Stereoselectivities in Enzymatic Syntheses of Fluorocitric Acid

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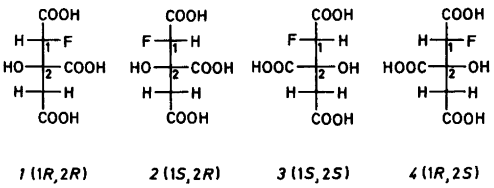

Fig. 1. Absolute configurations of the fluorocitric acids formed in reactions catalyzed by citrate (*si*)-synthase. The reaction with fluoroacetyl-CoA yields *1* together with a diastereomeric minor product (2 −3% of *1*) which is probably *2*. The reaction with fluorooxaloacetate yields *3* and *4* in the ratio 35:65.

Citrate (*si*)-synthase (EC 4.1.3.7) catalyzes the biosynthesis of citric acid from acetyl-CoA and oxaloacetate. The enzyme can also accept fluoroacetyl-CoA as a substrate and a synthesis of toxic fluorocitric acid occurs when fluoroacetic acid, or a compound which can be biologically degraded to it, is administered to living organisms.[1,2] Only one stereoisomer of fluorocitric acid has been detected in *in vitro* experiments and the absolute configuration 1*R*,2*R* * (*1*), proposed by Carrell *et al.*,[3] has recently been proved.[4,5] The use of fluorooxaloacetate, which is also accepted as a substrate by citrate synthase, offers another possibility of obtaining fluorocitric acid by an enzymatic synthesis.[6] Initially only one stereoisomer was found in this reaction, but a recent investigation revealed that two diastereomeric forms of fluorocitric acid are produced in the approximate ratio of 2:1.[7] The initially detected isomer was found to be enantiomeric with the acid formed in the reaction with fluoroacetyl-CoA.[1]

We have reinvestigated these two enzymatic syntheses of fluorocitric acid. The crude reaction mixture obtained in the reaction with fluoroacetyl-CoA was acidified and treated with diazomethane and the mixture of trimethyl esters was analyzed by capillary column GLC. At the retention time of authentic[5] trimethyl (1*R*,2*S*)-fluorocitrate there was a small peak which was not found in a control experiment without enzyme. The trimethyl ester of the previously known product *1* (1*R*,2*R*) was eluted later. In six separate experiments the minor component amounted to 2−3% of the major product *1*. In the mass spectra of diastereomeric trimethyl fluorocitrates the peaks at $m/z = 101$, 160, 161 and 162 show approximately the same relative intensity for the two compounds (37, 0.3, 100 and 7%, respectively).[5] If the minor component is a trimethyl fluorocitrate, the ratio of the intensity for this compound at any of these four m/z values to the intensity at the same m/z value in the mass spectrum of the trimethyl ester of *1* should be constant. After purification of the sample by HPLC, a GLC-MS-SIR (selected ion recording) investigation was undertaken at $m/z = 101$, 160, 161 and 162 and a good constancy in the intensity was obtained: 18, 19, 19 and 18% at the four m/z values, respectively. The GLC, HPLC and MS characteristics of this minor component, taken together with the fact that it is formed enzymatically, strongly indicate that it is a fluorocitric acid belonging to the 1*RS*,2*SR* racemic pair. For mechanistic reasons it is probable that the minor isomer and *1* are epimeric at the fluorine-bearing carbon and that the former thus has the 1*S*,2*R* configuration (*2*).

There are two main ways in which the minor isomer *2* may be formed: directly in the enzymatic synthesis or by non-enzymatic epimerization of *1*, either in the reaction mixture or during work-up or analysis. The reverse epimerization *2*→*1* is excluded since deuterium is retained in *1* formed from (2*R*)-[2-$^2H_1$]fluoroacetyl-CoA[7] but should be lost during epimerization. No increase of *2* was observed after a tenfold prolongation of the reaction time; the weakly alkaline conditions thus caused no epimerization. No formation of diastereomeric acid was seen after a prolonged treatment of (1*S*,2*S*)-fluorocitric acid with stronger hydrochloric acid than that used in the work-up of the enzymatic synthesis. Since authentic trimethyl (1*S*,2*S*)-fluorocitrate on GLC analysis showed the presence of less than 0.2% of diastereomeric ester, no significant epimerization occurs during GLC analysis. We therefore conclude that *2* is formed in the enzymatic synthesis rather than by a non-enzymatic epimerization of *1*.

A reinvestigation of the citrate synthase mediated synthesis of fluorocitric acid from fluorooxaloacetate has also been carried out and, in agreement with previous findings,[7] two diastereomeric acids were detected in the ratio 35:65. These acids were isolated and characterized as

* The numbering is that of 2-hydroxy-1,2,3-alkanetricarboxylic acids.

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molybdate(VI) complexes by CD spectroscopy. Comparison with spectra of reference acids of known configuration[5] gave the absolute configurations of the enzymatically formed fluorocitric acids as 1*S*,2*S* (*3*, minor isomer) and 1*R*,2*S* (*4*, major isomer). The fluorocitric acids *3* and *4* are those expected[7] to be formed in the reaction with fluorooxaloacetate if acetyl-CoA attacks only the *re* face of the fluorooxaloacetate keto carbonyl group (corresponding to the *si* face in oxaloacetate), if both (*R*)- and (*S*)-fluorooxaloacetate can react, and if no inversion of configuration at C-2[8] occurs later in the synthesis. Evidently the reaction with the *R* isomer is the more easy one.

The absolute configuration of *1*,[4,5] combined with the finding[7] that the enzyme selectively abstracts the *pro-S* hydrogen of the fluoroacetyl group during the synthesis of *1*, establishes that an inversion of configuration occurs in the fluoroacetyl group in the synthesis. An inversion also occurs in the reaction with acetyl-CoA.[9,10] The detection of a second isomer of fluorocitric acid (probably *2*) means that the *pro-S* selectivity is only partial (97–98 %), provided that an inversion also occurs in the formation of *2*. A remarkable difference is seen between fluoroacetyl-CoA and propionyl-CoA with regard to their *pro-S*/*pro-R* selectivities. The *pro-S* hydrogen in fluoroacetyl-CoA is the more reactive, but the stereochemically analogous (*pro-R*) hydrogen in propionyl-CoA is the *least* reactive by a factor of about 60.[11,12] The detailed interpretation of this effect requires a knowledge of the topological features of the active site of citrate (*si*)-synthase; studies of the enzyme are being pursued by X-ray methods.[13]

Experimental. GLC of trimethyl fluorocitrates was performed using a Carbowax 20 M fused silica capillary column (50 m × 0.2 mm) mounted in a Hewlett-Packard 5830 A gas chromatograph (electronic integration system) run in the splitless mode (90–190 °C, 20 °C/min). The retention times of the 1*RS*,2*SR* and 1*RS*,2*RS* stereoisomers (17.86 and 18.46 min, respectively) represent a base-line resolution. GLC-EI-MS-SIR (selected ion recording) was performed using the same column mounted in a Finnigan 4000 instrument. HPLC separations of the isomeric trimethyl esters were carried out on a silica gel column (Partisil 10, Reeve Angel, 25 cm × 4.6 mm) with 2,2,4-trimethylpentane–ethyl acetate (5:2) as eluant and using RI-detection. Under the conditions used, the retention times were 6.0 and 7.2 min for the 1*RS*,2*SR* and 1*RS*,2*RS* isomers, respectively, also this representing a base-line resolution. ^{1}H NMR spectra were recorded on a JEOL JNM-FX 100 spectrometer.

Diethyl fluorooxaloacetate was synthesized according to a method given for the difluoro analogue.[14] After distillation (b.p. 78 °C at approximately 100 Pa) it was obtained in a 25 % yield and in a purity exceeding 99 % (GLC, NMR). ^{1}H NMR ($CDCl_3$, TMS): δ 5.94 (d, C*H*F, *J* 47.4 Hz), 4.40 (2H,q), 4.33 (2H,q), 1.40 (3H,t), 1.32 (3H,t).

Fluorooxaloacetic acid. The diethyl ester was hydrolyzed with acetic acid–hydrochloric acid for 25 days at +5 °C.[15] After freeze-drying, the acid was crystallized as in Ref. 16 but working in the temperature interval +20 to −20 °C; m.p. 87 −92 °C (decomp.); lit. m.p. 86–87 °C[15] and 82 −85 °C (decomp.).[17] In the ^{1}H NMR spectrum (D_2O, sodium 2,2-dimethyl-2-silapentane-5-sulfonate as reference) the title compound gave a doublet (J_{HF} 46.9 Hz) at δ 5.26 (*cf.* Ref. 17). Unhydrolyzed ester groups were present to the extent of approximately 9 mol % and only a minute amount (≈ 1 %) of fluoropyruvic acid was present, δ 4.52 (d, J_{HF} 47 Hz) (*cf.* Ref. 18).

Enzymatic synthesis with fluorooxaloacetate. The reaction mixture (11 ml) contained acetyl-CoA (10 mM), fluorooxaloacetate (16 mM), 5,5′-dithiobis(2-nitrobenzoic acid) (17 mM) and pig heart citrate synthase (Boehringer, 50 μg/ml) in 0.05 M Tris-HCl, pH 8.0. The mixture was kept overnight at 23 °C, acidified to pH ≈ 1 with 2 M hydrochloric acid and washed twice with ether to remove 5-nitro-3-thiolobenzoic acid and its corresponding disulfide. Concentration of the aqueous layer to near dryness at 30 °C, addition of excess ethereal diazomethane, drying with $MgSO_4$, filtration and concentration gave a crude product (10 mg) which contained the trimethyl esters of *3* and *4* in the ratio 35:65. After purification by HPLC, the esters weighed 2.7 and 5.8 mg, respectively, corresponding to a total yield of 31 %. The ^{1}H NMR spectra were indistinguishable from those of authentic samples.[5] Hydrolysis of the esters and characterization of the acids as molybdate(VI) complexes by CD were performed as described.[5] CD of minor isomer (*3*) (nm, [Θ] × 10^{-4}): 275, +0.95; 250, −1.2; 234, +1.2; 218, −1.3. CD of major isomer (*4*): 274, +0.70; 250, −0.77; 235, +0.69; 218, −1.4.

Enzymatic synthesis with fluoroacetyl–CoA. Fluoroacetic anhydride (20 μl) was dissolved in tetrahydrofuran (1.5 ml) and part of the solution (200 μl) was added to coenzyme A (14 μmol) in 1 % aqueous $NaHCO_3$ (1 ml). After 40 min at 0 °C, this solution of fluoroacetyl-CoA was transferred in 10 portions during 1 h to a mixture (23 °C) containing oxaloacetic acid (21 μmol), pig heart citrate synthase (Boehringer, 125 μg/ml) and 5,5′-dithiobis(2-nitrobenzoic acid) (21 μmol) in 0.1 M Tris–HCl (pH 7.8, 3.15 ml). The final pH 7.0 was reached after 15 h. Work-up and analysis were performed as above.

When more enzyme was used (360 μg/ml) a higher yield (≈ 20 %) and a purer product was obtained. The relative amount of the minor component was, however, unchanged (2–3 % of *1*).

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