Bacterial defluorination of 4-fluoroglutamic acid

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Abstract Fluorinated amino acids are used as enzyme inhibitors, mechanistic probes and in the production of pharmacologically active peptides. Because enantiomerically pure 4-fluoroglutamate is difficult to prepare, the selective degradation of the L-isomer is a potentially convenient method of obtaining D-4-fluoroglutamate from the racemate. In this paper, we describe our investigations on the degradation of 4-fluoroglutamate by bacteria. Fluoride ion was detected in resting-cell cultures of a number of bacteria that were incubated with racemic 4-fluoroglutamate. Analysis of the culture supernatants by chiral gas chromatography–mass spectrometry revealed that only the L-isomer was degraded. The degradation of 4-fluoroglutamate was also examined in cell-free extracts of Streptomyces cattleya and Proteus mirabilis, and it was observed that equimolar concentrations of fluoride ion and ammonia were generated. The activity was located in the soluble fraction of cell extracts, thus is not related to the L-2-amino-4-chloro-4-pentenoic acid dehydrochlorinase previously identified in membrane fractions of P. mirabilis.

Keywords Amino acid · Bacteria · Dehalogenation

Introduction

Substitution of hydrogen by fluorine in biological molecules can result in major changes in their electronic properties with only minor steric effects, and fluorinated compounds are finding increasing applications as therapeutic agents and molecular probes to examine enzyme–substrate or receptor–ligand interactions. Consequently, the proportion of pharmaceutical compounds that contain fluorine has increased from 2% in 1970 to approximately 18% today (Isanbor and O’Hagan 2006). Fluorinated amino acids, such as 4-fluoroglutaric acid, are of biological interest; the individual stereoisomers of this compound have been employed as inhibitors of glutamate decarboxylase (Drsata et al. 2000) and most notably in the preparation of fluorinated derivatives of the anti-cancer compound methotrexate (Harte et al. 1996; McGuire et al. 1991). Multi-gram quantities of the racemic mixture of all four stereoisomers can be prepared via Michael reaction (Tolman 1993), but obtaining enantiomerically pure 4-fluoroglutarate is more difficult and hence expensive. Kokuryo et al. (1996) resolved L-erythro and L-threo-4-fluoroglutarate acid from the racemic mixture, separating the diastereomers of N-chloroacetyl derivatives by recrystallisation and subsequent resolution of the enantiomers using aminoacylase. Resolution of the erythro and threo diastereomers was achieved by preferential crystallisation of the dimethyl and diisopropyl derivatives (Tolman et al. 1993); further resolution into the individual enantiomers was achieved by forming the diastereomeric salts with chiral amines, in the case of the erythro forms, or making the N-acetyl derivative to resolve the threo enantiomers (Tolman and Simek 2000). Tolman and Simek (2000) used purified E. coli glutamate decarboxylase to generate enantiomerically pure 4-amino-2-fluorobutyric acid and D-4-fluoroglutarate. However, the use of purified enzymes and the addition of cofactors, in this case pyridoxal 5-phosphate, have significant cost implications if done on a large scale. Asymmetric syntheses of the individual stereoisomers from...
hydroxyproline (Hudlicky 1993) and pyroglutaminol (Konas and Coward 2001) have been reported with low yields.

Some biological dehalogenating activities are known to be stereospecific and have found application in the resolution of racemic mixtures of commercially important compounds; for example, a pseudomonad containing a stereoselective haloacid dehalogenase is used in the production of S-2-chloropropionic acid, which is an important chiral building block for the manufacture of pharmaceuticals (Ishige et al. 2005). In this paper, we describe the resolution of D-4-fluoroglutamate from the racemate via selective defluorination of the L-isomer by bacteria.

**Materials and methods**

**Bacterial strains and culture conditions**

*S. cattleya* NRRL 8057 was grown in 250-ml Erlenmeyer flasks containing 50 ml of the medium described by Reid et al. (1995), and typically harvested after 6–8 days growth. *Proteus mirabilis* IFO 3849 was grown for 18 h at 30°C in a medium described by Moriguchi et al. (1987). *Escherichia coli* IMD 1, *Bacillus subtilis* IMD 7, *Streptomyces calvus* NCIMB 12240 and *Streptomyces coelicolor* A3(2) were cultured in either glucose–yeast extract medium or tryptone soya broth for 18 h.

Cells were harvested by centrifugation and washed twice with phosphate buffer (10 mM, pH 7) and re-suspended at a concentration of 0.3 g wet cells/ml buffer or distilled water. Resting-cell assays composed of a cell suspension plus 4-fluoroglutamate (2 mM) in a final volume of 3 ml and were incubated for 48–96 h at 30°C.

**Preparation of cell-free extracts**

Cell extracts of the various bacterial strains were prepared from washed cells re-suspended in phosphate buffer pH 7 containing 2-mercaptoethanol (0.01%). The cells were disrupted by passage through a French pressure cell (1,000 psi); cell debris was removed by centrifugation (40,000×g), and the supernatant was decanted and stored at 4°C. Protein concentration was determined using the Coomassie blue binding method (Bradford 1976). Enzymatic defluorination was determined by incubating the cell extract in phosphate buffer (100 mM, pH 8) with 4-fluoroglutamate (1–2 mM) in a final volume of 1 ml at 30°C. The assays were terminated with the addition of trichloroacetic acid (50 μl).

Membrane fractions of *P. mirabilis* and *S. cattleya* were prepared by ultracentrifugation of the cell-free extract (100,000×g) at 4°C for 60 min (Sorvall Discovery M120SE), the supernatant (soluble fraction) was removed, and the pellet (membrane fraction) was re-suspended in

| Bacterium                  | Fluoride ion concentration (mM) |
|---------------------------|---------------------------------|
|                           | Whole cells  | Cell-free extract |
| *Streptomyces cattleya*   | 0.74         | 0.38               |
| *Proteus mirabilis*       | 0.97         | 0.60               |
| *Escherichia coli*        | 0.91         | 0.31               |
| *Bacillus subtilis*       | 0.94         | 0.03               |
| *Streptomyces coelicolor* | 0.89         | 0.17               |
| *Streptomyces calvus*     | 0.60         | 0.12               |
phosphate buffer (pH 8). Both fractions were assayed for dehalogenating and deaminating activity by incubating them with either L-2-amino-4-chloro-4-pentenoic acid (2 mM) or 4-fluoroglutamate.

**Analytical methods**

Defluorination of 4-fluoroglutamate was determined by measurement of the free fluoride ion concentration using an Orion fluoride ion-selective electrode according to the method of Cooke (1972) and by $^{19}$F nuclear magnetic resonance (NMR) spectroscopy of culture supernatants and cell-free extracts (400 μl) containing D$_2$O (200 μl) to provide a lock signal, using a Varian 400-MHz spectrometer. Ammonia concentration was assessed using an ammonia assay kit (Sigma) and following the manufacturer’s instructions. Chiral analysis of the 4-fluoroglutamate remaining in resting-cell supernatants and enzyme assays was conducted using an Agilent 6890 gas chromatograph coupled to a 5973 mass-selective detector. The N-trifluoroacetyl methyl esters of the 4-fluoroglutamate enantiomers were prepared by adding a mixture of methanol and acetyl chloride (9:1 v/v, 500 μl) to lyophilised aliquots (500 μl to 1 ml) of enzyme assay mixture and resting-cell supernatants. After standing for 3 h, the solvent was removed under a stream of He gas. Trifluoroacetic acid anhydride (50 μl) and dichloromethane (300 μl) were added, and the reaction was left to stand for 15 min; the solvent was removed, and fresh dichloromethane was added (200 μl). Samples (1 μl) were injected (20:1 split) onto a Chirasil-L-val column (25 m×0.25 mm×0.12 μm) using He as the carrier gas. The column temperature was held at 60°C for 3 min then heated to 190°C at a rate of 3°C/min.

Degradation of L-2-amino-4-chloro-4-pentenoic acid (L-ACP) was determined by gas chromatography–mass spectrometry (GC-MS) analysis of the isobutoxycarbonyl isobutyl ester, which was prepared according to the method described by Sobolevsky et al. (2003), and comparing samples against a standard curve of L-ACP. Enzyme assay (80 μl) plus norvaline (0.25 mM) as an internal standard was mixed well with isobutanol (30 μl), pyridine (10 μl) and isobutyl chloroformate (30 μl). The derivatives were extracted into chloroform (500 μl) by vigorous shaking, and 1 μl was injected (splitless) onto a HP-1 column (12 m×0.25 mm×0.33 μm). The column temperature was held at 50°C for 2 min then heated to 300°C at a rate of 10°C/min; the mass spectrometer was operated in the scan mode.

**Chemicals**

DL-ERYTHRO/threo-4-fluoroglutamate was purchased from Fluorochem (Derbyshire, UK) and DL-threo-4-fluoroglutamate and L-threo-4-fluoroglutamate from Apollo Scientific (Cheshire, UK) and Matrix Scientific (Columbia, USA). L-2-Amino-4-chloro-4-pentenoic acid was a gift from Prof. Mitsuaki Moriguchi (Department of Applied Chemistry, Faculty of Engineering, Oita University, Japan). All the other chemicals used were acquired from Sigma-Aldrich.

**Results**

Defluorination of DL-erythro/threo-4-fluoroglutamic acid by whole cells

Resting-cell cultures of various bacteria were prepared and incubated with 2 mM 4-fluoroglutamate, and the defluorination of the compound was assessed by measuring the concentration of free fluoride in the supernatant (Table 1). All of the bacteria examined could degrade up to 50% of the racemic 4-fluoroglutamate. Resting-cell cultures of S. cattleya and P. mirabilis were

| Bacterium  | Defluorination (μM $F^-$ released/min) | Deamination (μM $NH_3$ released/min) |
|------------|----------------------------------------|------------------------------------|
| S. cattleya| 0.29                                   | 0.25                               |
| P. mirabilis| 5.2                                    | 4.8                                |

The cell extracts were dialysed overnight to remove excess ammonia then incubated with 1 mM L-threo-4-fluoroglutamate.

![Fig. 2 Chromatograms from the separation of N-trifluoroacetyl methyl ester of DL-threo-4-fluoroglutamic acid before (upper chromatogram) and after (lower chromatogram) incubation with resting cells of Proteus mirabilis IFO 3849](image-url)
also incubated with L-threo-4-fluoroglutamate, and complete defluorination was observed; thus, it could be inferred that only the L-erythro/threo isomers were degraded by the bacteria examined. The supernatants from the P. mirabilis and S. cattleya cultures that had been incubated with DL-threo-4-fluoroglutamate were also examined by $^{19}$F NMR, which revealed approximately equimolar amounts of fluoride (broad singlet, δ = −118 ppm) and 4-fluoroglutamate (heptet, δ = −178.5 ppm; Fig. 1); this confirmed the measurements from the fluoride ion-selective electrode and demonstrated that the remaining 4-fluoroglutamate was not degraded to another fluorinated compound nor taken up by the cell but remained in the culture supernatant. Further analysis of the culture supernatant by chiral GC-MS, after derivatisation of 4-fluoroglutamate to the N-trifluoroacetyl methyl ester, established that only the D-enantiomer was present at the end of the incubation period (Fig. 2). Thus, resting cells of bacteria could be used to resolve racemic mixtures of DL-4-fluoroglutamate by selectively degrading the L-isomer and leaving the D-isomer untouched.

### Cell-free defluorination of 4-fluoroglutamic acid

The degradation of L-4-fluoroglutamate in whole-cell cultures might be explained by deamination to 4-fluoro-α-ketoglutarate and subsequent entry to the citric acid cycle where it may be processed to fluoromalate, which undergoes spontaneous non-enzymatic defluorination (Teipel et al. 1968; Reid et al. 1995). However, when 4-fluoroglutamate was incubated with cell-free extracts of the various strains, the free fluoride ion could be measured in the assay mixtures of P. mirabilis, E. coli and S. cattleya (Table 2), indicating that these bacteria have a direct enzymatic defluorinating capability. The B. subtilis cell-free extract did not effectively degrade 4-fluoroglutamate, suggesting that either there is no direct defluorinating activity in this strain or the enzyme is more labile compared with that in other bacteria. No defluorination was detected in boiled cell-free extracts, and most of the defluorinating activity (approx. 70%) was recovered after dialysis, indicating that the reaction does not require any co-factors or co-substrates. It was also observed that there was an equimolar concentration of ammonia and fluoride in assays with P. mirabilis and S. cattleya cell extracts (Table 2), suggesting that defluorination of the substrate is linked to deamination. Moriguchi et al. (1987) discovered an unusual enzyme activity in the membrane fraction of P. mirabilis, which catalysed the deamination and dehalogenation L-2-amino-4-chloro-4-pentenoic acid (Fig. 3). Given the similarities in both the nature of the substrates and the reactions catalysed, it was thought that the same enzyme was responsible for the deamination and defluorination of 4-fluoroglutamate in S. cattleya and P. mirabilis. However, when 4-fluoroglutamate was incubated with the membrane and soluble fractions of cell extracts from the two bacteria, defluorinating activity was predominantly observed in the soluble fraction (Table 3). Degradation of L-ACP, measured by GC-MS analysis of the isobutoxycarbonyl isobutyl ester, was only detected in P. mirabilis extracts. These data indicate that the enzyme responsible for dechlorination and deamination of L-ACP does not act on 4-fluoroglutamate and that S. cattleya does not have an enzyme that degrades L-ACP.

### Discussion

The catabolism of fluoro-aromatic compounds has been studied in several strains of bacteria, which classically employ enzymes that degrade non-halogenated aromatic compounds but have sufficiently relaxed specificities to transform fluorinated derivatives of the natural substrates (Harper and Blakely 1971; Boersma et al. 2004). Reports of bacterial degradation of aliphatic fluorinated compounds are largely confined to the enzymatic cleavage of the C–F bond of fluoroacetate, catalysed by a specific dehalogenase (Nataranjan et al. 2005). In this paper, we describe the degradation of the fluorinated amino acid 4-fluoroglutamate by several bacteria and demonstrate a novel cell-free defluorination/deamination reaction. An enzyme that catalyses a similar reaction, the dechlorina-

### Table 3 Defluorination of 4-fluoroglutamate and dechlorination of L-ACP in soluble and membrane fractions of S. cattleya and P. mirabilis lysate

| Bacterium      | Defluorination (μmol F⁻ released/min per mg protein) | Membrane | Soluble | Dechlorination (μmol ACP degraded/min per mg protein) | Membrane | Soluble |
|----------------|------------------------------------------------------|----------|---------|------------------------------------------------------|----------|---------|
| S. cattleya    | 0.02                                                 | 0.06     | ND      | ND                                                   | ND       | ND      |
| P. mirabilis   | ND<sup>a</sup>                                        | 0.16     | 6.85    | 0.8                                                 |          |         |

<sup>a</sup> Not detectable
tion and deamination of L-2-amino-4-chloro-4-pentenoic acid, was previously identified in *P. mirabilis* (Moriguchi et al. 1987), but its ability to degrade fluorinated substrates was not reported. While cell-free extracts of *P. mirabilis* can degrade 4-fluoroglutamate, we have shown that the defluorinating/deaminating activity is in the soluble fraction of the cell and is not related to the dechlorinating/deaminating activity, which is located in the cell membrane. 4-Fluoroglutamate is not found in the natural world, although one of the bacteria that degrades it, *S. cattleya*, is known to biosynthesise fluorooacetate and 4-fluorothreonine (Deng et al. 2004), and initial speculation was that the dehalogenation reaction might be part of a specific protective mechanism in this organism. However, as 4-fluoroglutamate is degraded by other bacteria, it is more likely that the dehalogenation is gratuitous, although further investigation is required to identify the enzyme(s) responsible. Earlier experiments demonstrated that *Streptomyces* glutamate oxidase will deaminate 4-fluoroglutamate, but no significant fluoride release was detected (Murphy 1998). The observation that dialysed cell-free extract is active also indicates that an enzyme such as glutamate dehydrogenase is not responsible for the biotransformation.

Bacteria that can specifically degrade the L-isomer of racemic 4-fluoroglutamate might find application as a method to conveniently generate D-4-fluoroglutamate from racemic mixtures. Current methods for the resolution or synthesis of the individual isomers are expensive, labour intensive and in most cases result in low yields (Dave et al. 2003). Bacteria can conveniently yield 50% D-4-fluoroglutamate in a single reaction vessel under relatively mild conditions.

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