CHARACTERISATION AND PURIFICATION OF INSIDE-OUT SUBMITOCHONDRIAL PARTICLES OBTAINED FROM PLANT MITOCHONDRIA

I. M. MØLLER+, A. BERGMAN, P. GARDESTRÖM, I. ERICSON and J. M. PALMER+

Department of Biochemistry, University of Umeå, 90187 Umeå, Sweden and *+Department of Botany, Imperial College, Prince Consort Road, London SW7 2BB, England*

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1. Introduction

For the study of membrane components facing the matrix side of the mitochondrion and for the study of membrane asymmetry in general, it is necessary to have inside-out vesicles. Previous studies on submitochondrial particles (SMP) from plant mitochondria have in general assumed that the SMP were 100% inside-out (e.g., [1,2]).

Here, we have produced SMP from *Arum maculatum* mitochondria by sonication and characterised them with respect to enzyme activities and contamination by matrix enzymes and outer membrane fragments. We have also determined the polarity of the particles by measuring the fraction of the vesicles which could oxidise reduced cytochrome *c*. By this assay the SMP were 84% inside-out.

Phase partitioning in dextran–polyethylene glycol (PEG) systems will separate material according to surface properties [3] and this method has been used to separate inside-out thylakoid membranes [4]. We have applied phase partitioning to the SMP fraction and obtained SMP which were 93% inside-out.

2. Materials and methods

2.1. *Isolation of mitochondria*

Mitochondria were isolated from spadices of *Arum maculatum* as in [5]. They were stored at −90°C until used.

Abbreviations: HMDA, hexamethylene diamine; MOPS, 3-(*N*-morpholino)propanesulphonic acid; PEG, polyethylene glycol; SMP, submitochondrial particles

2.2. *Production of submitochondrial particles*

Mitochondria were thawed and diluted 2-fold with 0.3 M sucrose, 10 mM MOPS, 5 mM $MgCl_2$ (pH 7.2) (sonication medium) to yield ~20 mg protein/ml. The suspension was sonicated for 4 × 5 s ('output control' = 6) at 4°C with a Sonifier Cell Disruptor B30, Branson Sonic Power Co. The sonicated mitochondria were diluted to 70 ml with sonication medium and intact mitochondria and large fragments were pelleted at 48 000 × *g*, 10 min. The pellet was resuspended in sonication medium to yield pellet 1. The supernatant was centrifuged at 105 000 × *g*, 60 min, the supernatant (supernatant 2) decanted and the pellet resuspended in 0.3 M sucrose (SMP). These SMP were stored at −20°C and used within a few days. A certain loss of cytochrome *c* oxidase activity was observed while the polarity remained unchanged.

2.3. *Assay for inner membrane polarity*

Cytochrome *c* oxidase activity was assayed in 0.3 M sucrose, 10 mM MOPS, 10 mM potassium phosphate, 2 mM $MgCl_2$ (pH 7.2) with 60 μM reduced cytochrome *c* as the substrate. The reaction, followed at 550 nm, was totally inhibited by KCN (3 mM) and was unaffected by antimycin A (0.4 μM). The activity was assayed with and without Triton X-100 (0.02%, w/v). The polarity was calculated assuming that the activity with Triton X-100 equalled total activity and that the activity without Triton X-100 was due only to right-side out inner membrane vesicles.

2.4. *Enzyme assays*

Isocitrate dehydrogenase (NAD^+-linked), malate dehydrogenase and antimycin A-insensitive NADH → cytochrome *c* reductase were assayed as in [6–8]. Succinate dehydrogenase was measured in

33 mM phosphate buffer (pH 7.4), 0.1% bovine serum albumin, 0.1 mM EDTA, 0.05 mM 2,6-dichlorophenol-indophenol, 1.4 mM phenazine methosulphate and 0.015% Triton X-100. The reaction was started with 20 mM succinate. In all cases both KCN (3 mM) and *m*-chlorobenzhydroxamic acid (1 mM) were present to prevent re-oxidation by the cytochrome oxidase and the alternative oxidase pathways, respectively [9].

2.5. *Protein determination*

Protein concentration was determined as in [10], except in the presence of PEG when the method in [11] was used.

2.6. *Two-phase system*

Two-phase separations were made using a phase system with the final composition: 6.8% (w/w) Dextran 500, 6.1% (w/w) PEG 4000, 0.7% HMDA-PEG 6000 (synthesized by the general method in [12]), 0.3 mol sucrose and 10 mmol potassium phosphate/kg final solution (pH 7.0). All experiments with phase systems were done at 4°C.

3. Results and discussion

3.1. *Characterisation of mitochondrial fractions*

The fractions obtained during the isolation of SMP (see section 2) were characterised with respect to protein content and the activity of marker enzymes: antimycin A-insensitive NADH → cytochrome *c* reductase for the outer membrane, cytochrome *c* oxidase and succinate dehydrogenase for the inner membrane and malate dehydrogenase for the matrix space. The results are presented in table 1.

The mitochondrial pellet (pellet 1) contained most of the cytochrome *c* oxidase, while most of the NADH → cytochrome *c* reductase and the malate dehydrogenase was found in the soluble fraction (supernatant 2). The SMP were enriched in cytochrome *c* oxidase by a factor of 2.1 per unit of protein or by a factor of 2.6 when expressed per unit of NADH → cytochrome *c* reductase associated with the outer membrane. Therefore the SMP fraction consisted of relatively pure inner membranes. Very little malate dehydrogenase was found in the SMP.

Recoveries for all activities were close to 100% with the exception of succinate dehydrogenase which was found to be somewhat unstable; ~1/3rd of its activity was lost during sonication and a further loss was observed during the centrifugation steps. However, the SMP were relatively enriched in this inner membrane marker (table 1).

3.2. *Polarity assay*

Fig.1 shows the effect of Triton on the release of isocitrate dehydrogenase activity from intact mitochondria and cytochrome *c* oxidase activity from SMP. Both enzymes reached maximal activity at 0.012% Triton and this release appeared to be independent of the amount of sample present (2 μg or 28 μg protein). Similar curves were obtained measuring succinate → $Fe(CN)^{3-}$ reductase activity in the presence of 0.4 μM antimycin A (not shown). It is of interest to note that in [13] the critical micellar concentration for

Table 1
Distribution of marker enzymes in mitochondrial fractions during SMP isolation

Fraction	Protein	Cytochrome *c* oxidase	Succinate dehydrogenase	NADH → cytochrome *c* (A/A-insensitive)	Malate dehydrogenase
I Mitochondria	~200 mg = 100%	~60 μmol/min = 100%	~30 μmol/min = 100%	~20 μmol/min = 100%	~2 × 10^4 μmol/min = 100%
II Sonicated mitochondria	108 ± 8% (3)	116 ± 6% (3)	69 ± 10% (3)	127 ± 26% (2)	92 ± 0% (2)
III Pellet 1	33 ± 9% (3)	65 ± 24% (3)	16 ± 4% (3)	22 ± 13% (2)	22 ± 9% (2)
IV SMP	19 ± 7% (3)	39 ± 11% (3)	35 ± 18% (3)	15 ± 3% (2)	1.4 ± 0.4% (2)
V Supernatant 2	47 ± 15% (3)	5 ± 4% (3)	5 ± 4% (3)	73 ± 8% (2)	71 ± 21% (2)
Recovery	99%	109%	56%	110%	95%

The separation of mitochondrial fractions and measurement of protein content and enzyme activities were as in section 2. 'Recovery' is the sum of the activities found in fractions III–V. Values are presented as average ± standard error (number of preparations is given in parentheses); A/A is antimycin A

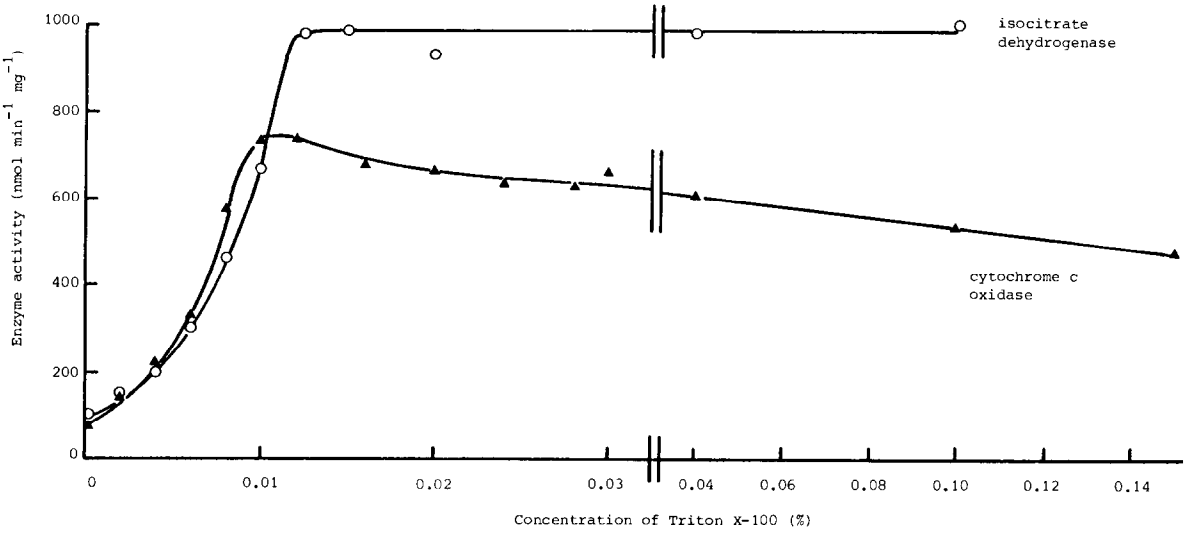

Fig.1. Effect of Triton X-100 concentration on the release of enzyme activities. Isocitrate dehydrogenase (NAD^+-linked) was measured on mitochondria (27 μg protein) and cytochrome *c* oxidase on submitochondrial particles (1.7 μg protein) as in section 2. The particles were always added after the Triton had been mixed into the assay mixture.

Triton X-100 was reported to be 0.015% (0.24 mM, M_r 643). The isocitrate dehydrogenase activity was not affected by high concentrations of Triton while a decrease in cytochrome *c* oxidase activity was observed above 0.03%. In the polarity assays measuring the activity of cytochrome *c* oxidase, 0.02% Triton X-100 was used. The average for 9 preparations of SMP was 84 ± 4% inside-out. The polarity of the SMP calculated from the cytochrome *c* oxidase assay need not be correctly correlated to the true polarity. This assay, like all polarity assays, is indirect and has certain disadvantages. The assay works in theory only with single membrane vesicles since added cytochrome *c* will not have access into multimembrane vesicles. The existence of multimembrane vesicles thus makes the assay overestimate the proportion of inside-out vesicles. It is also possible that some cytochrome oxidase has been inverted in some membranes by sonication ('mixed polarity' vesicles). If this is the case 100% inside-out cannot be reached with the assay and the proportion of inside-out vesicles (totally inside-out with respect to other properties) will be underestimated. Despite these problems with the quantification of the assay we think it is a suitable method to follow enrichment of inside-out vesicles upon fractionation of the original SMP preparation. In principle, the same polarity assay was used on rat liver SMP produced by sonication [14] which were found to be ~50% inside-out.

3.3. *Purification of inside-out SMP*

We attempted to purify inside-out SMP with the phase partition technique [3]. Several compositions of phase system were tried. A phase system with some of the PEG as HMDA · PEG (PEG–NH–$(CH_2)_6$–NH_2) proved useful as inside-out particles preferred the bottom phase and right side-out particles the top phase (fig.2). In a similar phase system without HMDA · PEG all particles partitioned to the bottom phase and the HMDA · PEG thus seems fairly specific for right side-out vescles. HMDA · PEG also specifically extracts intact spinach leaf mitochondria into the upper phase (P. G., unpublished). By extracting the bottom phase three times with pure upper phase, enriched inside-out particles can be obtained (93 ± 2%, n = 3). Further extractions did not increase the enrichment of inside-out particles. The yield was 15–20% protein and 30–50% cytochrome *c* oxidase activity of that in the SMP (fig.2). Overall yield of purified inside-out SMP (from mitochondria) was 3 ± 1% protein and 12 ± 3% cytochrome *c* oxidase for 4 preparations. The top phase contained particles with a lower inside-out ratio (fig.2).

Rat liver SMP were enriched from 47% inside-out to 60% inside-out polarity by using cytochrome *c* affinity chromatography [14]. Judged by this result the phase separation technique appears to be better suited for the selective purification of inside-out SMP.

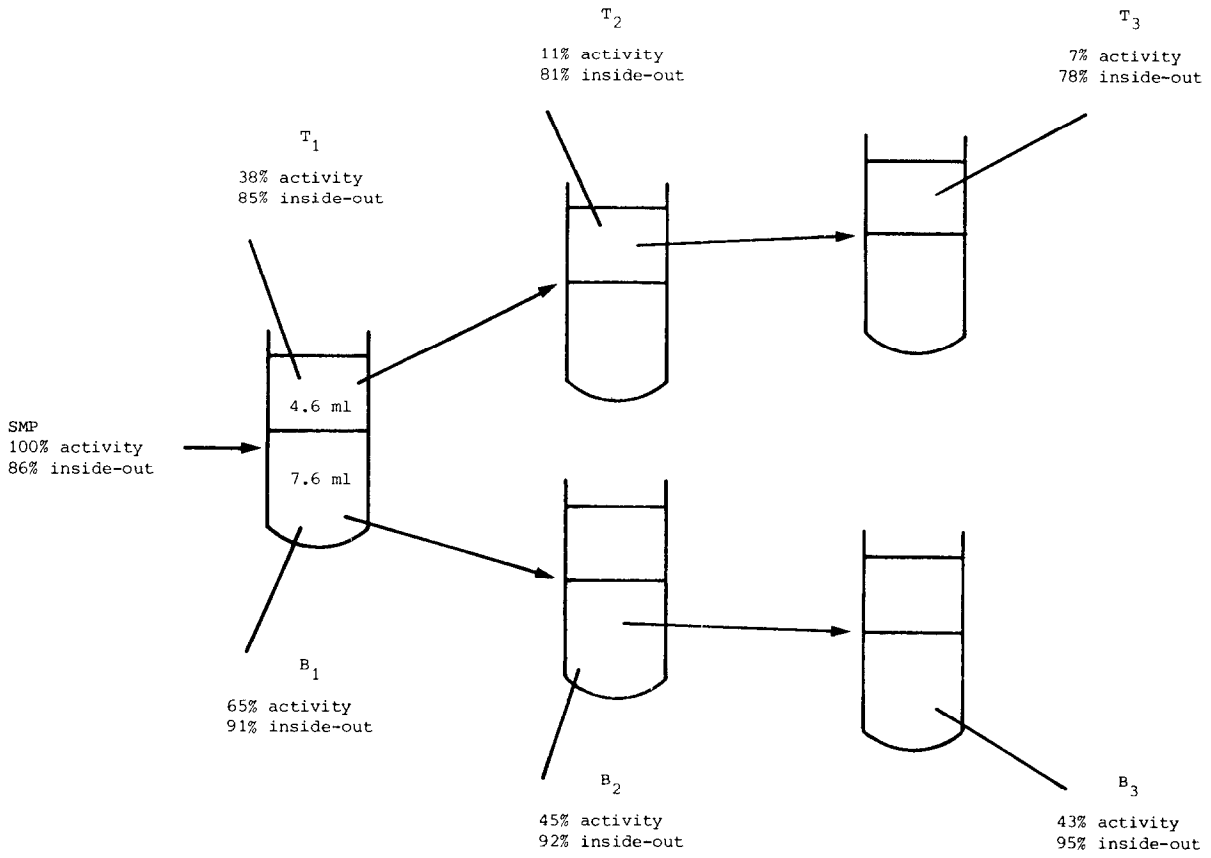

Fig.2. Batch separation of submitochondrial particles. Submitochondrial particles (8.4 mg protein, 4.4 μmol cytochrome *c* oxidase act./min) were thoroughly mixed into the phase system and the mixture centrifuged at 600 × *g*, 2 min. The resulting top phase (T_1) and the bottom phase together with the interface (B_1) were collected and repartitioned with pure bottom phase and top phase, respectively, yielding T_2 and B_2. The procedure was repeated yielding T_3 and B_3. Cytochrome *c* oxidase activity and the polarity of the SMP were assayed (section 2) by taking out samples from the individual phases. Similar results were obtained with two other preparations of SMP.

However, since different sources of SMP were used this can only be a tentative conclusion.

When the SMP were subjected to counter-current distribution (fig.3) two main peaks were obtained (CCD 1,3). The SMP in the peak fractions differ in polarity and CCD 1 clearly corresponds to B_3 (fig.2) both consisting of ~95% inside-out particles. CCD 3 corresponds to T_3 (fig.2) and has a comparable lower inside-out ratio. The material between the two peaks shows intermediate polarity. If the material were composed of two homogenous populations of vesicles (inside-out and right side-out) two distinct peaks would be expected in the counter-current distribution diagram [15]. As this is not the case it indicates that the material is heterogeneous. One explanation of this may be the presence of 'mixed polarity' vesicles.

4. Summary

(i) SMP produced by sonication of *Arum* mitochondria contained relatively little contamination by matrix enzymes or by outer membrane fragments. The particles were 84% inside-out as estimated by the access of cytochrome *c* to cytochrome *c* oxidase.

(ii) The SMP could be purified to yield a 93% inside-out fraction by applying the dextran/PEG two-phase separation technique.

(iii) With a purified inside-out preparation of SMP it will now be possible to study the localisation and kinetic behaviour of enzymes on the inside of the inner membrane of plant mitochondria.

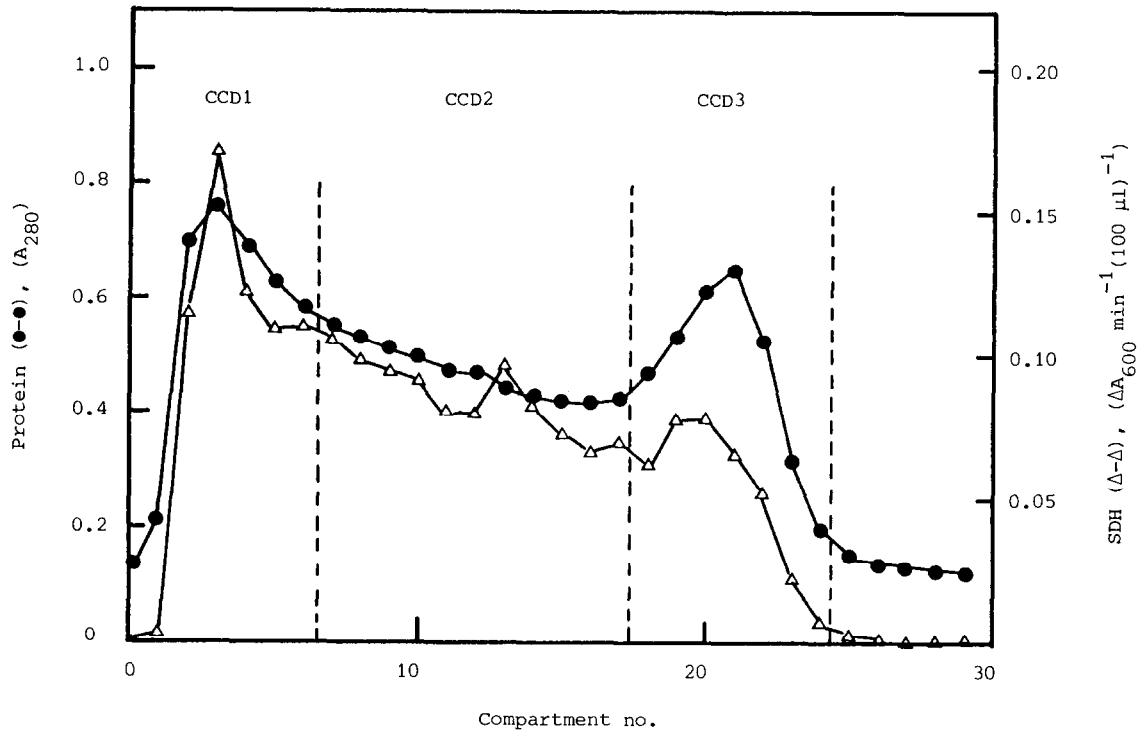

Fig.3. Counter-current distribution of SMP. Counter-current distribution (CCD) was performed using an automatic CCD apparatus as in [3,15]. There were 29 transfers with 40 s shaking time and 6 min settling time. The interface was held stationary together with the bottom phase. Fractions 1–6 (CCD1), 7–17 (CCD2) and 18–24 (CCD3) were pooled and the polarity measured (section 2). Yield of inside-out SMP was 94%, 92% and 84% for the 3 fractions, respectively. SDH is succinate dehydrogenase.

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