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

Studies with specifically tritiated (2*R*)-methylmalonyl-CoA as substrate

Peter F. LEADLAY* and James Q. FULLER

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

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(2*R*)-Methyl[2-³H]malonyl-CoA was used as the substrate for methylmalonyl-CoA epimerase from *Propionibacterium shermanii*, under conditions where the (2*S*)-methylmalonyl-CoA product was removed enzymically as fast as it was formed, and the fate of the label was monitored at different extents of reaction. Very little, if any, tritium is found attached to the C-2 position in the (2*S*)-epimer product (isolated as propionyl-CoA). Evidently, the hydrogen atom of the new C—H bond in the product is essentially solvent-derived. The rate of tritium release into the solvent is lower than the rate of product formation, and shows a primary kinetic tritium-isotope effect on k_{cat}/K_m of 2.3 ± 0.1 . The specific radioactivity of the remaining substrate rises slowly during the epimerase-catalysed reaction, and this provides an independent estimate of the primary kinetic tritium-isotope effect on k_{cat}/K_m of 1.6 ± 0.5 . These results, taken together, indicate that the mechanistic pathway of the epimerase-catalysed reaction resembles that established for proline racemase [Cardinale & Abeles, (1968) *Biochemistry* 7, 3970–3978], in which two enzyme bases are involved in catalysis. One base removes the proton from the substrate, the second provides the new proton, and there is no fast isotopic exchange between enzyme-bound intermediates and solvent protons.

Methylmalonyl-CoA epimerase (EC 5.1.99.1 classified as a racemase) catalyses the interconversion of (2*R*)- and (2*S*)-methylmalonyl-CoA. In both mammalian tissues and in the propionate-producing bacteria, the carboxylation of propionyl-CoA specifically produces the (2*S*)-epimer, whereas the (2*R*)-epimer is the specific substrate for the co-enzyme B-12-dependent mutase, which converts it into succinyl-CoA (Rétey & Lynen, 1964; Sprecher *et al.*, 1966). Methylmalonyl-CoA epimerase may also play a role in the biogenesis of macrolide antibiotics such as the erythromycins, the aglycones of which are constructed largely from methylmalonyl-CoA precursors (for review see Corcoran, 1982).

By analogy with the non-enzymic epimerization of methylmalonyl-CoA, a simple mechanism has been proposed (Overath *et al.*, 1962), involving the removal of the proton at C-2 from either substrate to form a thioester-stabilized carbanion, and the subsequent re-protonation of this species. Mechanistically, this links methylmalonyl-CoA epimerase with those enzymes, typified by malate synthase, thiolase and the acyl-CoA carboxylases, in which the

abstraction of a proton from C-2 of a CoA ester is followed by the formation of a new C—C bond. In all these reactions, the mechanism may involve a carbanion or enol intermediate. Alternatively, bond cleavage and bond formation may be concerted.

Two findings are consistent with a mechanism for methylmalonyl-CoA epimerase that involves proton transfers: the reaction does not involve an inter- or intra-molecular migration of CoA (Mazumder *et al.*, 1962), and isotopic exchange of the hydrogen atom at C-2 of methylmalonyl-CoA is accelerated by the presence of epimerase (Overath *et al.*, 1962; Mazumder *et al.*, 1962; Allen *et al.*, 1963). The existence of this exchange allows two types of tritium-isotope experiment to be performed, which provide complementary information about the mechanism of proton transfer (Albery & Knowles, 1976). First, the reaction of specifically labelled (2*R*)-methyl[2-³H]malonyl-CoA may be studied, and the fate of the tritium label monitored during the epimerase-catalysed reaction. These experiments are described in the present paper. Secondly, the epimerase-catalysed reaction may be performed in tritiated water, with (2*R*)-methylmalonyl-CoA as substrate,

* To whom correspondence should be addressed.

and the appearance of tritium label in either residual (2*R*)-methylmalonyl-CoA or the product can be examined after partial reaction, as described in the following paper (Fuller & Leadlay, 1982).

Materials and methods

Materials

Propionyl-CoA, avidin and phosphate transacetylase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), DEAE-cellulose was from Cambrian Chemicals (Croydon, Surrey, U.K.) and CNBr-activated Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden). Tritiated water (5 Ci/ml) and [1-¹⁴C]propionyl-CoA (50 mCi/mmol) were from Amersham International (Amersham, Bucks., U.K.). Methylmalonyl-CoA transcarboxylase was purified from *Propionibacterium shermanii* by the method of Wood *et al.* (1977), except that extracts were fractionated with poly(ethylene glycol) 6000 before chromatography on Bio-Gel A 1.5 m. The transcarboxylase was freed from traces of the epimerase by passage through short columns of anti-(methylmalonyl-CoA epimerase) immunoglobulin G covalently bound to Sepharose (see below). 2-Oxoglutarate dehydrogenase from *Escherichia coli* was kindly given by Dr. G. Hale (Department of Pathology, University of Cambridge). Avidin was coupled to CNBr-activated Sepharose by the method of Bodanszky & Bodanszky (1970). A 1 ml portion of this avidin-Sepharose was found to be capable of binding 180 nkat of methylmalonyl-CoA transcarboxylase activity. Assays were performed as described in the following paper (Fuller & Leadlay, 1983). Other materials have been described previously (Leadlay, 1981).

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

Immunological methods

Antibodies to methylmalonyl-CoA epimerase were raised in New Zealand White rabbits. Purified epimerase (300 µg) in 0.5 ml of 0.12 M-NaCl/0.02 M-KH₂PO₄/0.06 M-Na₂HPO₄ buffer, pH 7.2, was emulsified with 1 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) and the emulsion was injected intramuscularly. After 6 weeks the immunization was repeated with Freund's incomplete adjuvant, and after a further 14 days the rabbit was bled from a marginal ear vein. The antiserum was characterized by double immunodiffusion (Ouchterlony, 1949), and the immunoglobulin G fraction was obtained by selective DEAE-cellulose adsorption. Purified immunoglobulin G was coupled to CNBr-activated Sepharose by the method of Cuatrecasas *et al.* (1968). Titration with the purified epimerase showed that 1 ml of this

immunoabsorbent would completely remove from solution up to 35 nkat of epimerase activity.

Preparation of (2*R*)-methyl[2-³H]malonyl-CoA

(*RS*)-Methyl[2-³H]malonyl-CoA was prepared by non-enzymic exchange between methylmalonyl-CoA and tritiated water. The incubation mixture contained 144 µM-(*RS*)-methylmalonyl-CoA, 18 mM-potassium phosphate buffer, pH 8.0, and tritiated water (4.5 Ci/ml) in a final volume of 0.22 ml. After the mixture had been heated in a sealed tube at 70°C for 30 min, the volatile tritium-labelled material was recovered by freeze-drying *in vacuo* in a closed system, and three 1 ml portions of water were successively added to the residue and removed by freeze-drying.

The removal of the (2*S*)-epimer was accomplished by specific enzymic decarboxylation to propionyl-CoA. (*RS*)-Methyl[2-³H]malonyl-CoA (3.86 µmol) was dissolved at 0°C in 10 ml of 10 mM-potassium phosphate buffer, pH 8.0, containing 40 mM-sodium pyruvate, 2 mM-NADH and malate dehydrogenase (60 µg/ml). Reaction was started by the addition of epimerase-free methylmalonyl-CoA transcarboxylase (40 µg/ml). When the reaction was complete, as judged by the fall in *A*₃₄₀ of a sample of the mixture held in a 1 mm-pathlength cuvette, unlabelled (*RS*)-methylmalonyl-CoA (14.5 µmol) was added and the reaction was again allowed to proceed to completion. The separation of (2*R*)-methyl[2-³H]malonyl-CoA from propionyl-CoA was done essentially as described for the preparation of the unlabelled (2*R*)-epimer (Fuller & Leadlay, 1983). The concentrated desalted product was stored, in several portions, under liquid N₂ until required.

Measurement of the primary kinetic tritium-isotope effect on k_{cat}/K_m

In these experiments, tritium radioactivity that had been released into the solvent during the epimerase-catalysed reaction was measured. The incubation mixture, at 0°C in 0.95 ml of 0.2 M-potassium phosphate buffer, pH 8.0, contained 1.05 mM-NADH, 12.6 mM-sodium pyruvate, malate dehydrogenase (25 µg/ml) and 0.52 mM-methyl[2-³H]malonyl-CoA [82% (*R*)-epimer, 3.53×10^5 d.p.m. total ³H radioactivity].

A 50 µl sample was taken for analysis (see below). Methylmalonyl-CoA transcarboxylase (final concn. 26 µg/ml) was added, and the removal of the (2*S*)-epimer was monitored spectrophotometrically at 340 nm in a 1 mm-pathlength cuvette. Another 50 µl sample was removed when the reaction was complete. Then the epimerase (10 µl of a solution of 60 ng/ml) was added, and at measured time intervals further 50 µl samples were withdrawn. Each 50 µl sample was applied to a column (0.7 ml) of DEAE-cellulose in a Pasteur pipette, which was pre-equil-

brated with 5 mM-ammonium bicarbonate at 4°C. Tritiated water was washed through the column with 1 ml of 5 mM-ammonium bicarbonate. The eluate was collected in a scintillation vial containing 10 ml of scintillant [toluene/Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (5 g/l)], and the radioactivity of the sample was counted in an LKB Rack-beta scintillation counter.

Measurement of the tritium content of (2R)-methyl[2-³H]malonyl-CoA remaining after partial reaction

The incubation mixture, at 0°C in 2.42 ml of 0.2 M-potassium phosphate buffer, pH 8.0, contained 0.83 mM-NADH, 12.6 mM-sodium pyruvate, malate dehydrogenase (25 µg/ml) and 0.64 mM-methyl[2-³H]malonyl-CoA [80% (R)-epimer, 5.4×10^5 d.p.m. total ³H radioactivity]. A sample (0.3 ml) was removed and methylmalonyl-CoA transcarboxylase (30 µg/ml) was added. After removal of the (2S)-epimer, another sample (0.3 ml) was taken. The epimerase (30 µl of a solution of 60 ng/ml) was then added, and the reaction was monitored spectrophotometrically as described above. At various apparent extents of reaction, further samples (0.3 ml) were removed, filtered through a small column (0.3 ml) of anti-(methylmalonyl-CoA epimerase) immunoglobulin G-Sepharose, at 0°C, and washed through with 5 mM-ammonium bicarbonate. Each sample was immediately frozen in liquid N₂ until it could be analysed as described in the legend to Fig. 4.

Measurement of the tritium content of propionyl-CoA product

When (RS)-methyl[2-³H]malonyl-CoA was used, the (2S)-epimer was first removed with epimerase-free transcarboxylase, and the (2R)-methyl[2-³H]malonyl-CoA was allowed to react *in situ*. The reaction conditions were essentially as already described for the preparation of the 2-³H-labelled (2R)-epimer. A rate-limiting amount of methylmalonyl-CoA epimerase was added, and the reaction was allowed to proceed to completion, as judged by the fall in A_{340} of a sample held in a spectrophotometer cuvette under the same conditions. Further additions of the epimerase to the cuvette produced no further fall in A_{340} . Avidin (200 µg/ml final concn.) was added to the reaction mixture, which was then applied to a column (1.5 cm × 23 cm) of DEAE-cellulose pre-equilibrated at 4°C with 0.15 M-ammonium bicarbonate. The column was washed with 180 ml of the same solution, and propionyl-CoA was eluted with a salt gradient formed from 100 ml each of 0.15 M- and 0.3 M-ammonium bicarbonate (Fig. 1). Peak fractions were assayed for propionyl-CoA, and samples (0.3 ml) were added to 3 ml of scintillant and their radioactivities counted as described above. A sample (1.3 µmol) of the (RS)-

methyl[2-³H]malonyl-CoA starting material was chromatographed on a column of DEAE-cellulose in the same way, except that a salt gradient from 0.2 M- to 0.3 M-ammonium bicarbonate was required to elute the CoA ester. Peak fractions were assayed for (total) methylmalonyl-CoA, and samples (0.2 ml) were taken and their radioactivities counted as described above.

Alternatively, (2R)-methyl[2-³H]malonyl-CoA [80% (R)-epimer, 4.35×10^5 d.p.m./µmol of (R)-epimer] was allowed to react with the epimerase in the presence of excess transcarboxylase, exactly as described above for the measurement of the tritium content of residual methylmalonyl-CoA, in a total volume of 2.4 ml. Samples were withdrawn after addition of the transcarboxylase, and after the epimerase-catalysed reaction had reached 50% of completion, as judged by the change in A_{340} of the solution. Propionyl-CoA and methylmalonyl-CoA were separated by chromatography on small columns (1.5 cm × 7 cm) of DEAE-cellulose as described above. Their concentrations were determined by measuring their absorbance at 260 nm (assuming $\epsilon = 1.54 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$), and samples (0.3 ml) of each fraction were taken and their radioactivities counted as before.

Results

It is important for the validity of the experiments described in the present paper that the epimerase should always be clearly rate-limiting. Otherwise (2S)-methylmalonyl-CoA will not be removed as fast as it is formed and it might be able to exchange isotope with the solvent. The initial activity ratio of the transcarboxylase to the epimerase was always greater than 20:1. Addition of more transcarboxylase or malate dehydrogenase did not increase the rate of reaction. Reactions were conducted in solutions of constant high ionic strength to minimize the dissociation of the transcarboxylase to less-active species (Wood *et al.*, 1977). The irreversibility of the trapping reaction was also ensured by the presence of high concentrations of sodium pyruvate and NADH, and by taking advantage of the favourable equilibrium of the reaction catalysed by malate dehydrogenase (Yoshida, 1965).

A second requirement in this present work is that methylmalonyl-CoA epimerase should not be active during the work-up and analysis of the reaction mixtures. Preliminary experiments showed that the epimerase was rather resistant to inactivation by those chemical methods (e.g. addition of organic solvent or sodium dodecyl sulphate) that would not also affect the tritium content of methylmalonyl-CoA. Finally, a method was adopted in which the epimerase and the transcarboxylase were removed from reaction mixtures by rapid filtration through a

small affinity column. This inevitably made the determination of the extent of reaction less precise, but control experiments showed that this method provided efficient quenching of the reaction.

Specificity of labelling of (2*R*)-methyl[2-³H]-malonyl-CoA was checked by incubation of the radiolabelled material at pH 8 for 20 min at 0°C with methylmalonyl-CoA epimerase (2 µg/ml) and re-isolation of the methylmalonyl-CoA by chromatography on DEAE-cellulose as described above. This treatment decreased the specific radioactivity of the methylmalonyl-CoA from 2.0×10^8 to 2.3×10^6 d.p.m./µmol, indicating that approx. 99% of the tritium was labilized in the presence of the epimerase. Incubation in the absence of the epimerase under identical conditions left the specific radioactivity of the methylmalonyl-CoA essentially unchanged, and less than 1% of the total radioactivity present was released into the solvent. This result presumably reflects the relative stability of methylmalonyl-CoA to epimerization under these conditions (see the following paper, Fuller & Leadlay, 1983) and also the operation of a large primary kinetic tritium-iso-

tope effect on proton abstraction (Arnstadt *et al.*, 1975).

Tritium transfer from (2R)-methyl[2-³H]malonyl-CoA to product

When this experiment was performed with (*RS*)-methyl[2-³H]malonyl-CoA the (2*S*)-epimer was decarboxylated to propionyl-CoA *in situ*, before the addition of a rate-limiting amount of methylmalonyl-CoA epimerase, as described in the Materials and methods section. The (2*S*)-epimer could reasonably be assumed to retain its tritium on decarboxylation (Cheung *et al.*, 1975). The epimerase-catalysed reaction was allowed to reach completion, so that, had complete transfer of tritium occurred, the specific radioactivity of the propionyl-CoA should have been the same as that of the starting material. The propionyl-CoA was found to have an average specific radioactivity of 8.6×10^5 d.p.m./µmol, compared with 1.75×10^6 d.p.m./µmol for the methylmalonyl-CoA (Fig. 1). Thus only 49% of the tritium has been retained in the propionyl-CoA, which is close to the result expected for a mechanism where

Fig. 1. DEAE-cellulose column chromatography of (a) (*RS*)-methylmalonyl-CoA and (b) the propionyl-CoA derived from it by decarboxylation in the presence of methylmalonyl-CoA epimerase and excess methylmalonyl-CoA transcarboxylase. (a) (*RS*)-Methyl[2-³H]malonyl-CoA was eluted from a column (1.5 cm $\times$ 23 cm) of DEAE-cellulose (HCO_3^- form), pre-equilibrated at 4°C with 0.2 M-ammonium bicarbonate, by using a linear salt gradient formed from 100 ml each of 0.2 M- and 0.3 M-ammonium bicarbonate. Fractions of volume 1.1 ml were collected at 2.3 min intervals. The absorbance at 260 nm (●) was monitored continuously. Immediately it was eluted, methylmalonyl-CoA was assayed (■), and samples (0.2 ml) were taken and their radioactivities counted (○) as described in the Materials and methods section. The specific radioactivity of the methylmalonyl-CoA (△) was reasonably constant across the peak. (b) Propionyl-CoA was eluted from a column identical with that used in (a), except that the column was pre-equilibrated with 0.15 M-ammonium bicarbonate and the salt gradient was formed from 100 ml each of 0.15 M- and 0.275 M-ammonium bicarbonate. Fractions of volume 1.1 ml were assayed for propionyl-CoA (●), and samples (0.3 ml) were removed and their radioactivities (○) counted as described in the Materials and methods section.

no transfer of tritium has occurred during the methylmalonyl-CoA epimerase-catalysed reaction.

The experiment was repeated with purified (2*R*)-methyl[2-³H]malonyl-CoA as described in the Materials and methods section. The propionyl-CoA isolated, after the epimerase-catalysed reaction had reached about 50% of completion, contained an amount of tritium that was detectable but too small to measure accurately (about 300 total d.p.m. of ³H above background). The residual methylmalonyl-CoA contained a total of approx. 7000 d.p.m. of ³H. Its specific radioactivity (5.9 × 10⁵ d.p.m./μmol) was appreciably higher than that of the starting material [4.5 × 10⁵ d.p.m./μmol of (*R*)-epimer]. The radioactivity found in the propionyl-CoA-containing fractions would represent an extent of transfer of, at most, 2–6%. Even this is probably an overestimate, because, in the control experiment containing the transcarboxylase but no epimerase, the propionyl-CoA apparently contained similar, very low, amounts of tritium. The label in this latter sample could have arisen by at least two routes: the slow direct decarboxylation of (2*R*)-methylmalonyl-CoA by the transcarboxylase (see below), and the presence of traces of (2*S*)-methyl[2-³H]malonyl-CoA not removed in the preparation of the starting material {the non-enzymic epimerization of (2*R*)-methyl[2-³H]malonyl-CoA during storage forms essentially unlabelled (2*S*)-epimer}. Again, these findings do not support the idea that tritium transfer occurs during the epimerase-catalysed reaction.

Tritium release to solvent during partial reaction

The tritium released from (2*R*)-methyl[2-³H]-malonyl-CoA during its reaction with methylmalonyl-CoA epimerase was assayed, as described in the Materials and methods section, at different extents of reaction. The extent of reaction was deduced from the change in absorbance of 340nm of a sample of the reaction mixture held in a spectrophotometer cuvette. Results were obtained from experiments with two widely different concentrations of epimerase (see Table 1). The data were fitted to the following equation (Melander, 1960; Northrop, 1975) relating the primary kinetic tritium-isotope effect ($k_{cat.}/K_m)^H/(k_{cat.}/K_m)^T$ to the fractional extent of reaction *f*:

$$\frac{(k_{cat.}/K_m)^H}{(k_{cat.}/K_m)^T} = \frac{\ln(1-f)}{\ln[1-f(S_p/S_0)]} \tag{1}$$

where *S_p* is the specific radioactivity of the product (total radioactivity released to solvent/nmol of propionyl-CoA produced) at a given fractional extent of reaction *f*, and *S₀* is the original specific radioactivity of the starting material. From the slope of the line obtained (Fig. 2), the apparent kinetic tritium-isotope effect on $k_{cat.}/K_m$ is 2.3 ± 0.1 (S.D., *n* = 13).

Table 1. Rate of release of tritium into the solvent during the methylmalonyl-CoA epimerase-catalysed reaction
For details see the text.

Extent of reaction (%)	10 ⁻³ × Tritium released into solvent (d.p.m./ml)	Propionyl-CoA formed (nmol/ml)	³ H released/propionyl-CoA formed (d.p.m./nmol) (<i>S_p</i>)
5.5	3.45	16.1	214
9	5.6	26.3	213
11	6.4	33.5	191
14	8.2	41.6	198
17	9.6	51.0	189
19.5	11.1	58.0	192
22.5	13.6	67.3	202
29	17.3	86.1	201
32	20.6	95.4	216
17	9.0	44.3	203
24	12.9	60.8	212
38.5	20.8	99.0	210
62.5	39.7	175.7	226

Fig. 2. Apparent primary kinetic tritium-isotope effect on proton release into solvent during the methylmalonyl-CoA epimerase-catalysed reaction with (2*R*)-methyl[2-³H]-malonyl-CoA as substrate

The data in Table 1 were plotted according to eqn. (1). The epimerase concentration was either 0.6 ng/ml (O) or 6 ng/ml (●), and the continuous line is the predicted result for an isotope effect of 2.3.

The low rate of (epimerase-independent) decarboxylation of (2*R*)-methylmalonyl-CoA was estimated in a control incubation with the transcarboxylase only, and was used to correct the observed extent of reaction. At the lower epimerase concentration used, the proportion of (2*R*)-methylmalonyl-CoA diverted in this way was estimated to be 15%. The assumption that this direct decarboxylation of (2*R*)-methylmalonyl-CoA occurred was checked in three ways. First, the reaction was found to be completely inhibited by avidin. Secondly, in control experiments no tritium was released into the medium in the above assays unless epimerase was added. Finally, the control incubation, with the transcarboxylase only, was allowed to proceed for 200 min, after which approx. 25% of the (2*R*)-methylmalonyl-CoA had been decarboxylated. A sample (10 μ l) was then mixed with carrier methylmalonyl-CoA and propionyl-CoA and subjected to analysis by high-pressure liquid chromatography on a reverse-phase column (C₁₈ μ -Bondapak, 10 μ m, 0.4 cm $\times$ 30 cm) eluted isocratically with 50 mM-potassium phosphate buffer (pH 5.3)/methanol (4:1, v/v).

Fractions (1 ml) were collected in scintillation vials, 10 ml of scintillant was added to each and the radioactivity of each sample was counted. Again, no tritium release to water was detected (approx.

Fig. 3. Variation in the specific radioactivity of the remaining (2*R*)-methylmalonyl-CoA during the course of the methylmalonyl-CoA epimerase-catalysed reaction of (2*R*)-methyl[2-³H]malonyl-CoA in unlabelled water. The measured specific radioactivity of remaining (2*R*)-methylmalonyl-CoA after 35% reaction is probably too low, because a 48 h delay before radioactivities of samples were counted allowed some loss of tritium by exchange.

3500 d.p.m. total would have been expected), and essentially all of the radioactivity that had been applied was co-eluted either with methylmalonyl-CoA (74%) or with propionyl-CoA (21%).

Tritium content of residual (2R)-methyl[2-³H]-malonyl-CoA after partial reaction

(2*R*)-Methyl[2-³H]malonyl-CoA was re-isolated, after partial reaction with methylmalonyl-CoA epimerase, as described in the Materials and methods section. Results were obtained for various extents of reaction, from 10 to 56%. The specific radioactivity of the re-isolated methylmalonyl-CoA gradually rose from its initial value S_0 as the extent of reaction f increased (Fig. 3). The separation of CoA esters is shown in Fig. 4 for an experiment in which the reaction was stopped after 51% of the (2*R*)-methylmalonyl-CoA had reacted. The results were corrected to allow for a small amount of direct decarboxylation of the (2*R*)-epimer by the transcarboxylase.

Fig. 4. DEAE-cellulose column chromatography of the remaining (2*R*)-methylmalonyl-CoA after partial methylmalonyl-CoA-catalysed reaction.

A portion (0.3 ml, containing approx. 70 000 total d.p.m. of ³H) of the reaction mixture was removed after the epimerase-catalysed reaction had reached 51% completion. The sample was filtered through a mini-column (0.3 ml) of anti-(methylmalonyl-CoA epimerase) immunoglobulin G-Sepharose and loaded on to a column (0.6 cm $\times$ 2 cm) of DEAE-cellulose (HCO₃⁻ form) pre-equilibrated with 0.175 M-ammonium bicarbonate. The column was washed with 10 ml of the same solution, and 1.1 ml fractions were collected. A linear salt gradient, formed from 12 ml each of 0.175 M- and 0.4 M-ammonium bicarbonate, was then used to elute the methylmalonyl-CoA. The A_{260} (●) of each fraction (1.1 ml) was measured, and a 1 ml sample of each fraction was taken and its radioactivity counted (○). For other details see the Materials and methods section.

Fig. 5. Apparent primary kinetic tritium-isotope effect on proton abstraction from (2*R*)-methyl[2-³H]malonyl-CoA during the methylmalonyl-CoA epimerase-catalysed reaction

The data of Fig. 3 were plotted according to eqn. (2), and the primary kinetic tritium-isotope effect was deduced from the slope of the straight line obtained.

The primary kinetic isotope effect on $k_{\text{cat.}}/K_m$ was calculated by using the following equation (Melander, 1960), which relates the isotope effect to the specific radioactivity of remaining substrate S_s , at a fractional extent of reaction f , and to the initial specific radioactivity of the substrate, S_0 (see Fig. 5):

$$\ln \left(\frac{S_s}{S_0} \right) = \left(\frac{(k_{\text{cat.}}/K_m)^T}{(k_{\text{cat.}}/K_m)^H} - 1 \right) \cdot \ln(1-f) \quad (2)$$

Discussion

There are three possible alternative fates for a tritium atom, originally attached to C-2 in the substrate (2*R*)-methylmalonyl-CoA, during the course of the epimerase-catalysed reaction. It may be released into the solvent, it may be found in the product (isolated as propionyl-CoA), or it may remain in residual substrate. The rate at which tritium is released to the solvent will be determined by the primary

kinetic tritium-isotope effect on proton abstraction and by the existence of any enzyme-bound species that can equilibrate with solvent protons faster than the rate of the overall reaction ('wash-out'). The presence of any tritium attached to C-2 in the product would represent a direct transfer of tritium from substrate to product. Again, the observed extent of transfer would depend on how rapidly enzyme-bound intermediates can equilibrate with the solvent. Similarly, the variation in specific radioactivity of residual (2*R*)-methylmalonyl-CoA during the reaction will reflect any competition between proton exchange with the solvent and conversion of enzyme-bound intermediates into product. It too will be affected by the operation of a primary kinetic tritium-isotope effect on $k_{\text{cat.}}/K_m$ (Northrop, 1975).

Analysis of the tritium content of the (2*S*)-epimer product, isolated as propionyl-CoA, provides the first important piece of information about the mechanism of reaction of methylmalonyl-CoA epimerase: the hydrogen atom of the new C-H bond is essentially derived from solvent protons. This has also been shown for proline racemase (Cardinale & Abeles, 1968), but it is in contrast with results obtained with mandelate racemase (Kenyon & Hegeman, 1979), which indicate the 'transfer' of some hydrogen isotope, originally in the substrate, to a stable position in the product. Presumably, in all these enzymes, the proton abstracted from the substrate becomes temporarily attached to an enzyme base. If this base is the only one involved in proton transfer, there will be a simple partitioning between exchange with solvent protons, and re-protonation of the carbanion intermediate to form enzyme-substrate or enzyme-product species.

The partitioning ratio between exchange and direct 'transfer' of the substrate hydrogen to the product can vary widely even among enzymes with identical mechanisms. [Thus triose phosphate isomerase shows only 3-6% transfer with dihydroxyacetone phosphate as substrate (Herlihy *et al.*, 1976), whereas glucose phosphate isomerase with glucose 6-phosphate as substrate shows up to 80% transfer under certain conditions (Rose, 1970).] When transfer is observed it indicates the existence of a carbanion intermediate, and, in those cases so far investigated, it is associated with the involvement of a single enzyme base. Transfer is also conceivable for an enzyme with two basic groups involved in catalysis, if these groups are in protonic equilibrium with one other, but not with the solvent, so that the proton originally abstracted gets a 'conducted tour' (Cram & Gosser, 1964). In proline racemase, however, where two bases are involved, there is no transfer. One base apparently acts to remove the substrate proton, and the second either in the same or in a subsequent step is responsible for re-protonation. In the reverse direction the roles of the two

bases are reversed (Rudnick & Abeles, 1975). This type of mechanism is clearly consistent with our finding that very little (if any) transfer occurs in methylmalonyl-CoA epimerase. However, as pointed out by Rose (1966), it cannot be distinguished, without further evidence, from a single-base mechanism where complete isotopic exchange occurs between enzyme-bound species and the solvent. It might also be objected that a very small extent of transfer (<5%) cannot be firmly excluded. Both of these alternatives would imply, though, that isotopic exchange between enzyme base(s) and solvent protons is extremely fast.

Essentially all of the tritium originally at C-2 in (2*R*)-methyl[2-³H]malonyl-CoA is released to the solvent during the epimerase-catalysed reaction, so it is informative to compare the rate of tritium release with the rate of product formation. The data (see Table 1 and Fig. 2) show that tritium release lags behind propionyl-CoA formation, and, if we assume that release of a proton either accompanies or follows the release of product, there is a very small primary kinetic tritium-isotope effect of 2.3 on $k_{\text{cat.}}/K_m$ for the epimerase-catalysed reaction (Fig. 2). The primary kinetic tritium-isotope effect is also responsible for the observed gradual rise in the specific radioactivity of the (2*R*)-methyl[2-³H]-malonyl-CoA substrate as the reaction progresses (Fig. 3). From these data the primary kinetic tritium-isotope effect on $k_{\text{cat.}}/K_m$ is 1.6, which is in reasonable agreement with the value obtained by monitoring the tritium content of the water. These results do not support the idea of a rapid isotopic equilibration between an enzyme-bound intermediate and solvent protons, which would allow tritium to be released faster than the overall turnover of the epimerase-catalysed reaction.

The results given in the present paper, when combined with the evidence in the following paper (Fuller & Leadlay, 1983), strongly suggest that the mechanistic pathway for the reaction catalysed by methylmalonyl-CoA epimerase resembles that proposed for proline racemase (Rudnick & Abeles, 1975), in which two enzyme bases are involved. One acts to remove a proton from the substrate, the other re-protonates with a second, solvent-derived, proton. There is no rapid isotopic exchange between an enzyme-bound intermediate and the medium.

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