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Some properties of mitochondria, mitoplasts and submitochondrial particles of different polarities from plant tissues

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Irrespective of the starting material, i.e. washed mitochondria, purified mitochondria or mitoplast from *Solanum tuberosum* L., submitochondrial particles of well-defined polarities can be generated by French press treatment in low-salt medium or by sonication in high-salt medium. The first treatment will result in submitochondrial particles which are more than 80% right-side-out (right-side-out particles), the second in submitochondrial particles that are more than 80% inside-out (inside-out particles). The isoelectric point (p*I* = 4.0) of the inside-out particles measured by cross-partition is distinctly different from the isoelectric points of the other mitochondrial membranes which exhibit p*I* values between 4.5 and 4.7. The surface charge density measured by 9-aminoacridine fluorescence varies in the same order from -27 mC · m^{-2} for Percoll-purified mitochondria to -51 mC · m^{-2} for both right-side-out and inside-out particles. Even though the charge densities for the two surfaces of the inner membrane are similar, inside-out particles are much more negatively charged at pH 7.0, since they are 6-times larger. These results clearly demonstrate that it is possible to obtain submitochondrial particles of various polarities and sizes which in turn constitute valuable tools for the study of lateral and transverse asymmetry of the inner mitochondrial membrane.

Introduction

A basic feature of biological membranes is their transverse asymmetry, which is of particular functional importance for energy-transducing membranes such as the inner mitochondrial membrane and the thylakoid membrane in chloroplasts. Experimentally, the transverse asymmetry of biological membranes can be analysed by membrane-impermeable probes, such as proteolytic enzymes, chemical labels or antibodies. However, such tools can give conclusive results only when both right-sided and inverted vesicles of a membrane are available. Biological membranes can also show a heterogeneity in the lateral distribution of membrane components. Such lateral heterogeneity has proven to be very important in thylakoid membranes, but its importance in mitochondrial membranes is still a matter of speculation. The isolation of right-side-out and inside-out vesicles from chloroplast thylakoids has led to rapid advances in our understanding of the transbilayer organisation and the lateral heterogeneity of the thylakoid membrane [1].

Abbreviations: S-PEG, sodium poly(ethylene glycol) sulfonate; TMA-PEG, trimethylaminopoly(ethylene glycol) bromide.

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The generation and isolation of submitochondrial particles of well-defined characteristics and polarities (mitoplasts, right-side-out and inside-out particles) may be of great value for many biochemical and biophysical studies of the asymmetry of plant mitochondria inner membrane.

Knowledge of the surface charge density of the particles seems to be of central importance for the understanding of many biochemical processes. Electrostatic effects associated with such surface charges may be widely involved in membrane–membrane association [2,3], membrane fusion [4,5], enzyme–substrate interaction [6,7], protein–protein interaction [8], and protein reconstitution into membranes [9].

In this work we have used the method of Kay et al. [10] to produce inside-out and right-side-out submitochondrial particles from plant mitochondria. Submitochondrial particles were generated from Percoll-purified potato tuber mitochondria and characterized with respect to size (electron microscopy and sedimentation properties) and surface properties, such as isoelectric point measured by cross-partition [11–13] and surface charge density (9-aminoacridine fluorescence). The properties of submitochondrial particles are compared to the properties of intact mitochondria and mitoplasts.

Materials and Methods

Preparation of mitochondria. Potato tuber (*Solanum tuberosum* L.) mitochondria were prepared as quickly as possible as described by Diolez and Moreau [14], and purified as described earlier by Petit et al. [15]. 2–3 kg of washed and peeled potato tubers were disrupted with a fruit-juice extractor (Moulinex, France). During tissue grinding the potato juice was continuously mixed with 500 ml of a medium contained 0.3 M mannitol, 30 mM Mops-KOH (pH 7.3), 3 mM EDTA, 25 mM cysteine and 0.3% (w/v) bovine serum albumin. During the mixing KOH was added to maintain the pH at 7.3. The final volume of the extract was 2.5 l. Starch in the extract was allowed to settle for 5 min. The extract was then filtered through four layers of a 120 μm and two layers of a 40 μm nylon net and centrifuged for 10 min at $400 \times g$ (Beckman, JA-10 rotor). The supernatant was centrifuged twice, first for 10 min at $2000 \times g$ and then for 20 min at $10000 \times g$. The final pellets were resuspended in a washing medium (grinding medium minus EDTA and cysteine) to a final volume of approx. 20 ml. This fraction is hereafter called washed mitochondria. About 5 ml aliquots of washed mitochondria were then layered on top of a discontinuous Percoll gradient. The gradient was prepared in a polycarbonate bottle (26 ml) and consisted of 6 ml 18%, 8 ml 23% and 6 ml 40% (v/v) Percoll in 0.3 M sorbitol and 10 mM Mops-KOH (pH 7.2). The gradient was centrifuged in a 70 Ti rotor (Beckman) for 30 min at $10000 \times g$. Mitochondria were collected as a white band on the interface between the 23% and 40% Percoll layers, then diluted 10-fold in media described below and pelleted by centrifuging at $10000 \times g$ for 10 min in order to remove Percoll. The pellet was resuspended in a small volume of the same medium as used for pelleting and referred to as Percoll-purified mitochondria. The yield of Percoll-purified mitochondria was 60 mg protein from 3 kg tubers.

Preparation of mitoplasts. Either Percoll-purified mitochondria, washed with 5 mM Mops (pH 7.2), 0.3 M sucrose or sucrose-purified mitochondria [16] were used. Mitoplasts were obtained by swelling mitochondria for 20 min at 4°C with slow, dropwise addition of 5 mM potassium phosphate (pH 7.2) and gentle stirring. Final osmolarity was about 35 mosM. The swelling was followed by contraction with 1.5 M sucrose (final osmolarity, 400 mosM) and gentle stirring for 20 min at 4°C. The suspension was homogenized with a Potter-Elvehjem homogeniser in order to separate the outer membranes from the mitoplasts. After centrifugation at $27000 \times g$ for 30 min the mitoplast pellet was resuspended in a few millilitres of the appropriate medium depending on the further treatment (i.e., French press or sonication).

Generation of submitochondrial particles. Before generation of submitochondrial particles, samples (wahsed or purified mitochondria or mitoplasts) were washed by 10-fold dilution with either a low-salt + EDTA medium, (0.3 M sucrose, 5 mM Mops (pH 7.2), 0.1% (w/v) bovine serum albumin, 5 mM EDTA) or with a high-salt medium, (same as the low-salt + EDTA medium but 20

mM or 50 mM $MgCl_2$ instead of EDTA). After centrifugation for 10 min at 10000 × *g* (washed or purified mitochondria) or for 30 min at 27000 × *g* (mitoplasts), the final pellets were resuspended in the same medium as used for the wash.

For the French press method, the samples were diluted with low-salt + EDTA medium to 3–5 mg protein · ml^{-1} (minimum of 5 ml) and treated at +4°C in an Aminco French press at 200 MPa with a flow rate of 5 ml · min^{-1}.

For sonication, the samples were diluted with high-salt medium to 2–2.5 ml (10–20 mg protein/ml) and treated at +4°C with a Branson model B30 Sonifier (equipped with a microprobe) for 4 × 10 s (50% duty cycle) at setting 6 with 50 s between bursts to cool.

After disruption samples were diluted to 160 ml with the medium used for disruption. Intact mitochondria and large membrane fragments were pelleted at 48400 × *g* for 10 min. The supernatant was centrifuged at 300000 × *g* for 60 min in a Beckman L5-65B centrifuge using a 70 Ti rotor. The pellets were rinsed twice with either 0.3 M sucrose/2 mM Mops (pH 7.0) for charge density measurements (to reduce ionic strength) or with 0.3 M sucrose/50 mM potassium phosphate (pH 7.2) for cross-partitioning (to reduce chloride content), and finally resuspended in the appropriate medium.

Enzyme assays. The cytochrome *c* oxidase activity was measured spectrophotometrically at 550 nm with 540 nm as reference wavelength in an Aminco dual-wavelength spectrophotometer (slit 1.0 nm). The assay medium consisted of 10 mM potassium phosphate (pH 7.2) and 0.02% Triton X-100. The reaction was started by the addition of 60 μM reduced cytochrome *c*. The specific absorbtivity used for cytochrome *c* was 21.1 $mM^{-1} \cdot cm^{-1}$.

Ascorbate/cytochrome *c*-dependent O_2 uptake was measured as described by Neuburger et al. [17]. NAD^+-specific malate dehydrogenase was assayed as in Ref. 18 with or without 0.02% Triton X-100 [25].

Antimycin-insensitive NADH–cytochrome *c* reductase was assayed according to Moreau and Lance [16].

Polarity assay. the polarity of submitochondrial particles was assayed by the activity of cytochrome *c* oxidase in 0.3 M sucrose/10 mM potassium phosphate (pH 7.2), and 60 μM reduced cytochrome *c* with or without 0.02% (v/v) Triton X-100. Polarity was calculated as: % right-side-out-particles = ((rate − Triton)/(rate + Triton)) × 100.

Determination of isoelectric points. The isoelectric points of Percoll-purified mitochondria, mitoplasts, right-side-out or inside-out particles were determined by cross-partitioning at 4°C. A relatively large electrostatic potential difference between the bulk phases was produced by including either a cationic or an anionic poly(ethylene glycol) derivative in the phase systems [12]. In one series of phase systems with varying pH, TMA-PEG was used, and in the other S-PEG. A final two-phase system weighed 2.0 g and contained 6.8% (w/w) Dextran T500 (Pharmacia), 5.1% (w/w) PEG 3350 (Union Carbide). 1.7% (w/w) TMA-PEG3350 or S-PEG3350 (Aqueous Affinity, Arlöv, Sweden), 0.3 M sucrose, 0.7–2.5 mM citrate, water to final weight of 1.9 g and 100 μl of sample in 0.3 M sucrose/50 mM potassium phosphate (pH 7.2).

At low pH the recovery of cytochrome *c* oxidase decreased. To correct for this a sample was withdrawn from the phase system immediately after mixing and the phase separation was accelerated by centrifugation at 150 × *g* for 5 min. A sample was withdrawn from the top phase. This sample and the sample from the mixed system were neutralized by mixing 1 : 1 with 0.1 M sucrose 50 mM potassium phosphate (pH 7.2) and the enzyme activity was measured. The remaining phase system was mixed 1 : 1 with 0.3 M sucrose and the pH was determined after adjusting to room temperature.

9-Aminoacridine fluorescence. The fluorescence of 9-aminoacridine was measured as described by Johnston et al. [19] with the modifications of Möller et al. [20] in a SPEX Fluorolog 222 instrument linked to a SPEX Data mate. Excitation wavelength was 398 nm (slit-width 2 nm) and the emission was measured at 456 nm (slit-width 2 nm). The samples were 0.08 mg protein · ml^{-1} in 0.3 M sucrose/2 mM Mops-KOH (pH 7.0, 1.22 mM K^+), 20 μM 9-aminoacridine. EDTA (100 μM) was added to obtain the lowest possible fluorescence. The fluorescence was then titrated

with either KCl or decamethonium bromide. At the end of the experiment 20 mM $MgCl_2$ was added to obtain the highest release of fluorescence quenching (F_{max}) [21]. The surface charge density was determined as described by Möller et al. [20] and the calculations were performed as in Chow and Barber [22].

Electron microscopy. Immediately after generation of purification, organelles or vesicles were fixed in suspension with 25% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 and left at 4°C for 24 h. The samples were post-fixed 10 min by 1% osmium tetroxide in the same buffer, dehydrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed with a JEOL 200 CX electron microscope.

Protein determination. Protein measurements were performed as described by Bradford [23] with bovine serum albumin as a standard protein.

Membrane potential measurement. The membrane potential was measured with a TPP^+ electrode as described by Diolez and Moreau [24].

Results

Electron micrograph of the washed mitochondria (Fig. 1A), the purified mitochondria (Fig. 1B) and the mitoplasts (Fig. 1C) portrayed the starting materials and the submitochondrial particles. The purified mitochondria showed no contamination with intact plastids or microbodies, in contrast to the washed mitochondria (especially peroxisome contamination). Both washed and purified mitochondria exhibited intact membranes and a dense matrix. The intactness of purified mitochondrial preparations measured as KCN-sensitive ascorbate cytochrome *c*-dependent O_2 uptake or as cytochrome *c* oxidase activity in the absence or presence of Triton X-100 was higher than 96%. Membrane potential and integrity of the purified mitochondria in the media used for the generation of submitochondrial particles are shown in Table I. The quality of the mitoplast preparation was dependent on the mitochondrial preparation used. Percoll-purified mitochondria gave rise to mitoplast preparation with an integrity of about 80–85%, measured as the latency of the NAD^+ malate dehydrogenase (EC 1.1.1.37) in absence or presence of Triton X-100. On the basis of the antimycin resistant NADH–cytochrome *c* reductase, such mitoplast preparations are 50–60% devoid of outer membrane. Better preparations (about 90% depleted of outer member) were obtained when mitoplasts were prepared from sucrose-purified mitochondria [16].

TABLE I

MEMBRANE POTENTIAL AND INTEGRITY OF MITOCHONDRIA IN THE DIFFERENT MEDIA USED FOR THE GENERATION OF SUBMITOCHONDRIAL PARTICLES

Values are given as mean values ± S.D. for four independent preparations in each group.

Medium	Membrane potential (mV)	Integrity (%)
Nornal [a]	228 ± 2	97 ± 3
+ EDTA (5 mM final)	200 ± 4	85 ± 4
+ $MgCl_2$ (20 mM final)	239 ± 3	96 ± 3

[a] Medium containing initially 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 $g \cdot l^{-1}$ bovine serum albumin in 10 mM phosphate buffer (pH 7.2).

Irrespective of the starting material, the generation of submitochondrial particles always reached a high percentage of right-side-out and inside-out particles in the low- and high-salt medium, respectively (Table II). The results are thus very consistent with those obtained for submitochondrial particles from Jerusalem artichoke mitochondria [10]. The percentage of right-side-out and inside-out particles varied slightly with the starting materials. Washed mitochondria gave the highest amount of both right-side-out particles (92% by French press) and inside-out particles (94% by sonication) compared to purified mitochondria and mitoplasts.

Previous speculations about differences in size of the submitochondrial particles [10,25] are confirmed in this work by both sedimentation and electron microscopy. For right-side-out particles yields, on both protein and cytochrome *c* oxidase basis, were very similar in the $105\,000 \times g$ and $300\,000 \times g$ pellets. Not less than about one-third of the recovered cytochrome *c* oxidase activity was found in the $300\,000 \times g$ supernatant (Table III). In contrast, the yield of inside-out particles

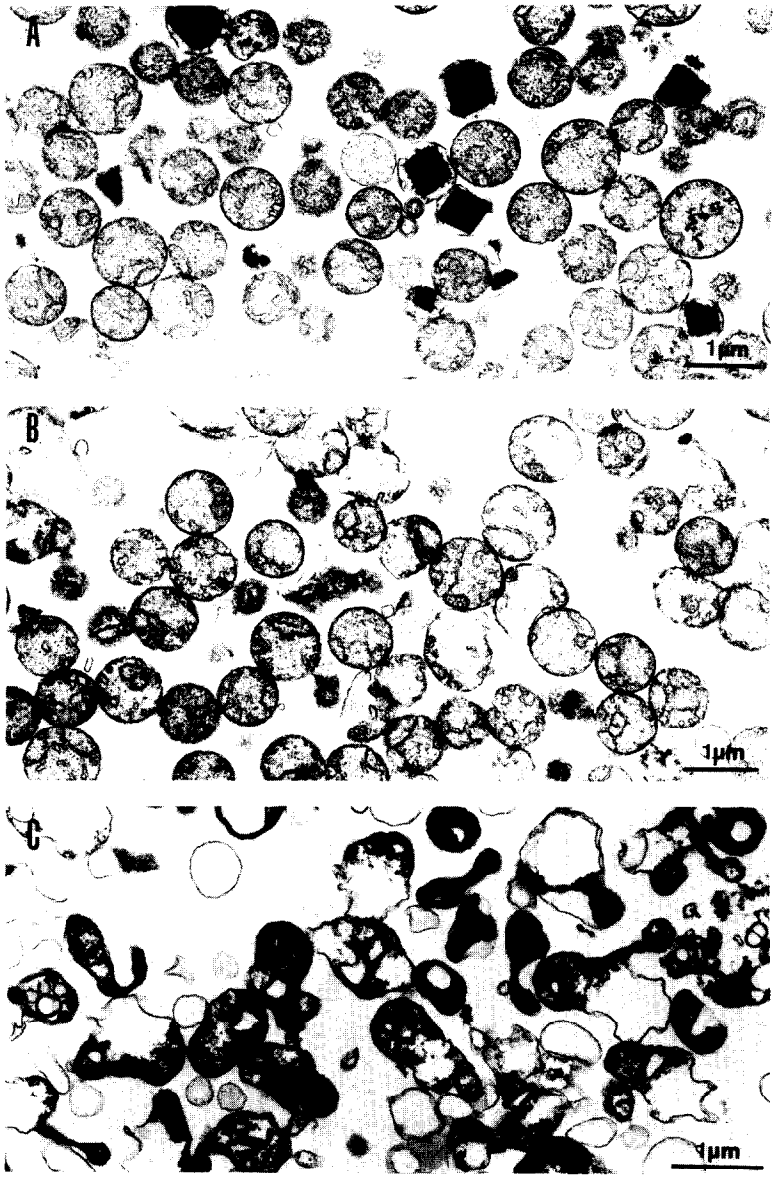

Fig. 1. Electron micrography of (A) washed mitochondria, (B) Percoll-purified mitochondria and (C) mitoplasts (50% devoid of outer membrane).

was about 10-times higher in the 105 000 × *g* pellet and no cytochrome *c* oxidase activity is found in the 300 000 × *g* supernatant (Table IV) and these large differences in sedimentation behaviour were confirmed by the size differences revealed by electron microscopy (Fig. 2A and B). The apparent size of right-side-out particles was 0.09 to 0.21 µm and that of inside-out particles 0.34 to 0.55 µm (submitochondrial particles pelleted at 300 000 × *g*) indicating that the average volume ratio between right-side-out and inside-out particles is about 26.

On a polarity basis, sedimentation at 300 000 × *g* gave a pellet of submitochondrial particles with the highest percent right-side-out (90%), whereas sedimentation of inside-out particles at 105 000 × *g* gave the highest value of inside-out particles (tables III and IV). The differences in polarity be-

TABLE II

PERCENTAGE OF RIGHT-SIDE-OUT PARTICLES GENERATED FROM WASHED MITOCHONDRIA AND MITOPLASTS OF *SOLANUM TUBEROSUM* L.

Values are given as mean ± S.D. for the number of independent preparations indicated in parentheses.

Particles	% Right-side-out particles	
	low-salt + EDTA medium (French press)	high-salt medium (sonication)
Washed mitochondria	92 ± 7 (3)	6 ± 3.9 (3)
Purified mitochondria	90 ± 10 (3)	14 ± 4.2 (3)
Mitoplasts (50% depleted of outer membrane)	82 ± 10 (4)	16 ± 6.7 (4)

tween the 105 000 × *g* and 300 000 × *g* pellets were larger for right-side-out than for inside-out particles. Taking into account both yield and polarity sedimentation at 300 000 × *g* was selected as a method of choice in further studies of sub-

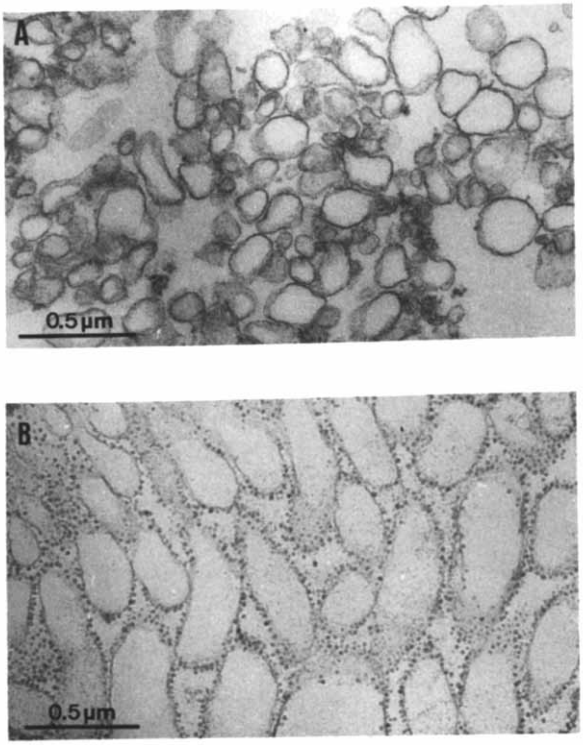

Fig. 2. Electron micrography of (A) right-side-out submitochondrial particles and (B) inside-out submitochondrial particles generated from Percoll-purified mitochondria.

mitochondrial particles in this work. It is, however, obvious that if a high percentage of the respective polarities is desired, it is more relevant to work with the submitochondrial particles sedimented between 105 000 × *g* and 300 000 × *g* for right-side-out particles and with the 105 000 × *g* fraction for inside-out particles.

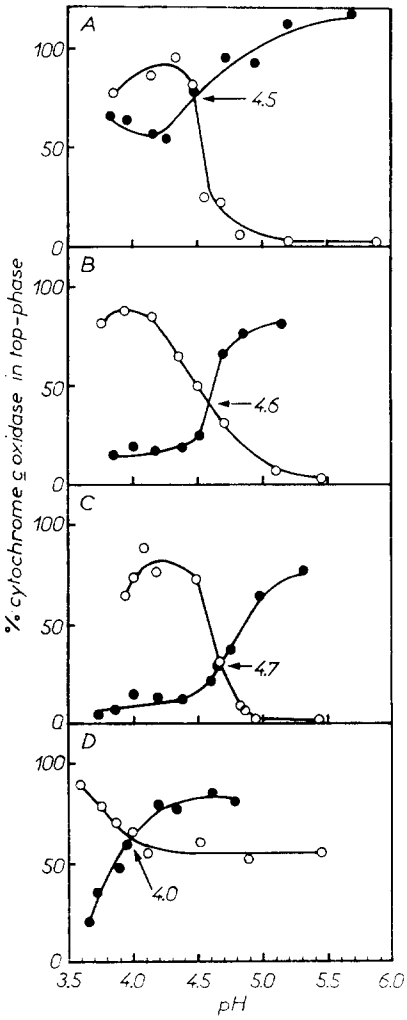

Fig. 3. Cross-partition of intact mitochondria (A), mitoplasts (B), right-side-out submitochondrial particles (C) and inside-out submitochondrial particles (D). Closed circles represent the series of phase systems with trimethylamino poly(ethylene glycol) (positively charged) and the open circles the series of phase systems with poly(ethylene glycol) sulfonate (negatively charged). The cross-point values given are mean values from two or three determinations, and the deviation was not more than ±0.05 pH unit. For precision on mitoplasts, see comments in the text.

TABLE III

SEDIMENTATION OF RIGHT-SIDE-OUT SUB-MITOCHONDRIAL PARTICLES

Right-side-out submitochondrial particles were generated by French press as described in Materials and Methods but without bovine serum albumin in the medium. Samples were diluted to 160 ml with the same medium as used for disruption, and large membrane fragments and intact mitochondria were pelleted at 40000 × *g* for 10 min. The supernatants was then centrifuged at 105000 × *g* for 60 min and then at 300000 × *g* for 60 min. The resulting pellets (P) were suspended in the appropriate medium for further experiments. % right-side-out submitochondrial particles is calculated as described in Materials and Methods. Values are given as means ± S.D. for three independent preparations in each group.

Fraction	% protein	Cytochrome *c* oxidase		% right-side-out particles
		μmol Cyt *c*/ min per mg protein	% total activity	
40000 P	3 ± 1.0	2.4 ± 1.1	4 ± 2.8	50 ± 17
105000 P	15 ± 0.6	4.3 ± 0.1	36 ± 2.8	73 ± 6
300000 P	18 ± 0.4	2.3 ± 0.2	23 ± 2.1	90 ± 10
300000 S	64 ± 1.7	1.1 ± 0.3	37 ± 2.8	73 ± 12

The results from cross-partition of mitochondria, mitoplasts (50–60% devoid of outer membrane) and right-side-out particle vesicles give access to the isoelectric points of the exposed membranes (Fig. 3). It has been shown that there is significant agreement between the isopartition point determined in aqueous dextran–poly(ethylene glycol) two-phase systems and the isoelectric point of particles obtained by other methods [11,12]. As can be seen in Fig. 3D, the isoelectric point measured (p*I* = 4.0) for inside-out particles is distinctly different from the isoelectric points for the other mitochondrial membranes. The outer surface of the outer membrane is slightly more acidic (p*I* = 4.5) (Fig. 3A) than the outer surface of the inner membrane (p*I* = 4.7) (Fig. 3C). Contaminating outer membranes in the mitoplast fraction (Fig. 3B) can explain the slightly lower isoelectric point for mitoplasts compared to right-side-out particles.

Measurements of surface charge density with the 9-aminoacridine method also show that the charge densities are widely different for all the membrane surfaces studied (Table V). The observed charge density for purified potato tuber mitochondria (-27 mC · m^{-2}) differs slightly from the value obtained for Jerusalem artichoke (*Helianthus tuberosus*) mitochondria (-33 mC ·

TABLE IV

SEDIMENTATION OF INSIDE-OUT SUBMITOCHONDRIAL PARTICLES

Inside-out submitochondrial particles were generated by sonication as described in Materials and Methods but without bovine serum albumin in the medium. After sonication samples were diluted to 160 ml with the same medium as used for disruption. Large membrane fragments and intact mitochondria were pelleted at 40000 × *g* for 10 min. The supernatant was then centrifuged at 105000 × *g* for 60 min and then at 300000 × *g* for 60 min. The resulting pellets (P) were suspended in the appropriate medium for further experiments. % right-side-out submitochondrial particles is calculated as described in Materials and Methods. Values are given as means ± S.D. for three independent preparations.

Fraction	% protein	Cytochrome *c* oxidase		% right-side-out particles
		μmol Cyt *c*/ min per mg protein	% total activity	
40000 P	41 ± 4.6	4.0 ± 1.7	60 ± 4.0	22 ± 6.9
105000 P	17 ± 5.9	6.0 ± 2.9	37 ± 4.4	11 ± 4.6
300000 P	2 ± 0.3	2.5 ± 0.6	2 ± 0.6	14 [a]
300000 S	40 ± 2.6	< 0.01	< 0.2	80 ± 16

[a] Only two measurements.

TABLE V

SURFACE CHARGE DENSITY OF PLANT MITOCHONDRIAL MEMBRANES

The net surface charge densities were measured by use of 9-aminoacridine fluorescence as described in Materials and Methods. The area per charge unit was also calculated for each charge surface density. OM, outer membrane.

Membrane	Surface charge density ($mC \cdot m^{-2}$)	Area per charge unit ($nm^{-2} \cdot (e^-)^{-1}$)
Mitochondria	−27	5.88
Mitoplasts (50% depleted of OM)	−35	4.32
Mitoplasts (90% depleted of OM)	−47	3.57
Right-side-out particles	−51	3.13
Inside-out particles	−51	3.13

m^{-2}) [20]. The difference may be explained by the occurrence of contaminating components in the latter preparation (not Percoll purified). The two mitoplast preparations of different quality with respect to contamination of outer membrane (the first depleted of 50–60% of the outer membrane and the other depleted by 90%) are more negatively charged, $-35\ mC \cdot m^{-2}$ and $-47\ mC \cdot m^{-2}$, respectively, than the outer mitochondrial membrane. The charge density observed seems thus to be dependent on the amount of outer membrane contamination. Hence, the charge density of the right-side-out particles ($-51\ mC \cdot m^{-2}$) may be representative of the charge density of the outer surface of the inner membrane. The measured surface charge density of the inside-out particles appears to be similar to that of right-side-out particles. However, because of the observed difference in size between the two types of vesicle, the number of charges per vesicles is about 9-times higher for the inside-out particles than for the right-side-out particles.

Discussion

Submitochondrial particles of various polarities have been produced from different mitochondrial preparations from *S. tuberosum*, i.e., washed mitochondria, purified mitochondria and mitoplasts (derived from Percoll- or sucrose-purified mitochondria). The results obtained are highly consistent with results obtained in a previous study with submitochondrial particles from Jerusalem artichoke mitochondria [10].

Irrespective of the starting material, submitochondrial particles of well-defined polarities can be generated by use of a French press in low-salt medium or by sonication in high-salt medium. The first method results in submitochondrial particle preparations which are more than 80% right-side-out, the second procedure results in more than 80% inside-out. Washed mitochondria yield the highest percentage of both right-side-out and inside-out particles, mitoplasts yield the lowest. The reasons for these differences are not known. The interaction between various membranes other than inner membrane during the generation of submitochondrial particles may, however, be of importance. Another possibility is a direct membrane effect on the polarity assay, which is indirect, being based on the latency of cytochrome *c* oxidase in the presence of Triton X-100. It can not be excluded that different amounts of the various membranes present during the assay, can, in part, buffer the Triton effect and thus influence the latency obtained.

The yield of potato tuber submitochondrial particles, both on a protein and a cytochrome *c* oxidase basis, is about 2-fold lower for inside-out particles than for right-side-out particles (Tables II and III). The yield of submitochondrial particles may be correlated with the proposed mechanism [10] for the formation of both right-side-out and inside-out particles. If the inside-out particles primarily derive from tightly stacked cristae regions and the right-side-out particles from the inner boundary membrane, the yield of the two types of vesicle may be dependent on the ratio of cristae region to boundary membrane. Indeed, in contrast with the animal mitochondria, plant mitochondria do not exhibit many cristae, which represent only a minor part of the surface of the inner membrane (Fig. 1A and 1B). Differences in conformational states of mitochondria and mitoplasts may also be of importance for the generation of submitochondrial particles. The conformation of the inner membrane appears to be similar for both washed and purified mitochondria,

whereas mitoplasts reveal a change in both conformation and number of cristae (Fig. 1). Submitochondrial particles derived from mitoplasts are less extreme with respect to polarity compared to submitochondrial particles derived from whole mitochondria. The differences in size and in size distribution between right-side-out and inside-out particles may also reflect the mechanism of submitochondrial particle generation.

The difference in isoelectric points between intact mitochondria and mitoplasts reflects differences in lipid composition [26,27] and polypeptide composition between the outer and inner mitochondrial membranes. The observed difference in cross-points between right-side-out and inside-out particles demonstrates differences in isoelectric points between the outer and inner surfaces of the inner membrane. This difference reflects the asymmetric molecular composition of the membrane. Such asymmetry is also seen when the partition of right-side-out and inside-out particles is compared at their respective cross-points. The partition, expressed as percentage of particles distributed to the top phase, at the isoelectric point, is dependent on surface properties other than charge, e.g., content of non-polar groups and content of non-charged polar groups. The partition values for right-side-out and inside-out particles differ significantly, being 30% and 60% respectively, and lower than the values (75%) for whole mitochondria.

Differences between the various mitochondrial membranes in the content of acidic and basic groups, demonstrated by cross-partition are also seen when the charge density at pH 7.0 is measured by the 9-aminoacridine method. The outer surface of the outer membrane has only half the value of those for the outer and inner surface of the inner membrane. Even though the charge densities for the two surfaces of the inner membrane are similar at pH 7.0, we can speculate that the chemical nature of the charged groups on both side of the inner membrane is different and because of this they exhibit different isoelectric points.

The composition of the medium used for generation of submitochondrial particles may influence some characteristics of the inner membrane. It is evident that mitochondria are more fragile in low-salt (+EDTA) medium than in high-salt medium with accompanying differences in electron transport (Table I). Interestingly, the presence of EDTA in the medium makes the inner membranes more susceptible to osmotic damage [28], whereas Mg^{2+} makes the inner membranes less susceptible [29]. From the cytochrome *c* oxidase measurements it can be seen that the formation of right-side-out particles is accompanied by a much larger decrease in activity compared to the formation of inside-out particles. Whether this is an effect of the medium or of the disruption pattern is not known.

Right-side-out and inside-out particles are generated in media of different ionic strength. It can thus not be excluded that the higher ionic strength used for generation of inside-out particles can cause some removal of loosely bound membrane components. It is not at present known to what extent such removal can be involved in the observed differences between right-side-out and inside-out particles.

The results presented in this paper clearly demonstrate that it is possible to obtain submitochondrial particles of various polarity and size from potato mitochondria and mitoplasts. As stated before [10,24] right-side-out and inside-out particles will be valuable tools in the studies of transverse asymmetry of the inner mitochondrial membrane. The two populations of submitochondrial particles may also prove to be of importance in ascertaining possible lateral molecular heterogeneity along the inner membrane. For those kinds of study the demonstrated access to submitochondrial particles of different sizes, possibly derived from different parts of the inner membrane, will be of great significance.

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