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RESOLUTION OF *PARACOCCUS DENITRIFICANS* MEMBRANE VESICLES OF DIFFERING CONFIGURATION USING AFFINITY CHROMATOGRAPHY

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1. Introduction

Membrane vesicles prepared from the plasma membrane of a variety of bacteria, and especially from *Escherichia coli* [1,2], have proved to be valuable systems for analysing the mechanism of both active transport and oxidative phosphorylation in bacteria [3,4]. The interpretation of data from experiments with such vesicles, however, is often subject to ambiguity [4] since three types of vesicle may in principle be formed upon disrupting the intact cell: right-side-out (RSO) vesicles, in which the components of the vesicle membrane have the same orientation as in the plasma membrane of the intact cell; inside-out (ISO) vesicles, in which complete inversion has occurred during their preparation so that the orientation of the vesicle membrane is the reverse of that of the plasma membrane of the intact cell; and scrambled vesicles, in which some components of the plasma membrane change their relative orientation during vesicle preparation so that the vesicles are not simply related, in a topological sense, to the intact cell.

After growth under appropriate conditions, osmotic lysis of spheroplasts derived from *Paracoccus denitrificans* cells produces a population of membrane vesicles which is believed to consist predominantly of ISO vesicles [5]. In order to characterise further these vesicles we have separated RSO from ISO vesicles by passing the vesicle preparation down an ADP-agarose column. The rationale was that the plasma membrane ATPase, which is expected to have a high affinity for ADP, would bind to the immobilised ligand, thus causing the column to retain only the ISO vesicles, in which the ATPase faces outward.

2. Materials and Methods

Paracoccus denitrificans (*Micrococcus denitrificans* NCIB 8944) was grown with succinate as carbon source and nitrate as added terminal electron acceptor [5]. Plasma membrane vesicles were prepared as described [5], with the modification of John [6]. They were resuspended in 10 mM Tris–acetate pH 7.3 containing 5 mM magnesium acetate. Membrane vesicle sidedness was determined using the assay of the latency of NADH-DCIP reductase activity described previously [5]. Oxygen uptake and protein were measured as described previously [7].

N^6-(6-Aminohexanoyl)-ADP-agarose (Cat. No. A1642) was purchased from Sigma (Lot No. 87C-8600).

3. Results

Table 1 shows that when a sample of *P. denitrificans* vesicles was applied to the ADP affinity column, a fraction of the preparation (Fraction A) was not retained by the column. Subsequent washing of the column with buffer did not cause elution of the retained vesicles, but when a pulse of ADP (sodium salt) was applied to the column, the retained vesicles (Fraction B) were eluted (Table 1).

It has been suggested that discrimination between

TABLE 1

Separation of *P. denitrificans* membrane vesicles using immobilised ADP 0.4 ml (3.0 mg protein) of a preparation of *P. denitrificans* membrane vesicles was applied to a 5 mm × 20 mm column containing 1 ml ADP- agarose pre-equilibrated with 10 mM Tris acetate/5 mM magnesium acetate pH 7.3. It was eluted with 2.5 ml of the same buffer. 0.5 ml fractions were collected, using an LKB Ultrorac fraction collector, at a gravity-induced flow rate of approx. 0.4 ml min^{-1}. After 5 fractions had been collected a pulse (0.5 ml) of 0.1 M ADP (sodium salt, pH 7.0) was applied to the column and further fractions collected. Fractions were pooled and assayed for protein and NADH dehydrogenase activities as described in Materials and Methods. The protein recovered represented about 85% of that applied to the column. The NADH dehydrogenase activities of the initial preparation were 48 (no bee venom) and 77 (plus bee venom) nmol min^{-1} mg $protein^{-1}$. All operations were carried out at room temperature, except that NADH dehydrogenase activities were measured at 30°C.

Fractions	mg protein	NADH dehydrogenase activity (nmol min^{-1} mg $protein^{-1}$)	
		No bee venom	+ Bee venom
1	<0.05	–	–
2 + 3 pooled	0.42	15	107
4 + 5 pooled	0.10	–	–
6, 7, 8 pooled	<0.05	–	–
9, 10, 11 pooled	1.95	55	83
12 + 13 pooled	0.15	–	–
14 + 15 pooled	<0.05	–	–

RSO and ISO vesicles may be made on the basis of the extent to which bee venom stimulates the NADH dehydrogenase activity of the vesicles [5]. The plasma membrane is effectively impermeable to (1 mM) NADH [8] so that RSO vesicles are not expected to oxidise added NADH unless the permeability of the membrane to NADH is increased, thus permitting access of NADH to its inward-facing dehydrogenase. Bee venom contains mellitin [5], a haemolytic agent with mild detergent properties, and thus destroys the integrity of the plasma membrane, enabling NADH to reach its dehydrogenase in the RSO vesicles.

Table 2 shows the results of experiments with four different preparations of *P. denitrificans* vesicles in which the stimulation of NADH dehydrogenase by bee venom was examined in (a) the original vesicle preparation, (b) the vesicles (Fraction A) that were

TABLE 2

Effect of bee venom on NADH dehydrogenase activity of unfractionated and fractionated *P. denitrificans* membrane vesicles

NADH dehydrogenase activities were measured as described in Materials and Methods. Bee venom was added to a final concentration of 50 μg ml^{-1}. Fraction A and Fraction B have the meanings indicated in the text.

Preparation	Sample	NADH dehydrogenase activity + Bee venom / NADH dehydrogenase activity – Bee venom
1	Initial	2.5
	Fraction A	6.9
	Fraction B	1.2
2	Initial	3.0
	Fraction A	6.6
	Fraction B	1.0
3	Initial	5.0
	Fraction A	>200
	Fraction B	2.8
4	Initial	1.8
	Fraction A	7.3
	Fraction B	1.5

not retained by the column, and (c) the vesicles (Fraction B) that were eluted from the column by ADP. It can be seen that the stimulation of NADH dehydrogenase activity by bee venom was always very much greater in fraction A than in either the original vesicle preparation or in fraction B. This suggests that fraction A is composed predominantly of RSO vesicles, whereas the ISO vesicles are retained by the column and eluted by ADP.

However, it is apparent from Table 2 that there was a good deal of variability in the proportions of RSO and ISO vesicles in the preparations, and only with preparation 3 was stimulation of NADH dehydrogenase by bee venom of fraction A as large as might be expected if the vesicles were entirely RSO. Furthermore, only in preparation 2 was no stimulation by bee venom of the NADH dehydrogenase activity of fraction B observed (Table 2). An explanation of these observations may be that both fractions A and B are usually contaminated by scrambled vesicles in which molecules of NADH dehydrogenase or of ATPase may have migrated topo-

logically during preparation of the vesicles. The RSO (fraction A) vesicles might be contaminated by vesicles in which the NADH dehydrogenase, but not the ATPase, is outward-facing, thus giving NADH dehydrogenase activity before the addition of bee venom. A stimulation by bee venom of the fraction B vesicles could be accounted for either if some NADH dehydrogenase molecules have everted so as to face the vesicle lumen in otherwise ISO vesicles or if some ATPase molecules have migrated to the outer surface of otherwise-RSO vesicles.

ISO vesicles from *P. denitrificans* exhibit respiratory control [7], and the effect of chromatography on this property of the vesicles was also examined. Assay of fraction B of preparation number 4 (Table 2) showed that the rate of oxygen uptake with NADH as substrate in the presence of ADP was 242 ng atom O min^{-1} mg $protein^{-1}$, and that the addition of 1 μg gramicidin D plus 30 mM ammonium acetate as uncoupler caused a stimulation to 600 ng atom O min^{-1} mg $protein^{-1}$, a 2.5-fold increase. The NADH oxidation rate of the original vesicles in the presence of ADP was 364 ng atom O min^{-1} mg $protein^{-1}$, and this was stimulated 2.7-fold to 993 ng atom O min^{-1} mg $protein^{-1}$ after addition of gramicidin D plus ammonium acetate. Thus the percentage stimulation of NADH oxidase activity upon adding an uncoupler is virtually unchanged by the chromatography treatment. With preparation 4 it was found that virtually all the protein applied to the column was recovered in fraction B, so that it was expected that the specific NADH oxidase activity in fraction B should have approximately equalled that in the original vesicle preparation. It seems, therefore, that although the coupling properties of the membrane, as judged by the stimulation of respiration by an uncoupler, are not noticeably damaged by the chromatography treatment, there is some inhibition of NADH oxidase activity. The succinate oxidase activity of fraction B of preparation 4 (415 ng atom O min^{-1} mg $protein^{-1}$) was found to be virtually the same as that of the original preparation (495 ng atom O min^{-1} mg $protein^{-1}$. Succinate oxidation was also found to support an uncoupler-sensitive enhancement in the fluorescence of 1-anilinonaphthalene-8-sulphonate in both the original vesicle preparation and in fraction B, but not, as expected, in fraction A.

To summarise, passage of *P. denitrificans* vesicles through an ADP affinity column separates the vesicles into fractions which are largely either ISO or RSO, although both fractions can be contaminated with what are suspected to be scrambled vesicles.

4. Discussion

There has been controversy concerning the orientation of membrane vesicles derived from bacterial cells by osmotic lysis [2,4]. In the case of *E. coli*, Hare et al. [9] (see also [10]) suggested that vesicle preparations contained approximately equal proportions of ISO and RSO vesicles, on the basis that an antibody to the membrane ATPase agglutinated the half of the vesicles which possessed NADH oxidase activity, although a differing interpretation has been placed on this observation by others [2]. The procedure proposed in the present paper has several advantages over the use of antibody to the ATPase for separating vesicles of opposite configuration: (1) the need for a purified preparation of ATPase and its antibody is obviated; (2) both the RSO and the ISO vesicles are recovered without significant alteration to their properties. We believe therefore that the use of immobilised ADP should be of value both for analytical and for preparative work. It might, for example, find application in the separation of RSO and ISO vesicles from a variety of bacteria, or in analysing the proposal [11] that certain proteins evert during osmolysis of *E. coli* spheroplasts to give scrambled vesicles.

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