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Characterisation of the Citrate Synthase Reaction with Propionyl-CoA

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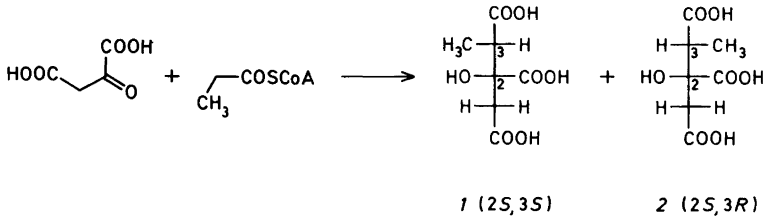
Experiments with propionyl-CoA stereoselectively deuteriated in the propionyl moiety demonstrate that the formation of (2*S*,3*S*)-methylcitric acid (*1*) catalysed by citrate (*si*)-synthase occurs with inversion of configuration in the propionyl moiety; the absolute configurations of the methylcitric acids *1* and *2* indicate a *si* attack on oxaloacetate. Deuterium in the *pro-S* position is exchanged for protium 60 times faster than deuterium in the *pro-R* position. Experiments with (*R*,*S*)-(2-$^2H_1$)propionyl–CoA * allowed the determination of isotope effects. For the enzymatic formation of *1*, a primary deuterium isotope effect $k_H/k_D=1.8$ and a secondary α-deuterium isotope effect $k_H/k_D=0.99$ were calculated; both are effects on V_{max}/K_M.

Propionyl–CoA reacts with oxaloacetate in a side reaction catalysed by citrate (*si*)-synthase (EC 4.1.3.7) to form two stereoisomeric methylcitric acids, *1* (2*S*,3*S*) and *2* (2*S*,3*R*) (Scheme 1); an erroneous configuration was initially ascribed to the latter isomer.[1,2] Normally the levels of *1* and *2* are low in the human blood but in the case of certain metabolic disorders *e.g.* propionic acidaemia, which are characterized by a reduced ability to metabolise propionate, elevated levels of methylcitrate are found.[3] Weidman and Drysdale followed the loss of tritium from two different samples of stereoselectively tritiated propionyl–CoA, which occurs in the presence of citrate synthase and oxaloacetate.[4] They showed that tritium in the *pro-S*-position * is exchanged for deuterium in D_2O 15 times faster than tritium in the *pro-R* position. The exchange reactions are over 1000 and 100 times faster respectively, than V_{max} for the condensation to methylcitrate.[4] We have studied this reaction using stable isotope labelling and analysis by mass spectrometry. While Weidman and Drysdale[4] analyzed the label of propionyl–CoA, we have also analyzed the label of the two methylcitric acids.

* All stereochemical specifications regarding labelled species of propionyl–CoA concern the propionyl moiety.



Scheme 1. Configurations of the methylcitric acids formed in the citrate synthase reaction with propionyl–CoA.

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Optically active monodeuteriated propionic acids were synthesized from ethyl (*S*)-lactate as shown in Scheme 2; (*R,S*)-(2-$^2H_1$)propionic acid was also prepared. After conversion into the anhydrides and subsequent reaction with coenzyme A, the three acids gave deuteriated analogues of propionyl–CoA. The optical purities of the CoA thiolesters of (*R*)- and (*S*)-[2-$^2H_1$]propionic acids were 96 and 89 % respectively, *i.e.* higher than for previously prepared samples;[5] the amounts of nondeuteriated material were 3 and 7 % respectively. The three CoA esters were incubated with citrate synthase and oxaloacetate in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) in order to inhibit the reverse reaction. The deuterium content of the remaining propionyl–CoA was determined at different times by derivatisation with aniline and MS analysis of the resulting propioanilide. The deuterium contents of methylcitric acids were measured by selected ion recording–EI–MS of the trimethyl esters using the intensities of the peaks at m/z=189 and 190 ($M^+-COOCH_3$). The molecular ion group was too weak to be useful and the base peak (m/z=157) group was unsuitable because it contained an isotope distribution which differed from that of the m/z=189 group. Evidently the loss of CH_3OH which occurs in the degradation m/z 189→157 is accompanied by a loss of CH_3OD.

RESULTS AND DISCUSSION

The loss of deuterium from propionyl–CoA during the course of its reaction with citrate synthase and oxaloacetate is shown in Fig. 1. An experiment with (*S*)–(2-$^2H_1$)propionyl–CoA gave k=4.0±0.9 h^{-1} (n=3) for the pseudo first-order rate constant for the deuterium–protium exchange in the *pro-S* position of propionyl–CoA; experiments with (*R*)-(2-$^2H_1$)- and (*R,S*)-(2-$^2H_1$)propionyl–CoA gave k=0.069±0.009 h^{-1} (n=7) for the exchange in the *pro-R* position. This ratio of 60 between the rates is considerably higher than that for the corresponding exchange of tritium for deuterium, which is 15.[4] As pointed out by Weidman and Drysdale,[4] a rate difference of this magnitude is compatible only with a retention of configuration in the exchange reaction.

From the results shown in Fig. 2 it is clear that the *pro-S* hydrogen of propionyl–CoA is selectively abstracted during the enzymatic synthesis of *1*. Considering the absolute configuration of *1*, it is therefore evident that the synthesis occurs with inversion of configuration in the propionyl moiety and probably also with *si* attack on oxaloacetate. The absolute configurations of *1*, *2* or fluorocitric acid do not give any information about the *re*/*si* stereochemistry of the addition to oxaloacetate unless it is known that no inversion of configuration occurs during the later stage of the synthesis (Ref. 13, note 8). Such an inversion has only been excluded for the synthesis of citric acid[7] itself, but not for fluorocitric or methylcitric acids. However, we tentatively assume that the syntheses of these latter acids are analogous.

The above results are analogous to those found for acetyl–CoA[6–10] and fluoroacetyl–CoA.[11–13] The deuterium contents found for *2* were not

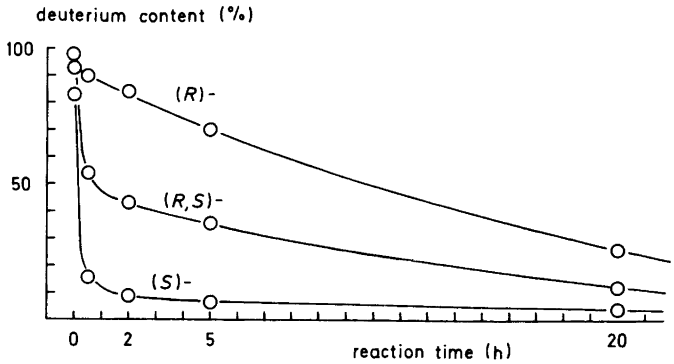


Fig. 1. Time course of the loss of deuterium from coenzyme-A thiol esters of (*R*)-, (*R,S*)-, and (*S*)-[2-$^2H_1$]propionic acid which occurs in the presence of citrate (*si*)-synthase and oxaloacetate.

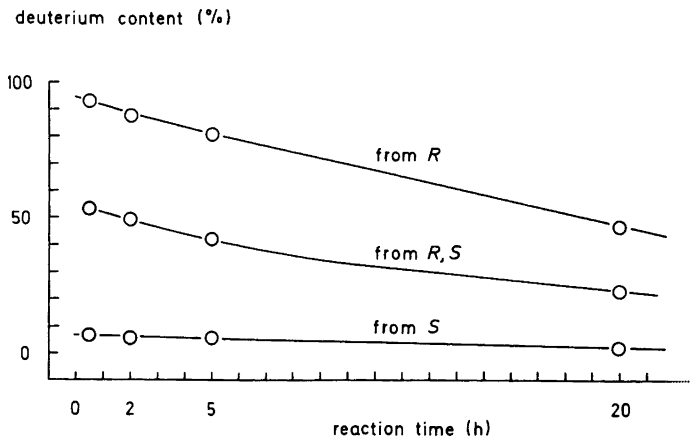


Fig. 2. Deuterium contents of *1* formed from (*R*)-, (*R,S*)-, and (*S*)-[2-$^2H_1$]propionyl–CoA.

reproducible, possibly owing to incomplete separation on GLC.

A virtually full retention of label is obtained in the formation of *1* during the initial phase of the synthesis (Fig. 2); owing to the isotope exchange reaction of propionyl–CoA the deuterium content of the methylcitrate formed gradually decreases. The high retention of label shows that the exchange of propionyl–CoA molecules between the solution and the active site of citrate synthase must be much faster than the isotope exchange reaction which presumably takes place at the active site. In the enzymatic synthesis of a particular stereoisomer of methylcitrate, say *1*, the ratio of the rate of formation of *1*-d_0 to that of *1*-d_1 from propionyl-CoA partially monodeuteriated in the nonreacting *pro-R* position should at any given moment be equal to $(k_H/k_D)\times$ [propionyl-d_0–CoA]/[propionyl-d_1–CoA], where k_H/k_D is a secondary deuterium isotope effect. Since the reaction involves an intermolecular competition between labelled and nonlabelled propionyl–CoA, the isotope effect is an effect on V_{max}/K_M.[14] For a time interval t_1–t_2 it seems reasonable to assume that, if the formation of *1* is linear with time, the ratio of nondeuteriated to deuteriated *1* formed during this interval can be calculated from eqn. (1), in which *a* is the fraction of deuterium label in propionyl-CoA at time t_1, *k* is the rate constant for the exchange of this deuterium for protium under the actual conditions, and k_H/k_D is the secondary deuterium isotope effect.

$$\frac{1\text{-}d_0}{1\text{-}d_1} = \frac{\int_{t_1}^{t_2} [1-a \exp(-kt)]dt}{\int_{t_1}^{t_2} a \exp(-kt)dt} \times k_H/k_D \qquad (1)$$

If (*S*)-[2-$^2H_1$]propionyl–CoA, which has deuterium in its reacting *pro-S* position, is added to the reaction mixture it becomes necessary to account for the new possibilities of forming *1*-d_0, either from the deuteriated propionyl–CoA itself or from propionyl-d_0–CoA formed from it in the isotope exchange reaction. In the new term accounting for the reaction with (*S*)-[2-$^2H_1$]propionyl–CoA, the primary deuterium isotope effect must be considered, and this, like the secondary isotope effect, is an effect on V_{max}/K_M. Although the formation of methylcitrate may alter the composition of the mixture of different species of propionyl–CoA, the yield of *1+2* after 20 h reaction is $\lesssim$5 % under the conditions used and the alteration should therefore be negligible.

Calculation of isotope effects. Studies of the reaction with (*R,S*)-(2-$^2H_1$)propionyl–CoA make it possible to calculate both a primary and a secondary isotope effect for the condensation leading to *1*. In a single experiment with this substrate, we determined the rate of the slow isotope exchange reaction as well as the deuterium contents of *1*. Measurements after 2 and 5 h reaction both gave the rate constant k=0.060 h^{-1}. The rate of the fast exchange reaction was assumed to be the same as that in a parallel experiment performed under the same condi-

tions: $k=4.0\ h^{-1}$. Three different species of propionyl–CoA served as substrate: propionyl-d_0–CoA, (*R*)-, and (*S*)-propionyl-d_1–CoA; the initial mole fractions were 0.03, 0.485 and 0.485 respectively. After 0.5 and 5 h reaction, the deuterium contents of *1* formed were 53 and 42 % respectively (Fig. 2). Consideration of the different species of propionyl–CoA which can give rise to *1*-d_0 and *1*-d_1, respectively and calculation of areas according to the preceding paragraph, setting $k_H/k_D=1/x$ and $k_H/k_D=1/y$ for the primary and secondary isotope effects, respectively, lead to the expressions:

$$\frac{47}{53}=\frac{0.015+0.007+0.105x+0.137}{0.235y}$$

$$\frac{58}{42}=\frac{0.150+0.360+0.121x+2.304}{2.065y}$$

which apply to the results at times $t=0.5$ and 5 h, respectively. The first term in the numerators comes from the reaction with propionyl-d_0-CoA present from the beginning, the second and fourth terms from the reaction with propionyl-d_0-CoA formed from (*R*)- and (*S*)-propionyl-d_1–CoA respectively during the progress of the reaction, and the third term from the reaction with (*S*)–propionyl-d_1–CoA; the denominators come from the reaction with (*R*)–propionyl-d_1–CoA. From these equations it is found that $1/x=1.8\pm0.3$, *i.e.* the primary isotope effect, and $1/y=0.99\pm0.03$, the secondary isotope effect. The error limits indicate the results of calculations based on estimated errors of ±1 % in the MS analyses. However, the total error limits are probably wider because of the low accuracy in the determination of the rate constants.

Previously reported deuterium isotope effects for citrate synthase mediated reactions with labelled acetyl-CoA represent combinations of primary and secondary effects. Kosicki and Srere[15] found $k_H/k_D=1.4$ from (approximate) V_{max} values for the separate reactions with acetyl–CoA and trideuterioacetyl–CoA. Cornforth and co-workers[16] calculated an intramolecular effect of $k_H/k_D=1.94$ for the reaction with the coenzyme A thiol ester of [2-$^2H_1$,$^3H_1$]-acetic acid. For the same reaction Klinman and Rose[17] gave the value 3.0; a different interpretation of their results leads, however, to the result $k_H/k_D=1.9$.

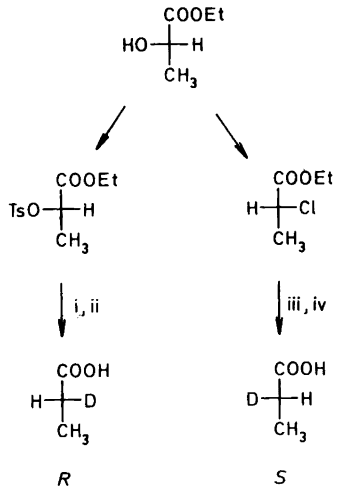
The primary deuterium isotope effect obtained for propionyl–CoA in this work is thus similar to previously found effects for acetyl–CoA, suggesting that the reaction steps which involve removal of an α hydrogen are partially rate-limiting to about the same extent. It should be remembered, however, that the difference in K_M between labelled and non-labelled substrates may not be negligible. Thus the value for K_M of acetyl–CoA is twice that of trideuterioacetyl–CoA in the citrate synthase reaction.[15] The fact that the secondary isotope effect is close to unity shows that the differences between protium and deuterium in size, electronegativity and bond bending frequency do not affect the citrate synthase reaction with propionyl–CoA to any significant extent.

EXPERIMENTAL

Propioanilides and trimethyl methylcitrates were analyzed by GLC–EI–MS–SIR (selected ion recording) using a UCON HB 5100 wall-coated capillary column (20 m) mounted in a Finnigan 4000 gas chromatograph-mass spectrometer. A complete separation of the diastereomeric trimethyl esters was obtained. Analyses were run in the splitless mode with temperature programming (110–170 °C; 10 °C/min). Optical rotations were measured on a Perkin-Elmer 241 polarimeter. The isotopic purity of D_2O was 99.7 %.

Synthesis of propioanilides. Deuterium-labelled propionic acids were converted into sodium salts as previously described[18] and these were treated with oxalyl chloride (0.55 mol equiv.) in benzene (25 °C, 15 h) to obtain the anhydride, which was distilled at 1.3 kPa. The anhydride (20–30 mg) and aniline (0.5 ml) were heated under N_2 atmosphere (100 °C, 1 h). After the solution had been cooled, 4 M hydrochloric acid was added to a final acidity of about pH 1 and the propioanilide was extracted with methylene chloride (3 ml). The organic layer was washed with water and finally with sodium hydrogen carbonate solution.

(R)-*[2-$^2H_1$]propionic acid.* The sodium salt was prepared (Scheme 2) as previously described[18] and showed $[\alpha]_{365}^{20}-3.19°$ (*c* 1.9, H_2O) which is 88.4 % of the calculated[19] maximum value of −3.61°. The anhydride showed α_D^{25} −1.81°; $\alpha_{365}^{25}-7.75°$ (neat). The propioanilide contained (MS) 2.9 % of non-deuteriated mate-



Scheme 2. Synthetic routes to (*R*)- and (*S*)-[2-$^2H_1$]propionic acids. Reagents: i, $NaBD_4$; ii, NaOH; iii, $LiAlD_4$; iv, CrO_3.

rial and should therefore be 92.8 % *R*, 4.3 % *S* and 2.9 % d_0.

(S)-*[2-$^2H_1$]propionic acid.* Reduction of ethyl (*R*)-2-chloropropionate[20] with lithium aluminium deuteride was carried out as being analogous to the reduction of ethyl (*S*)-2-tosyloxypropionate.[18] No trideuterio-2-propanol could be detected (GLC) in the crude reaction mixture (*cf.* Ref. 18). Spinning-band distillation gave (1,1,2-$^2H_3$)-1-propanol (65 %; GLC purity >99 %); $\alpha_D^{25.0}$ −0.010°; $\alpha_{365}^{25.0}$ −0.156° (neat). Oxidation with Pt/O_2 in aqueous sodium hydrogen carbonate (23 °C, 6 d) and work-up as previously described[18] afforded sodium (*S*)-[2-$^2H_1$]-propionate (88 %); $[\alpha]_D^{25.0}$ +0.72°; $[\alpha]_{365}^{25.0}$ +2.57° (*c* 8.4, water), *i.e.* 72.8 % of the calculated maximum[19] value. The propioanilide contained (MS) 7.2 % of non-deuteriated material and should thus contain 82.8 % *S* and 10.0 % *R* isomer.

(R,S)-*(2-$^2H_1$)propionic acid* was prepared from ethyl 2-bromopropionate, zinc dust and D_2O-DCl in THF (*cf.* Ref. 21 for a similar method), followed by alkaline hydrolysis (1 M NaOH, 2 h, 65 °C). After acidification, the acid was extracted with ether and converted into the propioanilide which showed (MS) 97.0 % d_1, 3.0 % d_0.

Enzymatic experiments. Reaction mixtures contained oxaloacetate (4.7 mM), propionyl–CoA (3.5 mM) prepared as described,[23] 5,5'-dithiobis-(2-nitrobenzoic acid) (6.7 mM) and pig heart citrate synthase (Boehringer, 1.5 μN) in a final volume of 4.2 ml 0.1 M Tris · HCl, pH 7.7. The mixtures were kept at 22 °C. Aliquots (≈1 ml) were withdrawn and the reaction stopped by the addition of 50 μl of perchloric acid (70 % in water). After filtration, the sample was stored at −18 °C overnight and then thawed to a 0 °C solution. Part (≈3/4) of the solution was acidified with 1 M hydrochloric acid to pH≈1, washed with ether (2×1 ml) and freeze-dried. Esterification with ethereal diazomethane, filtration and concentration gave samples of trimethyl methylcitrate. The remaining part (≈1/4) was freeze-dried and allowed to react with aniline as described above to obtain propioanilide.

Calculation of deuterium contents. A SIR–MS of unlabelled propioanilide was recorded for reference purposes on the same occasion as the measurements on the labelled samples and the actual relative intensities within the molecular ion group were used in the calculation of deuterium contents. Typical relative intensities are: *m/z* 150 (8.6 %), 149 (87.9 %) and 148 (3.5 %). It is assumed that these relative intensities are also valid for isotopically pure (2-$^2H_1$)propioanilide. In the MS analysis of mixtures of propioanilide and (2-$^2H_1$)propioanilide, the mole fraction x of deuteriated propioanilide was calculated according to the equation:

$$\frac{\text{abundance } m/z \text{ 150}}{\text{abundance } m/z \text{ 149}} = \frac{87.9x+8.6(1-x)}{87.9(1-x)+3.5x}$$

Deuterium contents of trimethyl methylcitrates were calculated in a similar manner. The unlabelled ester usually showed the following relative intensities: *m/z* 190 (7.6 %), 189 (88.4 %) and 188 (2.9 %).

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