Binding of concanavalin A to the outer membrane of potato tuber mitochondria detected by flow cytometry

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Purified and intact mitochondria isolated from potato tuber show a specific concanavalin A (Con A) binding as deduced from fluorescence labelling studies in the presence of the specific sugar α-D-methylmannose. This specific binding also occurs in mitoplasts, indicating the presence of Con A-binding sites on the inner mitochondrial membrane. In contrast, the binding of wheat germ agglutinin appears non-specific. Flow cytometry with purified mitochondria enables assessment of the binding by individual mitochondria. Thus, three mitochondrial subpopulations were identified by their reactivity towards fluorescent Con A.

Concanavalin A *Glycoconjugate* *Mitochondria* *(Potato tuber)* *Flow cytometry*

1. INTRODUCTION

There has recently been development of mitochondrial research addressing the interaction of these organelles with other compartments of the eukaryotic cell. As Schatz has noted, "one promising approach is to study the mitochondrial outer membrane" [1] since such interactions, including the relationship to the cytoskeleton [2], protein import [3,4], and mitochondrial DNA control of the expression of cell surface antigens [5,6], implicate outer membrane proteins.

Several lines of evidence derived from mammalian cells have indicated that glycoproteins are components of mitochondria [7–9]. With respect to the cytoplasmic side of the membrane of intracellular organelles such as mitochondria, conflicting results have been reported. Biochemical studies have shown that liver mitochondria contain moderate amounts of carbohydrates [10–12] and that the outer membrane carbohydrate content, especially mannose, is about 5-times higher than that of the inner membrane and may be responsible for the binding of these membranes to Con A [7,9]. However, in several electron microscopic studies dealing with conjugated lectins stained with ferritin, no labelling was seen on the outer membrane of mitochondria from nerve cells [13], rat liver cells [14,15] and from rabbit polymorphonuclear leukocytes [16].

Regarding plant mitochondria, except the report of Mannella and Bonner [17] associating polysaccharides with outer mitochondrial membrane from *Phaseolus aureus*, there is a lack of information about glycoconjugates associated with mitochondrial membranes. Here, fluorescein-conjugated Con A has been used to reveal specific Con A-binding glycoproteins in the outer membrane of intact potato mitochondria, and in the inner membrane of these mitochondria using mitoplasts. Furthermore, 3 subpopulations of mitochondria could be identified by the reactivity towards F-Con A.

Abbreviations: $DiOC_6(5)$, 3,3'-dihexyloxadicarbocyanine iodide; F-BSA, bovine serum albumin-fluorescein isothiocyanate; F-Con A, concanavalin A-fluorescein isothiocyanate; F-WGA, wheat germ agglutinin-fluorescein isothiocyanate; Mops, 4-morpholinopropanesulfonic acid; PBS, phosphate-buffered saline; TPP^+, tetraphenylphosphonium

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Such a result underlines the particular interest of using flow cytometry. Although the technique has been applied to mitochondria in the cytoplasm [18], this is, we believe, the first application to isolated mitochondria.

2. MATERIALS AND METHODS

2.1. *Preparation of mitochondria*

Potato tubers (*Solanum tuberosum* L.) were obtained from a local market. Mitochondria, prepared as in [19], were layered on Percoll step gradients containing 0.3 M mannitol, 0.1% BSA (fraction V, Sigma) and 10 mM Mops (pH 7.2). Each gradient comprised 2 ml of 80% (v/v), 4 ml of 40%, 3 ml of 23% and 3 ml of 10% Percoll mixture in a 15 ml tube. Two gradients were centrifuged for 35 min at 13200 × *g* in an SW27 rotor (Beckman). Purified mitochondria were collected from the 23–40% interface and washed in 0.1 M PBS (pH 7.2) with 0.1% BSA to remove Percoll. Mitoplasts were obtained by osmotic shock after swelling and by pelleting at 10000 × *g* to remove the outer membrane.

2.2. *Spectrofluorimetric determination of lectin binding*

Purified mitochondria (0.5 mg protein) were mixed with 250 μg/ml F-Con A (Sigma) containing 1.9 mol FITC per mol lectin in 0.1 M PBS-BSA. After 30 min incubation at 25°C, the mitochondria were washed, the pellet treated with the chaotropic agent MAC 19 (French patent 82-05-005 kindly provided by M. Monsigny) and the lectin-linked fluorescein released into the supernatant determined spectrofluorimetrically. Three controls were performed in order to ascertain the specificity of the labelling. Firstly, the mitochondria were preincubated with 250 μg/ml α-methyl-D-mannose for 30 min at 25°C and then incubated with Con A. Secondly, mitochondria incubated without Con A and mannose but treated in the same manner were examined. Thirdly, incubation in fluorescein-labelled BSA gave an estimate of non-specific binding. Similarly, mitochondria were mixed with 250 μg/ml F-WGA (Sigma) containing 2.2 mol FITC per mol lectin, with or without preincubation in 250 μg chitobiose/ml.

2.3. *Cytofluorimetric determination of Con A binding*

Mitochondria were prepared without MAC 19 and diluted to 2–5 μg protein/ml. The EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) was equipped with an argon laser (Spectra-Physics 2025-05). A standard 76 μm nozzle was used: smaller nozzles did not improve resolution. The sheath was water, at 13 lb/inch^2. For fluorescein, emission filters were 515 nm LP interference, 515 nm LP absorbance, and 560 nm SP interference (eliminating any Raman emission). For rhodamine 123, $DiOC_6(5)$ and safranin, emission filters were 540 LP interference and 540 LP absorbance. Excitation was 488 nm for fluorescein, rhodamine 123 and $DiOC_6(5)$, and 514 nm for safranin. The dyes were: 10 μM rhodamine 123 (aqueous, Kodak); 2.5 μM $DiOC_6(5)$ (ethanolic, a gift from Paul Horan, available from Molecular Probes) and 20 μM safranin O (aqueous, Sigma).

2.4. *Metabolic activity*

Membrane potential was measured with a TPP^+ electrode [20,21]. Intactness of the outer mitochondrial membrane was estimated by the successive measurement of cytochrome-*c* oxidase activity in a hypotonic and an isotonic medium. Oxygen uptake was measured by polarography [19]. Mitochondria were examined by electron microscopy on a Jeol CX200 using standard fixation procedures.

3. RESULTS AND DISCUSSION

3.1. *Condition of the mitochondria*

By electron microscopy, two distinct and intact membranes (outer and inner) were seen. As measured by the cytochrome-*c* oxidase method, the mitochondria exhibited a high degree of integrity of the outer membrane (intactness 95–97%). Respiratory activities (succinate oxidation rates in phosphorylating conditions) were 238 ± 22 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein associated with respiratory control ratios of 4.2 ± 0.3 ($n = 6$). A 220–230 mV membrane potential was generated under these conditions [21]. Such intact and active preparations are necessary if one is to examine specifically the outer membrane (eventually in relation to physiological states), and subsequently to examine the inner membrane with mitoplasts.

Table 1

Con A and WGA binding on mitochondrial outer membrane expressed as relative fluorescence derived from fluorescein analogs, and autofluorescence of mitochondria

	Arbitrary fluorescence units
Con A	264
α-M + Con A	23
WGA	42
CH + WGA	30
F-BSA	35
Mitochondria alone	16

Excitation, 495 nm; emission, 520 nm. Means of 9 replicates. CH, *N,N'*-diacetylchitobiose; α-M, α-D-methylmannose

3.2. *Spectrofluorimetric assay*

From table 1 it is evident that such intact mitochondria bound Con A, this binding being specific inasmuch as preincubation with α-D-methylmannose reduced the binding by 95%. WGA binding was slight, being of the same degree as the non-specific F-BSA binding.

3.3. *Flow cytometry*

Flow cytometry differs conceptually from bulk measurement in a fluorimeter cuvette, since each mitochondrion is measured individually as it moves past the laser beam [22]. Thus one obtains a set of individual values constituting a population described not only by its mean but also by quantitative differences between mitochondria (the frequency distribution) and even qualitative differences (positive and negative subpopulations).

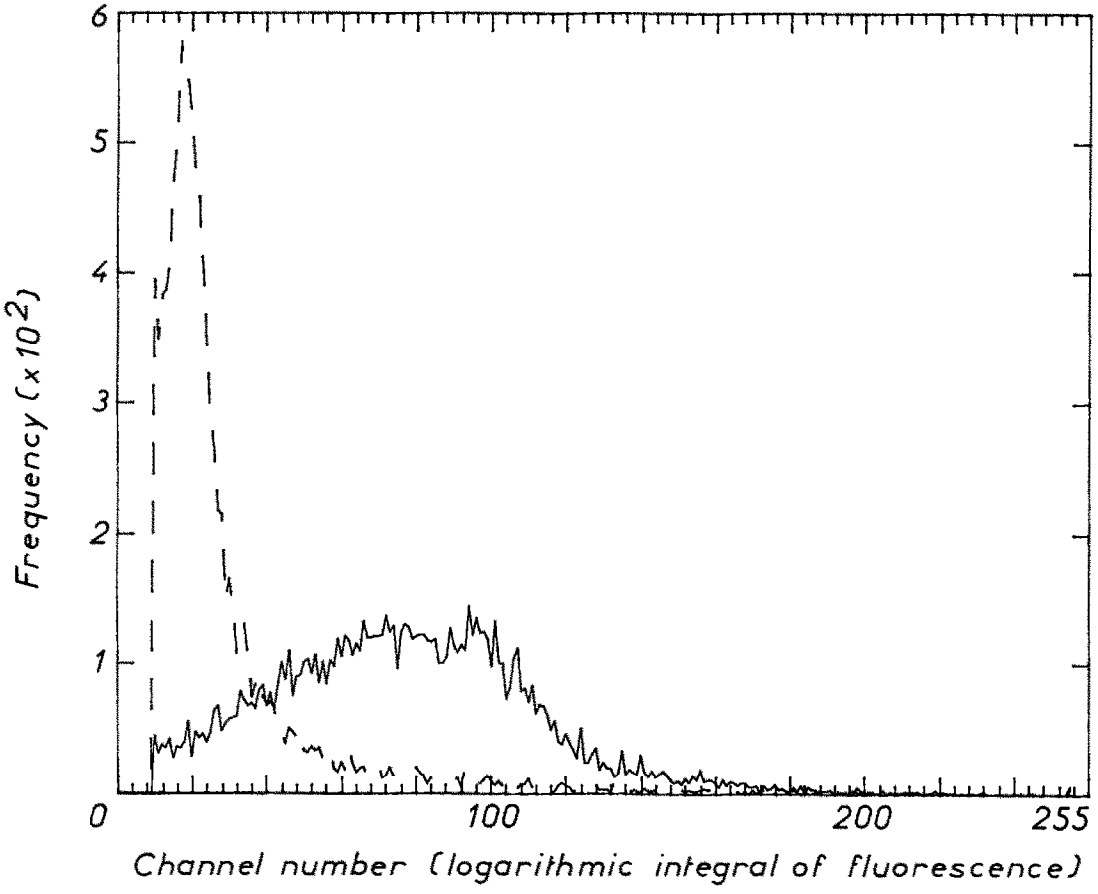

Fig.1. Fixation of safranin by isolated mitochondria (2–5 μg protein · ml^{-1}). Mitochondria alone (– – –) or with 20 μM safranin (——).

Isolated mitochondria stained with potential-sensitive dyes can be observed by flow cytometry (fig.1) and the size distribution obtained (fig.2). Similar results were obtained with each dye. Mitochondrial size and fluorescence were at the lower limit of resolution. In fig.2, two histograms of forward light scatter are superimposed. One curve is from standard beads of 0.305 and 0.804 μm. The left half of the 0.305 μm peak includes noise from deflection of the laser by the stream itself: this constant noise can be recorded for an identical period and subtracted from such a histogram in deferred time to obtain a 'noise-corrected histogram'. The key procedure to minimize this optical noise is to close the laser aperture to its minimal opening and to raise power (760 mW in this case). A further precaution was to maintain the maximal permitted count rate (e.g. 5000 objects$\cdot s^{-1}$) to maximize the signal/noise ratio (noise$\cdot s^{-1}$ being constant). The second histogram in fig.2 is the frequency distribution of forward light scatter from chilled mitochondria: this is coherent with a mean diameter of 0.6 μm as measured in electron micrographs.

These histograms were obtained by gating on the logarithm of integral fluorescence (i.e. only fluorescent objects were retained in the analysis). To gate objects on forward light scatter is impractical because, observed through this parameter alone, small mitochondria are indistinguishable from noise. Furthermore, it is unacceptable to gate out some of the mitochondria along with noise (us-

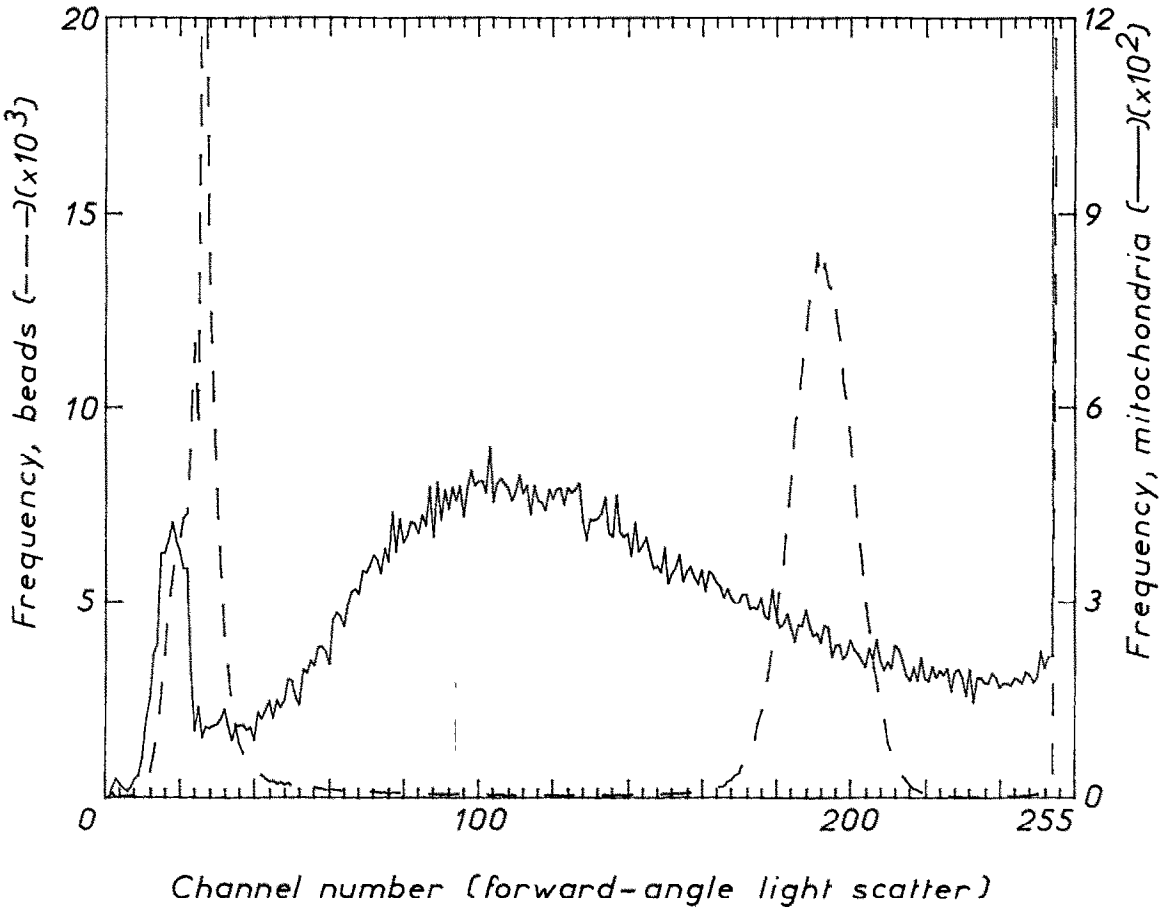

Fig.2. Relative intensities of forward angle light scatter for standard beads (---; 0.305 and 0.804 μm, Coulter) and chilled potato mitochondria (——). Only objects emitting fluorescence after staining with $DiOC_6(5)$ are included in this distribution: technically, light scatter was gated on logarithmic integral of fluorescence.

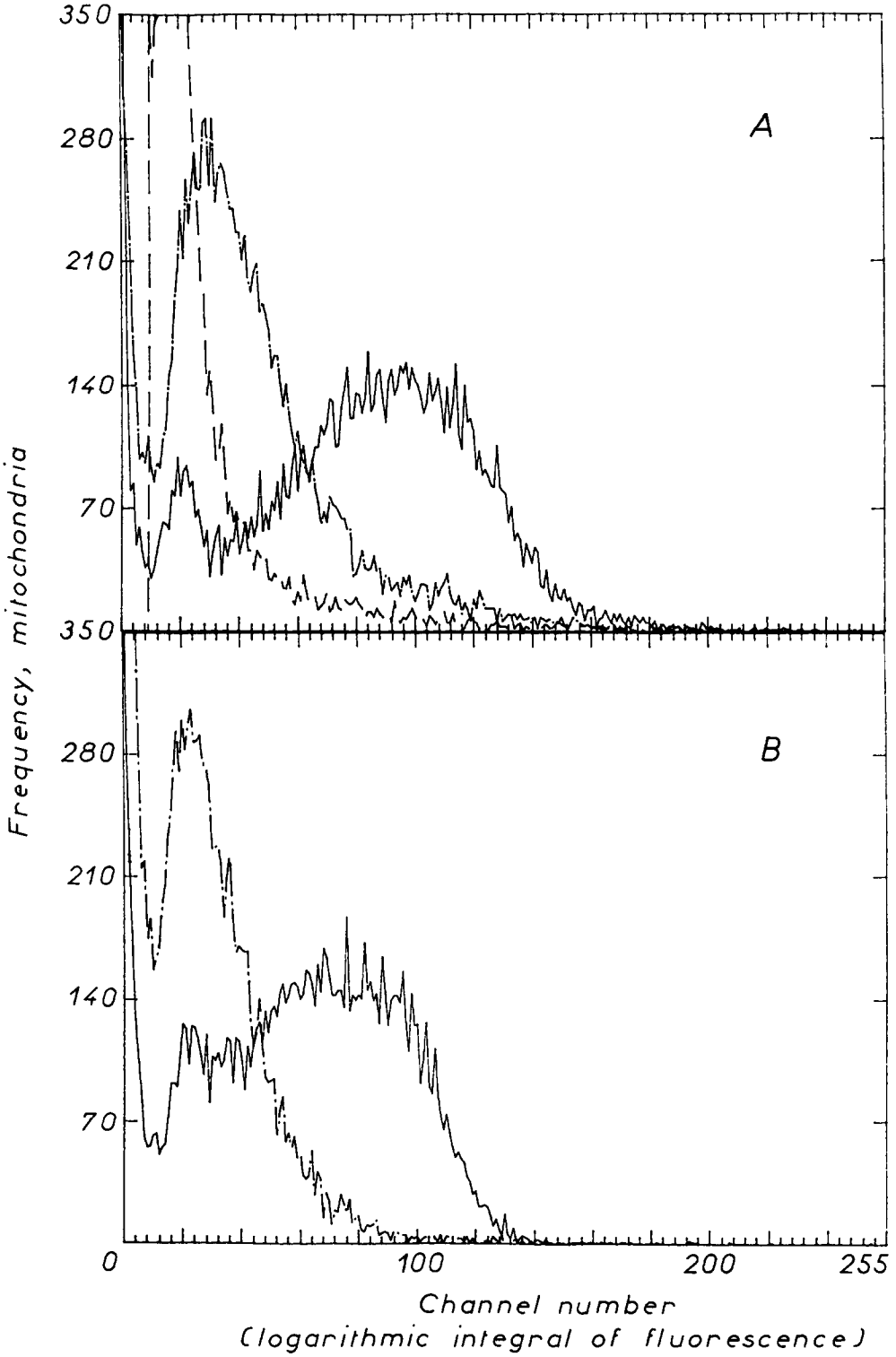

Fig.3. Fixation of F-Con A by potato mitochondria (A) or mitoplasts (B) without (——) and with (- - - -) α-D-methylmannose. Autofluorescence of unstained mitochondria is also shown (in A, – – –). The photomultiplier was at 1150 V. When the modal intensities in A are converted to a linear scale, the relative fluorescence values are: unstained, 1; Con A with mannose, 1.4; Con A alone, 8.3.

ing forward light scatter), since the results would no longer concern the whole population.

3.4. *Heterogeneity of Con A binding*

Fig.3A presents the raw histograms for mitochondria labelled with F-Con A with or without α-D-methylmannose. Mitochondria differ strongly in their capacity to bind the lectin: the extreme values differ 50-fold (the X-axis is logarithmic). In addition, a distinct subpopulation (22% of the whole) is unmarked. Mitochondria preincubated in α-D-methylmannose have a low modal fluorescence, but the right-hand skew of the distribution reveals that part of the population has retained considerable Con A. This non-specific binding is echoed in the similar distribution found with F-BSA or F-WGA (not shown). Although F-WGA binds to mitochondria, we have not yet been able to establish unambiguously the presence of specific binding.

Mitoplasts exhibit a specific binding of F-Con A on the inner membrane (fig.3B).

The mitochondrial suspension thus has subpopulations which may be classified by the intensity of fluorescence in the presence of F-Con A as follows. 22% of mitochondria did not show any significant binding (relative to the background autofluorescence). Of the 78% that did bind Con A, one class (49% of the suspension) displayed significant specific Con A binding; in the remainder the intensity of binding was not significantly different from the broad distribution of non-specific fixation (with α-D-methylmannose). These percentages and the tests of significance were obtained with a curve-fitting routine ('Immuno' of the Epics MDADS system) which models non-reactive populations (sequentially, on autofluorescence and non-specific fluorescence) such that the histogram of Con A-treated mitochondria may then be compartmentalised into its constituent parts.

Thus potato mitochondria differ in their degree of expression or availability of surface glycoproteins. Extension of this approach may help in understanding the subpopulations of mitochondria coexisting in the cell. The capacity of flow cytometry for multiparametric analysis might be explored to relate the presence or absence of available receptors on individual mitochondria to their different stages of development or competence.

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