, lactate dehydrogenase and nicotinamide adenine dinucleotide (reduced form) (NADH), while that for estimating (2*S*)-alanine contained L-alanine dehydrogenase, nicotinamide adenine dinucleotide (NAD) and hydrazine. Hydrazine forms the pyruvate hydrazone irreversibly and, thus, prevents the back-reaction. Both determinations were performed by measuring the change in absorbance at 340 nm due to the consumption [for (2*R*)-alanine] or formation [for (2*S*)-alanine] of NADH. Therefore, it was possible to monitor the chirality of the alanine produced from the decarboxylation reaction throughout the course of the incubation. This facility was particularly important because SHMT is able to catalyse the reacemisation of (2*R*)-alanine upon prolonged incubation.[13]

Analyses by these methods revealed that, for each enzyme, the cytosolic SHMT and the *E. coli* wild-type and H228N mutant, only (2*R*)-alanine was formed initially, Fig. 1. This result was verified by using a second chirality determination in which the alanine produced after a 6 h incubation period was isolated, purified and then converted into its *N*-(1*S*,4*R*)-camphanoyl derivative. The ^{1}H NMR spectra of the camphanamides derived from different incubations were then compared with those for authentic samples of (2*S*)-*N*-camphanoylalanine **2** and (2*R*)-*N*-camphanoylalanine **3** whereupon it became evident that only (2*R*)-alanine was produced, Fig. 2.



The finding that the decarboxylation gives only one enantiomer of the product indicates that the protonation of the quinonoid intermediate and the decarboxylation are both stereospecific. However, the result gives no clues as to which carboxy group is cleaved, or any information on upon which face of the coenzyme protonation occurs.

In order to address these questions the 2*R* and 2*S* enantiomers of 2-amino-2-methyl[1-^{13}C]malonic acid (**1**, C^A = ^{13}C, C^B = C; and C^A = C, C^B = ^{13}C) were synthesized (full details have been reported elsewhere)[22] and each enantiomer was incubated with each of the cytosolic rabbit liver enzyme and the *E. coli* wild-type and mutant SHMT in the presence of PLP. The alanines produced upon decarboxylation were purified by TLC on cellulose, together with unchanged substrate, and were examined by ^{1}H and ^{13}C NMR spectroscopy, Fig. 3. The spectra indicated that for each enzyme (2*R*)-2-amino-2-methyl-[1-^{13}C]malonate (**1**, C^A = ^{13}C, C^B = C) was converted into unlabelled alanine (δ_H 1.21, d, $J_{2\text{-H},3}$ 6.8 Hz) while the (2*S*)-[1-^{13}C]enantiomer (**1**, C^A = C, C^B = ^{13}C) was converted into [1-^{13}C]alanine (δ_H 1.21, dd, $J_{2\text{-H},3}$ 6.8, $J_{3\text{-H},C\text{-}1}$ 4.0 Hz). It is, therefore, apparent that the *pro-R* carboxy group of the substrate is lost during the decarboxylation. Since the alanine products must possess (2*R*)-absolute stereochemistry, *vide supra*, it is evident that the decarboxylation process occurs with retention of configuration. This is the expected stereochemical course for a decarboxylation substrate for SHMT in which the *pro-S* carboxy binds at the same site in the enzyme as does the carboxy group of (2*S*)-serine. Note that the hydroxymethyl group of serine is replaced by a proton with retention of configuration. Our new data are consistent with the proposal that the decarboxylation of the external aldimine and the protonation of the quinonoid intermediate should occur on the 4′-*si*-face of the coenzyme, Scheme 5.

The results of this stereochemical study are also consistent with the idea that decarboxylases and SHMT, which possess similar active-site peptides (S[or T]-X-H-K-), also possess similar active-site structures. Since it has been proposed that the histidine residue in decarboxylases provides a binding site for the carboxylate moiety of the substrate-PLP aldimine during decarboxylation (and also that it serves as an acid for the protonation of the quinonoid intermediate at C^α),[8] the equivalent histidine residue in SHMT (H-288) may interact

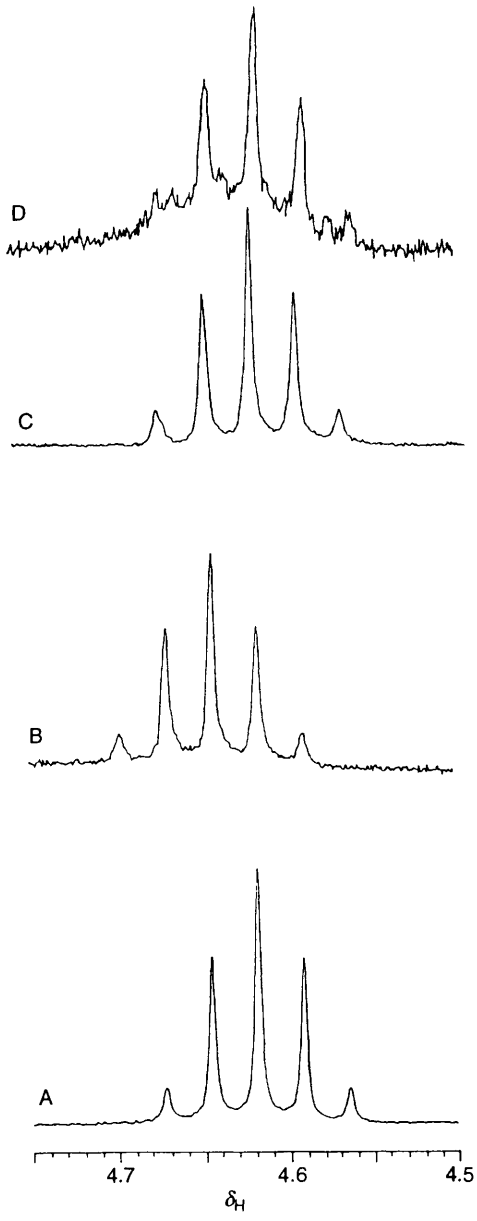


Fig. 2 Partial 270 MHz ^{1}H NMR spectra of *N*-camphanoyl- (2*R*)- and (2*S*)-alanines **2** and **3** showing the signals due to the C-2 protons of the alanine moiety. A, Unlabelled *N*-camphanoyl-(2*R*)-alanine **3**; B, Unlabelled *N*-camphanoyl-(2*S*)-alanine **2**; C, *N*-camphanoylalanine prepared from alanine isolated from the incubation of 2-amino-2-methylmalonic acid with *E. coli* wild-type SHMT (the H228N mutant gave a similar result); D, *N*-camphanoylalanine prepared from alanine isolated from the incubation of 2-amino-2-methylmalonic acid with cytosolic SHMT. See Experimental section for details. The samples derived from the decarboxylation of 2-amino-2-methylmalonic acid show a quintet centred at δ 4.62 and are coincident with the signals of the sample derived from (2*R*)-alanine.

with the hydroxymethyl of the serine-PLP aldimine in the retroaldol cleavage of serine and the *pro-R* carboxy group of 2-amino-2-methylmalonic acid during its decarboxylation. However, it seems unlikely that the histidine residue serves as a general acid for the protonation of the appropriate quinonoid intermediates at C^{α}, since the rates of both reactions for the wild-type enzyme are similar with the H228N mutant. Furthermore, the microscopic reverse reaction, deprotonation at C^{α} for the aldimine derived from (2*R*)-alanine occurs just as readily in the H228N mutant as it does in the wild-type enzyme.[13]

The results described in this paper are not in accord with Palekar, Tate and Meister's results[4] and the very different

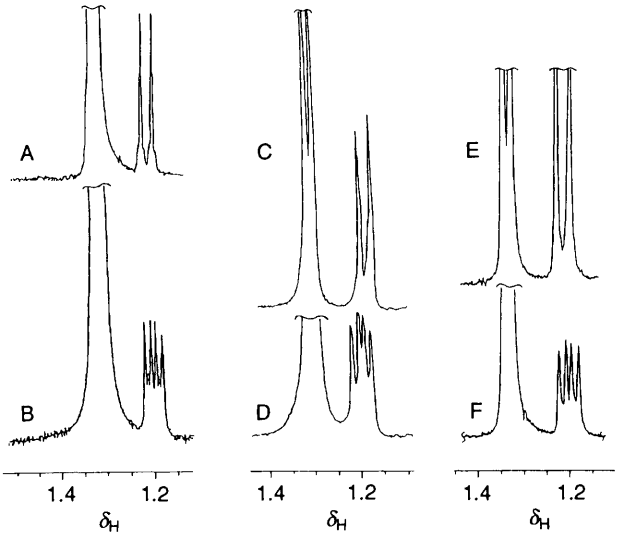


Fig. 3 Partial 270 MHz ^{1}H NMR spectra of the alanine products and recovered starting materials from the incubation of (2*R*)-2-amino-2-methyl[^{13}C]malonic acid with: A, cytosolic SHMT; C, *E. coli* SHMT; and E, *E. coli* H228N SHMT; and from the incubation of (2*S*)-2-amino-2-methyl[1-^{13}C]malonic acid with: B, cytosolic SHMT; D, *E. coli* SHMT; and F, *E. coli* H228N SHMT. The absence of a ^{13}C-coupling pattern in the signals due to the methyl group of alanine (δ 1.2) alanine samples derived from (2*R*)-2-amino-2-methyl[1-^{13}C]malonic acid indicate that the *pro-R* carboxy group is cleaved during the decarboxylation.

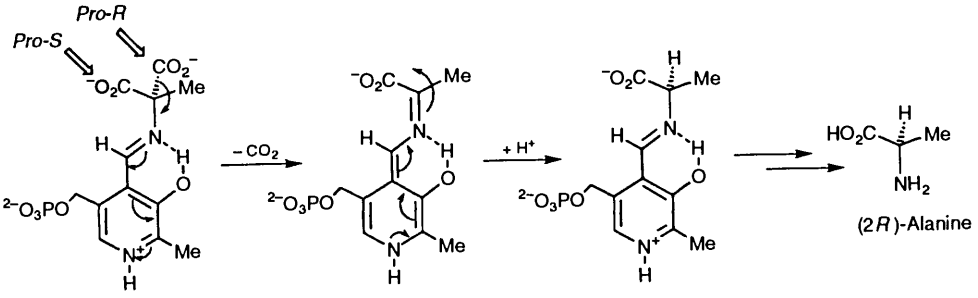
conclusions are difficult to comprehend. The earlier findings may best be rationalised in terms of racemisation of 2-aminomalonic acid prior to decarboxylation, but the possibility that the bulkier 2-amino-2-methylmalonate substrate is limited to one of the two proposed binding conformations for 2-aminomalonic acid (Scheme 4, conformation **B**) cannot be discounted.

The results of experiments designed to re-examine the stereochemical course of the decarboxylation of 2-aminomalonic acid by SHMT are described in the following paper.

Experimental

Elemental analyses were carried out at the microanalytical laboratory, University College, London. Specific rotations were determined on an Optical Activity Ltd AA-100 polarimeter using a 5 cm pathlength cell at 21 °C, and are given in units of 10^{-1} deg cm^2 g^{-1}. All ^{1}H NMR spectra were recorded on either a JEOL JNM-GX270 (270 MHz), a Bruker AM360 (360 MHz) or a Varian VXR500 (500 MHz) spectrometer. Aqueous solutions were referenced using either the H^{2}HO signal (at δ 4.61), the signal for 1,4-dioxane (at δ 3.66), or the sodium salt of 3-(trimethylsilyl-[2,2,3,3-^{2}H$_4$]propionic acid [TMS(Na)] signal (at δ 0.0). *J* Values are given in Hz. ^{2}H NMR spectra were recorded at 55.3 MHz on a Bruker AM360 spectrometer and are quoted in ppm relative to C^{2}HCl$_3$ (at δ_D 7.27). $\{^1H\}$ ^{13}C NMR spectra were recorded at 67.9 MHz, and chloroform (δ_C 77.20), 1,4-dioxane (δ_C 67.40), pyridine (δ_C C-2 and C-6 149.8) or methanol (δ_C 47.00) were used as chemical-shift references, as indicated. Mass spectra and accurate mass measurements were recorded on a Kratos MS 30 or a VG 70 250 SE spectrometer. Major fragments are given as percentages of the base peak intensity (100%). Fast-atom bombardment (FAB) spectra were recorded using glycerol as matrix. pH Measurements were performed using a Jenway PNA2 pH meter.

Enzymes other than SHMT were obtained from Sigma Chemical Co., Poole, UK and were used without further purification. Serine hydroxymethyltransferases were provided by Professor Verne Schirch of the Virginia Commonwealth University.



Scheme 5

2-Amino-2-methylmalonic Acid **1**.—Following the method of Bailey *et al.*,[18] liquid bromine (24 g, 150 mmol) was added slowly to a stirred solution of 2-methylmalonic acid (17.7 g, 150 mmol) in 2.0 mol dm^{-3} NaOH (250 cm^3). Once the bromine colour had disappeared, the pH of the solution was adjusted to 1.8 by using conc. HCl and the solution was extracted with diethyl ether (3 × 150 cm^3). The combined extracts were then concentrated under reduced pressure, and the oily residue of bromomalonic acid was taken up in 33% aq. ammonia (260 cm^3). This solution was then maintained at 46 °C for 72 h in a sealed flask. The ammonia was then removed by lyophilisation and the residual powder was taken up in the minimum amount of water, the mixture was filtered, and the solution was then applied to an Amberlite IR120 (H^+) ion-exchange column (3 × 30 cm). The required 2-amino-2-methylmalonic acid was eluted from the column with water. The ninhydrin-positive fractions were pooled and lyophilised to give a solid, which was recrystallised from water–acetone to give the required compound **1** (12.8 g, 64%), m.p. 282–284 °C (Found: C, 36.0; H, 5.3; N, 10.5. Calc. for $C_4H_7NO_4C$, 36.09; H, 5.30; N, 10.52%); ν_{max}(Nujol)/cm^{-1} 3000–2600 (OH, NH), 1730 (C=O) and 1605 (NH); δ_H[270 MHz; ^2H_2O; TMS(Na)] 1.69 (3 H, s, 2-Me); δ_C(67.9 MHz; ^2H_2O; ref. MeOH) 17.83 (C-3'), 61.94 (C-2) and 169.04 (C-1,3); *m/z* (FAB – glycerol–water) 226 $[M + H + glycerol]^+$ and 134 $[M + H]^+$.

(2S,1'S,4'R)-N-*Camphanoylalanine* **2**.—(2*S*)-Alanine (0.5 g, 5.6 mmol) was dissolved in 2 mol dm^{-3} NaOH (5 cm^3) and the solution was shaken with a solution of (1*S*,4*R*)-(−)-camphanoyl chloride (1.34 g, 6.16 mmol) in toluene (5 cm^3) for 15 min, and then was stirred for 2 h at room temperature. The toluene layer was separated, and the aqueous solution was extracted once with dichloromethane (5 cm^3) which was discarded. The aqueous solution was then adjusted to pH 2 by using 5 mol dm^{-3} HCl, and extracted with dichloromethane (3 × 5 cm^3). The combined organic layers were then dried ($MgSO_4$), and concentrated under reduced pressure to give an oil which crystallised on scratching. Camphanic acid was removed by column chromatography on silica (1:9; EtOAc–CH_2Cl_2) to give (2S,1'S,4'R)-*camphanoylalanine* **2** as a crystalline solid, which was recrystallised from diethyl ether–hexane (0.65 g, 43%), m.p. 146–148 °C (Found: C, 57.8; H, 7.1; N, 5.3%; M^+, 269.1257. $C_{13}H_{19}NO_5$ requires C, 57.98; H, 7.11: N, 5.20%; M^+, 269.1263); $[\alpha]_D$ −15.6 (*c* 1.0, $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3420 (NH), 2990 (CH), 1795 (C=O, lactone), 1730 (C=O, acid), 1680 and 1530 (C=O, amide); δ_H(270 MHz; C^2HCl_3) 0.93 (3 H, s, 4'-Me), 1.11 and 1.12 (each 3 H, s, 7'-Me), 1.51 (3 H, d, *J* 7, 2-Me), 1.63–2.55 (4 H, m, 5'- and 6'-H_2), 4.64 (1 H, quintet, *J* 7, 2-H), 5.90 (1 H, br s, 1-OH) and 7.00 (1 H, d, *J* 7, 2-NH); δ_C(67.9 MHz; $C_5{}^2H_5N$) 9.82, 16.63, 18.44, 28.34, 30.57, 31.16, 48.75, 53.89, 55.06, 92.55, 167.01, 175.26 and 178.35; *m/z* (EI) 269 (M^+, 29.3%), 254 ($[M - CH_3]^+$, 3.2), 224 ($[M - CO_2H]^+$, 58.3) and 83 (100).

(2R,1',S,4'R)-*Camphanoylalanine* **3**.—This was prepared in a similar manner to diastereoisomer **2**, but starting with (2*R*)-alanine to give the *camphanamide* **3** (0.57 g, 38%) as a crystalline solid, following chromatographic purification on silica and recrystallisation, m.p. 157 °C (Found: C, 58.0; H, 7.2; N, 5.2%; M^+, 269.1262); $[\alpha]_D$ −23.2 (*c* 1.0, $CHCl_3$); ν_{max}($CHCl_3$)/cm^{-1} 3420 (NH), 2980 (CH), 1784 (C=O, lactone), 1734 (C=O, acid), 1673 and 1529 (C=O, amide); δ_H(270 MHz; C^2HCl_3) 0.97 (3 H, s, 4'-Me), 1.09 and 1.12 (each 3 H, s, 7'-Me), 1.51 (3 H, d, *J* 7, 2-Me), 1.61–2.60 (4 H, m, 5'- and 6-H_2), 4.62 (1 H, quintet, *J* 7, 2-H), 6.40 (1 H, br s, 1-OH), 6.90 (1 H, d, *J* 7, 2-NH); δ_C(67.9 MHz; $C_5{}^2H_5N$) 9.83, 16.76, 17.80, 29.16, 30.65, 31.16, 48.71, 53.86, 55.04, 92.36, 167.34, 170.52 and 178.54.

Serine Hydroxymethyltransferase.—Lyophilised SHMT (100 mg) was dissolved in potassium phosphate buffer (10 cm^3; 20 mmol dm^{-3}, pH 7.3) containing (2*S*)-serine (20 mmol dm^{-3}), dithiothreitol (1 mmol dm^{-3}) and ethylenediamine tetraacetic acid (EDTA) (0.1 mmol dm^{-3}). The resulting solution was incubated for 5 min at 50 °C. Insoluble material was removed by centrifugation and then the supernatant was dialysed overnight against potassium phosphate buffer (1000 cm^3; 20 mmol dm^{-3}, pH 7.3) containing dithiothreitol (1 mmol dm^{-3}) and EDTA (0.1 mmol dm^{-3}) to remove any unbound PLP. The solution was then divided into 1 cm^3 fractions and was stored at −30 °C until required.

Determination of Enzyme Activity (L-allo-*Threonine Assay*).[19] —SHMT (1–10 mm^3 of 10 mg cm^{-3} solution) was added to a 1 cm^3 cuvette containing [L-*allo*-](2*S*,3*S*)-threonine (1.33 mg, 10 mmol dm^{-3}), dithiothreitol (0.15 mg, 1 mmol dm^{-3}), PLP (2.65 μg, 10 μmol dm^{-3}), NADH (0.21 mg, 0.3 mmol dm^{-3}) and yeast alcohol dehydrogenase (35 units) which had been equilibrated at 30 °C. The amount of enzyme activity was determined by measuring the decrease in absorbance at 340 nm due to the oxidation of NADH (λ_{max} 340 nm [ε 6220 dm^3 mol^{-1} cm^{-1}]). One unit of enzyme activity is the amount of enzyme required to convert one micromole of L-*allo*-threonine into acetaldehyde and glycine per minute.

Incubation Conditions for 2-Amino-2-methylmalonic Acid and SHMT.—Incubations contained 2-amino-2-methylmalonic acid (10 mg, 75 mmol), PLP (1 μmol), and SHMT (2 units) (either the rabbit liver cytosolic or *E. coli* enzyme) in potassium phosphate buffer (300 mmol dm^{-3}, pH 7.50) in a total volume of 2.0 cm^3. The reactions were maintained at 30 °C for 4 h during which time several small aliquots of the solution were removed for analysis by TLC or for determination of the chirality of the products (see below). Control incubations were identical except that the enzyme was omitted.

After alanine had been tentatively identified as the decarboxylation product, a large-scale incubation was performed under conditions identical with those described above and the alanine product was isolated from the incubation solution after denaturation of the protein and then purified by preparative TLC (PLC) on cellulose with 4:1 MeOH–aq. ammonia (0.88 g

cm^{-3}) as the developer; for details see below. The ^1H NMR spectrum of the sample in ^2H_2O was identical with that for an authentic sample of alanine.

Determination of the Stereochemistry of the Decarboxylation Product.—*(a) Using enzyme assays for* (2R)- *and* (2S)-*alanine.*[21] (2R)-*Alanine assay.* 2-Amino-2-methylmalonic acid (10 mg, 75 mmol) was incubated with SHMT, as described above, and 50 mm^3 aliquots of the incubation solution and of a control experiment containing no enzyme were removed at 30 min intervals over the period of the reaction, ~4 h. The samples were adjusted to pH 10.0, to prevent non-enzymic decarboxylation, and were then heated at 100 °C for 1 min to inactivate the SHMT. The samples were then added to an assay mixture containing D-amino acid oxidase (6 units), lactate dehydrogenase (5 units), catalase (40 units) and NADH (0.3 mmol dm^{-3}) in aminotris(hydroxymethyl)methane (Tris) buffer (100 mmol dm^{-3}, pH 8.3) such that the total volume was 1 cm^3. The decrease in absorbance at 340 nm due to the oxidation of NADH was recorded and was used to calculate, against known standards, the amount of (2*R*)-alanine present in the original incubation. (At 340 nm the oxidation of NADH to NAD caused a change in absorbance of 6220 dm^3 mol^{-1} cm^{-1}.) The concentrations of (2*R*)-alanine formed during incubations, as depicted in Fig. 1, were corrected for non-enzymic decarboxylation.

(2S)-*Alanine assay.* Exactly as described above, 50 mm^3 aliquots of the 2-amino-2-methylmalonic acid incubation solution and of a control experiment were removed, adjusted to pH 10.0, and then heated. For the determination of (2*S*)-alanine, the samples were added to an assay mixture containing L-alanine dehydrogenase (5 units), hydrazine hydrate (5 mm^3) and NAD^+ (10 mmol dm^{-3}) in 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (100 mmol dm^{-3}, pH 9.0) to a total volume of 1 cm^3. The increase in absorbance at 340 nm due to the reduction of NAD^+ was recorded and was used to calculate the amount of (2*S*)-alanine present in the original incubation. The concentrations of (2*S*)-alanine formed during incubations, as depicted in Fig. 1, were corrected for non-enzymic decarboxylation.

The formation of pyruvate (produced *via* abortive transamination of the coenzyme) was also followed by using the lactate dehydrogenase and NADH assay. The amount of pyruvate formed never exceeded the starting concentration of PLP but, in the case of the cytosolic SHMT, formed rapidly enough to inactivate the enzyme. Activity could be restored by the addition of PLP.

(b) *By* ^1H *NMR spectroscopic comparison of the diastereoisomeric* N-(1S,4R)-*camphanamide derivatives.* 2-Amino-2-methylmalonic acid (50 mg, 0.38 mmol) and SHMT (4 units) were incubated in buffer (2.0 cm^3) for 6 h at 30 °C in a similar manner to that described above. In the experiment in which cytosolic rabbit liver SHMT was used, further aliquots of PLP (100 nm^3 of 10 mmol dm^{-3} solution in incubation buffer) were added after 2 and 4 h. After 6 h, each reaction mixture was adjusted to pH 10.5 by using 2 mol dm^{-3} aq. KOH and the protein was precipitated by addition of absolute ethanol. After centrifugation (3000 rpm for 20 min) the supernatant fraction was concentrated under reduced pressure (< 40 °C) to give a residue. This was dissolved in the minimum amount of water and applied to two cellulose PLC plates (20 × 20 cm, 0.5 mm thick). These were developed using 4:1 MeOH–aq. ammonia (0.88 g cm^{-3}) and were visualised by spraying the edge of the plates with ninhydrin solution. The band containing the alanine was removed from the plate and was stirred with ethanol–water (3:2; 25 cm^3) for 3 h. The cellulose was removed by filtration through Celite and the filtrate was concentrated under reduced pressure. The residual solid was dissolved in water (1 cm^3) and the solution was adjusted to pH 11.0 by using 2 mol dm^{-3} aq. NaOH. A freshly prepared solution of (1*S*,4*R*)-camphanoyl chloride (21.6 mg, 0.1 mmol) in toluene (1.5 cm^3) was added and the mixture was shaken vigorously for 15 min and then stirred for 2 h. The resulting mixture was diluted with both water (5 cm^3) and dichloromethane (5 cm^3) and the organic phase was separated and discarded. The aqueous phase was adjusted to pH 2.0 (5 mol dm^{-3} HCl) and was extracted with dichloromethane (3 × 5 cm^3). The combined organic layers were dried ($MgSO_4$), and concentrated under reduced pressure, and the chirality of the isolated alanine was then deduced by comparison of the 270 MHz ^1H NMR spectra with those of *N*-(1*S*,4*R*)-camphanoylalanines of known chirality at C-2 of the amino acid moiety (see Fig. 2).

Decarboxylation of (2R)- *or* (2S)-2-*Amino-2-methyl*[1-^{13}C]-*malonic Acid with SHMT.*—Incubations contained either (2R)- or (2*S*)-2-amino-2-methyl[1-^{13}C]malonic acid (10 mg, 75 μmol) together with PLP (1 μmol) and SHMT (2 units) in potassium phosphate buffer (300 mmol dm^{-3}, pH 7.50) in a total volume of 2.0 cm^3. The reactions were maintained at 30 °C for 4 h and were terminated by adjusting the pH to 10.5 with 2 mol dm^{-3} aq. KOH. The protein was precipitated by addition of absolute ethanol and was removed by centrifugation (3000 rpm for 20 min). The supernatant solution was concentrated under reduced pressure (< 40 °C) to give a residue, which was dissolved in deuterium oxide for ^1H and ^{13}C NMR spectrocopic analysis (Fig. 3). This entire experiment was performed using rabbit liver cytosolic SHMT, *E. coli* SHMT and the *E. coli* mutant H228N.

^1H NMR spectroscopy indicated that 10, 35 and 35% of the total substrate was decarboxylated by the cytosolic rabbit liver, the *E. coli* wild-type and the *E. coli* H228N mutant enzyme, respectively. The identity of the labile carboxy group was assigned from an examination of the coupling pattern for the methyl signal of the alanine products in the ^1H NMR spectra (*i.e.*, the presence or absence of a ^{13}C coupling; see Fig. 3), and from the ^{13}C NMR spectra. Control experiments which were conducted in the absence of SHMT showed that negligible decarboxylation occurred over a period of 96 h.

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