# STUDIES ON METHYLMALONYL ISOMERASE

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## SUMMARY

The partial purification (approx. 23-fold) and assay of the methylmalonyl isomerase system from bovine-liver mitochondria is described. The purified isomerase system catalyzes the isomerization of enzymically-synthesized [1-$^{14}$C]- and [3-$^{14}$C]methylmalonyl-CoA to form [1-$^{14}$C]- and [4-$^{14}$C]succinyl-CoA, respectively. This is in agreement with the proposal of EGGERER *et al.*[1] that isomerization involves rearrangement of the thiolester group.

## INTRODUCTION

EGGERER *et al.*[1] and SWICK *et al.*[2] have reported that [2-$^{14}$C]methylmalonyl-CoA is converted by enzymic isomerization to [3-$^{14}$C]succinyl-CoA. It was observed by PHARES *et al.*[3] that enzymic isomerization of [1-$^{14}$C]methylmalonyl-CoA in the presence of [2-$^{14}$C]propionyl-CoA yielded succinate (derived from succinyl-CoA) labeled principally in the carboxyl- and not the methylene-carbons. These experiments rule out intermolecular transcarboxylation between methylmalonyl-CoA and propionyl-CoA, and support instead apparent thiolester carbonyl migration, or transpropionation as suggested by STERN AND FRIEDMAN[4]. Although it has been assumed by many workers that the thiolester carbonyl of methylmalonyl-CoA is converted during isomerization to the thiolester carbonyl of succinyl-CoA, this point has not been definitely established. The labeling experiments mentioned do not rule out the possibility that CoA transfer occurs prior to thiolester carbonyl migration, or subsequent to carboxyl group migration. In view of the recent suggestion[5] that enzymic racemization of methylmalonyl-CoA precedes isomerization, this point becomes an even more important consideration since racemization could be a result of CoA transfer.

The present investigation reveals that a methylmalonyl isomerase system purified from bovine liver catalyzes an isomerization in which the carboxyl group and thiolester carbonyl of succinyl-CoA are derived from the carboxyl group and thiolester carbonyl, respectively, of methylmalonyl-CoA.

Abbreviations: DBCC, dimethylbenzimidazolylcobamide coenzyme; POPOP, 1,4- bis-2-(phenyloxazolyl)-benzene; PPO, 2,5-diphenyloxazole.

## MATERIALS AND METHODS

$KH^{14}CO_3$ was obtained from Volk Radiochemical Corporation, sodium[1-$^{14}$C]propionate from Research Specialties Co., DEAE-cellulose from Eastman Organic Chemicals, glutathione and Alumina C$\gamma$ from Sigma Chemical Co., coenzyme A and ATP from Pabst Laboratories, Sparkleen detergent from Fisher Scientific Co., and Thixotropic Gel Powder, PPO, and POPOP from Packard Instrument Co., Inc. Bovine liver mitochondrial propionyl carboxylase was provided by D. R. HALENZ of this laboratory. Dimethylbenzimidazolylcobamide coenzyme was generously supplied by Dr. K. FOLKERS of Merck Sharp and Dohme Research Laboratories. Succinamic acid, prepared by reaction of excess $NH_4OH$ with recrystallized succinic anhydride, was treated with Amberlite IRA-120 resin ($H^+$ phase) to remove $NH_4^+$, and recrystallized from ethanol until chromatographically pure. [1-$^{14}$C]propionyl-CoA was prepared by the method of SIMON AND SHEMIN[6] using [$^{14}$C]propionic anhydride formed by the reaction of propionyl chloride with sodium [1-$^{14}$C]propionate. Unlabeled propionyl-CoA and succinyl-CoA were also prepared by the method of SIMON AND SHEMIN. Coenzyme A derivatives were assayed by the hydroxamate method[7]. Protein was determined spectrophotometrically according to the method described by LAYNE[8]. The following systems were used for chromatography: ethanol–$NH_4(OH)$–water (80:5:15, v/v); isoamyl alcohol saturated with 4 *N* formic acid; *n*-amylformate–formic acid–water (7:2:1, v/v); and *n*-butanol–acetic acid–water (4:1:5, v/v).

All quantitative measurements of radioactivity, with the exception of isomerase assays, were conducted with a Packard Tri-carb liquid scintillation spectrometer. Radioactive areas on chromatograms were located with a chromatogram strip counter. Hydroxamates were located with 1.67 % $FeCl_3$ in 3 *N* HCl, organic acids with 0.2 % brom-cresol-purple in alcoholic formaldehyde, and amino acids with 0.3 % ninhydrin in 95 % EtOH.

## EXPERIMENTAL

### *Isomerase assay*

Methylmalonyl isomerase assays were conducted by a modification of the method described by FLAVIN AND OCHOA[9], which involved coupling of the propionyl carboxylase and methylmalonyl isomerase systems. The complete reaction mixture included (in $\mu$moles): Tris (pH 8.5) 80; ATP, 2.0; $MgCl_2$, 2.0; GSH, 2.5; propionyl-CoA, 0.4; $KH^{14}CO_3$ (specific activity, 0.17 $\mu$C/$\mu$mole), 7.2; DBCC, $3 \cdot 10^{-3}$; and propionyl carboxylase (specific activity, 25 units/mg)[10], 0.3 mg; and methylmalonyl isomerase (up to 0.37 units) in a total volume of 0.55 ml. DBCC and methylmalonyl isomerase were added following a 10-min preincubation of all other reaction components at 37°. The complete reaction mixture was then incubated an additional 20 min at 37°. The reaction was terminated with 0.15 ml of 20 *N* $H_2SO_4$, and after the addition of 0.05 ml of 6 % succinic acid and centrifugation, a 0.65 ml aliquot of the supernatant was oxidized by heating at 100° with 0.8 ml of 6 % $KMnO_4$ for 10 min. 0.2 ml, then 0.3 ml of 1.5 *M* $Na_2CO_3$ containing "Sparkleen" detergent (5 mg/ml) were added to the acidic solution to permit evolution of excess $^{14}CO_2$.

Following centrifugation, a 0.6 ml aliquot was placed on an aluminum planchet, covered with a circle of lens paper, dried on the steam bath, and the amount of per-

manganate-stable radioactivity (succinate) determined using a gas-flow counter. Isomerase was omitted in negative controls. The isomerization reaction followed zero-order kinetics with up to 0.37 units of isomerase under the conditions described. One unit of isomerase, as defined by BECK *et al.*[11], catalyzes the isomerization of 1.0 $\mu$mole/h of methylmalonyl-CoA to succinyl-CoA. Specific activity is expressed as units of isomerase/mg of protein.

*Purification of isomerase*

All purification procedures were conducted at 0–4°. Ten g of bovine liver mitochondrial acetone powder, prepared according to the procedure of LANE AND HALENZ[12], were extracted by stirring for 30 min with 200 ml of 0.004 *M* Tris (pH 7.3), containing 0.005 *M* GSH. The extract was then centrifuged at 12800 × *g* for 10 min, and the supernatant (approximately 191 ml) brought to 46% saturation by slow addition of saturated ammonium sulfate, pH 8.0.*

After centrifugation, the supernatant was brought to 60% saturation with saturated ammonium sulfate. The precipitate, following centrifugation, was redissolved in 75 ml of 0.004 *M* Tris buffer (pH 7.2), and then dialyzed for 8 h against two 8-l changes of the same buffer. After dilution of the dialyzed enzyme solution to a protein concentration of 3.8 mg/ml, alumina C$\gamma$ (20.2 mg/ml) was added to produce a gel to protein ratio (mg/mg) of 0.5. The suspension was stirred slowly for 30 min, centrifuged, and the enzyme eluted from the gel by slow stirring for 1 h with about 130 ml of 0.2 *M* phosphate buffer (pH 7.8). The eluate, to which 0.002 *M* GSH was added after centrifugation, was dialyzed for 12 h against 4.9 l of distilled water which resulted in a final phosphate concentration of 0.005 *M*. The dialyzed enzyme (127 mg of protein) was then applied to a 2.5 × 30 cm DEAE-cellulose column previously equilibrated[13] with 0.005 *M* potassium phosphate (pH 7.0). Stepwise gradient elution was accomplished by placing 190 ml of 0.005 *M* phosphate (pH 7.0) in a mixing chamber attached to the column, and introducing the following buffers (all pH 7.0) into a separatory funnel attached to the mixing chamber: 0.005 *M* phosphate, 60 ml; 0.05 *M* phosphate, 200 ml; 0.1 *M* phosphate, 300 ml; 0.09 *M* phosphate and 0.1 *M* NaCl, 300 ml; and 0.08 *M* phosphate and 0.2 *M* NaCl, 300 ml. Elution was conducted at a flow rate of 5 ml per minute, and 15 ml fractions were collected. Fractions were assayed for isomerase activity and protein. Fractions (numbers 32–52) containing most of the eluted isomerase activity were pooled and dialyzed for 15 h against sufficient ammonium sulfate solution, pH 7.4, to produce 65% saturation at equilibrium. The precipitate, collected by centrifugation, was stored under 65% saturated ammonium sulfate at 0°. The purification data are summarized in Table I.

*Enzymic isomerization of [1-$^{14}$C]methylmalonyl-CoA and [3-$^{14}$C]methylmalonyl-CoA*

Highly-purified propionyl carboxylase was employed to generate [1-$^{14}$C]methylmalonyl-CoA (tube 1) from [1-$^{14}$C]propionyl-CoA and $HCO_3^-$, and [3-$^{14}$C]methylmalonyl-CoA (tube 2) from propionyl-CoA and $H^{14}CO_3^-$. The [$^{14}$C]methylmalonyl-CoA served as substrate for purified methylmalonyl isomerase. Subsequently, succinamic acid, prepared from the resulting succinyl-CoA was isolated and chemically degraded to determine the distribution of radioactivity. Reaction mixtures contained (in

* Ammonium sulfate was saturated at room temperature and neutralized with $NH_4OH$ so that, when diluted 5-fold, the specified pH was obtained.

$\mu$moles): potassium phosphate, pH 6.5, 200; ATP, 16; GSH, 5; $MgCl_2$, 16; DBCC, $1.5 \cdot 10^{-2}$; propionyl carboxylase (specific activity, 264 units/mg)[12], 22.4 units; and DEAE-cellulose-purified isomerase (specific activity, 9.8 units/mg), 17.4 units. In addition, tube 1 contained 3.5 $\mu$moles of [1-$^{14}$C]propionyl-CoA (0.87 $\mu$C/$\mu$mole) and

TABLE I

PURIFICATION OF METHYLMALONYL ISOMERASE

| *Treatment* | *Protein (mg)* | *Enzyme activity units* | *Recovery (%)* | *Specific activity (units/mg)* |
|---|---|---|---|---|
| (1) Acetone powder extract | 2100 | 1535 | 100 | 0.73 |
| (2) 0.46–0.60 $(NH_4)_2SO_4$ fraction | 502 | 1286 | 84 | 2.6 |
| (3) Dialyzed alumina C$\gamma$ eluate | 132 | 1175 | 76.5 | 8.9 |
| (4) Pooled DEAE-cellulose column fractions | 22 | 371 | 23.5 | 16.9 |
| (5) Precipitated DEAE-cellulose column fractions | 8.9 | 87 | 5.7 | 9.8 |

15 $\mu$moles of $KHCO_3$; tube 2 contained 2 $\mu$moles of $KH^{14}CO_3$ (5 $\mu$C/$\mu$mole), 1.1 $\mu$moles of propionyl-CoA and 80 $\mu$moles of Tris. After preincubation of all other components for 4 min at 37°, isomerase and DBCC were added, and the reaction mixture was incubated for an additional 15 min at 37° in a final volume of 3.15 ml. The enzymic reaction was terminated and amides of the acyl-CoA derivatives formed[1,11] by addition of 0.4 ml of 15 *M* $NH_4OH$. The reaction mixture from tube 1 was adjusted to pH 1.0, gassed with $CO_2$, and then both reaction mixtures were adjusted to pH 4.5.

Randomization of label in succinyl-CoA (via succinate) is possible through enzymic deacylation followed by reacylation, or CoA transfer. These interfering enzyme activities, although not absent in the DEAE-cellulose-purified isomerase used in the labeling experiments, were greatly reduced in comparison to isomerase from earlier stages of purification. In order to approximate the extent of deacylation occurring under the conditions used in the labeling experiments described above, DEAE-cellulose-purified, and ammonium sulfate-purified isomerase were incubated with the same reaction mixture under the same conditions used in tube 2 of the labeling experiment. Also, in order to determine the extent of succinate incorporation into succinyl-CoA, an aditional tube in which [1,4-$^{14}C_2$]succinate (0.13 $\mu$moles, 7.6 $\mu$C/$\mu$mole) and succinyl-CoA (0.79 $\mu$moles) were substituted for $KH^{14}CO_3$ and propionyl-CoA, was incubated simultaneously. The reaction was terminated by the addition of 1200 $\mu$moles of neutralized hydroxylamine, and the carboxylic and hydroxamic acids were then isolated by the procedure of STADTMAN AND BARKER[14], followed by chromatography on Whatman 3MM paper using the isoamyl alcohol–formic acid solvent system.

Analysis of the chromatograms revealed that the DEAE-cellulose-purified isomerase catalyzed the deacylation of 22 % of the [$^{14}$C]succinyl-CoA formed, and the incorporation of 2.7 % of the added [$^{14}$C]succinate into succinyl-CoA. The deacylase activity and the ability to catalyze the incorporation of succinate into succinyl-CoA of the DEAE-cellulose-purified enzyme were reduced 3.6-fold and 5.4-fold, respectively, compared to the ammonium sulfate-purified enzyme.

*Isolation and characterization of succinamic and methylmalonamic acids*

The reaction mixtures (previously adjusted to pH 4.5) were extracted with 150 ml of warm acetone and, following centrifugation, the supernatants were filtered and concentrated to dryness. The residue was redissolved in water, applied by streaking to sheets of Whatman 3MM paper, and the chromatograms developed descending for 16 h (solvent was permitted to run off chromatograms) using the ethanol-ammonia solvent system. The radioactive bands were located, and eluted with water. The chromatograms exhibited the following radioactive bands: succinate (11 cm from the origin), a mixture of succinamic and methylmalonamic acids (29 cm from the origin), and propionamide (41 cm from the origin; from extracts of tube 1 only). Succinamic and methylmalonamic acids were eluted together, and then completely resolved by rechromatography on Whatman 3MM paper using the ascending *n*-amylformate–formic acid solvent system. After elution with water, aliquots of succinamic acid and methylmalonamic acid were hydrolyzed in 6 *N* HCl at 100° for 3.5 h and their paper chromatographic properties compared with unhydrolyzed aliquots and with co-chromatographed authentic succinic, methylmalonic, and succinamic acids. The details of the paper chromatographic characterization of the isolated methylmalonamic and succinamic acids are summarized in Table II.

TABLE II

CHARACTERIZATION OF DERIVATIZED REACTION PRODUCTS

| Compound | $R_F$ | | | |
|---|---|---|---|---|
| | Unhydrolyzed | | Acid hydrolyzed | |
| | Solvent 1* | Solvent 2** | Solvent 1* | Solvent 2** |
| Authentic succinic acid | 0.43 | 0.65 | — | — |
| Authentic succinamic acid | 0.30 | 0.32 | — | — |
| Authentic methylmalonic acid | 0.57 | 0.80 | — | — |
| Enzymically synthesized succinamic acid | 0.30 | 0.32 | 0.42 | 0.66 |
| Enzymically synthesized methylmalonamic acid | 0.42 | 0.49 | 0.55 | 0.80 |

* *n*-Amylformate–formic acid solvent system.
** Isoamyl alcohol–4 *N* formic acid solvent system.

*Degradation of [$^{14}$C]succinamic acids*

The [$^{14}$C]succinamic acids derived from the differentially labeled succinyl-CoA's, and isolated as described in the previous section, were subjected to HOFFMAN degradation[1,11] in order to ascertain the distribution of $^{14}$C-activity. An aliquot of [$^{14}$C]succinamic acid and 20 $\mu$moles of unlabeled authentic succinamic acid were mixed and dried in the degradation vessel. After purging with $CO_2$-free air, 0.8 ml of NaOBr (prepared by reacting 0.035 ml of $Br_2$ with 20 ml of $CO_2$-free 2 *N* NaOH at 0°) were introduced, and the reaction continued at 70° for 2 h in a closed system. The reaction was terminated by the addition of 0.16 ml of 12 *N* HCl followed by 0.3 ml of water. The $CO_2$ evolved from the reaction was trapped in $CO_2$-free 2 *N*

NaOH, precipitated as $BaCO_3$[15] and the washed $BaCO_3$ was dried and counted as a suspension in 10% thixotropic gel, 0.4% PPO, 0.01% POPOP in toluene. In order to determine the extent of degradation of succinamic acid, the degradation mixture was neutralized to pH 4.5, diluted to volume, and aliquots were assayed for β-alanine by the method of TROLL AND CANNAN[16] using β-alanine as standard. The remaining volume was desalted on a washed column (1 × 15 cm) of Amberlite IRA-400 resin in the $OH^-$ phase, evaporated to dryness, redissolved in water, and chromatographed ascending on Whatman 3MM paper using the butanol-acetic solvent system in order to remove any ungraded succinamic acid. The β-alanine was eluted, and the aliquots were assayed for radioactivity and β-alanine. Total radioactivity as β-alanine was calculated from the specific activity of the β-alanine and from the extent of succinamic acid degradation. The distribution of radioactivity in the succinamic acids is summarized in Table III. It is evident from these data that the radioactivity associated with carbons 1 and 3 of methylmalonyl-CoA is found, after enzymic isomerization, associated predominantly with carbons 1 and 4, respectively, of succinyl-CoA.

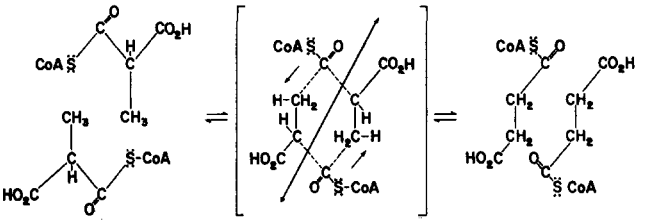
TABLE III

DISTRIBUTION OF RADIOACTIVITY IN SUCCINAMIC ACIDS

| *Origin of succinamic acid degraded* | *Radioactivity (disintegrations/min)* | | |
|---|---|---|---|
| | | *In degradation products* | |
| | *Total degraded* | *β-alanine (from carbons 2, 3 and 4 of succinyl-CoA)* | *$CO_2$ (from carbon 1 of succinyl-CoA)* |
| Enzymic isomerization of [1-$^{14}$C]methylmalonyl-CoA | 37 700 | 5 500 | 35 100 |
| Enzymic isomerization of [3-$^{14}$C]methylmalonyl-CoA | 47 900 | 35 900 | 14 500 |

## DISCUSSION

The present investigation indicates that during the enzymic isomerization of methylmalonyl-CoA the thiolester carbonyl (originating from carbon 1 of propionyl-CoA) and the free carboxyl group (originating from $CO_2$) of methylmalonyl-CoA become the thiolester carbonyl and free carboxyl group, respectively, of succinyl-CoA. This finding rules out possible transcarboxylation followed by CoA transfer, or CoA transfer prior to migration of the thiolester carbonyl, and substantiates the proposal of EGGERER *et al.*[1] that thiolester carbonyl migration occurs. PHARES *et al.*[3] had previously demonstrated that intermolecular transcarboxylation from methylmalonyl-CoA to propionyl-CoA is not catalyzed by methylmalonyl isomerase. No evidence is available at the present time which indicates whether thiolester carbonyl migration occurs inter- or intramolecularly. A concerted mechanism for the isomerization of methylmalonyl-CoA which involves intermolecular thiolester carbonyl transfer is suggested. The mechanism, shown below, is consistent with the labeling experiments reported herein and elsewhere.



In view of the recent report by MAZUMDER AND SASAKAWA[5] it is of interest to note that apparent isomerase activity (1.23 units/ml) of the pooled DEAE-cellulose chromatographic fractions was greater than that of the most active chromatographic fraction (tube 38, 0.94 units/ml). This apparent discrepancy could be accounted for if the broad peak of apparent isomerase activity eluted from the column were actually composed of two partially resolved enzyme peaks (possibly racemase and isomerase), both of which are required for the formation of succinyl-CoA from enzymically-synthesized methylmalonyl-CoA.

Using methylmalonyl isomerase prepared from sheep kidney cortex as described by BECK *et al.*[11], KOSOW[17], of this laboratory, was unable to demonstrate the isomerization of ethylmalonyl-CoA or dimethylmalonyl-CoA.

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