The reaction of 4-deoxy-4-fluoro-D-glucose with an outer membrane protein of *Pseudomonas putida*

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1. INTRODUCTION

Glucose-grown or cell-free extracts of *Pseudomonas putida* metabolise 3FG to 3FGA and 3F2KGA [1]. These fluorinated metabolites are produced by the enzymes glucose oxidase (EC 1.1.3.4) and gluconate dehydrogenase (EC 1.1.99.3) which are considered to be located on the outer surface of the cytoplasmic membrane [2,3]. Using membrane vesicles prepared from glucose or succinate grown *P. putida* we have shown that 3FG is transported by the same active-transport system as glucose [4]. 3FG [5] and 4FG act as catabolite repressors in lactose-grown *Escherichia coli* [6]. In the above studies with 3FG or 4FG the carbon-fluorine bond remains intact. Here, we wish to report that:

(i) Unlike 3FG, the isomeric 4FG is not transported or oxidized by whole cells of glucose grown *P. putida*; instead an extensive release of F^- occurs.
(ii) Fluoride release from 4FG is dependent upon the presence of an inducible/repressible protein which is associated with the outer membrane of the organism.
(iii) Fluoride release is inhibited in the presence of D-glucose.

A preliminary account of some of these results has appeared in [7].

Abbrevations: 3FG, 3-deoxy-3-fluoro-D-glucose; 3FGA, 3-deoxy-3-fluoro-D-gluconic acid; 3F2KGA, 3-deoxy-3-fluoro-2-keto-D-gluconic acid; 4FG, 4-deoxy-4-fluoro-D-glucose; 4F2KGA, 4-deoxy-4-fluoro-2-keto-D-gluconic acid; α-Me4FG, methyl 4-deoxy-4-fluoro-α-D-glucopyranoside; NEM, *N*-ethyl maleimide; PMS, phenazine methosulphate; DCIP, 2,6-dichloroindophenol; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Chemicals*

Crystalline 4FG 4-deoxy-D-glucose and α-Me-4FG were synthesized as in [8]. NEM, DTT, chloramphenicol, lysozyme, ribonuclease A, deoxyribonuclease I, DCIP, PMS and all other carbohydrates, chemicals and reagents were obtained from Sigma (St Louis MO).

2.2. *Culture conditions and organism*

Pseudomonas putida ATCC 12633 was routinely maintained on the appropriate carbon source–mineral salts agar medium as in [4] except that cells were harvested from glucose or succinate after 15 h (late exponential phase). Protein determination was as in [9].

2.3. *Preparation of the cytoplasmic and outer membrane*

The cytoplasmic membranes (vesicles) from glucose-grown *P.putida* were isolated as in [4]. Isolation of an outer membrane fraction was based on a modified procedure [10]. Glucose grown whole cells were harvested after 15 h by centrifugation (Sorvall Superspeed RC2-B) at 10 000 × g for 10 min at 30°C and washed with 0.1 M potassium phosphate buffer (pH 7.1). The resulting cells (4 g wet wt) were suspended in 40 ml 20% sucrose and the following ice-cold reagents: 20 ml 2 M sucrose, 10 ml 0.1 M potassium phosphate (pH 7.1), 10 ml 0.01 M $MgSO_4$, 61 mg DTT and 40 mg lysozyme. The outer membrane fraction was isolated from this mixture as in [10] and characterized by measuring the activities of glucose, gluconate and succinate dehydrogenases using PMS and DCIP as electron acceptors. SDS gel electrophoresis was also carried out as in [10].

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2.4. *Respirometric and chromatographic studies*

Cell-free extracts of glucose grown *P. putida* were prepared by sonication [11]. The extent and rates of respiration of 4FG was carried out by the manometric method using a Gilson differential respirometer as in [4]. Thin-layer chromatography was as in [11].

2.5. *Kinetics and inhibition of fluoride release*

(i) *In the absence of chloramphenicol:* Fluoride ion was determined by a fluoride electrode (Orion Research, Cambridge MA). The reaction was initiated by the addition of known amounts of 4FG (1 ml) to 3 ml of a glucose- or succinate-grown suspension of whole cells (5–8 mg protein/ml) all in 100 mM phosphate buffer (pH 7.1), and incubated in a rotary shaker at 30°C. At various time intervals fluoride release from 4FG was determined (fig.1). A similar protocol was used in the presence of 1 mM DTT with the appropriate cell envelope fractions (table 3). The inhibition of fluoride release from 4FG using glucose-grown whole cells was obtained by the simultaneous addition of 2.5 mM 4FG and 5 mM of the appropriate sugar or 10 mM NEM (final conc.) to a 2 ml cell suspension (15–20 mg protein) all in phosphate buffer (pH 7.1). After incubation as above fluoride release was determined after 30 and 60 min intervals (table 2).

(ii) *In the presence of chloramphenicol:* The extent of fluoride release was also determined after glucose grown cells (5–8 mg protein/ml) had been pre-incubated with chloramphenicol (1 mg/ml) all in phosphate buffer (pH 7.1) for 1 h at 30°C. Samples of 3 ml were removed and incubation continued for 24 h in the presence of various amounts of 4FG (fig.2). The initial rates of fluoride release were the average of duplicate determinations and the Lineweaver-Burk plots [12] were estimated by linear regression of the kinetic data (fig.3).

3. RESULTS AND DISCUSSION

Cell-free extracts or membrane vesicles prepared from glucose-grown *P. putida* oxidize 4FG to the extent of 2 g atoms oxygen/mol with retention of the C–F bond (table 1). Thin-layer chromatography of vesicle or cell-free extracts before and after complete oxidation indicated that 4FG (R_F 0.62) was converted to a slower-moving reducing component (R_F 0.42) which is consistent with the formation of 4F2KGA. These results are similar to those obtained with 3FG [1] and show that 4FG is a substrate for glucose oxidase and gluconate dehydrogenase. With whole cells, however, 4FG elicits a release of fluoride without any detectable respiration (table 1). Thus with 0.5 mM 4FG 100% fluoride release occurs and no 4FG or de-fluorinated product can be detected by chromatography. The rate and extent of this fluoride release is repressed with cells grown on succinate (fig.1). Further support for the presence and synthesis of protein for the de-fluorination of 4FG was provided when glucose- or succinate-grown cells were preincubated with chloramphenicol prior to their exposure to 4FG. Such treatment reduced fluoride release after 24 h to 11% and 2.5%, respectively (fig.1). These values presumably reflect the amount of endogenous

Table 1

The respiration of 4FG and fluoride ion release by whole cells, cell-free extracts and vesicles prepared from *P. putida*

Added 4FG (μmol)	Net oxygen consumption (μl, endogenous subtracted)			mol oxygen/mol substrate oxidized			nmol F . h^{-1} . mg protein $^{-1}$			% Fluoride released after 24 h (24 mg protein)
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)
1	0	23	21	0	1.1	0.95	3.0	0	0	100
2	0	45	48	0	1.0	1.1	6.0	0	0	100
5	0	108	118	0	1.0	1.1	12.2	0	0	95
10	0	n.d.	212	0	n.d.	1.0	22.8	0	0	91
20	0	n.d.	n.d.	0	n.d.	n.d.	32.4	0	0	88

n.d., not determined; (1) whole cells; (2) cell-free extracts; (3) vesicles

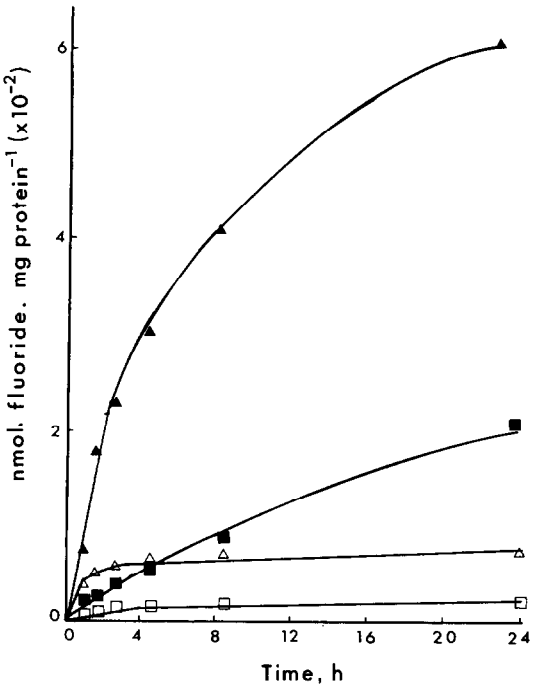

Fig.1. The effect of chloramphenicol on fluoride release from 4FG by glucose and succinate grown *P. putida:* (▲) glucose grown; (■) succinate grown; (△) glucose grown + chloramphenicol; (□) succinate grown + chloramphenicol; cells were pre-incubated with chloramphenicol before exposure to 2.5 mM 4FG.

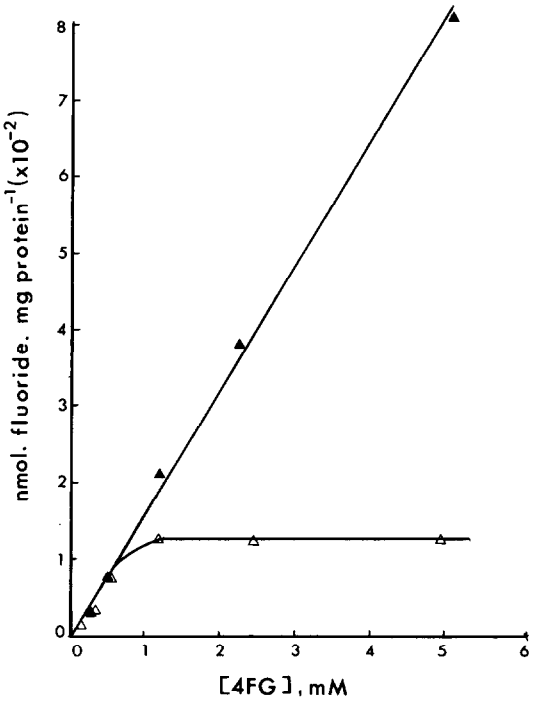

Fig.2. Fluoride release *vs* [4FG] for glucose-grown and chloramphenicol-treated *P. putida*: (▲) glucose grown; (△) glucose grown + chloramphenicol; all measurements made with 24 mg total cell protein.

protein which reacts with 4FG. To estimate this amount of protein a plot of fluoride release against various concentrations of 4FG with a fixed amount of total cell protein (24 mg) using glucose grown chloramphenicol treated cells was undertaken. Up to 1.25 mM 4FG results in the production of 133 nmol fluoride/mg protein and this value remains constant with increasing concentrations of 4FG (fig.2). From this data one may calculate that 2.4% of the total protein (57.6 μg in 24 mg) has reacted with 4FG. The fluoride release with chloramphenicol-treated cells is irreversible since washing the cells after exposure to saturating concentrations of 4FG and re-exposure to 4FG elicits no further significant fluoride release.

A comparison of the effect of various sugars on the release of fluoride from 2.5 mM 4 FG indicates that the most effective protection of fluoride release is afforded by D-glucose, D-gluconate and 2-keto-D-gluconate (table 2). L-Glucose, α-Me4FG, methyl α- or β-D-glucopyranoside, D-galactose and maltose provide no protection. As might be anticipated 4-deoxy-D-glucose is a poor inhibitor, which confirms the important stereo-specificity and hydrogen bonding requirement at C_4 for the binding of sugars with the D-gluco-configuration as well as 4FG to the protein. Furthermore, the complete protection of fluoride release afforded by NEM suggests the importance of protein –SH groups in the de-fluorination reaction. This reaction displays saturation kinetics with K_m = 3.6 mM and V_{max} = 1 nmol fluoride . mg protein^{-1} . min^{-1} (fig.3). These results would be consistent with an initial fast equilibrium binding of 4FG (by fluorine-hydrogen bonding) to protein followed by a slow nucleophilic displacement of fluoride, possibly by –SH group participation of the protein which then becomes co-valently attached to the sugar. The location of fluoride release in *P. putida* was established by fractionation of the cell envelope using a

Table 2

The effect of various sugars and NEM on fluoride release from 4FG in *P. putida*

2.5 mM 4FG + 5 mM sugar or 10 mM NEM added to incubate[a]	% Inhibition of fluoride release after (min)	
	30	60
L-Glucose	0	0
α-Me4FG	0	0
D-Galactose	0	0
2-Deoxy-D-glucose	57	55
D-Glucose	81	91
4-Deoxy-D-glucose	30	28
D-Gluconate (K^+ salt)	90	90
2-Keto-D-gluconate (Ca^{2+} salt)	90	87
Methyl α-D-glucopyranoside	0	0
Methyl β-D-glucopyranoside	0	0
Maltose	0	0
NEM	100	100

[a] Whole cell suspensions (15–20 mg protein) in phosphate buffer (pH 7.1) incubated at 30°C (total vol. 3 ml)

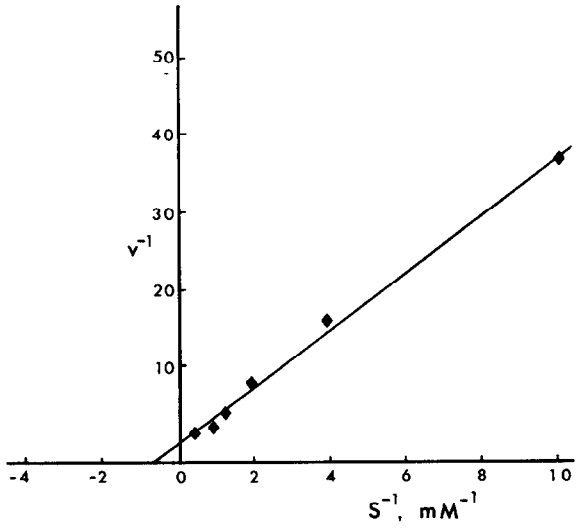

Fig.3. Lineweaver-Burk plot of fluoride release from 4FG by chloramphenicol treated glucose grown *P. putida*: *v*, initial rate of fluoride release (nmol fluoride . mg protein^{-1} . min^{-1}); *S*, [4FG] in mM.

Table 3

Fluoride release from 4FG and various fractions of the cell envelope of *P. putida*

Fraction[a]	nmol fluoride/ mg protein
Supernatant from spheroplast	20
Spheroplasts	80
Vesicles	19
Cell-free extract	6
Outer membrane	300
Whole cells	358

[a] Fractions were incubated 24 h at 30°C in phosphate buffer (pH 7.1) with 2.5 mM 4FG final conc. (see section 2)

modified procedure [10]. The criteria for the purity of the outer membrane was based on the low specific activities of glucose, gluconate and succinate dehydrogenases (14, 7.5 and 1 nmol reduced DCIP. mg protein $^{-1}$. min^{-1}, respectively) compared with the respective activities of these enzymes in the cytoplasmic membrane (303, 177 and 6.5). Additionally, the SDS gel electrophoresis of the outer membrane gave a distribution of 5 protein bands similar to those reported for *P. aeruginosa* [10]. Therefore, it is evident (table 3) that the site of de-fluorination occurs predominately in the outer membrane fraction. Fractions produced by osmotic shock [13] of whole cells failed to release fluoride from 4FG.

These results indicate the presence of an inducible repressible protein associated with the outer membrane of *P. putida* which reacts with 4FG. The isolation, mechanism of fluoride release and possible function of this protein in relation to glucose transport remains to be elucidated. Since 4FG meets the criteria of an affinity label [14] we have synthesized D-[6-^{3}H]4FG to assist us with these studies [15].

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