Proton transfer in methylmalonyl-CoA epimerase from *Propionibacterium shermanii*

The reaction of (2*R*)-methylmalonyl-CoA in tritiated water

James Q. FULLER and Peter F. LEADLAY*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

(Received 3 March 1983/Accepted 23 May 1983)

The reaction catalysed by methylmalonyl-CoA epimerase from *Propionibacterium shermanii* was studied in tritiated water, in the direction with (2*R*)-methylmalonyl-CoA as substrate, under 'irreversible' conditions. After partial reaction, even when most of the substrate had been converted into product (isolated as propionyl-CoA) essentially no solvent tritium appeared in residual (2*R*)-methylmalonyl-CoA. The product, however, did contain tritium, and the specific radioactivity of the (2*S*)-epimer was deduced to be 0.33 times that of the solvent. These results provide further support for the mechanism proposed for the epimerase-catalysed reaction in the accompanying paper [Leadlay & Fuller (1983) *Biochem. J.* 213, 635–642], in which two enzyme bases act respectively as proton donor and acceptor. The observed low discrimination against solvent tritium entering the product can be accounted for by a mechanism in which the release of product is slow, and the re-protonation step on the enzyme is reversible, without leading to isotopic exchange with the solvent.

Methylmalonyl-CoA epimerase (EC 5.1.99.1, classified as a racemase) from *Propionibacterium shermanii* catalyses the interconversion of (2*R*)- and (2*S*)-methylmalonyl-CoA, an essential step in the production of propionic acid by these bacteria. The epimerase has been purified to apparent homogeneity (Allen *et al.*, 1963; Leadlay, 1981). It is a dimer of subunit M_r 16500 and requires no added cofactors or metal ions for activity. As discussed in the preceding paper (Leadlay & Fuller, 1983), the observation that there is very little (if any) 'transfer' of tritium label from (2*R*)-methyl[2-³H]malonyl-CoA to the product during the epimerase-catalysed reaction means that the hydrogen atom of the new C–H bond is essentially solvent-derived. This can be explained if an enzyme base that is involved in removal of a proton from C-2 of (2*R*)-methylmalonyl-CoA is itself in protonic equilibrium with the solvent. Alternatively, the finding can be accommodated by a mechanism in which two separate enzyme bases are involved. One removes the hydrogen atom from C-2 of (2*R*)-methylmalonyl-CoA, and the second furnishes the new proton. This type of mechanism has been established for proline racemase (Cardinale & Abeles, 1968; Rudnick & Abeles, 1975).

A feature of this second mechanism is that the proton removed from the substrate is 'protected' from

the solvent and does not exchange with solvent protons until the product has been released. A sensitive test for such an exchange in the present case is to perform the methylmalonyl-CoA epimerase-catalysed reaction with unlabelled (2*R*)-methylmalonyl-CoA in tritiated water, under conditions where the (2*S*)-product is removed enzymically as fast as it is formed. Any appearance of tritium label in unchanged substrate would rule out a mechanism of the type proposed for proline racemase. It might also allow a quantitative description of the way in which an exchanging intermediate partitions, between going on to form product and returning to substrate (Maister *et al.*, 1976; Fletcher *et al.*, 1976). Secondly, there will be isotopic discrimination against solvent tritium in the step where the new C–H bond is formed, and we can investigate this by measuring the rate at which solvent tritium enters the product. In the present paper both types of experiment are reported, with (2*R*)-methylmalonyl-CoA as substrate.

Materials and methods

Materials

Deionized distilled water and A.R.-grade reagents were used, except where otherwise stated. Other materials used are described in the preceding paper (Leadlay & Fuller, 1983).

* To whom correspondence should be addressed.

Manipulations involving volatile tritium-labelled material were performed in a well-ventilated fume hood.

Preparation of (2R)-methylmalonyl-CoA

This was obtained by removal of the (2S)-epimer from (2RS)-methylmalonyl-CoA epimerase. A typical incubation mixture contained, in a final volume of 5 ml, 10 mM-potassium phosphate buffer, pH 8.0, 20 mM-sodium pyruvate, 1.6 mM-NADH, 0.93 mM-(RS)-methylmalonyl-CoA and malate dehydrogenase (80 µg/ml). Reaction was initiated by the addition of methylmalonyl-CoA transcarboxylase (final concn. 16 µg/ml), and the reaction was monitored to completion by measuring the fall in A_{340} in a 2 mm-pathlength cuvette.

The reaction mixture was passed slowly through a 2 ml column of avidin-Sepharose, pre-equilibrated, at 4°C, with 10 mM-potassium phosphate buffer, pH 8.0. The eluate and washings were then applied to a column (1.5 cm × 22 cm) of DEAE-cellulose that had been pre-equilibrated with 0.2 M-ammonium bicarbonate at 4°C. The column was washed with 65 ml of the same buffer, and CoA esters were eluted at 60 ml/h with a linear gradient formed from 70 ml of 0.2 M-ammonium bicarbonate and 70 ml of 0.275 M-ammonium bicarbonate. Methylmalonyl-CoA was eluted near the end of the gradient, and was quickly pooled and concentrated at 4°C, by using an ultrafiltration unit (Amicon, Woking, Surrey, U.K.) fitted with a 76 mm-diam. UM-05 membrane. The CoA ester was effectively desalted by adding further portions of cold 10 mM-potassium phosphate buffer and continuing the ultrafiltration (a method due to Professor S. J. Wakil, quoted by Sedgwick *et al.*, 1977). The concentrated desalted solution (approx. 2 ml) was stored under liquid N₂ until required.

Assays

(2S)-Methylmalonyl-CoA was assayed at 0°C at pH 8.0, with the use of epimerase-free methylmalonyl-CoA transcarboxylase and malate dehydrogenase. (2R)-Methylmalonyl-CoA was assayed by subsequent addition of methylmalonyl-CoA epimerase to the same cuvette. Propionyl-CoA was assayed by an adaptation of the method of Garland (1965) for acetyl-CoA: propionyl-CoA was hydrolysed to CoA in the presence of arsenate and phosphate transacetylase, and the CoA was allowed to react *in situ* with NAD⁺ and 2-oxoglutarate in the presence of 2-oxoglutarate dehydrogenase. Other assays were as previously described (Leadlay, 1981).

Stability of (2R)-methylmalonyl-CoA

(2R)-Methylmalonyl-CoA, after purification on DEAE-cellulose, consisted of 90–95% (2R)-epimer

by enzymic assay. On storage, the proportion of (2S)-epimer gradually increased. Samples of freshly prepared (2R)-methylmalonyl-CoA were incubated at various pH values, at 0°C, and after measured time intervals the proportion of (2R)-epimer was assayed as described above (see Fig. 1).

Tritium fractionation factor for (RS)-methylmalonyl-CoA

(RS)-Methylmalonyl-CoA, dissolved in 10 mM-potassium phosphate buffer, pH 7.0, was heated with tritiated water at 70°C for 30 min in a sealed tube. Samples were taken and diluted for counting of radioactivity as described in the preceding paper (Leadlay & Fuller, 1983). The mixture was then freeze-dried to recover the tritiated water, and several portions of distilled water were added to the residue and subsequently removed by freeze-drying. The methyl[2-³H]malonyl-CoA was purified on a column (1.5 cm × 20 cm) of DEAE-cellulose, at 4°C, as described in the preceding paper (Leadlay & Fuller, 1983). It was clearly separated from a small amount of propionyl-CoA. The values for specific radioactivity reported in Table 1 are averages for peak fractions containing methylmalonyl-CoA.

In another experiment, (RS)-methylmalonyl-CoA (3.5 µmol) was dissolved in 0.5 ml of 10 mM-potassium phosphate, pH 8.0, containing tritiated water (0.08 Ci/ml) and methylmalonyl-CoA epimerase (0.12 µg/ml). After incubation for 16 h at 20°C, samples were taken as before for determination of the specific radioactivity of the solvent. The mixture was cooled to 0°C, and the epimerase was removed by filtration through a small column of anti-(methylmalonyl-CoA epimerase) immunoglobulin G-Sepharose. The isolation and analysis of the radio-labelled methylmalonyl-CoA were performed as before, and the results are shown in Table 1.

Tritium content of (2R)-methylmalonyl-CoA remaining after partial reaction with epimerase in tritiated water

In a typical experiment, 1.35 ml of a solution of methylmalonyl-CoA [1.43 µmol, 80% as the (2R)-epimer] and 0.19 ml of 2 M-potassium phosphate buffer, pH 7.5, were added to 0.1 M-potassium phosphate buffer, pH 8.0, containing sodium pyruvate (50 µmol) and malate dehydrogenase (60 µg), to give a final volume of 2.5 ml and a final pH of 8.0. The mixture was held on ice, and the (2S)-epimer present was removed by the addition of epimerase-free methylmalonyl-CoA transcarboxylase (5 µl of a 2 mg/ml solution). The reaction was monitored by placing a portion of the mixture in a 2 mm-pathlength cuvette in a spectrophotometer cell holder pre-equilibrated with ice-cold water, and observing the decrease in A_{340} . At the end of the reaction, 50 µl of tritiated water (5 Ci/ml) and 20 µl of a dilute solution

(5 $\mu\text{g/ml}$) of methylmalonyl-CoA epimerase were added in rapid succession to the reaction mixture. A portion of this, in a well-sealed cuvette, was used to monitor the epimerase-catalysed reaction. Samples were also taken for accurate determination of the specific radioactivity of the mixture. After a suitable time interval, the reaction mixture was applied to a small column containing 1 ml of anti-(methylmalonyl-CoA epimerase) immunoglobulin G–Sephadex that had been layered on top of 2 ml of avidin–Sephadex and pre-equilibrated at 0°C with 10 mM-potassium phosphate buffer, pH 8.0. The contents of the cuvette were allowed to reach the end point of the epimerase reaction. Usually, additional epimerase was added to speed this up. Control experiments showed that the affinity column was completely effective in removing the epimerase activity, as long as the ‘dwell time’ of each part of the load in the column bed was at least 1 min. The time at which the reaction was stopped was arbitrarily taken as the point when all the mixture had been allowed to enter the column bed. The column was washed with 3.5 ml of 10 mM-potassium phosphate buffer, pH 8.0, and the eluate and washings were combined. Tritiated water was removed by freeze-drying the mixture at 0°C *in vacuo* in a closed system.

The residue was immediately dissolved in 7 ml of ice-cold 2.5 mM-ammonium bicarbonate and was applied to a column (1.5 cm $\times$ 21 cm) of DEAE-cellulose pre-equilibrated with 0.2 M-ammonium bicarbonate. The column was washed with 100 ml of the same bicarbonate solution, and the methylmalonyl-CoA was then eluted at 72 ml/h by a linear salt gradient formed from 100 ml of 0.2 M-ammonium bicarbonate and 100 ml of 0.3 M-ammonium bicarbonate (see Fig. 3). Samples of representative fractions were assayed enzymically for methylmalonyl-CoA, and samples (0.3 ml) were placed in 3 ml of scintillant [toluene/Triton-100 (2:1, v/v) containing 2,5-diphenyloxazole (5 g/l)] and their radioactivities counted in an LKB Rack-beta liquid-scintillation counter. Quench correction was made by using the external-standard ratio method. The counting efficiency for tritium was approx. 45%.

Tritium content of propionyl-CoA product after partial reaction with epimerase in tritiated water

Methylmalonyl-CoA [1.09 μmol , 85% (2*R*)-epimer] was added to an incubation mixture, at 0°C, that contained, in a final volume of 20 ml, 0.2 M-potassium phosphate buffer, pH 8.0, 1.3 mM-NADH, 30 mM-sodium pyruvate and malate dehydrogenase (40 $\mu\text{g/ml}$). Methylmalonyl-CoA transcarboxylase was added and the fall in A_{340} of a sample of the mixture was monitored (in a 1 mm-pathlength cuvette). When the A_{340} became constant, tritiated

water (final concn. 22.5 mCi/ml) was added, followed by a rate-limiting amount of methylmalonyl-CoA epimerase (5 ng/ml). Samples (20 μl) were taken and diluted for counting of radioactivity. At measured time intervals samples (4 ml) were taken, and the epimerase and the transcarboxylase were removed by filtration through a small affinity column as described in the preceding paper (Leadlay & Fuller, 1983). Each filtered sample was frozen and freeze-dried to remove volatile tritium-labelled material. The residue was then taken up in ice-cold 2.5 mM-ammonium bicarbonate and applied to a column (1.5 cm $\times$ 20 cm) of DEAE-cellulose pre-equilibrated at 4°C with 0.15 M-ammonium bicarbonate. The propionyl-CoA was eluted with a linear salt gradient formed from 100 ml of 0.15 M-ammonium bicarbonate and 100 ml of 0.275 M-ammonium bicarbonate. Propionyl-CoA was identified by its position of elution and by enzymic assay. The concentration of propionyl-CoA in each fraction was conveniently quantified by its u.v. absorption, since earlier experiments had shown a good correspondence between propionyl-CoA concentration and A_{260} , assuming a molar absorption coefficient, under these conditions, of $1.54 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

To obtain the tritium content of propionyl-CoA at the end of the epimerase-catalysed reaction, a separate reaction mixture was set up, containing, at 0°C, in a final volume of 10 ml, 0.2 M-potassium phosphate buffer, pH 8.0, 0.1 mM-(*RS*)-methylmalonyl-CoA, 20 mM-sodium pyruvate, 0.2 mM-NADH and malate dehydrogenase (40 $\mu\text{g/ml}$). Methylmalonyl-CoA transcarboxylase (20 $\mu\text{g/ml}$) was added, and the progress of the reaction was continuously monitored as described above. After the removal of the (2*S*)-methylmalonyl-CoA, tritiated water (final concn. approx. 2.5 mCi/ml) was added, followed by a rate-limiting amount (15 ng/ml) of methylmalonyl-CoA epimerase. Portions of the reaction mixture were removed for accurate determination of the specific radioactivity of the solvent. At the end of the reaction, the entire reaction mixture was treated with avidin (final concn. 10 $\mu\text{g/ml}$) and freeze-dried to remove most of the volatile tritium-labelled material. The residue was taken up in ice-cold 2.5 mM-ammonium bicarbonate, and CoA esters were separated on a column (1.5 cm $\times$ 20 cm) of DEAE-cellulose, as described above. Fractions were assayed enzymically for propionyl-CoA as soon as possible after elution from the column. Duplicate samples (0.3 ml) were also taken and their radioactivities counted as described above. Fractions containing residual propionyl-CoA were pooled, and triplicate samples were taken and their radioactivities counted (average 2930 d.p.m./ml). The rest was twice freeze-dried and re-dissolved in the original volume of ice-cold distilled water. Further samples were then taken for

scintillation counting as before (average 2915 d.p.m./ml).

Results

Stability of (2R)-methylmalonyl-CoA

Reaction conditions for studying methylmalonyl-CoA epimerase must be carefully chosen so as to minimize the rate of non-enzymic epimerization. Initially, the conditions for preparing (2R)-methylmalonyl-CoA were chosen by reference to the data of Overath *et al.* (1962), who used ^1H -n.m.r. spectroscopy to measure the rate of exchange of solvent deuterium with the C-2 hydrogen of methylmalonyl thiol esters. In the present work, epimeric purity of the isolated (2R)-methylmalonyl-CoA was checked enzymically as described in the Materials and methods section. In agreement with the results obtained by Overath *et al.* (1962), and with those obtained by Arnstadt *et al.* (1975) and Sedgwick *et al.* (1977) with malonyl-CoA, the rate of epimerization is low at pH 8.5, but becomes very high at lower pH values (see Fig. 1).

The upper limit for the pH to be used in kinetic experiments was defined by the limits of stability of the coupling enzyme, methylmalonyl-CoA transcarboxylase, which tends to disassociate into less-active species above pH 8.0 (Wood *et al.*, 1977). All kinetic experiments were conducted at 0°C, because the non-enzymic epimerization is, as expected, much faster at room temperature (Arnstadt *et al.*, 1975; Sedgwick *et al.*, 1977; P. F. Leadlay & J. Q. Fuller, unpublished work). Both epimerization and hydrolysis of methylmalonyl-CoA were found to be negligibly slow under the conditions chosen for the kinetic experiments.

Tritium fractionation factor for (RS)-methyl[2- ^3H]-malonyl-CoA

In preliminary experiments, to establish conditions for the preparation of (RS)-methyl[2- ^3H]-malonyl-CoA (see the preceding paper, Leadlay & Fuller, 1983), unlabelled (RS)-methylmalonyl-CoA was allowed to equilibrate with tritiated water, either by brief heating in buffer at pH 7.0, or in the presence of purified methylmalonyl-CoA epimerase. The specific radioactivity of the re-isolated methylmalonyl-CoA was consistently found to be close to that of the solvent (Table 1). The specificity of labelling was confirmed as described in the preceding paper (Leadlay & Fuller, 1983). The data in Table 1 provide values of Φ_{RS} , the tritium fractionation factor for C-2 of (RS)-methylmalonyl-CoA. Φ values represent the extent to which ^3H fractionates between a particular hydrogenic site and a corresponding site on a solvent molecule (Kresge, 1964; Alberty, 1975). When $\Phi > 1$, ^3H prefers the site, and the specific radioactivity will exceed that of the solvent.

Fig. 1. Stability of (2R)-methylmalonyl-CoA to epimerization

Experiments were performed, as described in the Materials and methods section, at 0°C at various pH values. The half-life for epimerization was calculated in each case from the initial slope, and is shown in parentheses after each pH value: pH 8.5 (123 h) (Δ); pH 7.1 (87 h) ($\blacktriangle$); pH 5.0 (38 h) ($\circ$); pH 1.5 (4.1 h) ($\bullet$).

Table 1. Tritium fractionation factor Φ_{RS} for methylmalonyl-CoA

$10^{-6} \times \text{Sp. radio-activity of solvent (d.p.m./}\mu\text{g-atom)}$	$10^{-6} \times \text{Sp. radioactivity of methylmalonyl-CoA (d.p.m./}\mu\text{mol)}$	Equilibrium fractionation factor Φ_{RS}
198*	204	1.03
148	162	1.09
1.79	1.75	0.98

Mean $\pm$ s.d. ... 1.03 $\pm$ 0.05

* Equilibration in the presence of methylmalonyl-CoA epimerase.

In connection with the use of the equilibrium perturbation method of determining isotope effects (Schimerlik *et al.*, 1975), Cleland and his co-workers have compiled experimental measurements of fractionation factors, and drawn up empirical rules that allow the prediction of fractionation factors (Cleland, 1980). These rules predict a tritium fractionation factor of 1.11 unless the thioester carbonyl group is treated as 'ketone-like', when the predicted value is 1.04. The latter is in better agreement with the experimentally determined fractionation factor (Table 1) for (RS)-methylmalonyl-CoA.

Isotopic content of propionyl-CoA product after partial reaction

The epimerase-catalysed reaction was performed in tritiated water, and the specific radioactivity of the

Table 2. Incorporation of solvent tritium into product during the methylmalonyl-CoA epimerase-catalysed reaction

For details see the text.

Extent of reaction (%)	$10^{-6} \times$ Sp. radio-activity of solvent (d.p.m./ μ g-atom)	$10^{-6} \times$ Sp. radio-activity of propionyl-CoA (d.p.m./ μ mol)	Kinetic product isotope effect*
49	52.4	37.9	3.39
70	52.4	44.4	3.1
81	52.4	49.7	2.45
100†	62.3	42.1	2.84

* Calculated from eqn. (1) as described in the text.

† (RS)-Methylmalonyl-CoA was used as substrate.

Fig. 2. DEAE-cellulose chromatography of propionyl-CoA produced by the reaction of (2R)-methylmalonyl-CoA in tritiated water, in the presence of methylmalonyl-CoA epimerase and excess methylmalonyl-CoA transcarboxylase

A sample (4 ml), removed from the reaction mixture (see the Materials and methods section) after the epimerase-catalysed reaction had reached 70% of completion, was filtered at 0–4°C through a small immuno-affinity column and freeze-dried, as described in the legend to Fig. 3. The sample was applied at 4°C to a column (1.5 cm $\times$ 19 cm) of DEAE-cellulose (HCO_3^- form). The column was washed with 100 ml of 0.15 M-ammonium bicarbonate, and CoA esters were then eluted by using a linear salt gradient formed from 100 ml each of 0.15 M- and 0.275 M-ammonium bicarbonate. Fractions (3 ml) were collected at 3 min intervals, and for each fraction A_{260} (●) was measured and duplicate samples (0.3 ml) were taken and their radioactivities (○) counted as described in the Materials and methods section.

product, isolated as propionyl-CoA, was measured at four different extents of reaction, as described in the Materials and methods section (see Table 2). A typical separation of CoA esters is shown in Fig. 2. Propionyl-CoA in these experiments is produced in three ways. First, propionyl-CoA originating from (2S)-methylmalonyl-CoA present in the starting

material will not be radiolabelled, since tritiated water is not added to the mixture until the (2S)-epimer has been completely removed by the epimerase-free methylmalonyl-CoA transcarboxylase. Secondly, (2R)-methylmalonyl-CoA will be converted in the presence of the epimerase into propionyl-CoA, by way of (2S)-methylmalonyl-CoA. Since we have shown in the preceding paper (Leadlay & Fuller, 1983) that the hydrogen atom at C-2 in the (2S)-epimer product of the epimerase-catalysed reaction is derived from the solvent, tritium will be introduced at this stage, to an extent governed by the size of any primary kinetic tritium-isotope effect. When methylmalonyl-CoA is decarboxylated to propionyl-CoA by the transcarboxylase, another solvent-derived hydrogen atom is introduced (Cheung *et al.*, 1975). Importantly, when the transcarboxylase-catalysed reaction is conducted in tritiated water, the propionyl-CoA is formed without isotopic discrimination against solvent tritium (Rose *et al.*, 1976; P. Leadlay, unpublished work). Finally, a small proportion of the (2R)-methylmalonyl-CoA substrate will be decarboxylated directly by the transcarboxylase, as discussed in the preceding paper (Leadlay & Fuller, 1983). Propionyl-CoA derived by this route will not be radiolabelled by the action of the epimerase. The observed ratio, R , of the specific radioactivity of propionyl-CoA to the specific radioactivity of the solvent is therefore related to the primary kinetic tritium-isotope effect (k_H/k_T) on product formation in the epimerase-catalysed reaction by the following equation:

$$R = \frac{c + b(1 + k_T/k_H)}{a + b + c} \quad (1)$$

where a , b and c are the relative amounts of propionyl-CoA formed by decarboxylation of (2S)-epimer before addition of isotope, by the linked action of the epimerase and the transcarboxylase on (2R)-methylmalonyl-CoA, and by the slow direct decarboxylation of (2R)-epimer respectively. The quantities a and b were measured directly from the spectrophotometric recorder trace, and c (approx. 3% of b) was deduced either from the rate of change in A_{340} before addition of epimerase, or from the absorbance change in a control incubation containing only epimerase-free transcarboxylase. It can be seen from Table 2 that the isotopic discrimination against solvent tritium in the epimerase-catalysed reaction does not vary in any systematic way with the extent of reaction. The average value found for k_H/k_T is 3.0 ± 0.3 .

Tritium content of (2R)-methylmalonyl-CoA substrate after a partial reaction

A series of experiments was performed, as described in the Materials and methods section, with (2R)-methylmalonyl-CoA as substrate, in tritiated

Fig. 3. Isolation of remaining (2*R*)-methylmalonyl-CoA after partial reaction with methylmalonyl-CoA epimerase in tritiated water

A reaction mixture in which the epimerase-catalysed reaction had reached 63% of completion was filtered through a small immuno-affinity column as described in the Materials and methods section. The filtered sample (7 ml) was rapidly freeze-dried to remove volatile tritium-labelled material, dissolved in 7 ml of ice-cold 2.5 mM-ammonium bicarbonate and applied to a column (1.5 cm $\times$ 21 cm) of DEAE-cellulose (HCO_3^- form) pre-equilibrated with 0.2 M-ammonium bicarbonate. The column was washed with 50 ml of the same bicarbonate solution, and CoA esters were eluted with a linear salt gradient formed from 100 ml each of 0.2 M- and 0.3 M-ammonium bicarbonate, in fractions of volume approx. 3 ml each, at 2.5 min intervals. Triplicate samples (0.3 ml each) were taken and their radioactivities (O) counted. The methylmalonyl-CoA concentration at this high extent of conversion was too low for accurate enzymic assay, so the concentration was estimated from the absorbance at 260 nm (●), assuming $\epsilon = 1.54 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The specific radioactivity of the solvent in this experiment was $1.9 \times 10^6 \text{ d.p.m./}\mu\text{g-atom}$.

water of very high specific radioactivity. The conditions used to ensure that the epimerase-catalysed reaction was irreversible were at least as stringent as those used in the work described in the preceding paper (Leadlay & Fuller, 1983). As a further check that (2*S*)-methylmalonyl-CoA is removed irreversibly, [^{14}C]propionyl-CoA was added as a tracer to a control incubation mixture (without tritiated water). The reaction was stopped after 60% of the methylmalonyl-CoA had reacted. Less than 1% of the added label was found to be associated with residual methylmalonyl-CoA after chromatography on DEAE-cellulose. The importance of ensuring complete irreversibility was underlined by the results of preliminary experiments where the epimerase and the transcarboxylase activities were more evenly matched. These appeared to show significant incorporation of solvent tritium into substrate at high extents of reaction (results not shown). It is evident, however, from Table 3 that, under the conditions described here, there is negligible exchange of solvent tritium into re-isolated (2*R*)-methylmalonyl-CoA, even when over 60% of the substrate has been converted into product. A typical isolation of remaining methylmalonyl-CoA is shown in Fig. 3.

The methylmalonyl-CoA epimerase-catalysed reaction is freely reversible, but by removing (2*S*)-methylmalonyl-CoA as fast as it forms, by using methylmalonyl-CoA transcarboxylase and malate dehydrogenase in the presence of excess pyruvate and NADH, the reaction can be studied, in the direction with (2*R*)-methylmalonyl-CoA as substrate, without interference from the reverse reaction.

In the preceding paper (Leadlay & Fuller, 1983) we have argued that the mechanistic pathway for methylmalonyl-CoA epimerase resembles that proposed for proline racemase (Cardinale & Abeles, 1968), in which two enzyme bases are involved in proton transfer, and the proton abstracted from the substrate is not able to undergo isotopic exchange with the solvent until the product is released. The data in Table 3 fully support this view, because they demonstrate that there is essentially no appearance of solvent tritium in (2*R*)-methylmalonyl-CoA, re-isolated after partial reaction, even after most of the substrate has been converted into product. An apparently plausible alternative explanation for these results, that an intermediate that can exchange with solvent protons partitions very unequally in favour of conversion into product, as against reverting to substrate, is unattractive because of the 'symmetrical' nature of the catalysed reaction. We therefore predict that, when the epimerization is studied in the reverse direction, there will also be very little labelling of (2*S*)-epimer by solvent tritium, even at high extents of conversion.

Table 3. Measurement of tritium content of residual (2*R*)-methylmalonyl-CoA after partial reaction with methylmalonyl-CoA epimerase in tritiated water

For details see the text.

Extent of reaction (%)	$10^{-6} \times \text{Sp. radio-activity of solvent (d.p.m./}\mu\text{g-atom)}$	$10^{-6} \times \text{Sp. radio-activity of remaining methylmalonyl-CoA (d.p.m./}\mu\text{mol)}$	Exchange (%)
12	2.87	14.2	4.9
24	1.65	380	2.3
26	1.44	468	3.2
36	1.57	666	4.2
56	1.42	327	2.3
63	1.90	854	4.5

As shown in the preceding paper (Leadlay & Fuller, 1983), the hydrogen atom of the new C–H bond in the (2*S*)-epimer product (isolated as propionyl-CoA) is essentially derived from the solvent during the epimerase-catalysed reaction. During the reaction of unlabelled (2*R*)-methylmalonyl-CoA in tritiated water, therefore, one might expect that there would be a large primary kinetic tritium-isotope effect on this protonation step. A very large tritium-isotope effect on protonation might also in principle account for the observed lack of incorporation of solvent tritium into the substrate after partial reaction. However, from the results in Table 3, the observed discrimination against solvent tritium is rather low ($k_H/k_T = 3.0$). Solvent tritium enters the product much faster than it enters the substrate under these conditions, as expected for a mechanism involving two catalytic bases (Rose, 1966). For triose phosphate isomerase, the absence of an expected primary kinetic tritium-isotope effect on protonation of an enzyme-bound intermediate has been ascribed to the intervention of a slow step after re-protonation (Maister *et al.*, 1976), which might be either a conformational change or the release of product. The existence of this slow step allows the protonation to be reversed, regenerating the intermediate, which in turn is able to exchange isotope with the solvent. The expected discrimination against forming a C–³H bond, as opposed to a C–H bond, is therefore nullified. In the present case the observed low discrimination against tritium can be explained in either of two ways, both of which imply the existence of two distinct enzyme bases involved in catalysis (see Scheme 1). First, the small discrimination against tritium could arise if, as in triose phosphate isomerase, the rate of product release is rate-limiting and the enzyme–product complex is therefore in isotopic equilibrium with some species that can exchange the proton(s), on the enzyme base that is providing the new hydrogen atom, with the solvent. From the other results given in the present paper, such a view implies that, once substrate has bound, one of the enzyme bases (B_2 in Scheme 1) is still able to exchange with solvent, but the other (B_1) is not. Since the roles of the two bases are interchanged for the reverse reaction, it is hard to explain how this difference could arise. The alter-

native view would be that neither enzyme base can undergo isotopic exchange with solvent protons except at the stage of free enzyme. Then, if B_2 , the base providing the solvent-derived proton, bears no other hydrogen atom in its conjugate acid form, one would expect that there should be no kinetic isotopic discrimination against solvent tritium at all, since there would be no choice offered between tritium and hydrogen. The specific radioactivity of the (2*S*)-epimer product would reflect the tritium fractionation factor for the enzyme base B_2 , which is likely to lie between about 0.5 and 1. This would be true whether or not the release of product was slow. On the other hand, if the enzyme base B_2 , which delivers the solvent-derived proton, bears either two or three hydrogen atoms in its conjugate acid form, there should be an intramolecular discrimination between these hydrogen atoms in the protonation step ($k_H/k_T = 8\text{--}20$). The observed low discrimination might then be caused either by restricted rotation of the conjugate acid form of B_2 , or by the subsequent product dissociation being sufficiently slow to allow some reversal of the protonation step. Our observation in the preceding paper (Leadlay & Fuller, 1983) that no solvent tritium is introduced into (2*R*)-methylmalonyl-CoA during the epimerase-catalysed reaction is most easily reconciled with initial proton abstraction by an enzyme base B_1 bearing no other hydrogen atoms.

There is precedent for the idea that proton transfer might be relatively fast without necessarily allowing isotopic exchange with the solvent. Thus a study on proton transfer in acetyl-CoA acetyltransferase from pig heart (Gilbert, 1981) showed that, in the direction of acetoacetyl-CoA synthesis from acetyl-CoA, the proton abstraction from acetyl-CoA is at equilibrium on the enzyme, although no proton exchange with the solvent is seen. For methylmalonyl-CoA epimerase with (2*R*)-methylmalonyl-CoA as substrate, $k_{\text{cat.}}/K_m$ under the conditions of these experiments is $2.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (P. F. Leadlay, unpublished work), which is not far off the diffusion-controlled limit. It is therefore possible that the dissociation of substrate (or product) could be partially rate-limiting in the reverse reaction. If bound substrate (or product) partitions unequally in favour of deprotonation, rather than dissociation

Scheme 1. Methylmalonyl-CoA epimerase-catalysed interconversion of (2*R*)- and (2*S*)-methylmalonyl-CoA

to free enzyme, this would certainly account for the observed low primary kinetic tritium-isotope effect on proton abstraction (see the preceding paper, Leadlay & Fuller, 1983). The small absolute value of the tritium-isotope effect on both proton abstraction and proton delivery makes it very unlikely that these are intrinsic effects, so it seems clear that they are being modulated by other, isotopically insensitive, steps.

We are grateful to Professor J. R. Knowles for helpful comments, and to the Science and Engineering Research Council for a research grant.

References

- Albery, J. W. (1975) in *Proton Transfer Reactions* (Caldin, E. & Gold, V., eds.), pp. 263–285, Chapman and Hall, London
- Allen, S. H. G., Kellermeyer, R., Stjernholm, R., Jacobson, B. & Wood, H. G. (1963) *J. Biol. Chem.* **238**, 1637–1642
- Arnstadt, K.-I., Schindlbeck, G. & Lynen, F. (1975) *Eur. J. Biochem.* **55**, 561–571
- Cardinale, G. J. & Abeles, R. H. (1968) *Biochemistry* **7**, 3970–3978
- Cheung, Y. F., Fung, C. H. & Walsh, C. (1975) *Biochemistry* **14**, 2981–2986
- Cleland, W. W. (1980) *Methods Enzymol.* **64**, 104–125
- Fletcher, S. J., Herlihy, J. M., Albery, W. J. & Knowles, J. R. (1976) *Biochemistry* **15**, 5612–5617
- Garland, P. J. (1965) *Biochem. J.* **97**, 857–865
- Gilbert, H. F. (1981) *Biochemistry* **20**, 5643–5649
- Kresge, A. J. (1964) *Pure Appl. Chem.* **8**, 243–258
- Leadlay, P. F. (1981) *Biochem. J.* **197**, 413–419
- Leadlay, P. F. & Fuller, J. Q. (1983) *Biochem. J.* **213**, 635–642
- Maister, S. G., Pett, C. P., Albery, W. J. & Knowles, J. R. (1976) *Biochemistry* **15**, 5607–5612
- Overath, P., Kellerman, G. M., Lynen, F., Fritz, H. P. & Keller, H. J. (1962) *Biochem. Z.* **335**, 500–512
- Rose, I. A. (1966) *Annu. Rev. Biochem.* **35**, 23–56
- Rose, I. A., O'Connell, E. L. & Solomon, F. (1976) *J. Biol. Chem.* **251**, 902–904
- Rudnick, G. & Abeles, R. H. (1975) *Biochemistry* **14**, 4515–4522
- Schimerlik, M. I., Rife, J. E. & Cleland, W. W. (1975) *Biochemistry* **14**, 5347–5354
- Sedgwick, B., Cornforth, J. W., French, S. J., Gray, R. T., Kelstrup, E. & Willadsen, P. (1977) *Eur. J. Biochem.* **75**, 481–495
- Wood, H. G., Chiao, J.-P. & Poto, E. M. (1977) *J. Biol. Chem.* **252**, 1490–1499