Stereochemical Course of Malonate Decarboxylation in *Malonomonas rubra*

Jason Micklefield,† Keith J. Harris,† Stefan Gröger,† Ursula Mocek,† Hubert Hilbi,‡ Peter Dimroth,‡ and Heinz G. Floss*,†

Department of Chemistry BG-10, University of Washington Seattle, Washington 98195
Mikrobiologisches Institut
Eidgenössische Technische Hochschule
ETH-Zentrum, CH-8092, Zürich, Switzerland

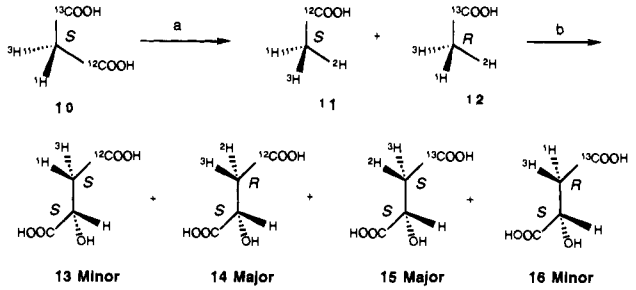
Received September 12, 1994

Malonomonas rubra can grow anaerobically on malonate as its source of carbon and energy.[1] A malonate decarboxylase in this organism decarboxylates malonate without the intermediate formation of malonyl-CoA. Studies[2] with crude extracts from *M. rubra* showed that the decarboxylase is a biotin-containing acetyl enzyme. It was postulated that malonate exchanges with the enzyme-bound acetyl group to give an enzyme-bound malonyl thioester which is decarboxylated, regenerating the acetyl enzyme. In this communication we report on the steric course of this decarboxylation.

The decarboxylation of malonate involves the conversion of a pro-prochiral molecule of the Caabb type[3] into another pro-prochiral molecule of the Caaab type; its stereochemical analysis requires the use of three different isotopic labels. Our general strategy involved decarboxylating the *R* or *S* isomer of chiral malonate, carrying ^{13}C in one carboxyl group and ^{3}H at one of the methylene positions, in $^{2}H_2O$ to give a pseudoracemate of chiral acetate in which one enantiomer contains ^{13}C and the other ^{12}C in the carboxyl group. Which enantiomer contains ^{13}C and which one ^{12}C can be determined by ^{3}H-NMR analysis upon conversion of the acetate sample into malate with the malate synthase system[4] (Scheme 1). The synthesis of the substrates (Scheme 2) involved preparation of (2*R*,3*S*)- and (2*S*,3*R*)-[1,4-$^{13}C_2$,3-$^{3}H_1$]malate **7** and **8**,[11] which served as configurationally stable precursors of (*R*)- and (*S*)-[1-^{13}C,2-^{3}H]-malonate **9** and **10**.[12,13]

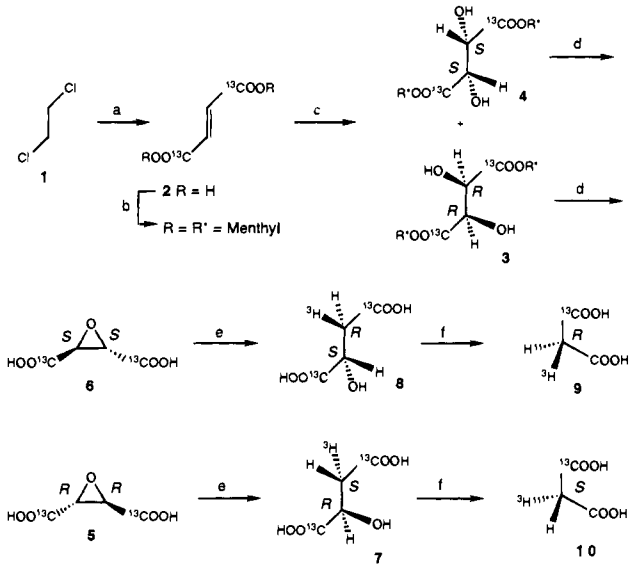
Immediately before the enzyme incubation a mixture of the potassium salt of (2*R*,3*S*)-[1,4-$^{13}C_2$,3-$^{3}H_1$]malate **7** and carrier (*R*)-malate (13.5 mg, 60 mCi) was oxidized with 1.58 equiv of potassium permanganate in 0.2 mL of deuterium oxide. After 5 min the mixture was filtered through a Celite/Chelex 100 (Bio-Rad)/charcoal column, to remove residual $KMnO_4$ and MnO_2, giving (*S*)-[1-^{13}C,2-^{3}H]malonate **10**.[14] An extract of *M. rubra* (1.0 mL) prepared in $^{2}H_2O$ phosphate buffer[16] was added to **10** in the $^{2}H_2O$ solution. This mixture was incubated at 30 °C for 20 min and quenched with dilute H_2SO_4, and the resultant acetic acid was isolated by lyophilization. The acetic acid was converted enzymatically to acetyl-CoA and incubated with malate synthase and glyoxylic acid to give malate with inversion of configuration,[4] which was subjected to ^{3}H-NMR analysis (320 MHz, ^{1}H and ^{2}H decoupled).

Scheme 1[a]



[a] (a) Malonate decarboxylase from *M. rubra*, in D_2O. Retention assumed. (b) (i) Acetate kinase, ATP. (ii) Phosphotransacetylase, CoASH. (iii) Glyoxylic acid, malate synthase.

Scheme 2[a]



[a] (a) (i) 18-Crown-6 ether, $K^{13}CN$, CH_3CN. (ii) KOH, MeOH, H_2O. (iii) Br_2, HBr. (iv) NaI, acetone.[5,6] (b) (−)-Menthol, H^+. (c) (i) OsO_4. (ii) Silica gel chromatography. (d) (i) HBr, acetic acid. (ii) Potassium menthoxide,[7] THF. (iii) KOH.[8,9] (e) $LiEt_3B^3H$.[10] (f) $KHCO_3$, $KMnO_4$, D_2O.

* Address correspondence to this author at the University of Washington.
† University of Washington.
‡ ETH Zürich.

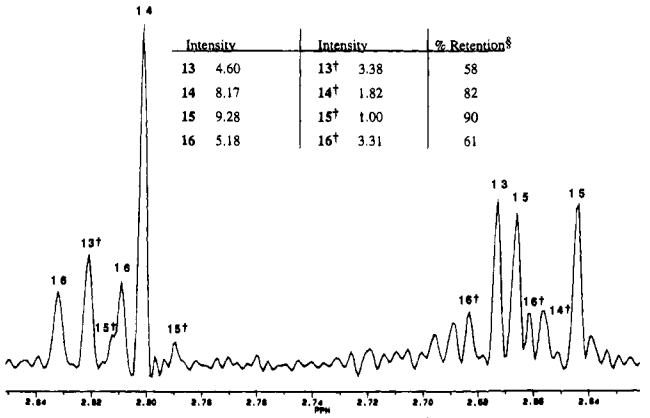
(1) Dehning, I.; Schink, B. *Arch. Mikrobiol.* **1989**, *151*, 427.
(2) Hilbi, H.; Dehning, I.; Shink, B.; Dimroth, P. *Eur. J. Biochem.* **1992**, *207*, 117.
(3) Floss, H. G.; Tsai, M. D.; Woodard, R. W. *Top. Stereochem.* **1984**, *15*, 253.
(4) Cornforth, J. W.; Redmond, J. W.; Eggerer, H.; Buckel, W.; Gutschow, C. *Nature* **1969**, *221*, 1212. Lüthey, J.; Rétey, J.; Arigoni, D. *Nature* **1969**, *221*, 1213.
(5) Jordan, P. M.; Spencer, J. B. *Tetrahedron* **1991**, *47*, 6015.
(6) Nystrom, R. F.; Loo, Y. H.; Leak, J. C. *J. Am. Chem. Soc.* **1952**, *74*, 3434.
(7) Mori, K.; Iwasawa, H. *Tetrahedron* **1980**, *36*, 87.
(8) Mattingley, P. G.; Miller, M. J.; Cooper, R. D. G.; Daugherty, J. *J. Org. Chem.* **1983**, *48*, 3556.
(9) Optical rotations: **5**, $[\alpha]_D = -117.0°$ (*c* 2.6, EtOH); **6**, $[\alpha]_D = 116.4°$ (*c* 2.6, EtOH). The literature value for (2*S*,3*S*)-epoxysuccinic acid is $[\alpha]_D = 117.8°$ (*c* 2.6, EtOH) (Ohhashi, J.; Harada, K. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2977).
(10) Andres, H.; Morimoto, H.; Williams, P. G. *J. Chem. Soc., Chem. Commun.* **1990**, 627.
(11) (2*R*,3*S*)- and (2*S*,3*R*)-[1, 4-$^{13}C_2$,3-$^{3}H_1$]malate were optically pure as determined by ^{3}H-NMR (320 MHz, ^{1}H decoupled: $\delta_{^3H}$ 2.82, dd, $^{2}J_{^{13}C^{3}H} = 7.4$ Hz and $^{3}J_{^{13}C^{3}H} = 4.3$ Hz).
(12) Huang, S.; Beale, J. M.; Keller, P. J.; Floss, H. G. *J. Am. Chem. Soc.* **1986**, *108*, 1100.
(13) Jordan, P. M.; Spencer, J. B.; Corina, D. L. *J. Chem. Soc., Chem. Commun.* **1986**, 911.

(14) A chemical yield of 35% for this oxidation was determined by oxidizing an unlabeled sample of malate under the same conditions and then converting the mixture of malonate, oxalate, and malate obtained, with the addition of a known amount of succinate as a standard, to the corresponding dimethyl esters followed by GC analysis. This yield does not account for any proton exchange of the malonate formed, which is known to be rapid depending upon pH.[15]

(15) (a) Sedgwick, B.; Cornforth, J. W.; French, S. F.; Gray, R. T.; Kelstrup, E.; Willadsen, P. *Eur. J. Biochem.* **1977**, *75*, 481. (b) Huang, S. Ph.D. Thesis, Purdue University, West Lafayette, IN, 1984.

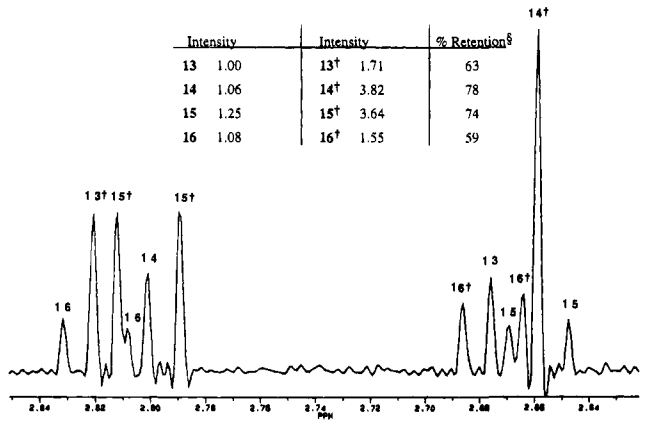
(16) Frozen wet cells (2 g)[2] were washed twice with deuterium oxide (Fluka, 99.8 atom % D) containing 50 mM potassium phosphate (pH 7.5), 0.5 M NaCl, 1 mM $MgCl_2$, 5 mM dithioerythritol, and 0.2 mM diisopropyl fluorophosphate. The pellets were then suspended in 5 mL of the same buffer (except 50 mM NaCl and DNase I were present) and ruptured as before[2] to give 4.3 mL of cell-free extracts (25.6 units/mL).

0002-7863/95/1517-1153$09.00/0



Intensity		Intensity		% Retention§
13	4.60	13†	3.38	58
14	8.17	14†	1.82	82
15	9.28	15†	1.00	90
16	5.18	16†	3.31	61

Figure 1. $^3H\{^1H,^2H\}$-NMR spectra (acetone-d_6) at 320 MHz (Bruker AF-300) of malate (4.6 mCi) derived from (*S*)-malonate **10**. The FIDs were accumulated with 4K data points in an unlocked mode with a pulse angle of 36°, an acquisition time of 0.913 s/FID, and a total time of 17.5 h. The data were processed with a Gaussian multiplication (GB = 0.8 and LB = −1.2) and zero-filled to 16K to enhance the resolution. Inset: Relative intensities of malate species derived from (*S*)-[1-^{13}C, 2-^{3}H]malonate. §Percent retention values for individual isotopomers.



Intensity		Intensity		% Retention§
13	1.00	13†	1.71	63
14	1.06	14†	3.82	78
15	1.25	15†	3.64	74
16	1.08	16†	1.55	59

Figure 2. $^3H\{^1H,^2H\}$-NMR spectra (acetone-d_6) at 320 MHz of malate (7.5 mCi) derived from (*R*)-malonate **9**. The FIDs were accumulated and processed as in Figure 1 (except total acquisition time was 2.7 h and GB = 0.7 and LB = −0.8 for processing). Inset: Relative intensities of malate species derived from (*R*)-[1-^{13}C,2-^{3}H]malonate. §Percent retention values for individual isotopomers.

As shown in Scheme 1, if the decarboxylation of (*S*)-[1-^{13}C, 2-^{3}H]malonate **10** occurs with complete retention of configuration and without proton exchange, then two species of chiral acetate, (*S*)-[2-$^2H_1$,2-^{3}H]- and (*R*)-[1-^{13}C,2-$^2H_1$,2-^{3}H]acetate **11** and **12**, will be formed in equal proportions, which in the malate synthase reaction will produce four isotopomers of tritiated malate, **13**−**16**. The isotopomers **13** and **16** will result from deuterium abstraction from acetates **11** and **12**, respectively, and will be minor products due to the primary kinetic deuterium isotope effect of the reaction ($K_H/K_D = 3.8$[17]). Removal of a proton from **11** and **12** will give **14** and **15**, respectively, which will be major products. Conversely, if the decarboxylation reaction proceeds with inversion of configuration, the ^{13}C will reside in the *S* rather than the *R* isomer of the acetate produced, resulting in the opposite C-3 epimers of malate (**13**†−**16**†)[18] being formed. All eight malate isotopomers can be distinguished by ^{3}H-NMR due to (a) the difference in chemical shift of the two methylene hydrogens,[19] (b) the deuterium isotope shift on tritium resonances in molecules retaining deuterium, and (c) the two-bond ^{13}C−^{3}H coupling observed in molecules carrying ^{13}C.

The ^{3}H-NMR spectrum of the malate obtained from (*S*)-malonate **10** is shown in Figure 1; from this spectrum can be calculated the relative proportions of the four isotopomers (**13**−**16**) and the corresponding diastereoisomers (**13**†−**16**†) (Figure 1 inset). It is evident that the set **13**−**16** predominates, i.e., the decarboxylation reaction proceeds predominantly in a retention mode. The intensity ratio of each diastereomeric pair, e.g., **13**:**13**†, gives a numerical value for the ratio of apparent retention vs inversion in the decarboxylation reaction. The ratios of the deuterated malate species, **14**:**14**† and **15**:**15**†, indicate a high degree, 82−90%, of retention stereochemistry. In all likelihood, the enzymatic decarboxylation is completely stereospecific, and the small degree of apparent inversion observed reflects slight racemization of the substrate malonate **10** during its preparation.[20] The ratios of the nondeuterated malate species, **13**:**13**† and **16**:**16**†, suggest a lower degree of stereospecificity, 58−61% retention, but this is probably an artifact due to several factors, like the presence of small amounts of ^{1}H (no more than 5%) in the ^2H_2O of the decarboxylation reaction mixture, a large solvent isotope effect, and some exchange of the methyl protons in the malate synthase reaction.[21]

The above results were confirmed by carrying out the same experiment with (*R*)-malonate **9** (Figure 2). In this case we expect malates (**13**†−**16**†) to predominate, and this is indeed observed. While the numerical values for the nondeuterated species agree closely with those from the previous experiment, the deuterated species seem to be formed with a slightly lower degree of retention. This parallels a slightly longer reaction time in the preparation of the (*R*)-malonate sample and thus is undoubtedly due to a somewhat higher degree of racemization (to produce HOOC−C^{2}H^{3}H−^{13}COOH) during the generation of the substrate.

In conclusion, the above results demonstrate that the malonate decarboxylase of *M. rubra* decarboxylates malonate with retention of configuration. This agrees with the stereochemistry observed for all other biotin-containing decarboxylases,[22,23] most of which act on CoA esters rather than on the free acids. It suggests that the *M. rubra* malonate decarboxylase may operate by a similar mechanism.

Acknowledgment. We acknowledge with gratitude the services of the National Tritium Labeling Facility, Berkeley (supported by NIH Grant RR 01237), the advice and assistance of Mr. Hiromi Morimoto and Dr. Phil Williams in the preparation of the tritiated malate samples, and the assistance of Mr. A. Gabriel Kastelle in carrying out the malate synthase reactions. Financial support for this work was obtained from the NIH (GM 32333 to H.G.F.) and SERC (NATO postdoctoral fellowship to J.M.).

JA9430208

(17) Lenz, H.; Wunderwald, P.; Buschmeier, V.; Eggerer, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 517. Lenz, H.; Eggerer, H. *Eur. J. Biochem.* **1976**, *65*, 237.

(18) The dagger (†) denotes C-3 stereochemistry opposite to that shown in Scheme 1. For example, **13** has the 2*S*,3*S* and **13**† the 2*S*,3*R* configuration.

(19) δ_H (acetone-d_6) of the *pro*-3*S* hydrogen of (2*S*)-malate is 2.68 ppm and of the *pro*-3*R* hydrogen 2.82 ppm.

(20) $t_{1/2}$ for proton exchange of malonate is 56 min at 30 °C, pH 7.5.[15b]

(21) Eggerer, H.; Klette, A. *Eur. J. Biochem.* **1967**, *1*, 447.

(22) Rétey, J.; Robinson, J. A. *Stereospecificity in organic chemistry and enzymology*; Verlag Chemie: Weinheim, 1982; pp 129−149.

(23) Hoffmann, A.; Dimroth, P. *FEBS Lett.* **1987**, *220*, 121. Dimroth, P. *Eur. J. Biochem.* **1981**, *115*, 353. Buckel, W. *Eur. J. Biochem.* **1986**, *156*, 259.