The Biosynthesis of Methylcitrate

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Citrate synthase catalyses the formation *in vitro* of two diastereoisomers of methylcitrate from propionyl-CoA and oxaloacetate. The proportion of diastereoisomers produced is temperature-sensitive. In the presence of oxaloacetate and a thiol trap, the enzyme catalyses the exchange of both α -protons of propionyl-CoA with $^2\text{H}_2\text{O}$. The *pro-S*-proton at the α -carbon atom is exchanged 15 times faster with $^2\text{H}_2\text{O}$ than is the *pro-R*-proton. The exchanges are over 1000 and 100 times faster respectively than V_{max} . These results are interpreted in the light of recent reports that 2*R*,3*S*- and 2*S*,3*S*-methylcitrate are produced by the enzyme-catalysed condensation and also excreted by patients with propionic acidemia.

Methylcitrate is excreted in the urine of patients suffering from propionic acidemia, a rare inborn error of human metabolism (Ando *et al.*, 1972). Its synthesis has been postulated to be the consequence of the accumulation of propionyl-CoA and subsequent condensation with oxaloacetate by analogy with the citrate synthase condensation of acetyl-CoA and oxaloacetate to form citrate.

We have shown previously that pig heart citrate synthase and propionyl-CoA form a binary complex with K_D of approx. 0.1 mM, and the addition of a saturating concentration of oxaloacetate to the enzyme-propionyl-CoA system lowered the K_D of propionyl-CoA by a factor of 20 (to 5 μM), indicating the formation of a propionyl-CoA-enzyme-oxaloacetate ternary complex with enhanced affinity for propionyl-CoA (Weidman & Drysdale, 1973; Weidman *et al.*, 1973). In these experiments the enzyme slowly catalysed a reaction between propionyl-CoA and oxaloacetate with a V_{max} about 10^{-4} times that of acetyl-CoA and a K_m of 16 μM for propionyl-CoA. The product was identified as methylcitrate. We concluded that a citrate synthase-catalysed condensation of propionyl-CoA and oxaloacetate could account for the production of methylcitrate observed in propionic acidemia.

The absolute configuration of methylcitric acids was recently reported by Brandänge *et al.* (1977a), who used g.l.c.-mass spectroscopy techniques to distinguish between the trimethyl esters of the diastereoisomeric methylcitric acids, that is the 2*R*,3*S*- and 2*S*,3*R*-enantiomers and the 2*R*,3*R*- and 2*S*,3*S*-enantiomers (the numbering is that of 2-hydroxypropane-1,2,3-tricarboxylic acids; see Fig. 1). The configurational assignments were based on the c.d. of the molybdate complexes of stereospecifically synthesized 2*S*,3*S*- and 2*R*,3*S*-methylcitrate. In this

Fig. 1. Structural formulae for the methylcitric acids found in the urine of patients with propionic acidemia

The formulae are to be regarded as Fisher projections, although the lowest-numbered chain members are not placed at the top.

system the carbon atom originating from the methylene group of propionyl-CoA is C-3 and the one originating from the carbonyl group of oxaloacetate is C-2. The methylcitrate obtained from the urine of a patient with propionic acidemia were shown to be a mixture of 2*S*,3*S*- and 2*R*,3*S*-methylcitrate in the approximate ratio of 2:1. Brandänge *et al.* (1977a) pointed out that, if propionyl-CoA attacked the *si* face of the carbonyl group of oxaloacetate as acetyl-CoA does (Hanson & Rose, 1963), the product would have the *S*-configuration at C-2. The methylcitrate with *R*-configuration at C-2 could result from a citrate synthase-catalysed condensation either via attack of propionyl-CoA on the *re* face of the carbonyl of oxaloacetate or through condensation of 3-methyl-oxaloacetate with acetyl-CoA (normal *si* attack). Attack on the *re* face of the carbonyl group of oxaloacetate has not previously been observed for

mammalian citrate synthases. Likewise, the production of 3-methyloxaloacetate would involve a hitherto undetected metabolic pathway. In either case, methylcitrate synthesis *in vivo* would be stereospecific with respect to the centre derived from propionyl-CoA (C-3), but not with respect to the one derived from oxaloacetate (C-2).

Brandänge *et al.* (1977b) have also shown that propionyl-CoA is a substrate for pig heart citrate synthase and have reported that the 2*R*,3*S*- and 2*S*,3*S*-diastereoisomeric methylcitrate are formed. Therefore it is likely that the molecules (I) and (II) (Fig. 1) produced by propionic-acidaemia patients are synthesized *in vivo* by a citrate synthase-catalysed condensation. The establishment of the absolute configuration of methylcitrate at C-3 assists us to interpret our observations on the exchange of the α -proton of propionyl-CoA that the enzyme catalyses in the presence of oxaloacetate or *S*-malate. We report these results along with observations on the proportion of molecules (I) and (II) excreted by propionic-acidaemia patients and the effect of experimental variables on production *in vitro* of methylcitrate catalysed by citrate synthase.

Experimental

Materials

Acyl-CoA esters were obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). Pig heart citrate synthase (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) was desalted and assayed as previously described (Weidman *et al.*, 1973). Propionyl-CoA carboxylase was purified from bovine liver mitochondria to a specific activity of 22 units/mg (Giorgio & Plaut, 1967), where 1 unit of activity is the amount of enzyme required to convert 1 μ mol of substrate/min. $^3\text{H}_2\text{O}$ (5 Ci/ml) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). All other materials were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Kinetics of the citrate synthase-catalysed exchange of the α -protons of propionyl-CoA with $^2\text{H}_2\text{O}$

Stock solutions of citrate synthase, propionyl-CoA, Tris base, and oxaloacetate were prepared in $^2\text{H}_2\text{O}$. Exchange mixtures (pD approx. 7.5) contained 10 μ mol of propionyl-CoA, 10 μ mol of 5,5'-dithiobis-(2-nitrobenzoic acid), 20 μ mol of oxaloacetate, 0.51 or 0.051 mg of citrate synthase, and 2.6 ml of $^2\text{H}_2\text{O}$ (total volume 5.0 ml). A sample (0.01 ml) was taken for an enzyme assay and the solution was divided into five equal-volume samples. After 5, 10, 20, 40 and 60 min at room temperature, each sample was treated as follows with 70% (w/v) HClO_4 : 0.05 ml of 70%

HClO_4 was added and the precipitated protein centrifuged; the supernatant was adjusted to pH 9–10 with 1 M-KOH, and the KClO_4 removed by centrifugation; the supernatant was freeze-dried and the residue taken up in 0.3 ml of water; the pH was adjusted to 12–13 with 1 M-KOH and the solution incubated at room temperature for 30 min; the precipitate was removed by centrifugation and the pH of the supernatant adjusted to approx. 1.0 with conc. HCl; the acid solution was then extracted three times with equal volumes of diethyl ether; the ether extracts were combined and treated with a stream of diazomethane and N_2 to form the methyl ester of propionic acid. The methyl propionate solutions were then analysed in a g.l.c.-mass spectrometer computer system with a column (1.22 m) of 15% SE-30 on Gas-Chrom Q (80–100) at 33°C. Methyl propionate and methyl [$2\text{-}^2\text{H}_2$]propionate had elution times of 3.5 min. The molecular ions were used to estimate the extent of ^2H labelling.

Preparation of stereospecifically ^2H -labelled and ^3H -labelled propionyl-CoA derivatives

Preparation of ^3H -labelled [$2\text{-}^2\text{H}$]propionyl-CoA with citrate synthase and oxaloacetate. Propionyl-CoA (5 μ mol) was incubated for 40 min in $^2\text{H}_2\text{O} + ^1\text{H}^3\text{HO}$ in the presence of oxaloacetate, 5,5'-dithiobis-(2-nitrobenzoic acid), and the appropriate amount of citrate synthase to exchange approx. 0.7 atom of ^2H into the α -position (ascertained by g.l.c.-mass spectrometry of the derived methyl propionate). The reaction mixture was then freeze-dried, the residue dissolved in dilute HCl, and again freeze-dried. The residue was dissolved in 1 ml of water and passed through a column (1 cm $\times$ 15 cm) of Sephadex G-10 equilibrated with water. The radioactive propionyl-CoA-peak fractions were pooled and purified by the method of Cha & Parks (1964). The specific radioactivity of the propionyl-CoA was 9.54×10^4 d.p.m./ μ mol.

Preparation of (R)-[$2\text{-}^3\text{H}$]propionyl-CoA with propionyl-CoA carboxylase. This material was prepared by the procedure of Prescott & Rabinowitz (1968) and purified by the method given above. The specific radioactivity was 5.27×10^5 d.p.m./ μ mol.

Analysis of diastereoisomeric methylcitrates

In vitro. Methylcitrate was prepared enzymically under the following conditions: 40 mM-Tris/HCl buffer, pH 7.5, 100 μ M-propionyl-CoA, 200 μ M-Tris oxaloacetate and 0.16 mg of citrate synthase/ml (1.6 μ M-enzyme). The enzyme was added last. The extent of reaction was estimated by removing portions (0.1 ml) to determine the free CoA concentration by dilution with 0.90 ml of Tris/HCl buffer, pH 7.5, con-

taining 0.22 mM-5,5'-dithiobis-(2-nitrobenzoic acid). The reaction was stopped by the addition of 1M- H_2SO_4 to bring the pH down to approx. 1.5. The sample was then extracted three times with 2 vol. of ether. The ether extracts were combined and evaporated to dryness under N_2 . A solution of diazomethane in ether was then added to form the trimethyl ester. The diastereoisomers were separated on a column (0.2 cm $\times$ 300 cm) of 3% ECNSS-M on Gas-Chrom Q (120 mesh) at 165°C with an He flow rate of 30 ml/min. The proportions of the diastereoisomers of trimethyl methylcitrate present in the samples was determined with a Finnigan 3300 gas chromatograph/mass spectrometer (Finnigan Corp., Sunnydale, CA, U.S.A.) operating in the multiple-ion-detection mode. The intensity of the most abundant peak in the mass spectrum of trimethyl methylcitrate (m/e 157) was measured as a function of time. The instrument was also operated in the scan mode to establish the identity of the two peaks obtained, representing the two diastereoisomers of trimethyl methylcitrate. The spectra obtained agreed with those published by Brandänge *et al.* (1977a).

In urine of propionic-acidaemia patients. Urine samples obtained from patients suffering from propionic acidaemia were first adjusted to pH 9 by the addition of KOH and then extracted twice with 2 vol. of ether. The pH of the aqueous layer was adjusted to about 1.5 by the addition of 1M- H_2SO_4 , and the sample treated as described above to yield the trimethyl ester of methylcitrate. The ester was then examined by g.l.c.-mass spectrometry to determine the methylcitrate diastereoisomer distribution.

Results and Discussion

Methylcitrate diastereoisomer proportions in urine of propionic-acidaemia patients

We have examined esterified methylcitrate isolated from the urine of three propionic-acidaemia patients with documented propionyl-CoA carboxylase deficiency. The trimethyl methylcitrate eluted from the g.l.c. column in two peaks as reported by Brandänge *et al.* (1977a). The relative proportions of the 2S,3S- to 2R,3S-methylcitrate in the urine from these patients were 2.1, 0.52, and 0.85 [with the assignments of g.l.c. peaks determined by Brandänge *et al.* (1977a)]. Although one of these patients yielded a similar ratio of the two diastereoisomers to that previously reported (2.0), the urine of the other two patients showed ratios that varied substantially from this value. Thus the ratio of methylcitrate diastereoisomers excreted in propionic acidaemia is variable between patients. We have no information on whether the ratio varies with time in a given individual.

Methylcitrate diastereoisomer proportions produced in vitro

We next examined the methylcitrate produced enzymically from propionyl-CoA and oxaloacetate at pH 7.5 and room temperature. Product analyses indicated that, although two diastereoisomers of methylcitrate were produced, the proportion remained constant over the full time course of the reaction. Further experiments indicated that the diastereoisomer proportions were independent of (a) pH in the range 7.2–7.8 at room temperature, (b) the presence or absence of 5,5'-dithiobis-(2-nitrobenzoic acid), which traps the CoA produced in the reaction, (c) the number of enzyme turnovers, and (d) oxaloacetate or propionyl-CoA (25–200 μM) concentrations. However, the diastereoisomer proportion is sensitive to the reaction temperature (Table 1). The predominant diastereoisomer can be changed by altering the reaction temperature from 10 to 70°C, implying that the heats of activation ($\Delta H^\ddagger$) for the formation of the two diastereoisomers differ. At present we are not able to describe this situation further, but note that the stereoselectivity increases with increasing temperature for the diastereoisomer that would be expected if the reaction shared the steric pathway of citrate synthesis from acetyl-CoA and oxaloacetate. This problem requires further study.

The results indicate that the two diastereoisomers would be synthesized in molecule (II)/molecule (I) mole ratio of about 1.7 at 37°C. Two of the patients with propionic acidaemia excreted methylcitrate with diastereoisomer ratios of approx. 2. In the other two patients the ratio deviates strikingly from this expected value (Brandänge *et al.*, 1977a and the present paper). The deviation could result from (1) environmental influences on the stereoselectivity of citrate synthase, which we have not been able to reproduce, (2) differential metabolism of the two

Table 1. Effect of reaction temperature on enzymically prepared methylcitrate diastereoisomer distribution

Temperature (°C)	Proportion (%)	
	Diastereoisomer I*	Diastereoisomer II†
10	60	40
20	59	41
25	53	47
30	49	51
40	37	63
50	33	67
60	21	79
70	16	84

* Retention time 7.44 min; 2R,3S-methylcitrate.

† Retention time 7.94 min; 2S,3S-methylcitrate.

diastereoisomers, or (3) other pathways of synthesis of methylcitrate that do not involve citrate synthase.

Propionyl-CoA exchange studies

As was pointed out above, production *in vivo* or *in vitro* of methylcitrate is stereospecific with respect to the centre derived from propionyl-CoA. To form methylcitrate with the *S*-configuration at C-3 requires that the *pro-S*-proton of propionyl-CoA should be removed in the reaction, if condensation goes with inversion at the α -carbon of propionyl-CoA (Klinman & Rose, 1971). Propionyl-CoA binds tightly to the enzyme in the presence of oxaloacetate, but is a very poor substrate compared with acetyl-CoA (Weidman & Drysdale, 1973). The rate-limiting step for acetyl-CoA-oxaloacetate condensation appears to be proton abstraction from the acyl methyl group of acetyl-CoA, because the enzyme catalyses no net exchange between acetyl-CoA and $^2\text{H}_2\text{O}$ in the presence of oxaloacetate and a thiol trap to stop the back reaction (Bove *et al.*, 1959; Eggerer, 1965). However, similar experiments with propionyl-CoA indicate that both α -protons are exchangeable with those of the medium in the presence of co-substrate oxaloacetate. No exchange of the α -protons on propionyl-CoA occurs in the absence of either the enzyme or oxaloacetate. Fig. 2 is a plot of the logarithm of the fraction unexchanged as a function of time for both protons. The first proton-exchange velocity is 15 times greater than that of the second (Table 2). Both α -proton-exchange velocities for propionyl-CoA are much faster than $V_{\max}$ for the catalytic reaction. In fact the larger exchange velocity

Table 2. Exchange velocities of the α -protons of propionyl-CoA on citrate synthase induced by oxaloacetate or *S*-malate compared with $V_{\max}$ for the catalytic reaction

Acyl-CoA	Exchange velocity ($\mu\text{mol}/\text{min per mg}$)	$V_{\max}$ of catalytic reaction ($\mu\text{mol}/\text{min per mg}$)
Propionyl-CoA		
First proton	6.6	0.005
Second proton	0.44	
Propionyl-CoA*	0.02	NR†
Acetyl-CoA	NE‡	180

* First proton in the presence of 0.02M-*S*-malate; no oxaloacetate or thiol trap present.

† No reaction.

‡ No exchange can be demonstrated; see the text.

Fig. 2. Exchange of the α -protons of propionyl-CoA on citrate synthase in the presence of oxaloacetate. Symbols: $\circ$, exchange of the first α -proton (0.01 mg of enzyme/ml); Δ , exchange of the second α -proton (0.1 mg of enzyme/ml).

is only a factor of 30 less than $V_{\max}$ for the normal substrate, acetyl-CoA, indicating that the rate-limiting step is different for the propionyl-CoA-oxaloacetate condensation. These exchange results suggest that the enzyme active site can accommodate propionyl-CoA with either its *pro-R*- or *pro-S*-proton correctly oriented for exchange, but with one orientation exchanging 15 times more readily than the other. Consistent with this conclusion is the observation that the α -proton of isobutyryl-CoA also exchanges with a velocity intermediate with those of the two α -protons on propionyl-CoA (results not shown). We assume that exchange occurs with retention of configuration at the α -carbon. If inversion occurred, the rates of exchange of the first and second protons should not differ greatly.

We have also found the *S*-malate, but not *R*-malate, induces the exchange of at least one α -proton of propionyl-CoA on the enzyme. However, the velocity of this exchange is about 300 times slower than that of the first proton when the inducer is oxaloacetate. These results with the enantiomeric malates are consistent with those of Eggerer (1965), who showed that *S*-malate induces the exchange of acetyl-CoA with the medium.

The large difference in velocities of exchange of the two α -protons of propionyl-CoA afforded an opportunity to examine the stereochemistry of the first proton exchange. Advantage was taken of the known steric course of the propionyl-CoA carboxylase reaction in which the *pro-R*-hydrogen atom on the α -carbon atom of propionyl-CoA is replaced by a carboxy group with retention of configuration (Rétey & Lynen, 1965). Two [α - ^3H]propionyl-CoA samples were prepared, one from propionyl-CoA and the carboxylase in $^1\text{H}^3\text{HO}$, and the other by exchange on citrate synthase in the presence of oxaloacetate in $^1\text{H}^3\text{HO}$, under conditions favouring exchange of the first proton. Both specifically ^3H -labelled propionyl-

Table 3. Release of ^3H from stereospecifically labelled $[2\text{-}^3\text{H}]\text{propionyl-CoA}$ derivatives during propionyl-CoA carboxylase-catalysed carboxylation

Two samples were set up, each containing 0.1 ml of 1 M-Tris, pH 8.0 (100 μmol), 10 mg of KHCO_3 (100 μmol), 3.7 mg of KCl (50 μmol), 0.01 ml of 1 M- MgCl_2 (10 μmol), 1.9 mg of phosphoenolpyruvate (4 μmol), 3.7 mg of ATP (5 μmol) and 0.01 ml of pyruvate kinase (2.2 units). To sample A was added 0.75 ml of propionyl-CoA (prepared from exchange with citrate synthase) (0.75 μmol , sp. radioactivity 9.54×10^4 d.p.m./ μmol). To sample B was added 0.75 ml of propionyl-CoA (prepared from carboxylation of propionyl-CoA, followed by decarboxylation in a ^3H medium in the presence of propionyl-CoA carboxylase) containing 0.21 μmol with a sp. radioactivity 5.27×10^5 d.p.m./ μmol , and 0.25 ml of propionyl-CoA carrier (0.575 μmol). The final volume of each sample was 0.87 ml. For controls, 0.2 ml was withdrawn from each sample. To start the reaction 0.2 ml of a solution containing 0.5 unit of propionyl-CoA carboxylase/ml was added. This gave final volumes of 0.87 ml containing 0.58 μmol of propionyl-CoA (citrate synthase) and 0.6 μmol of propionyl-CoA (propionyl-CoA carboxylase). The experiment was run at room temperature. At 55 min, samples were withdrawn containing 0.35 ml; they were frozen and the reaction was allowed to continue until 165 min. All samples were then freeze-dried, and samples of the distillate counted for radioactivity.

Source of $[2\text{-}^3\text{H}]\text{propionyl-CoA}$	Reaction time (min)	^3H released (%)	Reaction (%)
Propionyl-CoA carboxylase	55	76	80
	165	77	94
Citrate synthase	55	2.5	72
	165	5.8	72

CoA molecules were converted into methylmalonyl-CoA with the carboxylase and release of ^3H to the medium was measured. The ^3H -labelled propionyl-CoA prepared by incubation with the carboxylase lost 77% of its ^3H to the medium, whereas the ^3H -labelled propionyl-CoA sample prepared from exchange on citrate synthase lost only 6% of its ^3H (Table 3). Thus the rapid proton exchange of the α -protons of propionyl-CoA on citrate synthase with oxaloacetate involves the *pro-S*-proton, which is the same proton as that removed in the condensation step leading to methylcitrate.

Clearly some step in the enzyme-catalysed reaction pathway subsequent to α -proton removal from propionyl-CoA is rate-limiting in the production of methylcitrate, since both α -proton-exchange velocities are much larger than V_{max} . The observation that the α -proton that exchanges with the higher velocity is the same proton that is removed in the condensation reaction may just be coincidental, or, on the other hand, may be a reflection of the stereochemical constraints in the rate-limiting step on proton abstraction.

We believe that a simple explanation of attack by propionyl-CoA on the *re* face of oxaloacetate would be that oxaloacetate can bind in productive ternary complexes with either its *re* or *si* face exposed to the incoming carbanion, by a 180° rotation for example. The unexpected increased stereoselectivity with increasing temperature would then reflect relative stabilization of one of these complexes over the other. Consistent with this argument are the observations of Brandänge *et al.* (1977b) that methylcitrate formation is less inhibited by butanedione modification of citrate synthase than is citrate formation, implying that close binding of oxaloacetate to the enzyme occurs only during citrate formation.

The above results indicate that the stereochemical course of citrate synthase catalysis is extremely sensitive to small changes in the substrate structure and suggest that differences in enzyme conformation affecting the ternary complex structure could play an important role in the proportion of diastereoisomeric methylcitrate produced from propionyl-CoA and oxaloacetate. Conceivably the citrate synthase/oxaloacetate/propionyl-CoA system will be useful for investigations of various proposed conformational effectors (e.g. ATP, succinyl-CoA) of the enzyme (Srere, 1974).

Beach *et al.* (1977) have concluded that none of the methylcitrate diastereoisomers is either a substrate for or an inhibitor of aconitase. Our observations on the tight binding of propionyl-CoA to the enzyme-oxaloacetate complex and on the kinetics of methylcitrate formation indicate that propionyl-CoA could function as an effective competitive inhibitor of citrate formation in mitochondria. The slowing of the citric acid cycle proposed by Ando *et al.* (1972) could thus be accomplished without involving inhibitory properties for methylcitrate.

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