Alamethicin permeabilizes the plasma membrane and mitochondria but not the tonoplast in tobacco (*Nicotiana tabacum* L. cv Bright Yellow) suspension cells

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The ion channel-forming peptide AlaM (alamethicin) is known to permeabilize isolated mitochondria as well as animal cells. When intact tobacco (*Nicotiana tabacum* L.) Bright Yellow-2 cells were treated with AlaM, the cells became permeable for low-molecular-mass molecules as shown by induced leakage of NAD(P)⁺. After the addition of cofactors and substrates, activities of cytosolic as well as mitochondrial respiratory enzymes could be directly determined inside the permeabilized cells. However, at an AlaM concentration at which the cytoplasmic enzymes were maximally accessible, the vacuole remained intact, as indicated by an unaffected tonoplast proton gradient. Low-flux permeabilization of plasma membranes and mitochondria at moderate AlaM concentrations was reversible and did not affect cell vigour. Higher AlaM concentrations induced cell death. After the addition of catalase that removes the H₂O₂ necessary for NADH

oxidation by apoplastic peroxidases, mitochondrial oxygen consumption could be measured in permeabilized cells. Inhibitor-sensitive oxidation of the respiratory substrates succinate, malate and NADH was observed after the addition of the appropriate coenzymes (ATP, NAD⁺). The capacities of different pathways in the respiratory electron-transport chain could thus be determined directly. We conclude that AlaM permeabilization provides a very useful tool for monitoring metabolic pathways or individual enzymes in their native proteinaceous environment with controlled cofactor concentrations. Possible uses and limitations of this method for plant cell research are discussed.

Key words: alamethicin permeabilization, mitochondria, plant cell survival, plasma membrane, respiratory enzyme, tonoplast.

INTRODUCTION

The role of mitochondria in cellular energy metabolism has been well established. Much of the research aimed at characterizing mitochondrial processes has relied on the development of efficient isolation methods that preserve the integrity of organelles [1,2]. The use of submitochondrial particles of defined purity and sidedness has allowed the characterization of alternative electron-transport pathways on the inner surface of the inner mitochondrial membrane [3]. The respiratory chain of plant mitochondria is more complex than that of animal mitochondria [4–6]. Besides being involved in primary metabolism and ATP production, plant mitochondria also take part in delivering precursors for biosynthesis, e.g. nitrogen assimilation [7]. Many of these processes also involve other cellular constituents: either other organelles, as is the case with photorespiration, or the surrounding cytosol. As an example of a direct interaction, several glycolytic enzymes co-purified with mitochondria isolated from *Arabidopsis* cells [8]. Such associations are difficult to preserve as functionally unperturbed during organellar isolation. Also, most membrane systems other than mitochondria and chloroplasts will, by necessity, be fragmented when tissues are homogenized, and thus cannot be isolated intact. Even mitochondria are not easily isolated from all tissues, e.g. no procedure has been published for isolating intact and functional mitochondria from *Arabidopsis* leaves with good yield and purity [9]. One reason for this might derive from

mitochondrial heterogeneity [10]. Due to dynamic changes in shape, mitochondria are not always short rods but extend into long tubular structures in response to low-oxygen tension in tobacco cell cultures [11] or when their division is impaired in *Arabidopsis* mutant plants [12]. Isolated organelles may therefore derive from a subpopulation with a distinct shape, and not necessarily be representative of the whole cell population. For these reasons, there is a need for methods that circumvent extraction of enzymes and organelles.

In plant cells, an alternative to isolating organelles would be to lower or abolish the permeability barrier of the PM (plasma membrane). For this, suspension-grown cells have the advantage compared with intact tissues of allowing an even treatment with the permeabilizing agent. Such a tool would permit the investigations of enzymes and organelles inside the intact cell. Yeast and mammalian cells have been permeabilized using detergents, osmotic shock, electrical pulses and antibiotic peptides [13–17]. A potentially suitable permeabilization agent for *in situ* studies of plant organellar activities is the channel-forming antibiotic AlaM (alamethicin). It is a 20 amino acid-residues-long hydrophobic peptide from the plant parasitic fungus *Trichoderma viride*, which is rich in aminoisobutyric acid, promoting a helical structure. Channel formation is voltage-dependent and, usually, multiple AlaM molecules form a barrel-like structure in the membrane surrounding the channel [18]. In synthetic membranes, AlaM insertion takes place only when the inner side of the membrane has

Abbreviations used: AlaM, alamethicin; BY-2, Bright Yellow-2; DTT, dithiothreitol; FW, fresh weight; n-PG, n-propyl gallate; NAD-GAPDH, NAD-glyceraldehyde-3-phosphate dehydrogenase; NAD-IDH, NAD-isocitrate dehydrogenase; NAD-MDH, NAD-malate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; PM, plasma membrane.

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a negative potential compared with the outer AlaM-exposed side [19]. The membrane potential of PM in intact plant cells is approx. -120 mV [20] and that of the inner mitochondrial membrane approx -200 mV [21], making these membranes good candidates for AlaM insertion, whereas such channels should be excluded from the positive, inside vacuolar membrane [22]. The channels formed by AlaM have been estimated to be 10 Å (1 Å = 10^{-10} m) in diameter with no real selectivity between univalent ions while mostly excluding bivalent cations [23]. After AlaM insertion, the size of the membrane potential usually decreases to the Donnan potential, approx. -50 to -70 mV in isolated mitochondria [21]. In animal and plant mitochondria, AlaM channels allow the passage of low-molecular-mass compounds such as dicarboxylates and nicotinamide nucleotides, but excludes folded proteins [24–26]. AlaM has previously been used for measuring Ca^{2+} -ATPase activity in human platelets [13], adenylate cyclase in mouse lymphocytes [27], glucose 6-phosphatase in rat liver microsomes [28] and for *in situ* assays of mitochondrial matrix enzymes in isolated rat liver and rat heart mitochondria [24]. Recently, AlaM was shown to be an efficient tool for *in situ* studies of the internal enzymes in mitochondria isolated from potatoes or pea leaves [26]. AlaM was here found to be better suited for quantifying internal NAD(P)H dehydrogenase activities than more disruptive methods such as sonication. Side effects in the form of partial cytochrome pathway inhibition were observed, but could be ameliorated by elevating the protein concentration [26].

In the present investigation, intact BY-2 (Bright Yellow-2) cells of tobacco (*Nicotiana tabacum* L.) were treated with AlaM, and the effects on the permeability of the PM, inner mitochondrial membrane and tonoplast were followed. We show that permeabilization of cells with low concentrations of AlaM resulted in a leakage of coenzymes, but without killing the cells. Higher AlaM induced a high flux of metabolite transport and the activities of glycolytic and tricarboxylic acid enzymes as well as the capacities of different electron-transport chain pathways could be determined *in situ*. The tonoplast, however, remained intact.

MATERIALS AND METHODS

Plant material and growth conditions

BY-2 tobacco cells (*N. tabacum* L. cv Bright Yellow) were cultivated in Murashige and Skoog basal medium [29] supplemented with 88 mM sucrose, 0.9 µM 2,4-dichlorophenoxy-acetic acid, 3 µM thiamine, 0.5 mM *myo*-inositol and 2 mM P_i . The initial pH of the medium was 5.0. Every 7th day, 2 ml of the cell suspension was subcultured into 50 ml of fresh medium in 250 ml Erlenmeyer flasks. The flasks were kept on a rotary shaker at 132 rev./min and 24°C in the dark. For all experiments, 40 mg FW (fresh weight) of cell suspensions in the mid- to late-exponential phase of growth were used (4–5-day-old cells). Cell growth was determined with each culture flask to assure similar cell densities during the course of the experiments. FW was determined on a 1 ml cell suspension per culture pelleted at 1500 g for 5 min, in triplicate.

Media

Permeabilization and activity measurements were always performed at a cell density of 40 mg FW · ml $^{-1}$ in either of two media: low-salt medium, 0.3 M sucrose, 20 mM Mops, 2.5 mM MgCl_2 and 1 mM EGTA (pH 7.2); and high-salt medium, 20 mM Mops, 100 mM KCl, 50 mM sucrose, 2.5 mM MgCl_2 , 1 mM EGTA and 5 mM P_i (pH 7.8). Catalase (EC 1.11.1.6; bovine liver) (Sigma, C-9322) was used at 192 units · ml $^{-1}$.

Oxygen consumption

Respiration was measured as oxygen consumption in a 1 ml Clark Oxygen Electrode (Rank Brothers, Cambridge, U.K.) at 24°C . Permeabilization of cells with various AlaM concentrations (see Figure 1) was performed in the low-salt medium. AlaM (Sigma) was dissolved in 95 % (v/v) ethanol to a stock solution of 10 mg · ml $^{-1}$ and stored at -20°C . Succinate, malate and NADH oxidation (see Figure 6 and Tables 1 and 2) were measured at 22 µg · ml $^{-1}$ AlaM in the high-salt medium unless otherwise stated. Final concentrations of the added reagents were: ATP, 1 mM; CoA, 0.5 mM; DTT (dithiothreitol), 5 mM; glutamate, 10 mM; malate, 20 mM; NAD^+ , 5 mM; pyruvate, 1 mM; and succinate, 20 mM. Complex I, complex III, complex IV and the alternative pathway were inhibited by the addition of 40 µM rotenone, 0.4 µM antimycin A, 1 mM KCN and 50 µM n-PG (n-propyl gallate) respectively.

NAD(P) $^+$ determination

Nicotinamide nucleotides were determined by enzymatic coupling [30] using a spectrofluorimeter (RF-5301PC; Shimadzu, Columbia, MD, U.S.A.) with excitation and emission wavelengths of 340 and 455 nm respectively. The slit width was 10 nm. Cells (40 mg FW) in 1 ml of low-salt medium without catalase were incubated with 0, 2.25, 5.5, 11, 22 and 44 µg · ml $^{-1}$ AlaM for 0–2 and 10 min on ice, with shaking, in the dark. The medium was then separated from the cells by filtration through a 0.45 µm membrane filter (Millex®-HA; Millipore, Bedford, MA, U.S.A.) using a syringe. The fluorescence intensity of the extracellular medium was measured both immediately after filtration and after 30 s incubation with 5.4 units · ml $^{-1}$ glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (EC 1.1.1.49; Sigma, G 5885), and 2 mM glucose 6-phosphate. This glucose-6-phosphate dehydrogenase uses both NAD^+ and NADP^+ as coenzyme. To correlate fluorescence intensities to NAD(P)^+ amounts, a standard curve between 0 and 2 µM NAD^+ was made using glucose-6-phosphate dehydrogenase and glucose 6-phosphate as above. NADP^+ gave an identical signal. Cells were also extracted using HClO_4 to obtain the total amount of nucleotides [31]. Determinations were conducted on cells from two separate cultures.

Spectrophotometrical measurements

Marker enzymes for cytoplasm and mitochondrial matrix were measured at 340–400 nm in an Aminco DW2a spectrophotometer, using a stirred cuvette (see Figure 3). The assay volume was 2 ml and temperature was 24°C . The assays were based on previously reported methods for measuring NAD-IDH (NAD-isocitrate dehydrogenase) [32], PEPC (phosphoenol pyruvate carboxylase) and phosphorylating NAD-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) [33]. For all three activities, the reaction mixture was supplemented with 100 mM KCl, 50 mM sucrose, 1 mM KCN, 50 µM n-PG and 1 mM EGTA. For NAD-IDH, the MgSO_4 concentration was doubled to 2 mM. NAD-MDH (NAD-malate dehydrogenase) was assayed as NAD-GAPDH but with P-glycerate kinase and 3-phosphoglyceric acid replaced with 2 mM oxaloacetate. All reactions were started by the addition of the metabolite substrate. At 22 – 44 µg · ml $^{-1}$ AlaM, the background rates (before the addition of the metabolite) was 30 – 55 nmol of $\text{NADH} \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$. At lower AlaM concentrations, the background was lower or non-existent but could not be clearly distinguished from noise caused by light scattering by cells in the stirred cuvette. We estimate that 10 % of the rates presented may be due to background. For determination of direct effects on NAD-GAPDH and PEPC, protein was extracted [34]

using an extraction buffer [35] without detergents. The desalted extracts were used immediately for the assay of NAD-GAPDH and PEPC.

Fluorescence microscopy

Cells ($40 \text{ mg} \cdot \text{ml}^{-1}$) in $200 \mu\text{l}$ of high-salt medium supplemented with $40 \mu\text{M}$ Acridine Orange, 2 mM ATP, 1 mM phosphoenol pyruvate and $50 \mu\text{g} \cdot \text{ml}^{-1}$ pyruvate kinase (Boehringer 109045, glycerol solution) were incubated with various AlaM concentrations for 10 min, or with 0.1% (v/v) Triton X-100 for 1 min. Fluorescence microscopy was performed with filters G-2A (510–560, DM 505, 520) using a Nikon-Optiphot-2 microscope, and images collected with an Olympus DP-70 digital camera (Olympus Optical, Tokyo, Japan). ATP and the ATP-regenerating system (phosphoenol pyruvate and pyruvate kinase) were added to maintain ATP-dependent H^+ transport across membranes such as the tonoplast also after PM permeabilization. The experiment was repeated twice with similar results, using cells from different cultures.

Cell-suspension growth after AlaM treatment

Cells (40 mg FW) in 1 ml of fresh growth medium were incubated with various concentrations of AlaM for 10 min. The cells were then separated from the medium by centrifugation at $1500 g$ for 5 min and washed once with fresh growth medium. In parallel, 1.5 ml aliquots of cell suspension from the same culture flask were withdrawn and cells were separated from the medium as above. The obtained conditioned medium was then used to resuspend the washed and pelleted AlaM-treated cells. Finally, the AlaM-treated cells in a total volume of 1 ml were subcultured into 25 ml of fresh medium in 100 ml Erlenmeyer flasks. The growth was monitored for 12 days by measuring attenuation D at 600 nm . The cultures from three flasks were used for the studies.

RESULTS AND DISCUSSION

AlaM permeabilizes plant cells

To investigate the effect of AlaM on plant cells, growing cells were harvested and their oxygen consumption registered during incubation with different concentrations of AlaM (Figure 1a). With increasing concentrations of AlaM, two effects were seen. A transient increase in oxygen consumption occurred, which came earliest at the highest concentration used ($44 \mu\text{g} \cdot \text{ml}^{-1}$). Thereafter followed a slower decrease in oxygen consumption, resulting in close to zero respiration within 5–10 min at the two highest AlaM concentrations used (22 and $44 \mu\text{g} \cdot \text{ml}^{-1}$). Cells treated with $11 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM showed a similar behaviour, but less rapidly, and did not stop respiring completely during the time the experiment was conducted. At lower concentrations of AlaM ($5.5 \mu\text{g} \cdot \text{ml}^{-1}$), only the increase in oxygen consumption was seen, and $2.25 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM had no consistent effect.

The initial rapid rise in oxygen consumption was somewhat puzzling, since the intracellular concentration of substrates for mitochondrial activities should decrease, rather than increase, as the cells were permeabilized. However, oxygen-consuming peroxidase-dependent redox cycles in the cell wall or at the outer surface of the PM [36] could make use of reductants [e.g. NAD(P)H] leaking out from the cytosol to reduce oxygen. To find out whether the initial AlaM-dependent rise in oxygen consumption of the cells was due to such apoplastic peroxidases, the assay was supplemented with catalase, which will metabolize the obligate intermediate H_2O_2 and therefore inhibit NAD(P)H oxidation through H_2O_2 -dependent peroxidases [36]. Indeed, in the presence of catalase, the initial rise in oxygen consumption was almost

Figure 1 Respiration of BY-2 cells during incubation with different AlaM concentrations

O_2 uptake was measured in the absence (a) and presence (b) of catalase in low-salt medium. AlaM was added at zero time. Rates are denoted as a percentage of the rate just before the addition of AlaM [$145 \text{ nmol of O}_2 \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$]. The graphs show averages of two different cultures. S.D. values varied between 2.5 and 12.4 %.

completely abolished (Figure 1b). These results indicate that the PM of BY-2 cells is permeabilized by AlaM, allowing NAD(P)H to escape the cell and become oxidized apoplastically. Consistently, addition of NADH to intact cells led to an immediate increase in the rate of oxygen consumption, with a magnitude similar to that induced by AlaM (results not shown). Concerning the subsequent slower inhibition of oxygen consumption, the pattern was similar with catalase added and in its absence; with higher AlaM, less time was needed for the oxygen consumption to decrease to zero. For $22 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM, a similar timing of respiratory decrease was seen in cells irrespective of age between early exponential and stationary phases (results not shown).

Nicotinamide nucleotides are released from cells during AlaM permeabilization

To determine whether nicotinamide nucleotides indeed had leaked out into the surrounding medium, cells were incubated for different time periods in the presence of various concentrations of

Figure 2 Nicotinamide nucleotide efflux from AlaM-permeabilized BY-2 cells

Cells were incubated with AlaM in a low-salt medium and after various times the solution was removed by filtration. Released NAD(P)⁺ was determined by reduction with glucose 6-phosphate and glucose-6-phosphate dehydrogenase. Error bars denote S.D. for two different cultures.

AlaM. The cells were removed by filtration and oxidized nicotinamide nucleotides (NAD⁺ and NADP⁺) released into the medium were determined enzymatically.

On addition of AlaM, a time- and concentration-dependent nucleotide leakage was observed. With the two highest concentrations of AlaM, released NAD(P)⁺ reached a maximum, approx. 14 nmol · (g FW)⁻¹, within 10 min. Approximately 50% of this leakage was observed using 11 µg · ml⁻¹ AlaM and even less using 5.5 µg · ml⁻¹ AlaM. At the lower concentrations, extracted nucleotides also increased over the duration of the incubation (Figure 2). In separate experiments, extraction for 10 min with Triton X-100 (0.1%, v/v) resulted in a similar release of nucleotides as with 44 µg · ml⁻¹ AlaM, 13.3 ± 1.4 and 16.4 ± 1.3 nmol · (g FW)⁻¹ respectively (mean ± S.D. for two separate cell cultures). This was roughly half of the HClO₄-extractable NAD(P)⁺ amount [29.1 ± 0.65 nmol · (g FW)⁻¹].

The lower NAD(P)⁺ value observed with AlaM and Triton X-100 is to be expected, since only free nucleotides will be released, i.e. enzymes in the permeabilized cells will still bind nucleotides. With HClO₄ extraction, enzymes are denatured, so enzyme-bound nucleotides will also be extracted. Additionally, NAD(P)⁺ may become degraded during permeabilization by AlaM and Triton X-100, for example by enzymes in the outer mitochondrial membrane [37]. The slight decrease in NAD(P)⁺ after 10 min incubation with 44 µg · ml⁻¹ (Figure 2) indicates degradation. Nevertheless, AlaM resulted in a release of nucleotides from the cells that may well correspond to all, or at least most of, the free cellular nucleotides in the cytosol and the mitochondria. The BY-2 cells are not green but contain undifferentiated plastids. It is likely that plastids also contribute to the pool of extruded NAD(P)⁺ at high AlaM concentrations since chloroplasts have an inner envelope with a positive outside membrane potential [38].

Does AlaM sequentially permeabilize PM and mitochondria?

The results in Figure 2 tentatively suggest that, with increase in concentration of AlaM, more intracellular compartments become permeabilized, most probably mitochondria and plastids not accessible at lower AlaM. The results thus agree well with the

Figure 3 Enzyme markers for AlaM permeabilization of the PM and mitochondrial inner membrane in BY-2 cells

Activities of NAD-MDH, NAD-IDH, NAD-GAPDH and PEPC. The main panel shows activities in cells permeabilized for 10 min with different AlaM concentrations (see the Materials and methods section). Enzyme activities after cell solubilization with 0.1% Triton X-100 for 1 min are depicted on the right. Virtually identical results were obtained using 0.05% Triton X-100 (results not shown). For NAD-GAPDH and PEPC measurements, protein was extracted from the same cultures as those used for this Figure. Activities of NAD-GAPDH and PEPC were 811 ± 147 and 75.3 ± 6.8 nmol of NADH · min⁻¹ · (g FW)⁻¹ respectively. The activities were less than 10% affected by the addition of 0.1% Triton X-100 or 44 µg · ml⁻¹ AlaM. Error bars denote S.D. for two (NAD-MDH, PEPC) or three (NAD-IDH, NAD-GAPDH) different cultures. Except for NAD-GAPDH and NAD-IDH, the data derive from separate sets of cultures.

results in Figure 1. During the first 10 min of treatment with 11 µg · ml⁻¹ AlaM, the PM is permeabilized enough to release mainly cytosolic nucleotides and mitochondrial respiration is only slightly affected. At higher AlaM concentrations, mitochondrial respiration stopped completely. Thus it was likely that the reduced respiration seen at higher AlaM concentrations was due to mitochondrial depletion of respiratory intermediates and coenzymes such as nicotinamide nucleotides.

To test whether AlaM indeed sequentially permeabilizes PM and mitochondria, we followed the activities of cytosolic and mitochondrial soluble respiratory enzymes, all of which have substrates and effectors that would be expected to move through the AlaM pore. The lowest concentration of AlaM used, 5.5 µg · ml⁻¹, induced clearly discernible, although low, activities of cytosolic NAD-GAPDH and PEPC that were absent from control cells. For AlaM values between 11 and 22 µg · ml⁻¹, the measurable activities increased significantly, with similar rates of approx. 400 nmol · min⁻¹ · (g FW)⁻¹ at 22 and 44 µg · ml⁻¹ AlaM (Figure 3). The mitochondrial matrix enzyme NAD-IDH responded in a highly similar manner to AlaM up to 22 µg · ml⁻¹. However, NAD-IDH continued to increase at 44 µg · ml⁻¹ AlaM (Figure 3), indicating that mitochondria become sufficiently permeabilized to support the high flux through NAD-IDH at somewhat higher AlaM concentrations than needed for the cytoplasmic enzymes.

When cells were permeabilized with Triton X-100, NAD-IDH and PEPC activities were relatively similar to the rates with AlaM, whereas a much higher activity of NAD-GAPDH was seen with Triton X-100. To determine whether this was a Triton X-100 or an AlaM effect, protein extracts were prepared from the cells. Of the maximum NAD-GAPDH activity measured in cells, 60% was present in the isolate, whereas only 14% of

the maximum PEPC activity in cells was recovered, indicating inactivation during extraction, for example due to dissociation [39]. Both enzymes were unaffected by Triton X-100 and AlaM after extraction. To investigate further whether permeability of the PM may still restrict the NAD-GAPDH activity even at high AlaM, we measured NAD-MDH in the NAD-GAPDH medium. However, the NAD-MDH activity at $44 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM was several-fold higher than the NAD-GAPDH activity, indicating that flux should not limit the measured NAD-GAPDH activity. NAD-MDH resides in both the cytoplasm and the mitochondrion and the response to AlaM is similar to that of NAD-IDH, although with higher activities throughout the AlaM concentration range (Figure 3). The maximum NAD-MDH activity in cells with AlaM was stimulated 50 % by Triton X-100. This is somewhat higher than that for NAD-MDH activity in isolated potato mitochondria, where Triton X-100 stimulated approx. 25 % more than AlaM [26]. This indicates that NAD-MDH is too fast to be substrate-saturated even at $44 \mu\text{g} \cdot \text{ml}^{-1}$, i.e. that the rate measured is close to the maximum flux of metabolites through the AlaM pore.

Less than $10 \text{ nmol of NAD(P)}^+ \text{ min}^{-1} \cdot (\text{g FW})^{-1}$ was released by treating the cells with $11 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM (Figure 2). It is therefore probable that the enzyme activities measured in the range below $22 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM (Figure 3) were limited by the flux of substrates and cofactors over the membranes. At sufficient AlaM, the activities instead reflect the *in situ* capacities of the respective enzymes under non-limiting conditions with respect to substrates and cofactors. The large difference between AlaM and Triton X-100 for NAD-GAPDH activity thus indicates that the enzyme is restricted within the cell by a component that is released by Triton X-100 but not by AlaM (e.g. a protein or non-transported metabolite).

Taken together, the measured enzyme activities show that AlaM allows a high flux of 1,3-bisphosphoglycerate, glyceraldehyde-3-phosphate, phosphoenol pyruvate, NAD^+ , NADH, oxaloacetate, malate, isocitrate and 2-oxoglutarate through the PM and, for the last six compounds, also through the mitochondrial inner membrane. For AlaM to permeabilize the PM and mitochondrial membrane consecutively, the peptide itself must also traverse the PM, possibly through its own pore. AlaM has been shown to allow peptide transport into yeast mitochondria [40].

AlaM does not permeabilize the vacuole

The tobacco cells used here are characterized by having a large vacuole, traversed by several strands of cytoplasm with vigorous cytoplasmic streaming (results not shown). To examine whether AlaM permeabilization also affected the vacuolar membrane, cells were incubated with Acridine Orange. This dye acts as a probe for the proton gradient, by being sequestered in acid compartments. This results in red fluorescence that also partially covers background green emission (Figure 4). For analysing AlaM-treated cells, ATP and an ATP-regenerating system were included during incubation. Thus Acridine Orange accumulation was not restricted by lack of ATP for H^+ transport across the tonoplast. Treating cells with $22 \mu\text{g} \cdot \text{ml}^{-1}$ of AlaM induced no difference in vacuolar Acridine Orange accumulation compared with control cells (red fluorescence). This clearly shows that the tonoplast is not permeabilized by this concentration of AlaM, but maintains a transmembrane proton gradient. Even at $44 \mu\text{g} \cdot \text{ml}^{-1}$ of AlaM, a significant fraction of the cells still accumulated Acridine Orange and most of the cells retained clearly distinguishable vacuoles. The differential distribution of Acridine Orange disappeared when the cells were instead treated with 0.1 % (w/v) Triton X-100. This treatment thus also destroyed the tonoplast, leaving only the background green fluorescence throughout the cell (Figure 4).

Figure 4 Effect of AlaM on accumulation of the ΔpH probe Acridine Orange in vacuoles of BY-2 cells

Cells were examined using fluorescence microscopy after incubation with various AlaM concentrations or 0.1 % Triton X-100 for 10 min in a high-salt medium supplemented with ATP and an ATP-regenerating system. Results shown are from one of three experiments with similar results, using cells from different cultures. Scale bar, $100 \mu\text{m}$. Red fluorescence indicates accumulation of Acridine Orange in acidic compartments, to be compared with the background green fluorescence from the cytoplasm.

The resistance of the vacuole to AlaM is most probably due to the positive inside membrane potential of the tonoplast ($+10$ to $+40 \text{ mV}$) [22] in contrast with the negative inside PM and mitochondrial inner membranes. Thus the observation that AlaM does not form channels when applied from the negatively charged side of model membranes [19] may also be valid in cells *in situ*.

AlaM causes cell death only at high concentrations

Since cells treated with up to $22 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM appeared to be structurally intact, we wanted to test the potential long-term effects of temporary permeabilization and loss of substrates and nucleotides. Cells were treated with different concentrations of AlaM for 10 min, pelleted by centrifugation and washed with growth medium to remove the AlaM. The cells were then resuspended in cell-free conditioned growth medium from their original culture, thus starting a new culture from a smaller cell mass [41], and recultured into new growth medium (see the Materials and methods section). No difference in growth was observed between control cells and cells that had been treated with 2.25 – $11 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM (Figure 5), i.e. under the conditions where the cells were not maximally depleted of cofactors (Figure 2). Cells treated with $22 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM showed delayed growth, but reached the same cell density approx. 2 days after control cells. Cells treated with $44 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM could not resume growth (Figure 5). These results show that a 10 min exposure to up to $11 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM caused no lethality, but at $44 \mu\text{g} \cdot \text{ml}^{-1}$, cell death sufficient to abolish further growth occurred. The intermediate effect at $22 \mu\text{g} \cdot \text{ml}^{-1}$ indicates growth retardation and/or death of a fraction of the cells. Since the cell cultures used have a normal daily

Figure 5 Effect of AlaM on the growth of BY-2 cell suspensions

Cells treated for 10 min with various concentrations of AlaM in the growth medium were recultivated and growth was monitored for 12 days by measuring A_{600} . Error bars denote S.E.M. for three different cultures.

growth yield of approx. 100% (results not shown) and, assuming no growth delay of individual cells, the 2 days delay in growth of the cell population indicates a cell survival of at least 25%. However, it is also possible that the cell survival is higher, but loss of coenzymes and low-molecular-mass metabolites delay the growth of the surviving permeabilized cells.

The results above indicate a relative cellular insensitivity to concentrations of AlaM that are sufficient to permeabilize the PM and mitochondrial inner membrane, allowing for example an NAD(H) exchange rate of $100\text{--}200\text{ nmol} \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$ (Figure 3). Such a permeabilization is expected to deplete rapidly the cytoplasm of free ATP, other nucleotides and metabolites and also depolarize the PM potential to the level of the Donnan potential. The lack of ATP will stop active processes and cause leakage of ions like Ca^{2+} from intracellular stores. Still, the ionic condition in the cytosol should be governed mainly by the external medium. Ca^{2+} present in the growth medium (3 mM) would be expected to diffuse into the cytoplasm, as reported for AlaM-treated bovine chromaffin cells [42]. The regeneration of the cell after permeabilization would include restoration of nucleotide and metabolite levels for energy metabolism and restitution of ionic homeostasis before growth can commence. The observed rapid growth recovery (Figure 5) indicates that this process is relatively fast. Possibly, resupply of cytoplasmic components is dependent on the remaining presence of some intact intracellular compartments (e.g. plastids) and, therefore, the use of higher AlaM concentrations leads to cell death. Clearly, the death of the cells is not correlated with vacuolar permeabilization. Short-term permeabilization of isolated potato mitochondria does not lead to cytochrome *c* extrusion [26], also making it less likely that the cell death induced here is due to an apoptotic-type process [43]. The AlaM permeabilization method may, however, be very useful for such studies.

AlaM, at a concentration of $10\text{ }\mu\text{g} \cdot \text{ml}^{-1}$, has been shown to be an elicitor of volatile terpenes and methyl salicylate synthesis, as well as causing rapid increase of endogenous jasmonic acid in lima bean plantlets [44]. In the present study, our results also indicate that direct AlaM effects on membrane permeability in exposed cells may be involved in the production of the response.

Figure 6 Oxidation of respiratory substrates by AlaM-permeabilized BY-2 cells

(a) Succinate oxidation; (b) malate oxidation; and (c) NADH oxidation. Note that 1 mM EGTA was present in the medium which removes Ca^{2+} and inactivates the NADH dehydrogenase on the outer surface of the inner mitochondrial membrane [4,5]. The numbers next to the traces are rates of oxygen consumption [$\text{nmol of O}_2 \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$] and were measured in the high-salt medium with catalase added. Pyr, pyruvate; Succ, succinate; A/A, antimycin A.

Mitochondria inside AlaM-permeabilized cells resume oxygen consumption as substrates and cofactors are added

As shown in Figures 1 and 2, AlaM caused the release of cofactors from the BY-2 cells as well as a complete inhibition of cellular respiration. The inhibition was caused by a permeabilization of the mitochondria and loss of respiratory substrates and cofactors from the matrix, as has previously been shown for isolated plant mitochondria [26]. When substrates and cofactors were added to isolated and AlaM-permeabilized mitochondria, they were able to cross the inner membrane and be metabolized [26].

The activities of soluble enzymes in both the cytoplasm and the mitochondrial matrix of AlaM-permeabilized cells could be determined provided that substrates and cofactors were present (Figure 3). To test whether oxygen consumption was also resumed, respiratory substrates and cofactors were added to cells permeabilized with $22\text{ }\mu\text{g} \cdot \text{ml}^{-1}$ AlaM in the presence of catalase (Figure 6). The addition of AlaM led to the same slow inhibition previously seen. However, after the addition of succinate and ATP, a high rate of oxygen consumption was observed (Figure 6a). The activity was substantially stimulated by ATP, also in the presence of $10\text{ }\mu\text{M}$ Ap_5A (P^1, P^5 -diadenosine 5'-pentaphosphate) (results not shown), which inhibits the adenylate kinase that otherwise may produce ADP [45]. ATP directly stimulates the

succinate dehydrogenase in mitochondria isolated from several plant sources, probably by interacting at the cytoplasmic side of the mitochondrial inner membrane [46].

AlaM-permeabilized cells only oxidized added malate slowly even in the presence of CoA and glutamate (results not shown). However, after the addition of NAD^+ , oxygen consumption was resumed to a rate similar to or higher than cellular respiration in unpermeabilized cells (Figure 6b). The malate oxidation was stimulated by glutamate (Figure 6b), whereas CoA had no effect (results not shown). After AlaM permeabilization, direct oxidation of NADH could also be measured, with rates generally higher than malate oxidation (Figure 6c). Oxidation of both malate and NADH was partially inhibited by rotenone, a specific inhibitor of the matrix-facing proton-pumping NADH dehydrogenase (complex I). This result further verifies the intracellular permeabilization of the inner mitochondrial membrane to NADH. It also indicates that both complex I and the internal rotenone-insensitive, Ca^{2+} -independent, low-affinity NADH dehydrogenase [47–49] were active under these conditions. The higher activity seen with NADH than with malate was due to a higher activity by the rotenone-insensitive enzyme in the former case (272 in Figure 6c versus 192 in Figure 6b), i.e. malate oxidation at pH 7.8 does not fully engage the rotenone-insensitive enzyme (Figure 6). The mitochondrial Ca^{2+} -dependent external NAD(P)H dehydrogenases [4,5] should be completely inactive under these conditions. The high-salt medium used contained 1 mM EGTA, which should be transported through the AlaM pore, chelating Ca^{2+} in the cytosol. Furthermore, AlaM strongly inhibits the external NADH dehydrogenase in isolated potato mitochondria [26] and this may also take place in an AlaM-permeabilized cell.

Oxidation of all substrates was efficiently inhibited by KCN, antimycin A and n-PG (Figure 6), which shows that both the cytochrome and the alternative pathway of the respiratory chain were active and that extramitochondrial oxygen consumption was low. The substrate oxidation described above was first measured in the low-salt medium with similar results, but with generally lower activities of oxygen consumption (results not shown). By changing the medium to high-salt medium, which has ion concentrations and a pH value more similar to intracellular conditions, higher activities were reached. When NADH oxidation was measured at different AlaM concentrations but otherwise as in Figure 6(c), the rate was approx. 30% higher at 22 and $32 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM than at 16 and 44, with similar rotenone sensitivity, 28–38%, at all concentrations (Figure 7). The decrease at $44 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM is consistent with the partial inhibition of complexes III and IV of the respiratory chain in isolated potato mitochondria at high AlaM concentrations and a similar inhibition was also observed with pea leaf mitochondria [26]. The maximum NADH oxidation at $22 \mu\text{g} \cdot \text{ml}^{-1}$ AlaM is similar to the maximum flux of NADH across the mitochondrial inner membrane as estimated by the NAD-MDH activity measured at the same AlaM concentration (2 mol of NADH/mol of oxygen; Figure 3). However, it is difficult to compare a reaction with one substrate to a reaction with two substrates with regard to AlaM channel flux capacity.

Determination of respiratory capacities in permeabilized cells

In cells, the capacities of cytochrome and alternative pathways are normally determined by consecutive additions of KCN and n-PG directly to normally respiring cells [50]. However, in many cases, the inhibitor first added does not decrease the rate, and close to complete inhibition is seen after the addition of the second inhibitor. This was the case for the cells analysed at the end of the exponential growth phase (Table 1), whereas cells in mid-exponential phase (Figure 6) show higher cell respiration that is

Figure 7 NADH oxidation by BY-2 cells at different AlaM concentrations in the assay

The cells were incubated for 10 min with the concentrations of AlaM indicated at which point the background rate of oxidation was below $50 \text{ nmol of O}_2 \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$. Then 1 mM NADH was added and, after a linear rate was achieved, consecutive additions of rotenone, KCN and n-PG were made. The averages between two separate cultures are shown with error bars denoting S.D.

Table 1 Cytochrome and alternative pathway capacities of control and AlaM-permeabilized BY-2 cells with NADH as substrate

Specific activities [$\text{nmol of O}_2 \cdot \text{min}^{-1} \cdot (\text{g} \cdot \text{FW})^{-1}$] were measured in the high-salt medium using cells in late exponential phase. Experiments were started with cell addition and followed by consecutive additions of the indicated compounds. Δ denotes the absolute change imposed by the addition, in $\text{nmol of O}_2 \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$. The rate + AlaM was determined 7 min after its addition. The values representing capacities of the cytochrome pathway (CP) and the alternative pathway (AP), as determined in intact cells and by the new method, are underlined. DTT was added during the experiments shown in the column to the right. Abbreviations are as in Figure 6.

	Control		+ DTT	
	Specific activity	Δ	Specific activity	Δ
Cells	137 ± 6			
+ KCN	137 ± 6	0		
+ n-PG	19 ± 3	<u>118 (AP)</u>		
Cells	134 ± 3			
+ n-PG	134 ± 3	0		
+ KCN	19 ± 5	<u>115 (CP)</u>		
Cells	133 ± 3		143 ± 6	
+ AlaM	15 ± 6		19 ± 4	
+ Pyr, ATP, NADH	279 ± 6		346 ± 13	
+ DTT	–		426 ± 16	
+ KCN	151 ± 1	128	186 ± 3	81
+ n-PG	35 ± 3	116	21 ± 5	<u>164 (AP)</u>
Cells	153 ± 10		130 ± 7	
+ AlaM	13 ± 4		17 ± 5	
+ Pyr, ATP, NADH	342 ± 10		361 ± 23	
+ DTT	–		426 ± 3	65
+ n-PG	303 ± 3	39	257 ± 16	169
+ KCN	19 ± 8	283	0	<u>257 (CP)</u>

affected already by the first inhibitor added (results not shown). If the inhibitor first added has no effect, it is actually the ubiquinone-reducing capacity or substrate supply in the mitochondria *in situ* that is measured rather than the ubiquinol-oxidizing capacities, which will be underestimated. Seeing that very high oxygen consumption could be measured in permeabilized cells after the addition of substrates, we investigated whether this might be used

Table 2 Capacities of cytochrome and alternative pathways during simultaneous NADH and succinate oxidation in AlaM-permeabilized BY-2 cells

Experimental details are same as in Table 1.

	Control		+ DTT	
	Specific activity	Δ	Specific activity	Δ
Cells	140 $\pm$ 3			
+ KCN	140 $\pm$ 3	0		
+ n-PG	19 $\pm$ 2	121 (AP)		
Cells	144 $\pm$ 5			
+ n-PG	144 $\pm$ 5	0		
+ KCN	20 $\pm$ 2	123 (CP)		
Cells	150 $\pm$ 6		143 $\pm$ 8	
+ AlaM	16 $\pm$ 7		11 $\pm$ 5	
+ Pyr, ATP, NADH, Succ	397 $\pm$ 19		410 $\pm$ 16	
+ DTT	–		495 $\pm$ 13	85
+ KCN	208 $\pm$ 11	189	293 $\pm$ 16	202
+ n-PG	30 $\pm$ 6	178	52 $\pm$ 8	241 (AP)
Cells	153 $\pm$ 3		145 $\pm$ 6	
+ AlaM	12 $\pm$ 6		18 $\pm$ 8	
+ Pyr, ATP, NADH, Succ	410 $\pm$ 26		397 $\pm$ 21	
+ DTT	–		475 $\pm$ 19	78
+ n-PG	260 $\pm$ 17	150	285 $\pm$ 23	190
+ KCN	26 $\pm$ 5	234	54 $\pm$ 7	232 (CP)

as a general method to determine respiratory chain (quinol-oxidation) capacities.

Cells were permeabilized with 22 $\mu\text{g} \cdot \text{ml}^{-1}$ AlaM, leading to virtual abolishment of oxygen consumption. After the addition of pyruvate, ATP and NADH, a rate was observed that was twice that of the control respiration in unpermeabilized cells (Table 1). The oxygen consumption in permeabilized cells oxidizing NADH was further increased by the addition of DTT, indicating that DTT activated the alternative oxidase under the conditions used. Stepwise additions of KCN and n-PG allowed the capacities of the cytochrome and alternative pathways to be determined. Under these conditions, the cytochrome pathway capacity [257 $\text{nmol} \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$] was more than twice the corresponding value as determined in unperturbed cells [116 $\text{nmol} \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$]. Also, the alternative pathway capacity was substantially higher in AlaM-permeabilized cells than in control cells [164 versus 118 $\text{nmol} \cdot \text{min}^{-1} \cdot (\text{g FW})^{-1}$ respectively]. Thus the capacities were severely underestimated when measurements were made in unperturbed cells.

In AlaM-permeabilized cells oxidizing NADH in the presence of DTT, both KCN and n-PG inhibited to the same extent, irrespective of whether the other inhibitor had previously been added, i.e. the rate after addition of NADH was equal to or greater than the sum of the quinol-oxidation capacities (Table 1). This indicates that the NADH dehydrogenases were able to saturate the ubiquinol-oxidizing pathways. However, such an additivity of the ubiquinol-oxidation pathways was not seen in all cultures. In Table 2, a separate experiment using both NADH and succinate as substrates is shown. Similar effects of AlaM, substrates, DTT, KCN and n-PG were observed, but the capacity of the alternative pathway was higher. In this experiment, the effect of KCN was higher in the presence of n-PG and vice versa (Table 2). This indicates that here, NADH and succinate together did not fully saturate the ubiquinol-oxidizing pathways. The residual rate after the addition of both KCN and n-PG was normally below 10 % of the total rates and not substantially increased in AlaM-permeabilized cells compared with intact cells (Tables 1 and 2), indicating that, in the presence of catalase, oxidation of NADH and succinate outside the mitochondrion is low. Oxidation of

succinate as the only substrate showed similar trends to NADH and NADH + succinate, but with a lower absolute rate (results not shown).

Using AlaM to permeabilize the PM and mitochondria, it is thus possible to assay for succinate oxidation, rotenone-sensitive and -insensitive NADH oxidation and the cytochrome and alternative pathway *in situ*. On AlaM permeabilization of potato mitochondria, partial inhibitory effects were seen at complexes III and IV of the electron-transport chain [26]. However, the inhibition could be reversed by using high protein concentrations in the assay. It is difficult to estimate to what extent the cytochrome pathway may be inhibited on intracellular permeabilization with AlaM, since the AlaM concentration and AlaM/protein ratio inside the cell are not known. However, considering the high rates determined as compared with the rate in intact cells (Tables 1 and 2), an inhibitory effect of 22 $\mu\text{g} \cdot \text{ml}^{-1}$ AlaM would have to be relatively small.

In late exponential phase, the sum of the capacities for quinol oxidation is more than double the respiration rate of the intact cells, indicating that the enzymes are present in excess and that the respiratory chain is working with an inadequate substrate supply and/or under conditions close to state 4. This is consistent with the suggestion that the alternative oxidase is mainly independent of enzyme concentration but instead kinetically controlled [51].

In the present study, the alternative oxidase activity was found to be stimulated by DTT (Tables 1 and 2), which activates by reducing the disulphide bridge that links the oxidase dimer in its oxidized form [52]. It has been argued that the enzyme is always fully reduced *in vivo* and that the thiol groups have no role in the *in vivo* regulation of the activity [51]. Our results indicate that oxidized alternative oxidase molecules are present in the AlaM-permeabilized cells (Tables 1 and 2), consistent with its *in vivo* presence in tobacco cells [53]. This makes DTT a necessary constituent of assays where the maximal alternative oxidase activity is to be measured.

Conclusions and perspectives

We here report that AlaM permeabilizes the PM and inner mitochondrial membrane, while leaving the tonoplast unaffected. This allows low-flux access to the cytoplasm and matrix for small molecules without killing or seriously disturbing the growth of the cells. To measure faster processes demanding a high flux of metabolites through the membranes, concentrations of AlaM that hamper cell viability and growth must be used. The results also show that the respiratory enzymes of the tricarboxylic acid cycle as well as the respiratory chain can be monitored inside AlaM-permeabilized mitochondria in permeabilized cells. Thus AlaM permeabilization provides a tool for determining the minimal cofactor requirements and capacities of substrate oxidation of both cytosolic and mitochondrial processes inside otherwise intact cells. The utility of AlaM is also illustrated by the observations that inactivation of enzymes like PEPC during extraction is avoided and that measurement of NAD-GAPDH is probably made under conditions retaining the restricting effect of cytoplasmic integrity (Figure 3), an observation that requires further investigation. Although AlaM may not be the method of choice to investigate the nature of this restriction, the observation points to the potential use of AlaM for finding discrepancies in the present understanding of cell enzymology.

It must be stressed that the BY-2 cells used here grow in strings (Figure 4), where AlaM in the medium has direct access to all cells. AlaM permeabilization is probably less suitable for cells growing in clumps, because the access of AlaM to deeper cell layers may be restricted. For use with other cell lines, the concentration dependence of AlaM permeabilization should be optimized

in each case. Since AlaM efficiency is influenced by the amount of biological material used, e.g. mitochondria [26], concentration dependence for AlaM must be determined at the cell concentration used.

As demonstrated for the electron-transport chain, AlaM permeabilization allows the determination of the catalytic capacity of complex multi-component enzymatic processes. Of crucial importance when using such a tool is that the assay for a certain enzyme or pathway is very specific. Enzyme activity assays involving coupling substrates in more than one step to, e.g. NADH production, as in ATPase assays [54], may work very well in isolated membrane fractions. However, in the complex system of the whole cell, assay conditions may artificially connect processes that normally are separate. Therefore, to avoid artifacts, it is important to include controls to check the dependence on all cofactors and substrates and/or sensitivity to specific inhibitors. Specifically to NADH-coupled activities, the residual NADH oxidation in the presence of KCN and n-PG may remove the NADH produced by an enzyme present in smaller amounts. Finally, it is important that assays have a relatively short duration. Permeabilization is expected to turn off gene expression but protein degradation may affect enzymatic capacities, especially if ATP is added, allowing proteasome-mediated degradation. Nevertheless, the method allows direct determination of enzyme and pathway activities in cells in the presence of defined cofactor concentrations, and assaying of the whole organelle or enzyme population of an organelle or enzyme and not only the part that can be purified. Thus the AlaM method may bridge the gap between analysis of isolated cell constituents and analysis of unperturbed cells and tissues by non-invasive techniques, as well as allowing the analysis of organelles that are difficult to isolate.

The survival of the cell after permeabilization of the PM indicates that the AlaM method also can be used for loading molecules into the cytoplasm of cells and for analysing their long-term effect on cell function. AlaM allows efficient loading of ions [23]. Although transport of folded proteins has not been observed [24–26], peptides up to 83 residues long have been loaded into yeast mitochondria through the AlaM pore [40]. This indicates that larger molecules may also be loaded into the living cell, for example when studying peptides interacting with cellular processes or regulatory proteins. This is a topic for further research.

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