Biosynthetic Pathway of Fluoroacetate in *Streptomyces cattleya*

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ABSTRACT

The biosynthetic pathway of fluoroacetate in *Streptomyces cattleya* NRRL 8057 was studied by ^{19}F–NMR and tracer experiments with various ^{14}C-labeled compounds. Radioactivity of ^{14}C-labeled glucose, glycerol, pyruvate, L-serine, β-hydroxypyruvate, succinate and L-aspartate was incorporated into fluoroacetate during incubation of them with growing and resting cells of *S. Cattleya* NTG29, a mutant strain overproducing fluoroacetate. Comparison of the ratio of radioactivity incorporated into fluoroacetate from the above compounds to that administered revealed the major biosynthetic pathway of fluoroacetate through β-hydroxypyruvate. Its C_2 and C_3 carbons were efficiently incorporated into fluoroacetate, but no significant incorporation of its C_1 carbon was found. Thus, β-hydroxypyruvate is probably fluorinated by replacement of the hydroxyl group by fluoride anion and then decarboxylated to form fluoroacetate.

INTRODUCTION

Toxic plants such as *Acacia georginae*[1] and *Gastrolobium grandiflorum*[2], and microorganism (e.g. *Streptomyces cattleya*) synthesize fluoroacetate from inorganic fluoride and accumulates in high concentration. The enzymatic system which catalyzes formation of the organic fluoride compound has not yet revealed.

Organic chloride, bromide and iodide compounds are produced by haloperoxidases, but no information is available about enzymes catalyzing incorporation of fluoride into organic molecules. A few reports described only a speculative biosynthetic pathway of fluoroacetate in plants[3]-[5]. The C-F bond may be formed either by addition or nucleophilic substitution with fluoride anion, but in either case fluoride anion must be dissociated from the water molecules tightly solvating. Alternatively, the C-F bond may be formed by addition of fluorine radical to an olefin, but enormous energy is required to produce fluorine radical from fluoride anion.

Sanada et al.[6] showed that *S. cattleya* NRRL 8057, a thienamycin producer, produces fluoroacetate

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and fluorothreonine from fluoride, and that both fluorine compounds are converted to each other during incubation with the mycelia. We here describe that fluoroacetate is synthesized through β-hydroxypyruvate, and then metabolized to fluorothreonine by the organism by means of ^{19}F-NMR and tracer experiments with various ^{14}C-labeled compounds.

MATERIALS AND METHODS

S. cattleya NRRL 8057 was obtained from Northern Utilization Research and Development Division, U. S. Department of Agriculture, and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to generate mutants overproducing fluoroacetate. *S. cattleya* NTG29 was the best producer, which produces about 0.1mg of fluoroacetate per ml of culture broth, and used throughout the experiments. The following growth media were used ; PSM Medium, 1% potato extract, 2% sucrose, 2% malt extract, and 0.058% (10mM) potassium fluoride (pH 6.5) ; K Medium, 2.5% soluble starch, 1.5% soybean flour, and 0.2% yeast extract (pH 6.3).

L-[U-^{14}C], DL-[1-^{14}C] and DL-[3-^{14}C] β-Hydroxypyruvates were prepared from the corresponding [^{14}C] serines by oxidative deamination with D-amino acid oxidase. The L-isomer was converted to the antipode *in situ* by catalysis of amino acid racemase with low substrate specificity[7]. The reaction mixture (0.2ml) containing [^{14}C] serine, 100 mM Tris HCl (pH 7.2), 3 units of D-amino acid oxidase, 5 units of amino acid racemase, and 1.3 units of catalase was incubated at 30°C for 70min, and then applied to a Dowex 50X8 (H$^+$) column (volume, 1 ml). The column was washed with 0.1ml of 100mM Tris HCl (pH 7.2) containing 10 μg of unlabeled β-hydroxypyruvate. The eluate was subjected to preparative HPLC (see below). [U-^{14}C], [3-^{14}C] and [1-^{14}C] β-Hydroxypyruvates were obtained with a yield of 78%, 76%, and 98%, respectively. Radioactivity of β-hydroxypyruvate was adjusted to 330 kBq/mmol by addition of an appropriate amount of cold β-hydroxypyruvate.

A solution containing [2-^{14}C] bromoacetate (9.25MBq ; 2.07GBq/mmol), 5.4 μmol of calcium carbonate, and 50 μmol of sodium hydroxide in 5ml of water was refluxed for 4h. [2-^{14}C] Glycolate was purified with a Dowex 50W×8 (H$^+$) column (5×10mm) and by preparative high performance liquid chromatography (HPLC) (see below) : yield, 23% ; 2.16MBq.

Culture broth (1ml) was centrifuged to remove mycelia. The supernatant solution was evaporated to dryness, and the residue was dissolved in 0.1ml of 20% H$_2$SO$_4$ and extracted twice with 0.5ml of ether. The extract was mixed with 0.1ml of methanol, and ether was evaporated by flushing with nitrogen gas. The remaining solution was filled up to 0.5ml with ether containing about 2% diazomethane (w/v). A 2-μl aliquot of the solution was analyzed by gas chromatography with a Shimadzu GC-14A Gas Chromatograph equipped with a flame ionization detector and a capillary column (0.25mm i.d. ×50m, ULTRON HR-20). Helium was used as a carrier gas at a flow rate of 1.4ml/min. The column temperature was kept at 50°C for 1 min, then raised to 71°C at a rate of 3.5°C/min. Fluoroacetate was identified by cochroma-

tography with methyl fluoroacetate, whose retention time was 6.3min.

The culture of *S. cattleya* NTG29 was started with inoculation of 5×10^4 spores in 5 μl of 20% glycerol to 5ml of Medium K supplemented with 10mM potassium fluoride, and the organisms were grown at 37°C with shaking for 5 days. ^{14}C-Labeled compounds were added to the culture, which was incubated for another 12h.

The tracer experiments with resting mycelia were carried out as follows. The mycelia grown as described above and washed three times with 50mM MES (pH 6.5) were suspended in the same buffer supplemented with 10mM potasium fluoride, and the mixture was shaken at 37°C for 4h. Labeled β-hydroxy-pyruvate was added to the suspension, which was then shaken at 37°C for another 3 or 12h.

The permeabilized mycelia were prepared as follows. The mycelia suspension was cooled in an ice bath, mixed with 0.2ml of benzyl alcohol, and stirred vigorously for 30sec. Mycelia were collected by centrifugation and suspended in 50mM MES buffer (pH 6.5) containing 10mM potassium fluoride. The suspension was shaken at 37°C for 4 h, and $[2-^{14}C]$ glycolate was added to the suspension, which was shaken for another 12h.

The mycelia were removed by centrifugation and washed with the same volume of water. The supernatant solution was combined and concentrated to about 5ml under reduced pressure. An equivolume of ethanol was added to the solution, and precipitate formed was removed by centrifugation. Ethanol was removed by evaporation, and 0.1g of NaCl was added to the solution. Then, the solution was three times washed with 10ml of ether. The aqueous layer was acidified by addition of 1ml of 20% H_2SO_4 and extracted 5 times with 20ml of ether. The ether extract was combined, mixed with 1ml of 0.1M Na_2CO_3, and evaporated under reduced pressure. The residue (about 1ml) was acidified with 1 μl of 65% $HClO_4$, and subjected to a preparative HPLC column, ULTRON PS-80H (0.8 $\times$ 30cm) (from Shinwa Kako, Kyoto, Japan) ; column temperature, 60°C ; a mobile phase, distilled water adjusted at pH 2.1 with H_2SO_4 ; flow rate, 1.0ml/min ; detection, 210nm. Fluoroacetate was isolated, dissolved in 10ml of Clear-sol I (from Nacalai Tesque, Kyoto, Japan), and radioactivity was measured with a Packard liquid scintillation counter A300CD.

RESULTS

S. cattleya NRRL 8057 was grown in PSM Medium supplemented with 10mM potassium fluoride at 28°C with shaking. Aliquots of the culture were taken at intervals and centrifuged. The supernatant solution was concentrated under reduced pressure, dissolved in 0.5ml of D_2O (99.8% ; from C.E SACLAY, GIF-SUR-YVETTE, France), and analyzed by ^{19}F-NMR in Varian VXR-200 spectrometer.

A triplet signal appeared at 217ppm, which was identified as the ^{19}F signal of fluoroacetate ; the authentic sodium fluoroacetate (from Nacalai Tesque, Kyoto, Japan) was used as a standard. *S. cattleya* NRRL 8057 was reported to produce 4-fluorothreonine, which shows a multiplet signal at 231.6ppm upon

^{19}F-NMR analysis[6]. A triplet signal was observed at 232ppm 3 days after appearance of the fluoroacetate signal in the culture, and was assigned to be the ^{19}F signal of 4-fluorotheronine. A few mutant strains of S. cattleya NRRL 8057 producing fluoroacetate at high concentrations did not produce a product showing the ^{19}F signal corresponding to that of 4-fluorothreonine. However, no strains showing only the fluorothreonine ^{19}F-NMR signal were obtained. These suggest that fluoroacetate is first produced and then metabolized to 4-fluorothreonine.
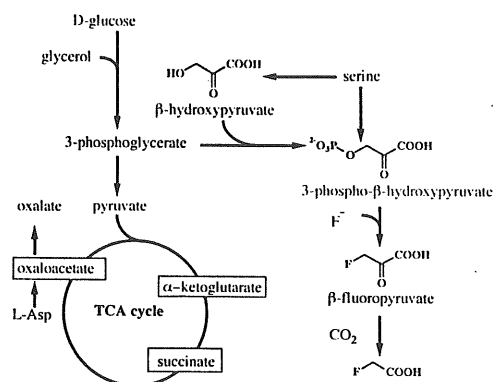
We examined the conditions for production of fluoroacetate by S. cattleya NTG29. S. cattleya NTG29 produced fluoroacetate efficiently in K Medium at fluoride concentrations between 5 and 20mM. Fluoride inhibited growth of the microorganism at higher concentrations thatn 50mM. Growth temperature also affected the productivity : fluoroacetate was produced twice more abundantly at 37°C than 28°C. The production of fluoroacetate was clearly observed 3 days after inoculation of spores (5×10^4) to K Medium (5ml) supplemented with 10mM potassium fluoride either at 28°C or 37°C.

Various ^{14}C-labeled compounds such as [U-^{14}C] glucose were added to the culture broth, and radioactivities of fluoroacetate and other metabolites produced extracellularly and intracellularly were measured (Table 1). Houck et al. reported that S. cattleya NRRL8057 produces oxalate at a high concentration based on a high activity of oxalacetate acetylhydrolase (EC 3.7.1.1)[8]. The present results are consistent with their hypothesis that oxalate is mainly produced from oxalacetate : radioactivities of [3-^{14}C]pyruvate, L-[U-^{14}C]aspartate, and [2, 3-^{14}C]succinate, which directly or indirectly participate in the TCA cycle, were effectively incorporated into oxalate. The activity of ^{14}C-glucose was incorporated into oxalate far less efficiently than the others. This is probably ascribed to the more distant metabolic situation of glucose from oxalacetate than pyruvate, aspartate, and succinate (SCHEME 1). α-Ketoglutarate and L-malate also were labeled with the radioactivity of pyruvate, aspartate, and succinate much more efficiently than with that of ^{14}C-glucose.

In contrast, the radioactivity of ^{14}C-glucose was incorporated into fluoroacetate much more effectively than that of pyruvate, aspartate, and succinate. This suggests that the precursor of fluoroacetate is metabolically closer to glucose than the metabolites participating in the TCA cycle (SCHEME 1).

We found that [U-^{14}C] glycerol is comparable to D-[U-^{14}C] glucose in the incorporation efficiency. L-[U-^{14}C] Serine and [U-^{14}C] β-hydroxypyruvate also showed high incorporation rates. Therefore, glycerol is probably metabolized to β-hydroxypyruvate through glycerate : β-hydroxypyruvate is derived also from L-serine and then converted to the precursor of fluoroacetate.

We determined incorporation rates of radioactivity of various ^{14}C-labeled β-hydroxypyruvates into fluoroacetate with the resting cells of S. cattleya (Table 1). Radioactivity of [U-^{14}C] β-hydroxypyruvate was incorporated into fluoroacetate at rate about 20% lower than that of the [3-^{14}C] compound. By contrast, only a slight radioactivity of [1-^{14}C] β-hydroxypyruvate was incorporated into fluoroacetate. These indicate that only the moiety of C_2 and C_3 carbons of β-hydroxypyruvate are incorporated into

D-glucose

glycerol

HO—COOH
O
β-hydroxypyruvate ← serine

3-phosphoglycerate → $^{3}O_3P$—O—COOH
O
3-phospho-β-hydroxypyruvate

F^-

oxalate　pyruvate

F—COOH
O
β-fluoropyruvate

oxaloacetate　　α-ketoglutarate

L-Asp　TCA cycle　　CO_2

succinate

F—COOH

SCHEME 1. Proposed pathway for fluoroacetate biosynthesis in *Streptomyces cattleya*.

Table 1. Incorporation of ^{14}C-labeled substrates into fluoroacetate and other compounds by *S. cattleya*

Precursors	Radioactivity fed (KBq)	Incorporation (%) into			
		oxalate	α-ketoglutarate	L-malate	fluoroacetate
Experiment 1					
D-[U-^{14}C]glucose	56	3.0	0.20	0.10	0.40
D-[1-^{14}C]glucose	56	3.0	0.04	0.09	0.32
D-[3,4-^{14}C]glucose	24	2.0	0.11	0.12	0.30
[3-^{14}C]pyruvate	63	6.9	0.76	0.83	0.06
L-[U-^{14}C]aspartate	48	6.6	0.79	0.20	0.08
[2,3-^{14}C]succinate	78	3.7	0.10	0.28	0.04
Experiment 2[b]					
[U-^{14}C]glycerol	81	nd[c]	nd	nd	0.42
L-[U-^{14}C]serine	49	nd	nd	nd	0.22
[U-^{14}C]β-hydroxypyruvate	39	nd	nd	nd	0.23
Experiment 3[d]					
[3-^{14}C]β-hydroxypyruvate	68	nd	nd	nd	1.7
[U-^{14}C]β-hydroxypyruvate	56	nd	nd	nd	1.3
[1-^{14}C]β-hydroxypyruvat	74	nd	nd	nd	0.07
Experiment 4[e]					
[2-^{14}C]glycolate	39	nd	nd	nd	0.07

[a]Labeled precursors were added to the culture broth 5 days after inoculation. The culture was incubated with shaking at 37°C for 12h, and then sonicated in a Biomic ultrasonic disintegrator for 10min. The mycelial debris was removed by centrifugation, and oxalate, α-ketoglutarate, L-malate, and fluoroacetate were isolated from the supernatant solution by preparative HPLC as described in Materials and Methods.

[b]Fluoroacetate was isolated from the culture broth as described in Materials and Methods.

[c]nd, not determined.

[d]The resting mycelia were used.

[e]The permeabilized mycelia were used.

fluoroacetate. However, these results as well as the lower incorporation rate of β-hydroxypyruvate in comparison with that of glycerol (Table 1 ; Experiment 2) are possibly due to instability of β-hydroxypyruvate : it is readily decarboxylated to form glycolate[9]. The hydroxyl group of β-hydroxypyruvate or its derived compound is probably replaced by fluoride to form fluoroacetate or its precursor in S. cattleya (SCHEME 1).

Glycolate is formed from β-hydroxypyruvate by oxidative decarboxylation[9]. Therefore, we examined incorporation of radioactivity of $[2-^{14}C]$glycolate into fluoroacetate with the permeabilized mycelia prepared as described above. The permeabilization affected the fluoroacetate productivity of the mycelia only slightly : the treated mycelia produced fluoroacetate about 30% less than the native one when determined by gas chromatography. However, only a little radioactivity of $[2-^{14}C]$glycolate was incorporated into fluoroacetate by incubation with the mycelia as shown in Table 1 : glycolate is unlikely to be a precursor of fluoroacetate.

DISCUSSION

Based on the observation that fluoroacetate is produced from 4-fluorothreonine by resting mycelia of S. cattleya, Sanada et al. proposed that fluorothreonine is synthesized from glycine and fluoroacetaldehyde through a reversible reaction catalyzed by threonine aldolase[6]. They suggested also that fluoroacetaldehyde is formed by reduction of fluoroacetate. Our results of ^{19}F-NMR studies showed that fluoroacetate is produced first and then converted to 4-fluorothreonine. However, we could not show the formation of fluoroacetaldehyde in the S. cattleya culture. Fluoroacetaldehyde, if produced, was probably lost by evaporation under the conditions we used.

Peters et al. reported that fluoroacetate was produced by incubation of pyruvate, potassium fluoride and ATP with a homogenate of Acacia georginae seedlings[3]. However, our results showed that pyruvate is not the precursor of fluoroaceate in S. cattleya. It may be possible that fluoroacetate is synthesized in A. georginae through a pathway different from that used by S. cattleya. Further studies including tracer experiments, however, are required to clarify the biosynthetic pathway of fluoroacetate in plants.

Halogen atoms are usually incorporated into organic compounds such as alkenes by haloperoxidase reactions in living systems. Vickery et al. speculated that fluoroacetate is formed from malonate, fluoride and hydrogen peroxide by a haloperoxidase[10]. However, the redox potential for reduction of hydrogen peroxide ($E_0 = +1.77v$) is insufficient to oxidize fluoride anion ($E_0 = -3.06v$) : the C-F bond cannot be formed by haloperoxidase reactions. Harper and O'Hagan postulated that fluoroacetate can be formed from phosphoglycolate or other activated glycolate species by nucleophilic displacement with fluoride under normal physiological conditions[5].

We have shown that only the moiety of C_2 and C_3 carbons of β-hydroxypyruvate are incorporated into fluoroacetate. This indicates that β-hydroxypyruvate is decarboxylated either before or after dis-

placement of the β-hydroxyl group by fluoride. Glycolate is produced by decarboxylation of β-hydroxy-pyruvate. However, radioactivity of [2-^{14}C] glycolate was incorporated into fluoroacetate far less efficiently than that of ^{14}C-β-hydroxypyruvate. Therefore, we here propose the following two possible pathways for the biosynthesis of fluoroacetate in *S. cattleya* (SCHEME 1). The β-hydroxy group of β-hydroxypyruvate is displaced by fluoride before it is decarboxylated : β-fluoropyruvate is formed from β-hydroxypyruvate, and then decarboxylated to produce fluoroacetate. Alternatively, β-hydroxypyruvate is converted to phosphoglycolate or other activated glycolate species, and then to fluoroacetate by nucleophilic displacement with fluoride. We are currently studying the fluoroacetate biosynthesis with various ^{13}C-labeled compounds such as glycolate and hydroxypyruvate by ^{13}C-NMR.

REFERENCES

1) Oelrichs, P. B., and McEwan, T. (1961) Nature 190 : 808

2) McEwan T. (1964) Nature 202 : 827

3) Peters, R. A., Shorthouse, M., and Ward, P. F. V. (1965) Life Sci. 4 : 749

4) Preuss, P. W., Colavito, L., and Weinstein, L. H. (1970) Experientia 26 : 1059

5) Yu, M. H., and Miller, G. W. (1970) Envirom. Sci. Technol. 4 : 492

6) Sanada, M., Miyano, T., Iwadare, S., Williamson, J. M., Arison, B. H. Smith, J. L., Douglas, A. W., Liesch, J. M., and Inamine, E. (1986) J. Anitibiotics 39 : 259

7) Soda, K., and Osumi, T. (1969) Biochem. Biophys. Res. Commun. 35 : 363

8) Houck, D. R., and Inamine, E. (1987) Archiv. Biochem. Biophys. 259 : 58

9) Walton, N. J., and Butt, V. S. (1981) Planta 153 : 225

10) Vickery, B., Vickery, M. L., and Kabeira, F. (1979) Experientia 35 : 299