Generation and purification of submitochondrial particles of different polarities from plant mitochondria

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Mitochondria were isolated from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers in a low-salt medium. Submitochondrial particles (SMP) produced by sonication in a low salt medium + 20 mM $MgCl_2$ were 18% right-side-out (RO) as judged by the latency of cytochrome *c* oxidase assayed ± Triton X-100. SMP produced by French press treatment in a low-salt medium + 5 mM EDTA to remove bound divalent cations were 98% RO. Less extreme treatments gave SMP of intermediate polarity. There was a positive correlation between the % RO-SMP (produced by sonication) and the % NAD^+-malate dehydrogenase enclosed within the SMP indicating that only RO-SMP contained trapped matrix. When a mixed population of SMP (45% RO) was applied to an aqueous polymer two-phase system, the top phase contained 76% RO-SMP and the bottom phase mostly inside-out SMP (26% RO). By analogy with the models for stacking of photosynthetic membranes, we propose that crista formation in the inner mitochondrial membrane is electrostatically regulated and SMP deriving from the closely stacked crista regions are inside-out.

Mitochondria (Plant) Polarity Sidedness Submitochondrial particle Two-phase partitioning

1. INTRODUCTION

Thylakoid membranes are found in granal stacks and stromal lamellae. When the membranes are disrupted, vesicles are formed from the appressed membrane regions and RO vesicles from the non-appressed regions. By phase partitioning of thylakoid vesicles it has been possible to separate IO and RO vesicles. This technique has led to rapid advances in our understanding of the lateral and transverse distribution and function of protein complexes and lipids in the thylakoid membranes (review [1]).

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Abbreviations: HMDA, hexamethylenediamine; IO, inside-out; MDH, malate dehydrogenase; Mops, 4-morpholinepropanesulfonic acid; RO, right-side-out; SDH, succinate dehydrogenase; SMP, submitochondrial particle(s)

It is possible to produce >90% IO-SMP by disruption of mammalian mitochondria under suitable conditions (e.g. [2]). However, SMP of mixed polarity have often been isolated from mammalian mitochondria (see [3,4] and references therein). SMP from plant mitochondria have generally been assumed to be 100% IO (e.g. [5,6]). In a few cases, the polarity of plant SMP has been tested directly and while turnip SMP were reported to be 100% IO [7], SMP from Jerusalem artichoke tubers [8] and *Arum maculatum* spadices [9] were only 51 and 84% IO, respectively.

We speculated that perhaps crista membranes 'stack' by analogy with thylakoid membranes and that such crista stacking – and thus the polarity of the SMP produced – might be electrostatically regulated as in thylakoids [10]. Here, we report on the successful application of this approach to the generation of RO-SMP, IO-SMP and SMP of intermediate polarity from Jerusalem artichoke

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tubers. A preliminary report of these findings has been presented [11].

2. MATERIALS AND METHODS

Mitochondria were isolated from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers essentially as in [12] except that 4 mM cysteine was used instead of $Na_2S_2O_5$. In the second wash, the medium was identical to that used in the subsequent disruption, containing 0.3 M sucrose, 5 mM Mops, pH 7.2, 0.1% (w/v) bovine serum albumin (low-salt medium) and where indicated 5 mM EDTA (low salt + EDTA) or 5 mM or 20 mM $MgCl_2$ (high salt). The final pellet was resuspended in the same medium.

For French press treatment, the mitochondria were diluted to 4–7 mg mitochondrial protein/ml and treated in an Aminco vessel at 200 MPa and a flow rate of ~5 ml/min.

Sonication of the mitochondria was done with a Branson model B-30 sonifier with a microprobe for 4 × 10 s (50% duty cycle) at setting 6 with 1 min between bursts to cool. Samples of 3–4 ml (10–20 mg/ml) were sonicated in graduated glass tubes.

After disruption, samples were diluted to 160 ml per 500 g peeled tubers with the medium used for disruption. Intact mitochondria and large membrane fragments were pelleted at 48400 × *g* for 10 min and discarded. The supernatant was centrifuged at 105000 × *g* for 60 min in a Beckman L5-65B centrifuge using a 70Ti rotor, the pellets rinsed twice with 0.3 M sucrose to reduce salt content for the phase partitioning (see below) and finally resuspended in 0.3 M sucrose using a hand homogenizer.

SDH was assayed as in [13]. Cytochrome *c* oxidase and NAD^+-specific malate dehydrogenase were assayed as in [14] ± 0.02% (w/v) Triton X-100 [9]. The latency was calculated as:

$$[(\text{rate} + \text{Triton}) - (\text{rate} - \text{Triton})] \times 100 / (\text{rate} + \text{Triton})\%$$

The % IO-SMP is defined as being equal to the % latency of cytochrome oxidase in SMP.

The different phase systems used in the experiments and specified in the figure and table legends were made up from stock solutions of 20% (w/v) Dextran 500 (Pharmacia, Sweden), 40% (w/v) PEG 4000 (Carbowax, Union Carbide), 10% (w/v) HMDA-PEG 6000 (a gift from Dr G. Johansson, University of Lund, Lund, Sweden) and appropriate stock solutions of sucrose and potassium phosphate buffer.

Protein was determined as in [15].

Cytochrome *c* was from Sigma and was reduced as in [8].

3. RESULTS

By varying the composition of the disruption medium as well as the method of disruption, SMP of widely different polarity could be generated

Table 1

The polarity and yield of SMP produced by French press treatment or sonication of Jerusalem artichoke mitochondria suspended in media of different ionic composition

Disruption method	Parameter	Medium			
		Low salt + EDTA	Low salt	High salt	
				5 mM $MgCl_2$	20 mM $MgCl_2$
French press	% RO	98 ± 1 (2)	81 ± 8 (2)	61 ± 7 (3)	38 (1)
	protein yield	24 ± 3 (2)	2.8 ± 0.5 (2)	4.7 ± 0.5 (3)	9 (1)
Sonication	% RO	54 ± 1 (2)	42 ± 5 (12)	20 ± 3 (4)	18 (1)
	protein yield	25 ± 0 (2)	12.4 ± 3.4 (11)	8.9 ± 4.2 (4)	21 (1)

Protein yield is in % of protein in mitochondria. Polarity is given in % RO-SMP. All data given as mean ± SD (number of independent preparations)

from Jerusalem artichoke mitochondria (table 1). As one extreme, French Press treatment in a low-salt medium + EDTA gave SMP which were completely RO. The % RO-SMP decreased progressively from low salt + EDTA via low salt to high salt for both disruption methods. The other extreme was obtained by sonication under high-salt conditions which yielded >80% IO-SMP (table 1). Other treatments gave SMP of mixed polarity but the % RO was always lower for SMP produced by sonication than for SMP from French press treatment. The yield of SMP was higher when disruption was under low-salt + EDTA conditions than under high-salt conditions (table 1). The outer membrane of plant mitochondria is more fragile in the presence of EDTA [16,17] and this may be true also for the inner membrane.

French press treatment of sonicated mitochondria gave a higher percentage of RO-SMP compared to sonication only. In contrast, sonication of mitochondria which had been French press-treated had little or no effect on the polarity of the SMP (table 2). From the results in table 1, one might have expected sonication to decrease the percentage RO-SMP.

For SMP produced by sonication, there was a strong negative correlation between the latency of cytochrome oxidase (indicating the % IO-SMP) and the latency of malate dehydrogenase, a soluble matrix enzyme (fig.1). Likewise, there was a positive correlation between the percentage latent malate dehydrogenase in SMP produced by sonication and the specific activity of malate dehydrogenase in the SMP (see legend to fig.1). Such correlations were not observed for SMP produced by French press treatment (fig.1, not shown).

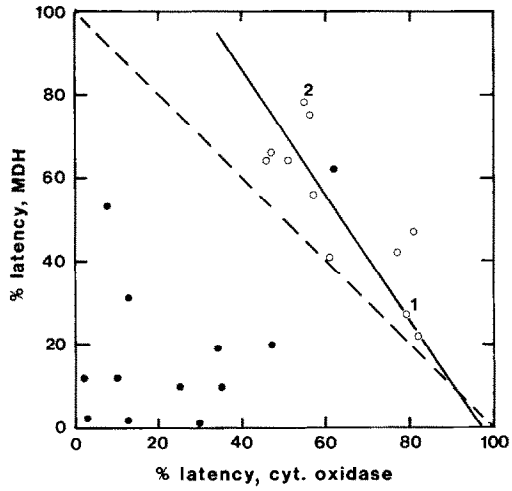

Fig.1. Correlation between % latency of cytochrome *c* oxidase (= % IO-SMP) and % latency of MDH. The specific activity of MDH was 2.4 and 7.1 $\mu mol \cdot mg^{-1} \cdot min^{-1}$ in the points marked 1 and 2, respectively, whereas the cytochrome oxidase activity was 2.02 and 2.15 $\mu mol \cdot mg^{-1} \cdot min^{-1}$, respectively. The broken line indicates the perfect correlation between the latencies. The continuous line was fitted by eye to the points obtained from SMP produced by sonication (○). (●) SMP produced by French press treatment.

Table 2

Sequential disruption of plant mitochondria with French press and sonication

Disruption	Medium	
	Low salt + EDTA	High salt (20 mM $MgCl_2$)
French press	97 (26)	38 (9)
then sonication	96 (27)	52 (21)
Sonication	53 (25)	18 (21)
then French press	76 (13)	52 (23)

No centrifugations were performed between disruptions by the 2 methods. Data are from one experiment and are given in % RO-SMP (yield in % protein compared to the mitochondria)

Aqueous polymer 2-phase systems have the ability to separate membrane vesicles with respect to differences in their surface properties [1,9,18]. The ability of such a system to discriminate between RO- and IO-SMP was tested. The 2-phase system separated these particles increasingly well as the concentration of polymers was increased (fig.2A). However, this was accompanied by an increasing distribution of the total amount of SMP to the bottom phase (fig.2B). This could partly be overcome by choosing a higher pH and by substituting 10% of the PEG 4000 with HMDA-PEG 6000 (fig.2C,D).

Using the optimized phase system, we separated RO- and IO-SMP from a mixed population. One separation was sufficient to obtain an average

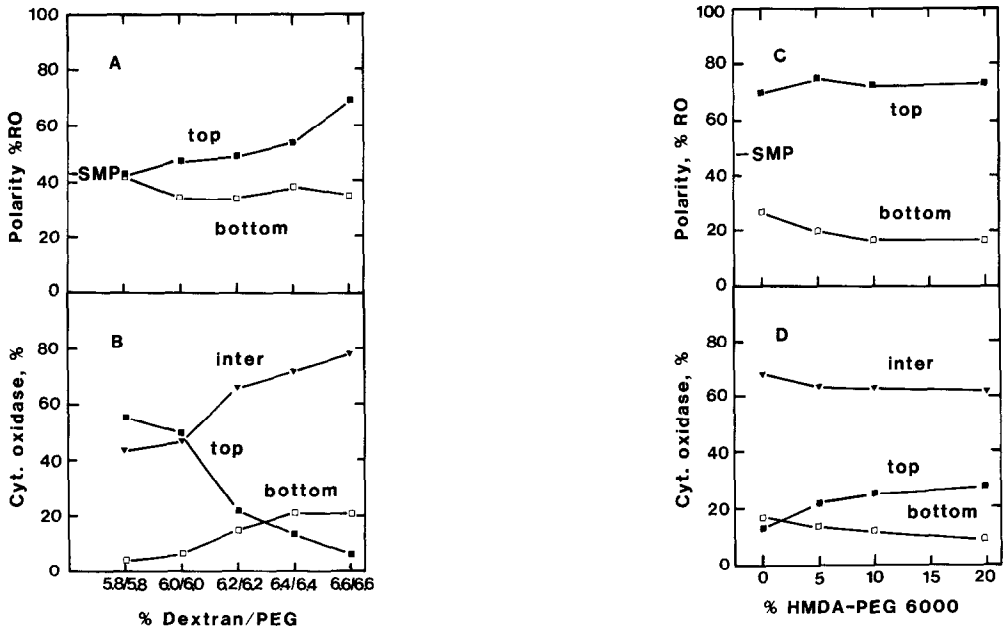

Fig.2. Optimization of the aqueous polymer 2-phase system for discriminating between IO- and RO-SMP. (A,B) Phase system contained Dextran/PEG 4000 as indicated plus 300 mmol sucrose·kg^{-1} and 10 mmol potassium phosphate·kg^{-1}, pH 7.0. (C,D) Phase system contained 6.6% (w/w) Dextran, 6.6% (w/w) PEG (HMDA-PEG 6000 as indicated, the rest PEG 4000), 300 mm sucrose·kg^{-1} and 10 mmol potassium phosphate·kg^{-1}, pH 8.0. In B and D, the curve for the interphase was calculated by subtracting the activities in the top and bottom phases from 100%.

purification of 76%/26% = 2.9-fold (table 3). The best purification was 3.6-fold. The specific activities of cytochrome oxidase and SDH were higher in the IO-SMP than in the RO-SMP. The opposite was true for MDH (table 3).

4. DISCUSSION

SMP of a wide range of polarities can be generated from Jerusalem artichoke mitochondria by selecting the right disruption conditions and

Table 3

Separation of RO- and IO-SMP from a mixed population of SMP

Fraction	% RO	Cytochrome oxidase	SDH	MDH
Mitochondria	–	2.0 ± 0.1	0.07 ± 0.02	16.2 ± 2.6
SMP	45 ± 3	2.3 ± 0.7	0.14 ± 0.03	4.5 ± 0.9
B_1	26 ± 1	3.3 ± 0.3	0.19 ± 0.04	3.7 ± 0.6
T_1	76 ± 7	2.2 ± 0.7	0.12 ± 0.04	9.7 ± 3.8

The optimized phase system (see fig.2) containing 6.6% (w/w) Dextran 500, 5.94% (w/w) PEG 4000, 0.66% (w/w) HMDA-PEG 6000, 300 mmol sucrose·kg^{-1} and 10 mmol potassium phosphate·kg^{-1}, pH 8.0, was used to separate SMP (produced by sonication in a low-salt medium) in a one-step procedure yielding a top phase (T_1) and a bottom phase (B_1). The data are means ± SD of 3 independent preparations. Results are expressed as μmol·mg^{-1}·min^{-1}

method of disruption (table 1). In a low-salt medium + EDTA, which removes membrane-bound divalent cations, the outer surfaces of the crista membranes are maximally negative [12,19,20] and would be expected to repulse each other [10]. Under these conditions few IO-SMP would be formed (fig.3A). On the other hand, under high-salt conditions the surface potential of the outer surface of crista membranes will be minimal in magnitude [12,20,21], adjacent cristae will be more likely to approach each other (stack) and the relative number of IO-SMP would be expected to be high (% RO-SMP low; fig.3B) by analogy with the situation for appressed and non-appressed thylakoid membranes [1].

The results in table 1 are consistent with the above model. Furthermore, if the model in fig.3 is correct, the RO-SMP should enclose matrix proteins whereas the IO-SMP should not. This is shown to be true in table 3 where the matrix enzyme MDH is enriched in the RO-SMP compared to the IO-SMP isolated from the same mixed population of SMP. The negative correlation between latency of cytochrome oxidase (= % IO-SMP) and latency of malate dehydrogenase (fig.1) also confirms this prediction. The ability to produce SMP of well-defined polarities will allow us to attack the problem of transverse distribution of protein complexes and lipids in the inner membrane of plant mitochondria.

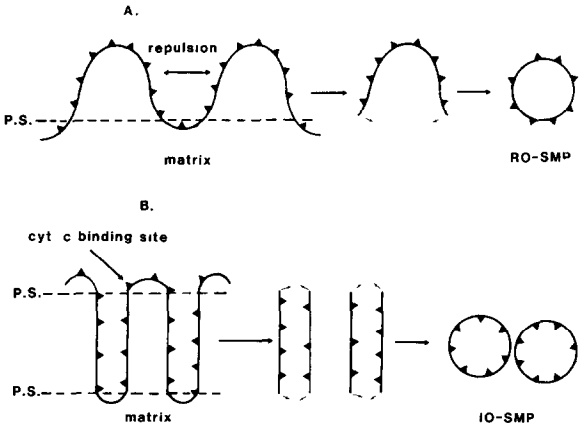

Fig.3. Proposed mechanism for the formation of RO-SMP under low-salt +EDTA/high negativity conditions (A) and of IO-SMP under high-salt/low negativity conditions (B). In A only one of the RO-SMP formed is shown. P.S., plane of shear.

From the above considerations it follows that in SMP of mixed polarity, IO-SMP will primarily derive from the tightly stacked crista regions whereas the RO-SMP are more likely to have been formed from the inner boundary membrane. It is therefore of great importance to be able to separate IO- and RO-SMP as done by phase partitioning (fig.2, table 3). The results confirm that phase partitioning is superior to a cytochrome *c* affinity column for separating SMP of different polarity (see [3,9]). The problem of lateral diffusion and/or lateral heterogeneity of protein complexes and lipids in the inner mitochondrial membrane [22] and its importance for the properties and regulation of electron transport have been treated in a series of elegant papers by the group of Hackenbrock (e.g. [23–25]). By using the separated sub-populations of SMP with opposite polarity and probably deriving from different membrane regions we will be able to address this problem from a different angle. In fact, the results in table 3 indicate that both cytochrome oxidase and SDH may be enriched in the IO-SMP, i.e. in the crista regions of the inner membrane of mitochondria suspended in a low-salt medium.

Heterogeneities in the distribution of matrix proteins and specific interactions between matrix proteins and inner membrane components may also be uncovered using RO-SMP to 'sample' the non-crista part of the matrix.

It is important to keep in mind that: (i) IO- and RO-SMP may not be the same size; (ii) vesicles of a given polarity may have quite a broad size distribution as indicated by results on thylakoid vesicles [26]; and (iii) that different treatments may give SMP which vary not only in mean polarity (table 1) but also in size distribution. We have arbitrarily chosen 105000 × *g* for 60 min to pellet the SMP and may, therefore, in some cases have failed to pellet the very smallest SMP. The problem of the size distribution of SMP merits further attention.

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