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The defluorination of 4-deoxy-4-fluoro-D-glucose in the cytoplasmic membrane of *Pseudomonas putida* †

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ABSTRACT

The metabolism of 5 mM 4-deoxy-4-fluoro-D-glucose in *Pseudomonas putida* to 4-deoxy-4-fluoro-D-gluconic and 4-deoxy-4-fluoro-D-*arabino*-2-hexulosonic acid is shown by ^{19}F NMR spectrometry to occur with concomitant release of fluoride from each of these substrates. Gluconate-grown *P. putida*, incubated with 4-deoxy-4-fluoro-D-[U-^{14}C]glucose until 50% fluoride ion is detected in the supernatant, gives 3,4-dideoxy-D-*glycero*-2-hexulosonic acid, identified by ^{13}C NMR, chemical, and spectrophotometric analyses. Although no fluoride release (F^-) from 4FG is detected in the outer membrane or periplasmic fractions of the cell envelope, substantial F^- is found in the cytoplasmic membrane (CM) fraction in the presence of such electron donors as L-malate. CM detergent extracts contain a glucose-inducible, succinate-repressible protein having an apparent molecular mass of 65 500. Reconstitution of CM extracts, derived from glucose- but not succinate-grown cells, with dipalmitoylphosphatidylcholine gives proteoliposomes which elicit F^- from 1 mM 4FG. This F^- release is totally inhibited by D-glucose but not by D-galactose. The 65 500 dalton protein is considered to be associated with the active D-glucose transporter system in *P. putida* and responsible for the defluorination of 4FG.

INTRODUCTION

There are only a few examples in biological systems in which the covalent carbon–fluorine bond is enzymatically broken and fluoride ion released. Those reported are mainly confined to bacteria found in soil and water[1]. Interestingly, fluoroacetic acid, a toxic natural product found in the plant species *Dichapetalum cymosum*[2], does not accumulate indefinitely and a haloacetate halohydrolyase

† Abbreviations: 4FG, 4-deoxy-4-fluoro-D-glucose: D-[U-^{14}C]-4FG, 4-deoxy-4-fluoro-D-[U-^{14}C]glucose; 4FGA, 4-deoxy-4-fluoro-D-gluconic acid: 4F2KGA, 4-deoxy-4-fluoro-D-*arabino*-2-hexulosonic acid; 3FG, 3-deoxy-3-fluoro-D-glucose; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PC, phosphatidylcholine (egg yolk); PMS, phenazine methosulphate; FAD, flavine adenine dinucleotide; Δp, proton motive force; $\Delta\psi$ and ΔpH, differences in electrical potential and pH respectively between the outside and the inside of the membrane.

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found in a soil pseudomonads is capable of defluorinating it[3]. Recently, it has been reported that *Pseudomonas cepacia*, a bacterial symbiont of *D. cymosum*, defluorinates fluoroacetate to glycolate by the same enzyme[4]. Thus, for natural products at least, a mechanism has evolved for the cleavage of the C–F bond and the removal of unwanted toxic material from the environment by microorganisms. In mammalian systems, some small but significant defluorination of fluoroacetate during metabolism in the rat has been observed, but the mechanism of C–F bond cleavage is not clear[5].

We have previously reported in vivo fluoride release from 3-deoxy-3-fluoro-D-glucose (3FG) in the locust[6]. To our knowledge, however, the defluorination of 4FG in *P. putida* is the first reported example of C–F bond cleavage of a synthetic fluorinated monosaccharide by a microorganism[7]. The mechanism of defluorination is regiospecific, since 3FG is metabolised without fluoride release in *P. putida*. Furthermore, the mechanism may be species-specific, since 4FG or 3FG are metabolised in *Escherichia coli*[8] without defluorination. Radiochemical studies confirmed that 4FG is defluorinated in *P. putida* and metabolised by this organism to a 5-carbon end-product, identified as 2,3-dideoxy-D-*glycero*-pentonic acid (2,3-dideoxyribonic acid). Based on these and previous kinetic studies[9], a mechanism for the initial interaction of 4FG with an inducible–repressible protein, prior to fluoride release, was suggested. In addition, a metabolic pathway involving a number of defluorinated intermediates was proposed[10]. In this communication we report: (*i*) in vivo ^{19}F NMR evidence of 4FG metabolism to 4FGA and 4F2KGA, which are also substrates for fluoride release in *P. putida*; (*ii*) further evidence to support the proposed pathway of defluorinated intermediates from 4FG; (*iii*) The membrane location and reconstruction of an inducible–repressible protein fraction into proteoliposomes that has significant fluoride release activity.

EXPERIMENTAL

General methods.—Crystalline D-[-U-^{14}C]-4FG (specific activity, 10600 dpm μmol^{-1}) and 4FG were obtained as previously reported[10]. Aqueous samples of 4FGA and 4F2KGA were obtained from 4FG by oxidation with partially purified enzymes[11]. A 2% (w/v) solution of phosphotungstic acid was obtained from the Public Health Laboratories (Windsor, ON) and copper grids, 200 mesh, from J.B. EM Services Inc. (Dorval, PQ). Deuterium oxide was obtained from MSD Isotopes (Division of Merck Frosst, Canada Inc., Montreal, PQ) and 5-mm borosilicate NMR tubes from Wilmad Glass Co. Inc. (Buena, NJ). Dupont 963 aqueous scintillation fluid was from Dupont NEM Research Products (Boston MA) and polyethylene liquid-scintillation vials were purchased from Fisher Scientific Co. (Fairlawn, NJ) Dowex 1×8 [Cl^-] (200–400 dry mesh A.S.T.M.) anion-exchange resin, PMS, FAD, L-malate, bovine serum albumin (Fraction V), Folin and Ciocalteu's phenol reagent, deoxycholic acid, sodium D-gluconate, D-*arabino*-hexulosonic 2-keto-D-gluconic) acid (hemicalcium salt), 3-deoxy-D-mannooctulosonate

(Kdo, ammonium salt), sodium periodate, 2-thiobarbituric acid, D-glucose 1-phosphate, succinic acid (disodium salt), orcinol, $MgCl_2 \cdot 6H_2O$, phenylmethylsulphonyl fluoride ammonium peroxysulphate (electrophoresis grade), Dalton Mark VI low-molecular-weight standards, SDS 6H high-molecular-weight standards, Coomassie Brilliant Blue G, 2-mercaptoethanol, DL-dithiothreitol, Ponceau S, 5-sulphosalicylic acid, egg phosphatidylcholine type II E (in EtOH), and octyl β-D-glucopyranoside were all obtained from Sigma Chemical Co. (St. Louis, MO). Bradford dye-reagent kit, SDS (electrophoresis grade), acrylamide, glycine, tris(hydroxymethyl)aminomethane hydrochloride (Tris), and TEMED (*N,N,N′,N′*-tetramethylene-diamine were obtained from Bio-Rad Laboratories (Richmond CA). Thin-layer chromatography (TLC) was performed on 20 × 20 cm silica gel sheets (0.2 mm thickness) from Eastman Kodak Co. (Rochester, NY) using 3:3:1 EtOH–AcOH–water and a 1:1 H_2SO_4–EtOH spray with subsequent charring for 20 min at 120°C for detection. Unless otherwise stated, all other chemicals and reagents were obtained from Fisher Scientific Co. (Fairlawn, NJ).

Culture conditions and organism.—Pseudomonas putida, biotype A (ATCC 12633) was routinely maintained at 30°C on a glucose–, gluconate–, or succinate–mineral salts agar medium and harvested after 15 h, as previously reported[12]. For large-scale cultures, a 12-L batch fermentation unit (Model SF-116, New Brunswick Scientific Co. Inc., Edison, NJ) was used. After inoculation of the fermentor, containing 1–2 L of mineral salts media, with a 4–8 h culture (50 mL), the batch culture was aerated (1.5 L min^{-1}), stirred at 400 rpm, and maintained under a slight positive pressure (0.5 lb in^{-2}) for 12 h at 30°C. At this time the cells are in mid- to late-logarithmic growth (optical density in the range 0.9–1.1 at 620 nm). Whole cells were harvested in sterile 1-L bottles by centrifugation at 4000*g*, for 20 min at 20°C using a J-6B centrifuge (Beckman Instruments Inc., Palo Alto, CA). The cell pellet was washed in 20–50 mM potassium phosphate buffer and reharvested. Typically, cell yields were 5–6 g wet weight L^{-1} of media. Protein determinations were based on the method of Bradford[13] (for whole cells), Lowry[14] (periplasmic fractions), or Peterson[15] (cytoplasmic membranes or proteoliposomes) using bovine serum albumin as a standard.

Fluoride release was detected with a fluoride-sensitive electrode (Orion Research, Cambridge, MA).

NMR spectroscopy.—^{13}C NMR spectra were obtained with a Bruker 300-MHz NMR spectrometer (Bruker Instruments, Bruker Canada, Milton, ON) in the Fourier-transform mode operating at 75 MHz. Spectra were obtained on samples in 5-mm NMR tubes at 23°C with D_2O as the solvent. Chemical shifts (δ) are reported in ppm relative to 1,4-dioxane. DEPT 135 and 90 analyses of ^{13}C NMR spectra were run overnight (~ 100 000 scans). ^{19}F NMR spectra were determined with the same instrument in conjunction with a ^{19}F–^{1}H dual probe at 30°C. Digital resolution (Hz/ppm) was 10.17. A line-broadening factor of 10–15 Hz was applied routinely for signal enhancement. Chemical shift values (δ) were relative to trichlorofluoromethane.

^{19}F NMR analysis of 4FG metabolism.—Glucose- or gluconate-grown *P. putida* was suspended in 50 mM potassium phosphate buffer, pH 7.5, to give a total volume of 25 mL and 10 mg protein mL^{-1}. Defluorination was initiated by the addition of 200 mM 4FG to give a final concentration of 5 mM and the suspension was incubated at 30°C with continous stirring. At 15-min intervals an aliquot (750 μL) was transferred to a 5-mm NMR tube and the ^{19}F NMR spectrum obtained as already described. The number of scans for each spectrum was 500–1000 at 30°C and the recycle delay time was 0.5 s. ^{19}F NMR data: 4FG, two sets of doublets each at δ 198.2, 198.5, and 200.2, 200.5. 4FGA, δ 208.1. 4F2KGA, single split peak, δ 201.5 and 201.8. Fluoride ion δ 118.2. (See Fig. 1.)

Liquid scintillation counting and measurement of $^{14}CO_2$.—Radioactivity of the various samples was with measured with a Beckman LS 7500 (Beckman Instruments Inc., Fullerton, CA) as previously reported[10] except that the scintillation fluid added (10 mL) to the sample (1 mL) was Formula 963 (Dupont, Canada). $^{14}CO_2$ was measured as described previously[10] with a Gilson differential respirometer (Gilson Medical Electronics, Middleton, WI) using glucose- or gluconate-grown *P. putida* (10 mg protein mL^{-1}) in a total volume of 3 mL.

Separation and isolation of radiolabeled metabolites.—The separation was performed on a borosilicate glass column of Dowex-1 × 8 (74 × 1.45 cm bed) borate anion-exchange resin, prepared as previously described[10]. The supernatant (3 mL), obtained from an incubation of 1 mM D-[U-^{14}C]-4FG with gluconate-grown *P. putida* (10 mg protein mL^{-1}) at 30°C until 50% fluoride ion had been released (80–100 min), was applied to the column. Various 1-mM sugar standards (Fig. 2) were added (1–2 mL) in the starting buffer (29 mM ammonium tetraborate–57 mM boric acid, pH 8.5–8.8) prior to the application of radiolabeled supernatant. Alternate 4-mL fractions of a linear gradient of ammonium tetraborate, consisting of starting buffer (250 mL) diluted in a linear manner with 0.5 M ammonium tetraborate (250 mL, pH 8.9–9.2), were collected at a constant flow of 0.5 mL min^{-1} and analysed for carbohydrate (1 mL) by orcinol–H_2SO_4 colourimetry[16] and radioactivity (250 μL) by liquid-scintillation counting. Fractionated peaks a–d (Fig. 2) were each pooled and ammonium tetraborate removed by repeated distillation and concentration to dryness with MeOH in vacuo. The resulting residues (metabolites) were solubilised in D_2O (500 μL) and used directly for NMR analysis. Preparative amounts of peak d (R_f 0.74) were obtained after pooling five column separations. ^{13}C NMR data for peak d gave: δ (proton decoupled) 23.9, 30.5, 50.5, 63.5, and 162.8. Peak d also gave positive colourimetric reactions with *o*-phenylenediamine[17] and periodate–thiobarbituric acid[18], which together constitute evidence for the presence of a 3-deoxy-2-hexulosonic acid. Based on these results and DEPT 135 and 90 editing of the NMR data, peak d was assigned the structure 3,4-dideoxy-D-*glycero*-2-hexulosonic acid (**1**, Fig. 3).

Cytoplasmic membrane fraction.—Isolation of this fraction from *P. putida* was based on a method adapted from Newman and Wilson[19]. Cultures (12 L) of cells grown on glucose, gluconate, or succinate were harvested at 2500*g* at 4°C. The cell

pellets were washed with sterile ice-cold 50 mM potassium phosphate buffer, pH 7.5 (500 mL), and the cells resuspended (0.2 g wet weight) in the same ice-cold buffer containing final concentrations of 1 mM dithiothreitol, 5 mM $MgSO_4 \cdot 7H_2O$ and hand homogenized before the addition of DNase (20 μg mL^{-1}) and 0.5 mM phenylmethylsulphonyl fluoride. The suspension of cells were disrupted by passage through a pre-chilled French pressure cell (American Instruments Co., Silver Spring, MLD) at 19 000–20 000 lb in^{-2} and collected in an ice-bath. After centrifugation (TY 30 rotor, Beckman Instruments, Fullerton, CA) at 11 700g at 4°C for 10 min, most of the supernatant was removed and the remaining cell debris recentrifuged. The supernatants were pooled and ultracentrifuged in a Beckman L8-55 at 140 000g (60 Ti rotor) at 4°C for 2 h. The resulting cytoplasmic membrane pellet was suspended in a minimal volume of sterile 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM dithiothreitol and 0.5 mM phenylmethylsulphonyl fluoride. After protein determination, the volume was adjusted to give a final protein concentration of 4–5 mg mL^{-1}. The membrane fraction was divided into 1-mL aliquots, frozen in liquid nitrogen and stored at −80°C.

Reconstitution of defluorinating activity into proteoliposomes.—Multilamellar liposomes were prepared from egg phosphatidyl choline (100 mg in EtOH) by established procedures[20]. The liposome suspensions (5 mg mL^{-1}) were stored in sterile 50 mM potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol at 4°C for periods no longer than 2 weeks. The viability of the liposomes was established by electron microscopy. One drop of a liposome suspension (1 mg mL^{-1}) was placed on a 200 mesh Formvar copper grid and an equal volume of 2% (w/v) phosphotungstic acid, pH 6.5, added and allowed to stand for 1 min. After drying in air, the grid was examined with a Phillips EM high resolution electron microscope (Eindhoven, The Netherlands). Proteoliposomes were prepared by a method based on the octyl glucoside dilution procedure of Racker et al.[21].

(a) Extraction of cytoplasmic membrane proteins. The cytoplasmic membrane fraction (1.25 mL containing 5 mg protein), 100 mM dithiothreitol (40 μL), 50 mM potassium phosphate buffer (2.5 mL), phosphatidylcholine (200 μL) from a stock solution (96 mg mL^{-1}), and a final concentration of 0.5 mM 2-mercaptoethanol, were rapidly blended (vortex mixer) in a small test-tube at 0°C. Octyl glucoside (33 μL) from a stock solution (15% w/v in 50 mM phosphate buffer) was added and the contents vortexed gently. The suspension was incubated at 4°C for 20 min, blended again, and centrifuged at 175 000g (60 Ti rotor) for 1 h at 4°C. The supernatant, containing the extracted membrane protein, was removed and the protein determined by the method of Peterson[15].

(b) Incorporation of membrane proteins into liposomes. A mixture of 2 mL of solubilised membrane protein (2–3 mg protein mL^{-1}), 1 mL liposomes (5 mg lipid), and 270 μL of 15% (w/v) octyl glucoside was blended (vortex mixer) and then incubated for 20 min at 4°C. The suspension was pipetted directly into 50 mM potassium phosphate buffer, pH 7.5 (30 mL), containing final concentrations of 1

mM dithiothreitol, 0.5 mM 2-mercaptoethanol, and vortexed gently at 25°C. The proteoliposome suspension was ultracentrifuged at 85 000*g* (60 Ti rotor) for 1.5–2 h at 4°C to give a proteoliposome pellet. Protein determination on an aliquot of the supernatant gave the protein not incorporated into the liposomes. Triplicate measurements on three separate extraction–reconstitution assays showed that 32% of the initial membrane protein was incorporated into the liposomes. The proteoliposome pellet was resuspended (final concentration, 4 mg protein mL^{-1}) in 50 mM potassium phosphate, pH 7.5 (3 mL), containing 12 mM $MgSO_4 \cdot 7H_2O$ and chloramphenicol (1 mg mL^{-1}) (PL-buffer). These mixtures were always filter-sterilized before use.

(c) Defluorination assay. Proteoliposomes (1 mL; 4 mg protein mL^{-1}) in PL-buffer, containing 10 mM L-malate–50 M FAD, were pipetted into a plastic vial and the volume adjusted to 1.99 mL with the same buffer. The suspension was incubated at 30°C for 20 min with constant shaking. Assays were initiated by the addition of 10 μL of 4FG (final concentration: 1 μmol 4FG 2mg protein^{-1} mL^{-1} PL-buffer) and fluoride release determined at 30°C in 1 h intervals. For glucose inhibition studies (Fig. 5), the same incubation procedure was used except that increasing [glucose] and the above [4FG] were added to initiate the assay.

SDS-PAGE analysis.—Cytoplasmic membrane proteins were analysed by SDS-PAGE using the discontinuous buffer system of Laemmli[22] and a large BRL gel apparatus (Bethesda Research Laboratories, Bethesda, MD). Routine analysis of membrane protein extracts in Tris buffer and 2-mercaptoethanol, required a 12% SDS-gel system and a 5% stacking gel. These gels were run at 60 V (stacking gel) for 2 h and then at 90 V (resolving gel) for 5 h. In general the gels were fixed and stained with Coomassie Brilliant Blue R 250. Molecular mass (daltons) was calculated using a Sigma plot version 4.1 (Jandel Scientific, Corte Madera, CA).

RESULTS AND DISCUSSION

Our earlier studies[7] showed that incubation of 1.0 mM 4FG with *P. putida* resulted in 100% fluoride release. The expected oxidation products 4FGA and 4F2KGA could not be detected because of the presence of membrane-bound glucose and gluconate dehydrogenases[11]. In cell-free extracts of this organism, however, no fluoride release could be detected and 4FG was oxidised to 4FGA and 4F2KGA. This result indicated that an intact cell envelope and/or membrane component was essential for carbon–fluorine bond cleavage to occur. The K_m values for fluoride release in whole cells[7] and oxidation of 4FG by the enzymes (D'Amore and Taylor, unpublished results) are 3.6 and 21.0 mM, respectively. This suggests that at lower concentrations, defluorination would be the preferred metabolic option whilst with increasing concentrations of 4FG, oxidation would compete with defluorination. The in vivo ^{19}F NMR analysis at higher concentrations of 4FG support this contention. Thus, after 15 min with 5.0 mM 4FG (peaks a, Fig. 1), the appearance of the fluoride ion (peak c) is concomitant with oxidation

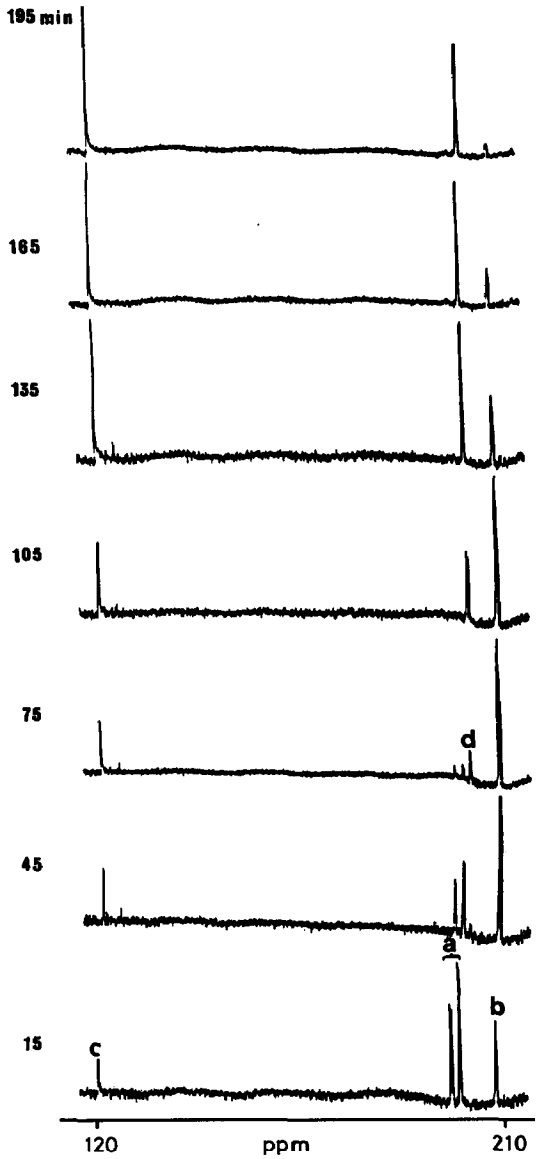

Fig. 1. In vivo ^{19}F NMR trace studies of 4FG metabolism in glucose-grown cells of *P. putida*. Whole cells of *P. putida* were incubated at 30°C with constant shaking between each NMR recording. Signals in order of appearance are: (a) 4FG; (b) 4FGA; (c) F^-; (d) 4F2KGA, (see Experimental).

and the appearance of a new signal (peak b) due to the formation of 4FGA. After 75 min incubation, the metabolism of 4FG to 4FGA is almost complete as shown by the reciprocal changes in the intensity of peaks a and b (Fig. 1). At this time, the appearance of a new signal (peak d) indicates the further oxidation of 4FGA to 4F2KGA. Subsequently, the reciprocal changes in intensity of peaks d and b, with

the corresponding increases in fluoride release, are evident after a further 2 h incubation period (Fig. 1). These results also indicate that 4FGA and 4F2KGA are substrates for the defluorinating protein, since their ^{19}F signals decrease with corresponding increases in the ionic ^{19}F fluoride ion signal. The protein that elicits fluoride release therefore, may be common to 4FG, 4FGA, and 4F2KGA.

In glucose-grown *P. putida* we have shown that loss of CO_2 and fluoride ion from 4FG results in the formation of the 5-carbon end product, 2,3-dideoxy-D-*glycero*-pentonic acid[10]. In an effort to identify earlier defluorinated metabolites, we have now used cells grown on gluconate as a sole carbon source. Such cells still retain the same capacity to release fluoride from 4FG but the extent of CO_2 formation is significantly decreased. Thus with glucose-grown cells, 5.2% of the initial radiolabel was released as CO_2 from D-[U-^{14}C]-4FG after a 2-h incubation with little change after 24 h. Under identical conditions with gluconate-grown cells, only 1.95% of the radiolabel was released after 2 h and 4.85% after 24 h. A similar decrease ($\sim$ 50%) in metabolic activity was observed with respect to the oxidation of 5 mM 4FG to 4FGA and 4F2KGA in gluconate grown cells.

In view of these results, large-scale incubations of gluconate-grown *P. putida* with 1mM [U-^{14}C]-4FG were undertaken until 50% fluoride release into the supernatant had occurred (1 h). Borate-ion exchange column chromatography of the supernatant allowed the separation and isolation of four radioactive components, peaks a–d (Fig. 2). The column elution times and R_f values of these components were different from the known end-product of 4FG metabolism, 2,3-dideoxy-D-*glycero*-pentonic acid[10]. TLC and ^{19}F NMR of component a indicated that it was unchanged 4FG, 75-MHz proton decoupled ^{13}C NMR of

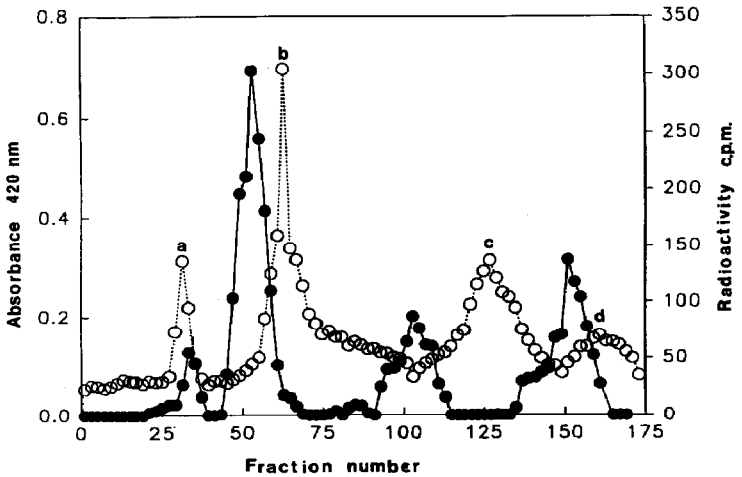

Fig. 2. Dowex 1×8 [borate^{2-}] column chromatographic analysis of the supernatant fraction after incubation of gluconate-grown *P. putida* in 1 mM D-[U-^{14}C]-4FG: ○———○, radioactivity; peak a, 4FG; peaks b, c, and d, defluorinated metabolites. ●———●, Non-radiolabelled standards in order of elution: 4FG; glucose; D-glucose 1-phosphate; D-glucose 6-phosphate.

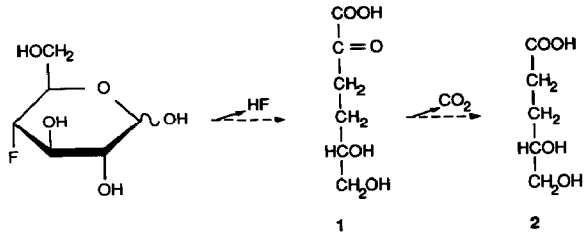

Fig. 3. Defluorinated metabolites of 4FG. **1** from gluconate and **2** from glucose-grown *P. putida*.

components b or c revealed 12 signals of similar intensity each of which were too complex to interpret. In contrast, the proton-decoupled ^{13}C NMR of d gave signals which could be distinguished on the basis of their relative intensities. Four intense resonances at 23.9, 30.5, 50.5, 63.5, and a weak signal at 162.8 ppm were observed. A DEPT 135-edited program of d indicated the presence of one positive-CH signal (δ 50.5) and three negative $-CH_2$ groups (δ 23.9, 30.5, and 63.5) which were assigned to C-5, C-3, C-4, and C-6 respectively. These assignments were confirmed by a DEPT 90 program which eliminated the $-CH_2$ signals and intensified the −CH signal (δ 50.5). The weak resonance at 162.8 ppm was attributed to the carboxyl −C=O group at C-1. The resonance signal for a keto-carbonyl group (range 190–205 ppm) was too weak to be detected. However, component d reacted with *o*-phenylenediamine to give an λ_{max} of 335 nm and a 330–360 nm extinction ratio of 1.4, which is consistent with 2-keto-aldonic acid structure[17]. Furthermore, d gave a periodate–2-thiobarbiturate colourimetric reaction with λ_{max}, 548 nm which is specific for carbohydrates with a 3-deoxy-2-ketoacid carbon skeleton[18]. On the basis of the NMR and spectrophotometric results, component d has been tentatively assigned the structure 3,4-dideoxy-D-*glycero*-hexulosonic acid (**1**). The presence of **1** as a metabolite precursor of **2** (Fig. 3) is consistent with our proposed pathway[10]. The structures of the 6-carbon precursors b and c remain to be elucidated.

From our earlier studies[7,10], it was known that defluorination of 4FG depended on a glucose-inducible–succinate-repressible protein located in either the periplasm, the outer membrane, or cytoplasmic membrane of *P. putida*. The involvement of the periplasmic proteins is considered unlikely since osmotically shocked cells[23], which removes major periplasmic proteins[24], still defluorinate 4FG. Likewise the outer membrane protein OprB involved with glucose transport[25] was excluded. Thus, growth of cells on gluconate, known to repress OprB, still allowed defluorination to occur.

Fluoride release from a cytoplasmic membrane fraction, prepared by a modified French-press procedure[19], was next examined. The results indicate that, compared with whole cells, 25% fluoride release still occurs with this membrane fraction. Of considerable interest is the observation that the provision of the natural electron donor, L-malate–FAD, increases the extent of fluoride release to 50% under

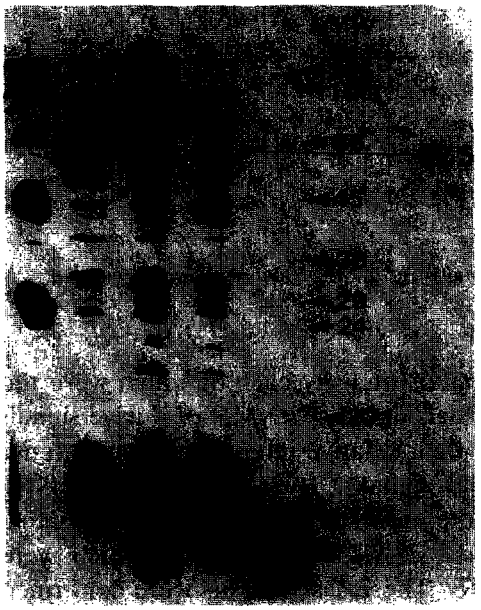

Fig. 4. SDS-PAGE of cytoplasmic membrane extracts from glucose-, gluconate-, and succinate-grown *P. putida*. Lanes 1 and 5, high- and low-molecular-mass markers in kilodaltons. Lanes 2, 3, and 4 membrane extracts (350 μg protein in each lane) from glucose-, gluconate-, and succinate-grown cells. The amount of protein with apparent molecular mass 65.5 kD is repressed in succinate-grown cells (lane 4).

identical conditions. It is known that the active transport of glucose, gluconate, and D-*arabino*-hexulosonate ("2-keto-gluconate") in the cytoplasmic membrane of *P. putida* is energy coupled via L-malate dehydrogenase and the electron-transport system[11,12] in accordance with chemiosmotic theory[26]. This respiratory couple provides the energy, Δp (composed of ΔpH and $\Delta\psi$) necessary to maintain intracellular sugar gradients. It would appear, therefore, that fluoride release from 4FG is energy coupled in some way by Δp to the sugar transport system in the cytoplasmic membrane. Some evidence to support this was provided when fluoride release in whole cells was extensively inhibited (> 90%) by making the external pH more alkaline (from 7.1 to 8.2 or adding 2,4-dinitrophenol which collapses ΔpH. It is noteworthy that defluorination does not occur after octyl glucoside extraction of the cytoplasmic membrane fraction. Presumably the topology and membrane protein integrity necessary for the chemiosmotic energy couple has been destroyed.

SDS-PAGE analysis of the cytoplasmic membrane fraction showed the presence of a glucose-inducible and succinate-repressible protein band with an apparent molecular mass of 65.5 kDa (Fig. 4, lanes 2 and 4, respectively). Since this was the only band to exhibit the same induction–repression pattern as that observed for fluoride release in whole cells[7], it was designated as the putative defluorinating protein. Conspicuous by the large decrease in electrophoretic mobility, this protein band appears to become cross-linked to itself or other membrane proteins (results

TABLE I

Fluoride release from 4-deoxy-4-fluoro-D-glucose (4FG) in proteoliposomes prepared from *P. putida* in the presence or absence of PC and L-malate

Procedures [a]	Glucose-grown	Gluconate-grown	Succinate-grown
Solubilisation (%) of initial protein concentration	52.6 (58.2)	49.5 (60.3)	54.2 (52.2)
Incorporation (%) [b] solubilised protein into liposomes	30.5 (32.2)	32.6 (30.3)	34.2 (38.2)
Fluoride release (%) [c] in proteoliposomes	20.0 (3.2) 2.5 [d]	22.0 (2.7) 2.3 [d]	2.5 (1.7) 1.5 [d]

[a] Values given for each procedure are the average of duplicate trials. [b] Incorporation (%) using a ratio of 0.8–1.2 mg protein:1 mg liposomes. [c] Fluoride release (%) measured after incubation time of 17–22 h in the presence of 10 mM L-malate–50 μMFAD with 1 μmol 4FG–2 mg proteoliposomes/mL–50 mM phosphate buffer, pH 7.5. Bracketed data were obtained in the absence of PC. All incubations contained chloramphenicol (1 mg/mL). [d] Fluoride release (%) in the absence of 10 mM L-malate–50 μM FAD.

not shown) in the absence of reducing agents (e.g., dithiothreitol). Supporting evidence for the existence of such active –SH groups is provided by the fact that total protection of fluoride release occurs after treatment of whole cells with *N*-ethylmaleimide[7].

Fluoride release activity was now reconstituted into liposomes by the detergent dilution procedure of Racker et al.[21]. The liposomes were made by established procedures known to produce multilamellar structures[20] with diameters of 3–5 μm. This was confirmed by electron microscopy. After initial solubilisation of the total cytoplasmic membrane protein by octyl glucoside, an average value of 32% of this solubilised protein was incorporated into liposomes under a variety of conditions (Table I) in the presence or absence of PC. The low fluoride release (2.5 and 1.7%) in proteoliposomes from succinate-grown cells is consistent with the known repression of fluoride release by whole cells grown on this carbon source. In proteoliposomes derived from glucose- or gluconate-grown cells, however, the 20–22% fluoride release was virtually eliminated in the absence of PC (Table I). A requirement for exogenous PC has been demonstrated elsewhere for the reconstitution of bacterial[19] and mammalian transport systems[27]. Thus the addition of PC may provide a protective and stabilising medium for the reconstitution of the defluorinating protein and associated components. Fluoride release in proteoliposomes is also dependent on the presence of the natural electron donor, L-malate. Thus in the absence of L-malate the fluoride release is reduced from 20–22% to ~ 2% (Table I). Similar results were obtained with the artificial electron donor, 0.1 mM PMS–20 mM ascorbate (results not shown). Again, with proteoliposomes derived from succinate-grown cells, in which the defluorinating protein has been

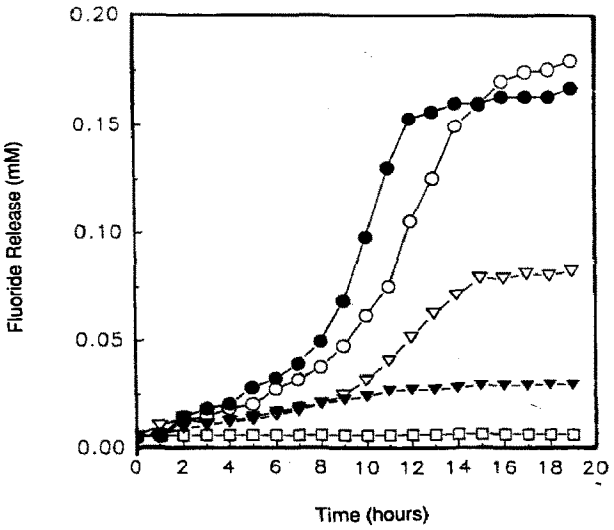

Fig. 5. The inhibition of the extent of fluoride release by glucose in proteoliposomes. Fluoride release in the presence of glucose: ●, 0.25 mM; ▽, 0.5 mM; ▼ 1.0 mM; □, 2.0 mM. ○, In the absence of glucose (see Experimental).

repressed, no fluoride release was observed in the presence or absence of electron donors.

In earlier studies[7], it was shown that fluoride release in whole cells of *P. putida* was most effectively inhibited by D-glucose, D-gluconate, and D-*arabino*-hexulosonate ("2-keto-D-gluconate") at twice the concentration (5.0 mM) of 4FG (2.5 mM). In the present studies with reconstituted proteoliposomes, D-glucose at half the concentration (0.5 mM) of 4FG (1.0 mM) was sufficient to cause 50% inhibition of fluoride release (Fig. 5). This direct inhibition, without the complexity of whole cell metabolism, suggests that 4FG binds to the same protein as D-glucose but with less affinity. The importance of the D-*gluco* configuration, especially at C-4, for binding to the defluorinating protein is illustrated by the effects of the C-4-epimer, D-galactose. Thus, in proteoliposomes, a two- or four-fold increase in concentration of D-galactose only gave a 6 and 10% decrease, respectively, in fluoride release from 1 mM 4FG.

Our results suggest the presence of an inducible–repressible protein that binds 4FG or glucose and which is associated with the glucose carrier protein known to be present in the cytoplasmic membrane of *P. putida*[12]. Subsequently, the chemiosmotic energy normally used for the active transport of glucose, gluconate, and "2-keto-D-gluconate"[11], is coupled to the defluorination of 4FG. Such a mechanism would appear to be common for 4FGA and 2K4FGA.

Further studies, involving protein isolation, sequencing and reconstitution, should permit a more detailed elucidation of the mechanism of carbon–fluorine bond cleavage in 4FG as well as a further understanding of the molecular basis of monosaccharide transport in this organism.

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