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Applications of aqueous two-phase partition to isolation of membranes from plants: A periodic NADH oxidase activity as a marker for right side-out plasma membrane vesicles

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Abstract

Phase separations using standardized mixtures of polyethylene glycol, dextran and potassium phosphate are used widely to prepare highly purified plasma membranes from plants and in the preparation of chloroplast subfractions. Other uses include the removal of right side-out plasma membrane vesicles as contaminants from Golgi apparatus, endoplasmic reticulum and tonoplast (vacuole membrane) fractions and separation of right side-out and inside-out plasma membrane vesicles. The higher degree of separation between plasma membranes into the upper phase and internal membranes into the lower phase is in large measure due to the fact that only plasma membranes are oriented cytoplasmic side in. Most other membranes are oriented cytoplasmic side-out. This property extends to separations of right side-out and inside-out plasma membrane vesicles and to the separation of right side-out and inside-out sub-mitochondrial particles. The inside-out vesicles partition into the lower phase whereas the right side-out vesicles remain in the upper phase. The lack of efficacy of aqueous two-phase partitioning in other types of membrane separations is apparently due to the fact that surface characteristics that may distinguish different internal membranes are not located at the cytosolic membrane surface. At present there are no direct enzymatic markers for right side-out plasma membrane vesicles from plants. Demonstrations of sidedness and estimates of fraction purity are based on measurements of latency of marker enzymes, e.g., ATPases, at the cytosolic surface. This report describes a periodic NADH oxidase as an enzyme marker for right side-out plasma membrane vesicles not requiring detergent disruptions of vesicles for measurement of activity.

Keywords: Aqueous two-phase systems; Partitioning; Enzymes; NADH oxidase

1. Introduction

Phase separations involve mixing of membranes with a mixture of polymers that themselves will separate into different phases. The procedure was first developed by Albertsson [1–3] and takes advantage of the different surface properties of membranes. Since different membranes and perhaps even the same membranes from different species may possess different surface charges, it was necessary initially to evaluate a series of polymer and salt solutions. Currently standardized mixtures of polyethylene glycol, dextran and potassium phosphate are utilized almost exclusively.

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To effect the separation, the phase system containing the membranes is inverted and returned upright 20–40 times and then centrifuged at 4°C at low speed for short times in a swinging bucket rotor to resolve the polymers into two phases. The phases can be re-partitioned or washed to yield further purification or collected directly.

The principal applications of aqueous two-phase partition in plants are for purification of plasma membranes, preparation of chloroplast subfractions and separation of right side-out and inside-out plasma membrane vesicles. Other uses include removal of plasma membrane contaminants from gradient purified Golgi apparatus, endoplasmic reticulum or tonoplast or to resolve preparations of right side-out and inside-out mitochondrial membranes. A new marker for right side-out plasma membrane vesicles, a periodic NADH oxidase, is described.

2. Materials and methods

2.1. Plant material and chemicals

Soybeans (*Glycine max* L. Merr. cv Wayne) were germinated and grown in moist vermiculite for 5 days in a dark cabinet. On day 5, hypocotyl segments were excised with a razor blade (2-cm sections from the hook downwards) and collected directly into cold distilled water. Homogenization was with a Waring blender at full speed (three 10 s bursts alternating with 10 s cooling) in 10-ml aliquots of the above isolation medium consisting of 0.25 *M* sucrose, 25 m*M* Tris–2-(*N*-morpholino)ethanesulfonic acid (MES), pH 7.5, 10 m*M* KCl and 1 m*M* $MgCl_2$ plus 1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 0.1% bovine serum albumin (BSA). After homogenization, the slurry was strained through a layer of Miracloth (Chickopee Mills, NY, USA) and the residue rewashed with 10 ml of fresh isolation medium. The strained suspensions were combined at the end and used for isolating a microsomal fraction by centrifugation. The first centrifugation to remove cell wall material, nuclei and other large particles was in a Sorval centrifuge with a HB-4 rotor at 10 000 *g* for 10 min. The supernatant was transferred to a Spinco SW-27 rotor and centrifuged at 60 000 *g* for 30 min. The microsomal pellets were suspended in 0.5 to 1 ml of 5 m*M* potassium phosphate buffer, pH 6.8, for two-phase partition.

Unless specified otherwise, all chemicals were from Sigma (St. Louis, MO, USA).

2.2. Isolation of right side-out plasma membrane vesicles by aqueous two-phase partition

The method used was that developed by Kjellbom and Larsson [4] for the isolation of plasma membranes from spinach leaves, but with a modified polymer composition of the two phases [5]. A 1-g amount of soybean microsomal membrane suspension was added to the two-phase system to give a final composition of 6.4% (w/w) Dextran T500 (Pharmacia, Piscataway, NJ, USA) and 6.4% (w/w) Carbowax (polyethylene glycol) 3350 (Fisher Scientific, Chicago, IL, USA), 0.25 *M* sucrose and 5 m*M* potassium phosphate, pH 6.8. The system was mixed by 40 inversions in 30-ml glass centrifuge tubes and separated into two phases by centrifugation (1000 *g* for 5 min). The upper phase, enriched in plasma membranes, was partitioned twice against a fresh lower phase and separated by centrifugation as before. The lower phase was repartitioned against a fresh upper phase, followed by a fresh lower phase to collect and wash additional plasma membranes. Finally, all upper phases were combined, diluted with cold distilled water or with 5 m*M* potassium phosphate buffer, pH 6.8 and centrifuged in a SW-27 rotor at 120 000 *g* for 45 min to pellet the isolated plasma membranes, which were finally resuspended in 0.5 to 1 ml of 25 m*M* Tris–MES containing 0.25 *M* sucrose or distilled water.

2.3. Isolation of inside-out plasma membrane vesicles produced by freezing and thawing

Plasma membrane vesicles were frozen and thawed six times followed by 30 passages through a 25 gauge hypodermic needle to invert some of the vesicles. The vesicles were then subfractionated by repeating the phase partition step of Section 2.2. The lower phase was enriched in inside-out vesicles. The upper phase contained the remaining right side-out vesicles. The vesicles were collected by centrifuga-

tion at 120 000 *g* for 45 min and resuspended in 25 m*M* Tris–MES, pH 7, containing 0.25 *M* sucrose.

2.4. Measurement of oxidation of NADH by plasma membrane vesicles

NADH oxidation was measured spectrophotometrically from the disappearance of NADH at 340 nm using a Hitachi U3210 spectrophotometer (Hitachi Instruments, Chicago, IL, USA). The assays were at 24°C with stirring by a magnetic stirring assembly. Individual reaction mixtures contained 1 m*M* KCN, 50 m*M* Tris–MES, pH 7, and 40–50 μg plasma membrane protein. After a 3-min equilibration period, 150 μ*M* NADH was added. Reaction rates at a full-scale absorbance of 0.06 were recorded with a linear recorder. The rates of NADH oxidation were calculated using a millimolar extinction coefficient of 6.22 for NADH. Addition of 1 μ*M* reduced glutathione (GSH) for 10 min followed by 0.03% hydrogen peroxide or 100 μ*M* oxidized glutathione (GSSG) was used to enhance the NADH oxidase activity.

3. Results

3.1. Isolation of plasma membranes

The primary utility of aqueous two-phase partition to plants has been for the isolation of plasma membranes directly from total microsomes according to Kjellbom and Larsson [4]. These applications have been reviewed extensively [3–6]. Normally, membranes are obtained in good yield (15 to 20%) and fraction purity (>90%). Contaminants include mitochondria and mitochondrial fragments (<2%), tonoplast (<5%) and Golgi apparatus (<1%).

3.2. Removal of plasma membrane contaminants during the isolation of Golgi apparatus and endoplasmic reticulum fractions

Morré and Anderson [7] described a procedure where all major plant membrane fractions may be prepared from a single homogenate (Fig. 1). Tissues (leaves or stems) were coarsely cut with scissors and finely chopped in a small amount of medium with razor blades. Homogenization was by mashing with mortar and pestle using 1-ml medium to 1-g fresh weight of tissue. The homogenization medium was 50 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 10 m*M* KCl, 1 m*M* EDTA, 10 m*M* ascorbate, 0.1% BSA and 5 m*M* dithiothreitol (pH 7.5) containing 0.4 *M* sucrose. The albumin, ascorbate, dithiothreitol and sucrose were added fresh to both homogenization and sucrose gradient solutions. The concentrated homogenates were squeezed through a single layer of Miracloth to remove unbroken cells, cell walls and debris. The homogenates were transferred to 35- or 50-ml centrifuge tubes and nuclei; starch and intact chloroplasts were pelleted by centrifugation for 10 min at 1000 *g* using a swing out rotor.

The 1000-*g* (10-min) supernatant was layered onto a discontinuous sucrose gradient consisting of 4 ml 37% (w/v) sucrose and 8 ml 21.5% (w/v) sucrose in 35-ml tubes. After centrifugation for 30 min at 22 000 rpm (65 000 *g*, Spinco SW-28 rotor), the membranes at the homogenate/21.5% sucrose interface (for endoplasmic reticulum) and at the 21.5/37% sucrose interface (for Golgi apparatus) were collected in separate tubes, resuspended in the clear yellow supernatant from the top of the gradient and pelleted by centrifugation for 20 min at 20 000 rpm (50 000 *g*, SW-28 rotor) or equivalent (30 min at 17 000 rpm, 35 000 *g*, SS-34 Sorvall rotor).

To remove contaminating plastids and plasma membranes, the pellets were resuspended in 0.5 ml 0.25 *M* sucrose containing 5 m*M* potassium phosphate, pH 6.8 and applied to 4 *g* two-phase systems consisting of 5.9% (w/w) polyethylene glycol (PEG 3350) and 5.9% (w/w) Dextran T-500 (Pharmacia, Uppsala, Sweden). After equilibration to ice bath temperature, the tubes were mixed by 40 vigorous inversions in the cold, re-equilibrated to ice bath temperature and the phases resolved by low speed (10 min, 750 rpm, 100 to 200 *g*) centrifugation. The upper phases (plus the interfaces) and lower phases were collected separately, diluted with homogenization medium minus sucrose and the membranes collected by centrifugation for 20 min at 20 000 rpm (50 000 *g*). The lower phase pellets contained the purified endoplasmic reticulum (homogenate/21.5% sucrose interface) or Golgi apparatus (21.5/37% sucrose interface).

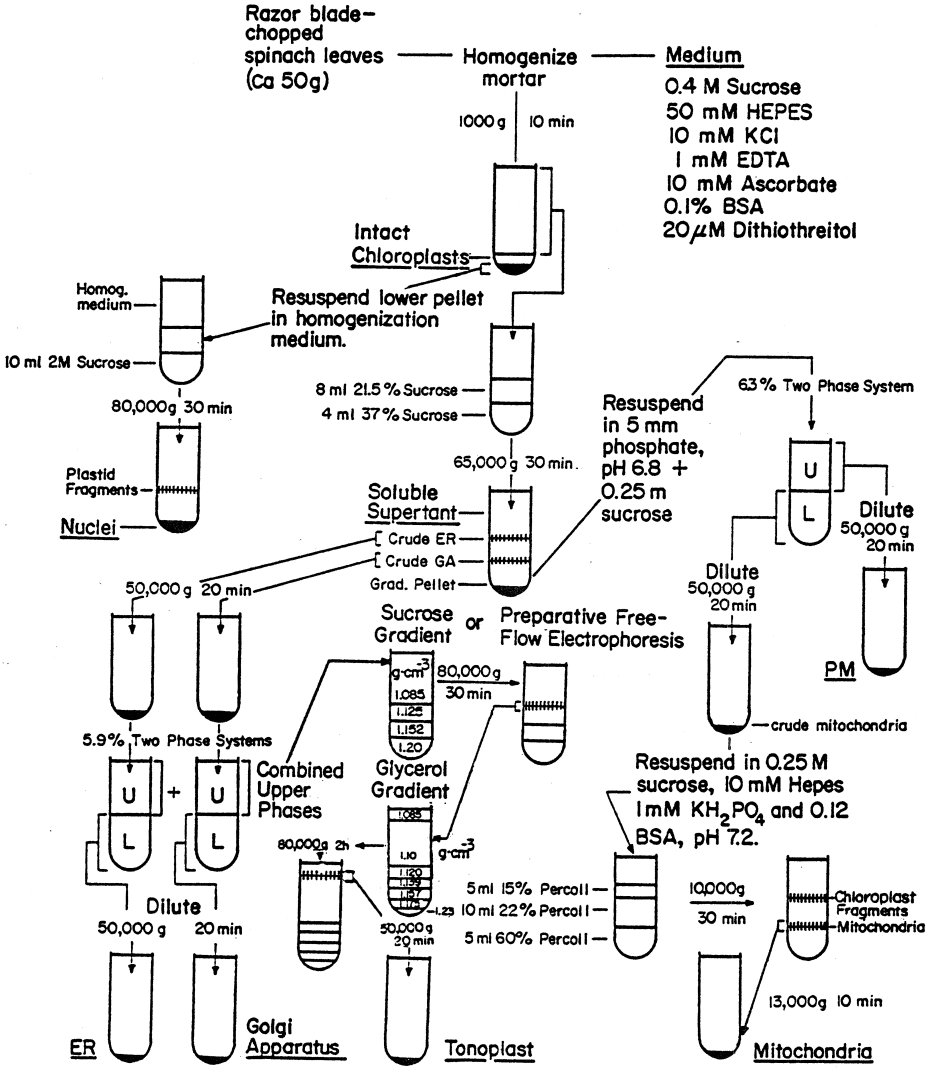

Fig. 1. Single homogenate fractionation procedure for plants yielding purified fractions of endoplasmic reticulum (ER), Golgi apparatus, nuclei, plasma membranes (PM), tonoplast, mitochondria, chloroplasts and soluble supernatant.

3.3. Separation of right side-out and inside-out plasma membrane vesicles

The plasma membrane vesicles prepared by aqueous two-phase partition have been previously shown to be predominantly right side-out [8] and to contain a NADH oxidase activity that was stimulated by auxins (Table 1) [9–12]. The vesicles prepared by aqueous two-phase partition, while predominantly right side-out, may contain some vesicles of a predominantly inside-out orientation and vice versa for the vesicles isolated by preparative free-flow electrophoresis [13,14]. Latency of ATPase activity was used as a measure of an enzymatic activity localized to the inside surface of the plasma membrane (Table 2). The ATPase activity of vesicles prepared by aqueous two-phase partition was 76–79% latent indicating up to 24% inside-out vesicles in these preparations. Similarly the latency of the inside-out vesicles purified by preparative free-flow

Table 1
Response to the synthetic plant growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D) of right side-out and inside-out plasma membrane vesicles prepared by freezing and thawing followed by aqueous two-phase separation and response to 0.01% Triton X-100[a]

Vesicle orientation	NADH oxidase (nmol/min/mg protein)	
	No addition	+1 μ*M* 2,4-D
(A) No Triton X-100		
Right side-out	2.9±0.5	4.9±0.6
Inside-out	9.3 ±1.2	9.8±1.5
(B) +0.01% Triton X-100		
Right side-out	11.8±0.7	15.2±1.9
Inside-out	12.0±1.0	15.7±0.9
(calculated from A; right side-out+inside-out)	12.2	14.7

[a] From DeHahn et al. [28]. Results are averages from four experiments±standard deviations.

electrophoresis was between 15 and 30%. This suggested that between 70 and 85% of the vesicles, but not all, were inside-out.

3.4. Plasma membrane-associated NADH oxidase as a marker for right side-out plasma membrane vesicles

Markers for mammalian plasma membranes include both cell surface-associated (γ-glutamyltranspeptidase, leucine amino peptidase, alkaline phosphatase) and cytosolic surface-located (ATPases, adenylate cyclase) markers [15]. These cell surface-associated markers are lacking in plants as in adenylate cyclase [16]. Therefore most papers dealing with aqueous two-phase-isolated plasma membrane vesicles from plants have used latent ATPase activities (comparing before and after detergent solubilization of the membranes) or glucan synthetase II [17] as enzymatic markers for the plasma membrane. Glucan synthetase also is latent (i.e., on the inside of the plasma membrane) and requires detergent solubilization for measurement of activity [18].

Because of the need for detergent treatment as part of the assay for the principal enzymatic markers for the plant plasma membrane, it would be helpful to have available an enzymatic marker for the direct assay of right side-out plasma membrane vesicles. The periodic, plasma membrane-associated oxidation of NADH provides such an activity.

A distinguishing characteristic of the plasma membrane-located NADH oxidase is its ability to oscillate with a regular periodicity of 24 min [19,20]. This oscillatory behavior is localized to the outer plasma membrane surface as shown by measurements comparing right side-out and inside-out plasma membrane vesicles (Fig. 2).

Only with right side-out vesicles was the periodic oscillation of activity seen that was stimulated by 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic plant growth regulator also used as a herbicide (Fig.

Table 2
Latency of ATPase activity of plasma membrane vesicles obtained from soybean hypocotyl microsomes by aqueous two-phase partition and free-flow electrophoresis in series[a]

Fraction	ATPase latency (%)
Aqueous two-phase partition	76–79
Preparative free-flow electrophoresis, inside-out	15–30
Preparative free-flow electrophoresis, right side-out	60–70

[a] From DeHahn et al. [28]. The ATPase latencies were determined from specific activities measured in the presence and absence of Triton X-100 at a near optimum Triton X-100-to-protein ratio of 1:1.8.

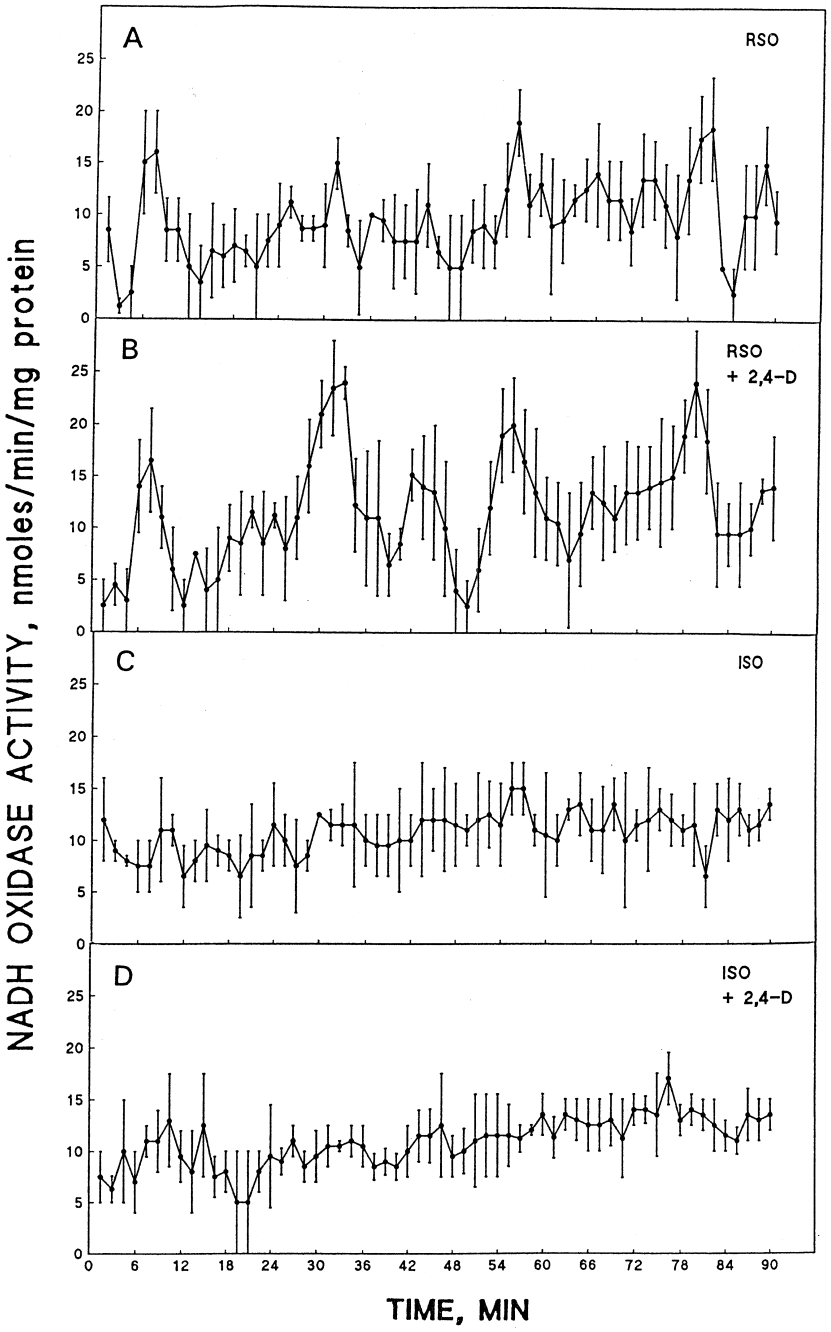

Fig. 2. Periodic variation in the rate of oxidation of NADH as a function of time over 90 min showing four maxima (6, 28.5, 52.5, 76.5 and 100.5 min). (A) With right side-out (RSO) vesicles with an average period length of 23.6±0.6 min per period. (B) In the presence of 1 μ*M* 2,4-D, a similar periodicity is seen but the rate of NADH oxidation is enhanced by a factor of about 1.7. (C) A similar variation in oxidation rate of NADH was not observed with preparations enriched in inside-out vesicles (ISO). (D) The activity of the inside-out vesicles also was unresponsive to 2,4-D (NADH is an impermeant substrate). Rates were determined over 1 min every 1.5 min.

2, panels A and B). With inside-out vesicles neither oscillatory behavior or 2,4-D stimulation was observed (panels C and D).

Alternatively, to prepare inside-out vesicles, plasma membrane vesicles, freshly prepared and stored frozen at −70°C were resuspended in the detergent Brij 58 ($C_{16}E_{20}$) to a final concentration of 0.5% (w/w) [21]. This treatment instantaneously produces 100% sealed, inside-out vesicles from preparations of 80–90% right side-out vesicles. The inside-out vesicles were collected by centrifugation at 120 000 *g* for 45 min and resuspended in 25 m*M* Tris–MES, pH 7, containing 0.25 *M* sucrose.

3.5. Other applications

Aqueous two-phase partitioning is used to separate intact chloroplasts from contaminating multiorganellar complexes [22]. Multiorganellar complexes consist of one or more chloroplasts, mitochondria, cytosol and peroxisomes all surrounded by plasma membranes. These complexes are formed by the rupture and reformation of plasma membrane vesicles that enclose cytoplasmic fragments during reformation. As with other two-phase procedures, the separation is based on delivery of the multiorganellar complexes into the upper phase based on the fact that the complexes are surrounded by plasma membranes.

Two-phase partition is used to remove chloroplast thylakoid membrane from isolated plant mitochondria [23]. When purified mitochondria are ruptured by sonication in low-salt medium, preparations of right side-out and inside-out membrane vesicles are obtained. These can be separated in a Dextran T500–PEG 3350/PEG 6000 two-phase system, the right side-out sub-mitochondrial particles enter the upper phase whereas the inside-out sub-mitochondrial particles enter the lower phase [23].

Both procedures using aqueous two-phase partition have been described for isolation of different regions of the thylakoid membrane of chloroplasts following rupture by sonication in hypotonic media. Enriched fractions include those of grana vesicles, stroma vesicles, margin vesicles and photosystem II vesicles from chloroplasts [24]. Most employ the standard dextran–PEG system but use different polymer concentrations, ions and pH.

4. Discussion

Aqueous two-phase partition has virtually revolutionized the preparation of the plasma membranes from both plant [1,3] and animal [25–28] sources. For plants, the preparation of plasma membranes by sucrose gradient centrifugation, preparative free-flow electrophoresis or counter-current distribution has been largely supplanted by batch procedures employing, for the most part, standard dextran–PEG two-phase systems. In the basic method, right side-out plasma membrane vesicles partition almost exclusively into the PEG-rich upper phase. Internal membranes (tonoplast, mitochondria, plastids, endoplasmic reticulum and Golgi apparatus) remain in the lower phase.

Highly purified plasma membrane vesicles can be everted by freezing and thawing accompanied by extrusion through a hypodermic needle into populations consisting of vesicles of reduced volume approximately half of which are inside-out and half of which are right side-out. Upon repartitioning, the right side-out vesicles once again enter the upper phase while the inside-out vesicles remain in the lower phase. Sidedness has been established by the localization of ATPase activity with the inside-out vesicles [8,13,14]. The external plasma membrane-associated NADH oxidase permits direct assay of right side-out plasma membrane vesicles without the need for detergent disruption (this report). Both ATP and NADH are impermeant substrates and are accessible only to enzyme exposed at the correct vesicle surface, inside-out for ATP and right side-out for NADH.

Other applications have been to remove plasma membrane contaminants from gradient purified endoplasmic reticulum and Golgi apparatus [7] and plasma membrane-enclosed cytoplasmic droplets from intact chloroplasts [22]. Dextran–PEG two-phase systems also have proven useful to separate inside-out and right side-out sub-mitochondrial particles [23] and to subfractionate chloroplast thylakoids [24].

There has been little progress to prepare membranes from plants other than to separate cytosolic from extra cytosolic surfaces. This requirement apparently limits further applications of aqueous

two-phase partition to membrane separations. Why extra cytosolic and cytosolic membrane surfaces should contribute such different properties to aqueous two-phase separations is not known. The differences are not due to acidic carbohydrates or sialoglycoproteins since these are largely lacking in plants. Even the presence of neutral carbohydrates or neutral glycoproteins do not provide an explanation since such molecules are largely lacking from inner mitochondrial membranes where separations of right side-out and inside-out sub-mitochondrial particles have been reported [23]. Some other property characteristic of the extra cytosolic membrane surface appears to determine efficacious partitioning in aqueous dextran–PEG two-phase systems.

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