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PROTEIN D1 – A GLUCOSE-INDUCIBLE, PORE-FORMING PROTEIN FROM THE OUTER MEMBRANE OF *PSEUDOMONAS AERUGINOSA*

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1. Introduction

The uptake of glucose by *Pseudomonas aeruginosa* probably involves two distinct inducible pathways with differing affinities for glucose. A high affinity (low K_m) system with a K_m value of 8 μM for glucose, is induced by growth on glucose but not by growth on gluconate, glycerol, succinate or citrate [1,2]. Stinson et al. [3,4] have described a periplasmic glucose binding protein which is induced in the same growth media, suggesting the involvement of this protein in the high affinity system. In addition, a low affinity (K_m = 1–2 mM) glucose transport system has been described and is induced by growth on glucose, gluconate and glycerol but is repressed by succinate and citrate [1,2].

Recently, Mizuno and Kageyama [5] described an outer membrane protein D which was considerably enhanced in cells grown in the presence of glucose. We demonstrated that protein D was in fact two polypeptides, one of which, protein D1, only appeared after growth of cells on glucose [6]. In this paper, we demonstrate that protein D1 is induced under growth conditions which result in induction of the high affinity glucose uptake system and the periplasmic glucose binding protein. Furthermore, protein D1 has been purified and shown to reconstitute sucrose glucose permeable pores in LPS-phospholipid vesicles. We postulate that the protein is an outer membrane glucose pore analogous to the *lamB* maltose pore of *Escherichia coli* [7,8].

2. Materials and Methods

P. aeruginosa PA01 strain H101 was used throughout and grown on BM2 minimal medium to an absorbance at 650 nm of 0.6 as previously described [6]. Carbon sources were added at a final concentration of 0.4% (w/v) for saccharides or 20 mM for the potassium salts of citric acid cycle substrates, pyruvate and gluconate. The methods of outer membrane isolation and sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis have been described [6,9].

For the purification of protein D1, outer membranes were isolated from 22 g (dry wt.) BM2 glucose grown cells without lysozyme treatment [6]. They were resuspended by sonication in 2% Triton X-100, 20 mM Tris–HCl buffer pH 8.0 at a protein concentration of 10 mg/ml and the Triton insoluble residue precipitated by centrifugation at 160 000 × g for 1 h. The pellet was resuspended by sonication in 2% Triton X-100, 20 mM Tris–HCl pH 8.0, 10 mM EDTA followed by centrifugation as described above. The supernatant (19.2 mg) was applied directly to a DEAE Sephacel column (1.8 × 7.3 cm) pre-equilibrated with 0.1% Triton X-100, 20 mM Tris–HCl pH 8.0, 10 mM EDTA (column buffer) and eluted with 20 ml column buffer, followed by 20 ml of 0.1 M NaCl in column buffer and 60 ml of a 0.1–0.25 M NaCl gradient in column buffer. The 2 ml column fractions were assayed for protein and run on an SDS polyacrylamide gel, and the fraction containing pro-

tein D1 (7.4 mg total) exhaustively dialysed against 5% ethanol to reduce Triton X-100 concentrations [10]. Protein F was purified as described previously [10].

Reconstitution of phospholipid-lipopolysaccharide (LPS) vesicles was as described in [9,10] except that synthetic dioleoyl phosphatidyl choline was used instead of *P. aeruginosa* phospholipids. This resulted in a two to five fold greater incorporation of radioactivity. Vesicles were collected by either: (a) column chromatography on Sepharose 4B, or (b) membrane filtration [9].

3. Results and Discussion

The outer membrane of cells grown on glucose contains a high level of protein D1 (Fig. 1, gel A; see also [6]. In contrast, the level of protein D1 in the outer membranes of succinate or gluconate grown cells was too low to observe (Fig. 1, gels B and C), even in heavily overloaded gels. These studies were extended to include cells grown on 30 different media. The appearance of protein D1 was induced by the addition of non-metabolizable inducers (e.g. galactose, 2-deoxyglucose and α methyl glucoside; Fig. 1, gel D) to BM2 pyruvate medium and repressed by the addition of citrate, 0.1% (w/v) proteose peptone No. 2, 0.2% (w/v) casamino acids and various amino to BM2 glucose medium. In addition, D1 was not induced by a variety of different carbon sources (Table 1). Overall, the results demonstrated that under all conditions where the high affinity glucose uptake system and the periplasmic glucose binding protein were induced [1–3,11,12], protein D1 was seen in SDS polyacrylamide gel electrophoretograms of outer membranes (Table 1). There was no such correlation between induction of protein D1 and the induction of the low affinity glucose uptake system [1,2], the mannitol, fumarate, glycerol or dicarboxylic acid uptake systems or a variety of hexose (including glucose) catabolizing enzymes [2,4,12]. Thus, while there is as yet no direct evidence supporting the involvement of protein D1 in glucose uptake, the co-regulation of this outer membrane protein with the high affinity glucose uptake system and a periplasmic glucose binding protein is highly suggestive.

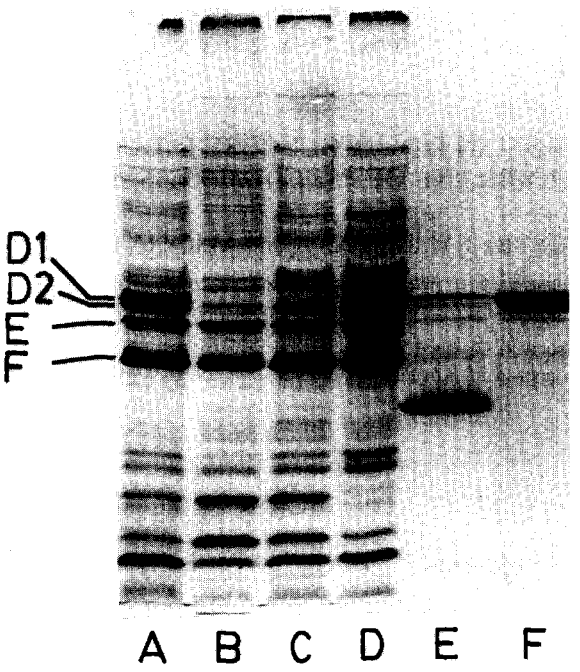

Fig. 1. Effect of carbon source on the production of outer membrane protein D1 by *P. aeruginosa*. Gels A–D, outer membranes of cells grown on the indicated carbon sources: A, glucose; B, succinate; C, gluconate; D, pyruvate + galactose. All samples were solubilized at 88°C for 10 min prior to electrophoresis. Gels E and F, purified protein D1 (fraction 37 of Fig. 2) solubilized at 30°C, 10 min and 88°C, 10 min, respectively. The heat modification of SDS polyacrylamide gel mobility was described earlier for protein D1 in whole outer membranes [6]. Note that even at 30°C a small amount of protein D1 is in the modified form.

Recently, we demonstrated that purified *P. aeruginosa* outer membrane protein F ("porin") could form pores in LPS-phospholipid vesicles with an exclusion limit for saccharides of around 6000 daltons [10]. Other outer membrane pore-forming proteins include porins from *E. coli* [13] and *Salmonella typhimurium* [14], the *lamB* protein from *E. coli* [7,8] and the 44000 dalton protein from *S. typhimurium* [15]. The latter two are particularly interesting since they are inducible by maltose and the *lamB* protein has been shown to mediate maltose uptake. In order to determine if protein D1 was able to form transmembrane pores in membrane bilayers we first purified the protein. The procedure used was derived from the observation that porin protein F

TABLE 1
Induction of outer membrane protein D1 in *P. aeruginosa* grown on various carbon sources: correlation with induction of high affinity glucose uptake and the periplasmic glucose binding protein

Growth substrate	Outer membrane protein D1 [a]	Periplasmic glucose binding protein [b]	Glucose transport [c]	
			High affinity	Low affinity
Glucose	+	+	+	+
Gluconate	–	–	–	+
Glycerol	–	N.D.	–	+
Pyruvate	–	–	–	–
Pyruvate + 2 deoxyglucose	+	+	+	N.D.
Pyruvate + galactose	+	+	+	N.D.
Pyruvate + α methyl glucoside	+	+	+	N.D.
Succinate	–	–	–	–
Acetate	–	–	–	–
Citrate	–	N.D.	–	–

[a] Estimated from SDS polyacrylamide gel electrophoretograms of outer membranes of cells grown on the indicated substrates; + protein induced to high levels; – protein D1 not visible in electrophoretograms. In addition the protein was induced to high levels with glucose + gluconate, glucose + 0.5% NaCl and glucose + phenylalanine + tyrosine as growth substrates, to intermediate levels with fructose, mannitol, glucose + gelatin, glucose + serine + alanine + glycine or glucose + asparagine + glutamine as growth substrates and was not observable in cells grown on malate, fumarate, 1% proteose peptone No. 2 with or without the addition of glucose, malate, succinate or citrate, glucose + 0.1% proteose peptone No. 2, glucose + 0.2% casamino acids, glucose + aspartate + glycine + valine, 1% tryptone + 0.5% yeast medium, or citrate + galactose.

[b] Taken from [3]. + means > 0.8 units/mg, – means ⩽ 0.15 units/mg. N.D., not determined.

[c] Taken from the data of [1–3,11,12]: + transport of glucose occurs by the indicated system; –, no transport of glucose; N.D., not determined.

remained insoluble in Triton X-100 + EDTA providing the peptidoglycan was intact due to specific binding of protein F to the peptidoglycan (Hancock, R.E.W., Carey, A.M., Irvin, R. and Costerton, W., manuscript in preparation). Thus, we were able to obtain a fraction containing all of the protein D1 in the outer membrane but virtually none of the protein F. When this fraction was bound to an ion exchange column and eluted with a salt gradient, the major peak fraction (Fig. 2) was a highly purified preparation of protein D1 (Fig. 1, gel F). Assuming quantitative yields, the isolation of 7.4 mg of purified protein D1 from $6 \cdot 10^{12}$ cells gives a minimum estimate of $2 \cdot 10^4$ molecules D1/cell. The only major contaminant present was LPS (22 μg of an LPS specific sugar 2 keto-3-deoxyoctanate per mg protein), but since this molecule was to be added in subsequent reconstitution experiments, we made no attempt to extract it.

We previously demonstrated that outer membrane-associated protein D1 was heat modifiable in that its mobility on subsequent SDS polyacrylamide gel electrophoresis was determined by the temperature of solubilization [6]. It was shown that the purified protein D1 was similarly heat modifiable (Fig. 1, cf. gels E and F), confirming the identity of the purified protein.

In order to assay for pore-forming ability, vesicles were reconstituted from purified protein D1, LPS and phospholipid and the leakage of sucrose or glucose, relative to a 20000 dalton dextran (NET 427A, Amersham Corp., Oakville, Ontario), measured. As controls, vesicles with LPS and phospholipid alone or with purified porin protein F instead of protein D1 were formed. The results, shown in Table 2, are expressed by the magnitude of a permeability index. They suggest that protein D1 is a pore-forming protein for glucose and sucrose. Preliminary experiments suggested that the trisaccharide raffinose could pass freely through protein D1 pores, but that an inulin of 5000 daltons could not.

We have observed that some outer membrane proteins are released by 0.2 M $MgCl_2$ washing, a treatment used to extract glucose binding and other peri-

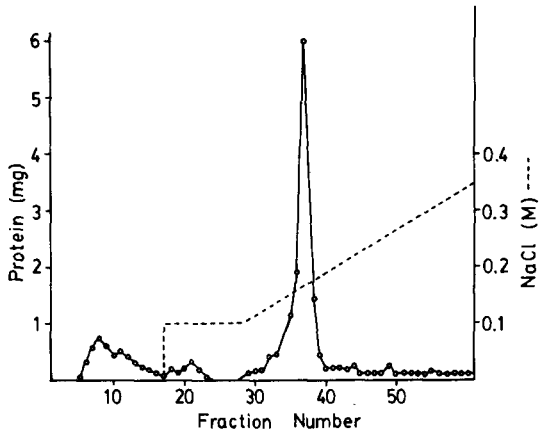

Fig. 2. Separation by DEAE-Sephacel chromatography of Triton X-100/EDTA soluble (lysozyme untreated) outer membrane proteins of *P. aeruginosa* PA01 strain H101 (o———o). The sample preparation and column buffer are described in Materials and Methods. The column was eluted with varying concentrations of NaCl in column buffer. Fractions 37 and 38 were substantially purified protein D1 preparations (see Fig. 1). Other fractions contained other proteins but little or no D1 (results not shown).

plasmic proteins from cells [3,4]. In order to distinguish outer membrane protein D1 from the glucose binding protein, binding assays were performed using the membrane filtration method described by Stinson and colleagues [4]. As a control, we assayed the binding activity of ammonium sulphate-precipitated, $MgCl_2$ extracts of BM2 glucose grown cells. This heterogeneous fraction, which should contain the glucose binding protein, bound 0.16 nmol glucose/mg protein in agreement with previous results [4]. In contrast, purified protein D1 bound less than 0.01 nmol glucose/mg protein, suggesting that it was not the glucose binding protein, and also that it is not a glucose specific permease.

The results presented here suggest that the uptake of glucose in *P. aeruginosa* shares several features with maltose (a glucose disaccharide) transport in *E. coli*. The maltose uptake system involves an inducible 50 000 dalton outer membrane protein (the *lamB* protein) which can reconstitute sucrose permeable pores in LPS-phospholipid vesicles [7], in addition to a 44 000 dalton coinducible periplasmic maltose binding protein [16]. In *lamB*-deficient mutants and at high external maltose concentrations, the porin of *E. coli* K-12 probably serves for maltose uptake (see [8] for discussion). Thus there are both high and low affinity maltose uptake systems in *E. coli*. For *P. aeruginosa* glucose transport, high and low affinity systems have also been demonstrated [1,2]. Based on the results presented here and by analogy to the mal-

Table 2
Reconstitution of sucrose and glucose permeable pores in LPS-phospholipid vesicles using purified outer membrane proteins D1 and F

Saccharide	Method [a]	Pore forming protein (nmol) [b]	Permeability index [c]
Sucrose	A	None	1.0
		D1 (1.0)	2.56
		F (1.1)	2.36
	B	None	1.0
		D1 (0.8)	4.96
		F (0.4)	4.23
Glucose	A	None	1.0
		D1 (1.0)	1.96
	B	None	1.0
		D1 (0.4)	2.0
		D1 (0.8)	3.97
		D1 (1.2)	24.14
	C	None	1.0
		D1 (0.8)	40.6

[a] Method A: collection of vesicles by Sepharose 4B column chromatography; Method B: collection of vesicles by membrane filtration; Method C: 0.5 μmol; *P. aeruginosa* phospholipids were substituted for 0.5 μmol dioleoyl phosphatidyl choline used in methods A and B. The differences in the permeability indexes are probably due to the preferential breakage of vesicles without pores during millipore filtration and the reduced equilibration time of vesicle contents with the external medium during column chromatography.

[b] The amount of protein added was calculated using the molecular weights for D1 and F of 50 500 and 35 300, respectively (average from Table 1 [6]).

[c] The permeability index as defined in [10].

$$\frac{[^3H]\text{dextran T20: } [^{14}C]\text{saccharide retained in the presence of added protein}}{[^3H]\text{dextran T20: } [^{14}C]\text{saccharide retained in the absence of added protein}}$$

The presence of a pore-forming protein leads to specific leakage of the [^{14}C]saccharide out of the vesicles, resulting in an enhanced [^{3}H]dextran T20: [^{14}C]saccharide ratio and thus a higher permeability index. The controls with no protein always give a permeability index of 1.0. In this assay three other partially purified outer membrane proteins, H1, G and I [10] did not appear to form pores.

tose transport system of *E. coli*, we propose that outer membrane protein D1 and the periplasmic glucose binding protein are co-regulated components of the *P. aeruginosa* high affinity glucose transport system. Furthermore, we hypothesize that the low affinity glucose transport system involves uptake across the outer membrane via the porin protein F. At present, we are attempting to test these hypotheses.

Acknowledgements

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