Phosphate is required to maintain the outer membrane integrity and membrane potential of respiring yeast mitochondria

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Abstract

In this study, the buffer requirements to maintain mitochondrial intactness and membrane potential in \textit{in vitro} studies were investigated, using gradient purified yeast mitochondria. It was found that the presence of phosphate is crucial for the generation of a stable membrane potential and for preserving the intactness of the outer membrane, as assessed by probing the accessibility of Tom40p to trypsin and the leakage of cytochrome \textit{b2} from the intermembrane space. Upon addition of respiratory substrate in the absence of phosphate, mitochondria generate a membrane potential that collapses within one minute. Under the same conditions, the mitochondrial outer membrane is disrupted. The presence of phosphate prevents both these phenomena. The $\Delta$pH component of the proton motive force appears to be responsible for the compromised outer membrane integrity. The collapse of the membrane potential is reversible to a limited extent. Only when phosphate is added soon enough after the addition of exogenous respiratory substrate can a stable membrane potential be obtained again. Within a few minutes, this capacity is lost. The presence of Mg$^{2+}$ prevents the rupture of the outer membrane, but does not prevent the rapid dissipation of the membrane potential. Similar results were obtained for mitochondria isolated and stored in the presence of dextran or BSA.

Introduction

For \textit{in vitro} studies using isolated mitochondria, it is important to know how the integrity, bioenergetics, and stability of the organelle are affected by the experimental conditions. In particular when studying the biogenesis of mitochondria, conservation of the membrane barrier properties of the mitochondrial membranes is a prerequisite for interpretation of the data.

Numerous studies have appeared on the bioenergetics of yeast mitochondria and the effects of energization on ultrastructural organization and inner membrane permeability. It has been reported that phosphate protects mitochondrial ultrastructure [Velours, Rigoulet and Guérin, 1977] and counteracts the yeast mitochondria permeability channel by inhibiting changes in inner membrane permeability under conditions of energization (for a review, see [Manon \textit{et al.}, 1998]). A role in the regulation of inner membrane permeability was also attributed to magnesium [Guérin \textit{et al.}, 1994; Jung, Bradshaw and Pfeiffer, 1997]. However, the implications of these findings for \textit{in vitro} studies on mitochondrial biogenesis are not generally appreciated. Although many investigators studying mitochondria \textit{in vitro} routinely add phosphate and magnesium to their buffers [Glick, 1995; Yaffe, 1991; Wagner \textit{et al.}, 1994], it is certainly not common practice (e.g. in [Swanson and Roise, 1992; Simbeni \textit{et al.}, 1993; Söllner, Rassow and Pfanner, 1991; Geissler \textit{et al.}, 2000], either both or one of these components were omitted).
There are no reports to date of a systematic analysis of the requirements for conserving outer membrane integrity in combination with a stable membrane potential in yeast mitochondria in response to energization. The aim of the present study is to define the experimental conditions required to maintain mitochondrial intactness and a stable membrane potential upon energization in \textit{in vitro} experiments using isolated yeast mitochondria. The effects of phosphate and magnesium were studied. The integrity of the outer membrane was monitored by assessing the permeability of this membrane to proteins of different sizes, \textit{i.e.} by the accessibility of Tom40p to trypsin (23 kDa) and the leakage of cytochrome \textit{b}2 (57 kDa) from the intermembrane space. In parallel, the membrane potential was monitored using the membrane potential sensitive fluorescent probe 3,3’-diethylthiadicarbocyanine iodide (diSC\textsubscript{2}(5)). It was found that phosphate is required to maintain the outer membrane integrity and membrane potential of respiring yeast mitochondria.

\textbf{Materials and methods}

\textit{Materials}

ECL reagents were purchased from DuPont NEN. Yeast extract and nycodenz were from Sigma, as was BSA (fatty acid content 0.001%). Zymolyase was obtained from Seikagaku (Japan). Dextran (M\textsubscript{r} 40,000) was supplied by Roth (Karlsruhe, FRG). DiSC\textsubscript{2}(5) was from Molecular Probes (Leiden, NL). All other chemicals were analytical grade.

\textit{Isolation of mitochondria}

The wild-type yeast (\textit{Saccharomyces cerevisiae}) strain D273-10B was grown aerobically to late log (OD\textsubscript{600} 4-5 (Perkin Elmer Lambda 18 UV/VIS spectrophotometer)) at 30°C in semi-synthetic lactate medium [Daum, Böhni and Schatz, 1982]. Spheroplasts were prepared using zymolyase as described previously [Daum, Böhni and Schatz, 1982]. The isolation and purification of mitochondria were based on published procedures [Daum, Böhni and Schatz, 1982; Gaigg \textit{et al.}, 1995; Glick and Pon, 1995]. The cellular homogenate was prepared using a buffer containing 10 mM MES, pH 6.0, and 0.6 M sorbitol (D buffer), to which 1 mM PMSF was added, and which was supplemented with 0.2\% (w/v) BSA or with 0.5\% (w/v) dextran instead. Crude mitochondria were isolated using D buffer and were further purified by nycodenz gradient centrifugation as reported [Glick and Pon, 1995], with some minor changes. The nycodenz gradient was made in D buffer and consisted of a layer of 18\% nycodenz overlaid with a 13.5\% instead of a 14.5\% (w/v) nycodenz layer. The final mitochondrial pellet was resuspended in 20 mM Hepes/KOH, pH 7.4 containing 0.6 M sorbitol (H/S buffer) including either 0.5\% (w/v) BSA [Kozlowski and Zagorski, 1988] or 5\% (w/v) dextran. Aliquots were snap-frozen with liquid nitrogen, stored at -80°C, and thawed only once before use. The concentration of mitochondria used in experiments is
always expressed as the protein concentration determined by using the BCA method (Pierce) with 0.1% (w/v) SDS added and BSA as a standard. H/S buffer was used in all experiments unless stated otherwise, without further addition of BSA or dextran.

Assessment of effects of incubation conditions on mitochondrial intactness

Mitochondria were thawed on ice before use. All samples were kept on ice unless stated otherwise. To investigate the effect of different conditions on the intactness of the mitochondria, 20 µg protein aliquots of the mitochondrial suspension were incubated for 10 min, on ice or at 20°C, in a volume of 100 µl H/S buffer, including additives as indicated. NADH and ethanol were used at concentrations of 2 mM or 1% (v/v) (170 mM), respectively. FCCP and valinomycin were added from stock solutions in ethanol to final concentrations of 20 and 5 µM, respectively (final ethanol concentration 1% (v/v)). Potassium phosphate (KH2PO4) and MgCl2 were added to concentrations of 2 and 10 mM, respectively.

The intactness of the mitochondrial outer membrane after these treatments was assessed by probing the accessibility of Tom40p to trypsin or, alternatively, by measuring the leakage of cytochrome b2 from the intermembrane space. In control experiments, digitonin (0.2% (w/v)) was added to destroy the outer membrane, or mitoplasts were prepared by diluting the mitochondria at least a factor of 10 in hypotonic buffer (20 mM Hepes/KOH, pH 7.4), and incubating for 20 min on ice [Jascur, 1991]. Trypsin was added from a 1 mg/ml stock solution in H/S to the samples to a final concentration of 100 µg/ml and after 20 min on ice the action of trypsin was stopped by TCA precipitation, as described below. For measuring leakage of cytochrome b2, samples were centrifuged for 12 min at 10,000 rpm (10,600 x g) in a microfuge at 4°C. Pellet and supernatant were separated and the pellet was resuspended in H/S buffer. TCA precipitations were performed by adding one volume of 10% (w/v) TCA and incubating on ice for 20 min. After centrifugation for 15 min at 14,000 rpm, the precipitated protein was washed with 50 µl of ice-cold acetone followed by 20 µl of ice-cold water. Protein pellets were dissolved in SDS-PAGE sample buffer, heated at 95°C for 5 min and aliquots were subjected to SDS-PAGE (10% gel) and Western blotting. The blots were decorated with antibodies raised against Tom40p and cytochrome b2. Protein bands were visualized by ECL according to the manufacturer’s instructions.

Measurement of mitochondrial membrane potential and respiration

Mitochondrial membrane potential was monitored by recording the fluorescence of the voltage sensitive dye diSC2(5) [Sims et al., 1974]. Fluorescence was measured on an Aminco SLM500C spectrofluorometer using excitation and emission wavelengths of 650 nm and 670 nm, respectively (bandpass 5 nm for both). The dye (final concentration 0.5 µM) was added from a 0.5 mM stock solution in DMSO to the mitochondria (final protein
Mitochondrial outer membrane integrity

concentration 40 µg/ml) suspended in H/S buffer. Respiratory substrates were added to final concentrations of 0.5 mM and 0.1% (v/v) (17 mM) for NADH and ethanol, respectively. To dissipate the membrane potential, either valinomycin was added from an ethanolic stock to a final concentration of 1 µM, or gramicidin D was added from a 1 mg/ml stock solution in DMSO to a final concentration of 1 µg/ml. Measurements were performed under continuous stirring in a final volume of 2 ml at a temperature of 20°C.

Respiratory activity of isolated mitochondria was monitored with an Oxygraph respirometer (Anton Paar K.G., Graz, Austria). 160 µg of mitochondria (on protein basis) were used in a volume of 2 ml H/S buffer at a temperature of 20°C.

Results

Characterization of the mitochondria

In conventional preparations of mitochondria, BSA is often included in the homogenization buffer, and also added to the final preparation for storage in the freezer [Daum, Böhni and Schatz, 1982] and to experimental samples [Glick, 1995] to protect the mitochondria. In our laboratory, we are investigating the import process of phosphatidylcholine into yeast mitochondria. Since BSA might interfere in in vitro studies on phospholipid transport because of its interaction with lipids, it was replaced with dextran in the isolation procedure. It is expected that dextran offers similar protection of mitochondria by maintaining a more physiological colloid osmotic balance across the outer membrane (based on e.g. [Wrogemann et al., 1985; Wicker et al., 1993]). Mitochondria isolated and stored in the presence of BSA (BSA mitochondria) or dextran (dextran mitochondria) were found to be similar in every respect in the present study, with the exception of the amount of endogenous respiratory substrate, which was consistently lower in dextran mitochondria than in BSA mitochondria, as will be explained later. All the results shown in this paper were obtained with dextran mitochondria, unless indicated otherwise.

The intactness of the isolated mitochondria was assessed by probing the accessibility of Tom40p to trypsin, and the leakage of cytochrome b2 from the intermembrane space. Tom40p cannot be degraded by trypsin when the mitochondrial outer membrane is intact [Hines et al., 1990]. Cytochrome b2, which is a soluble protein located in the intermembrane space [Daum, Böhni and Schatz, 1982], can only leak out when the outer membrane is disrupted. Figure 1 shows that after incubation of mitochondria with trypsin (lane 4), only a small portion of Tom40p is degraded to a lower molecular weight band (designated Tom40p*). In contrast, in mitoplasts (lane 2) and in mitochondria treated with digitonin (lane 3), trypsin completely converted all of the Tom40p to Tom40p* and also digested all of the cytochrome b2. Upon centrifugation of mitochondria, cytochrome b2 is quantitatively retained in the mitochondrial pellet (cf. lanes 7 and 8), while in mitoplasts, cytochrome b2 is
released into the supernatant (cf. lanes 5 and 6). These results indicate that during isolation, a small fraction of the mitochondria is broken, rendering Tom40p accessible to trypsin. However, after storage, no additional membrane rupture occurs, since there is no leakage of intermembrane space proteins into the supernatant. The isolated mitochondria are estimated to be 90-95% intact, similar to what has been reported by others [Kaput et al., 1989].

Figure 1. The intactness of isolated mitochondria. The intactness of the mitochondrial outer membrane was probed by subjecting the mitochondria to trypsin treatment. The degradation of Tom40p (to Tom40p*), and of cytochrome $b_2$ was visualized by Western blotting (left panel). Alternatively, the mitochondria were subjected to centrifugation to examine whether cytochrome $b_2$ is released from the mitochondria. Pellets (P) and supernatants (S) were subjected to Western blotting (right panel). Controls include disruption of the outer membrane by hypotonic shock, or solubilization of the mitochondria by digitonin, prior to trypsin treatment or centrifugation. For more details, see the 'Materials and methods' section.

Effects of energization

We investigated the effects of energization by addition of a respiratory substrate. Figure 2A shows the oxygraph trace of mitochondria in buffer at 20°C, displaying a certain low basal level of respiration, depending on the amount of endogenous substrate, and a rapid increase upon addition of exogenous respiratory substrate. Both ethanol and NADH (not shown) result in increased oxygen consumption. These results indicate that the mitochondria are capable of respiration and can metabolize exogenous substrate. Figure 2B depicts the membrane potential measurement performed under similar conditions (20°C). Addition of the membrane potential sensitive probe to mitochondria leads to a certain fluorescence level. Upon addition of respiratory substrate (ethanol and NADH have a similar effect) the fluorescence decreases, indicating that a membrane potential is generated. However, the fluorescence level returns to the initial value within minutes, demonstrating that the membrane potential is unstable. This was found with ethanol as well as with NADH (not shown). The membrane potential could not be regained by addition of an extra portion of substrate, indicating that depletion of substrate is not the cause of the instability of the membrane potential (not shown).
Figure 2C shows how the mitochondrial intactness is affected by a 10 min incubation under these conditions. The intactness of the outer membrane is no longer maintained, as can be inferred from the complete accessibility of both cytochrome b2 and Tom40p to trypsin (lane 3), as well as from the appearance of cytochrome b2 in the supernatant after centrifugation (cf. lanes 8 and 9). This was found with ethanol as well as with NADH (not shown). However, the intactness of the mitochondria is maintained when the incubation is performed without addition of exogenous substrate (lanes 1, 4 and 5) or with substrate at 0°C (lanes 2, 6 and 7). These results indicate that, in a simple buffer containing Hepes and sorbitol, respiration is responsible for rupture of the outer membrane, and that a stable membrane potential is not obtained under these conditions.
To investigate whether the generation of a proton motive force (pmf) induced by respiration in mitochondria is the cause of the rupture of the outer membrane, the uncoupler FCCP was added. Figure 3A shows that dissipation of the pmf by FCCP prevents the rupture of the outer membrane, since Tom40p and cytochrome b2 are not degraded by trypsin (lane 1), and cytochrome b2 is retained in the mitochondrial pellet upon centrifugation (cf. lanes 3 and 4). This indicates that the (temporary) generation of a pmf leads to the rupture of the outer membrane.

Figure 3. Effects of dissipating the pmf and the membrane potential. A: Western blot analysis of the mitochondrial outer membrane intactness. Mitochondria were incubated for 10 min under the conditions indicated. Ethanol was used as a respiratory substrate. The pmf was dissipated by the addition of FCCP (f), and the membrane potential was dissipated by the addition of valinomycin (v). Subsequently, the intactness of the outer membrane was probed as described in the legend of Figure 1 by trypsin treatment (left panel) or by centrifugation (right panel, P: pellet; S: supernatant); B: membrane potential measurement using the membrane potential sensitive fluorescent dye diSC2(5). At the start of the measurement, diSC2(5) is added to mitochondria in buffer. The arrows indicate the addition of valinomycin and respiratory substrate, and of gramicidin to check the dissipation of the membrane potential.
The pmf has two components, a pH gradient ($\Delta \text{pH}$) and a membrane potential ($\Delta \Psi$) [Rottenberg, 1979]. The membrane potential can be dissipated separately using valinomycin in the presence of $K^+$ ions [Rottenberg, 1989]. Figure 3B shows that, in the presence of valinomycin and ethanol as a respiratory substrate, the mitochondria are completely unable to form even the temporary membrane potential depicted in Figure 2B, indicating that the potassium concentration in the buffer is sufficient for valinomycin to dissipate the membrane potential. Figure 3A shows that under these conditions the rupture of the mitochondrial membrane is not prevented, as judged from the complete accessibility of Tom40p and cytochrome $b_2$ to trypsin (lane 2) and the release of cytochrome $b_2$ into the supernatant after centrifugation (cf. lanes 5 and 6). This strongly suggests that the $\Delta \text{pH}$ component of the pmf is responsible for the damaging effect on the mitochondria.

Buffer requirements for maintenance of mitochondrial integrity and membrane potential

We investigated whether phosphate or magnesium could fulfill the system’s requirements for maintaining outer membrane integrity and a stable membrane potential upon respiration. Figure 4A shows that after respiration in the presence of either phosphate or $Mg^{2+}$, Tom40p is not accessible to trypsin, and that cytochrome $b_2$ is not degraded by trypsin (lanes 1 and 2) and is retained in the pellet upon centrifugation (cf. lanes 3 and 4, and lanes 5 and 6). The membrane potential measurements under both conditions are depicted in Figure 4B. In the presence of phosphate, a stable membrane potential is obtained and maintained for at least an hour if the substrate is replenished (not shown). The membrane potential can be dissipated by addition of either valinomycin or gramicidin. $Mg^{2+}$ (which causes an increase in the initial fluorescence level probably due to decreased association of the probe with the membranes) does not prevent the collapse of the membrane potential shortly after the addition of exogenous respiratory substrate. These data indicate that phosphate and $Mg^{2+}$ are both able to prevent the rupture of the outer membrane, while phosphate, but not $Mg^{2+}$, is required for maintenance of a stable membrane potential.

Effect of late addition of phosphate

We investigated whether addition of phosphate following the addition of respiratory substrate could result in recovery of the membrane potential. Figure 5 depicts the fluorescence measurements in which phosphate was added at the indicated time points before and after the start of respiration. Addition just prior to the addition of ethanol results in a stable potential (A), as when phosphate is included in the buffer from the start of the experiment (cf. Figure 4B). When BSA mitochondria were used, addition of phosphate always resulted in a small decrease in fluorescence (data not shown), most likely due to the presence of some endogenous respiratory substrate which seemed to be absent from dextran.
Figure 4. Effects of phosphate and magnesium. A: Western blot analysis of the mitochondrial outer membrane intactness. Mitochondria were incubated for 10 min under the conditions indicated in the presence of phosphate ($P_i$) or magnesium ($Mg^{2+}$). Ethanol was used as a respiratory substrate. Subsequently, the intactness of the outer membrane was probed as described in the legend of Figure 1 by trypsin treatment (left panel), or by centrifugation (right panel, P: pellet; S: supernatant); B: membrane potential measurement using the membrane potential sensitive fluorescent dye diSC$_2$(5). At the start of the measurements, diSC$_2$(5) is added to mitochondria in buffer in the presence of phosphate ($P_i$) or magnesium ($Mg^{2+}$). The arrows indicate the addition of respiratory substrate, and of gramicidin to dissipate the membrane potential. The fluorimeter settings were adjusted for the measurement in the presence of $Mg^{2+}$ to be able to measure the higher level of fluorescence.

When phosphate is added 30 s (B) or 60 s (C) after the addition of respiratory substrate, the fluorescence decreases again to the level obtained when phosphate was present before addition of respiratory substrate (A), indicating that the membrane potential can be recovered. However, when phosphate is added 2 min after the addition of respiratory substrate, only a small decrease in fluorescence is observed (D), and after 5 (E) or 10 min (F) no decrease in the fluorescence is observed, indicating that the membrane potential is no longer recovered. The rate at which the mitochondria undergo the transient formation of the unstable membrane potential and its subsequent dissipation varies slightly from experiment to experiment (as can be seen from Figure 5) and also between different batches of
mitochondria (not shown). Therefore, there is probably also variation in the time allowed for recovery of the membrane potential by addition of phosphate.

Since we found that magnesium protects the mitochondria from outer membrane rupture, we also tested the reversibility of the membrane potential dissipation in the presence of magnesium. The addition of phosphate after 10 min was unable to recover the membrane potential in the presence of Mg\(^{2+}\), while in the presence of Mg\(^{2+}\) addition of phosphate 1 min after addition of respiratory substrate resulted in recovery of the membrane potential as it did in the absence of Mg\(^{2+}\) (not shown). Therefore, it can be concluded that the addition of phosphate within a short time period after initiation of respiration is important for the recovery of the membrane potential, while Mg\(^{2+}\) plays no role in this recovery.

Figure 5. Effect of late addition of phosphate. The membrane potential was measured using the membrane potential sensitive fluorescent dye diSC\(_2\)(5). At the start of the measurements, diSC\(_2\)(5) is added to mitochondria in buffer. The arrows indicate the addition of phosphate (P\(_i\)), ethanol (e) and gramicidin D (g). Phosphate was added before (A), or 30 s (B), 60 s (C), 2 min (D), 5 min (E), or 10 min (F) after the addition of respiratory substrate.
Discussion

The aim of the present study is to define the conditions required to maintain mitochondrial intactness and sustain a stable membrane potential upon energization in in vitro experiments using isolated yeast mitochondria. The outer membrane integrity was monitored by assessing the permeability of this membrane to proteins, i.e. by the accessibility of Tom40p to trypsin and by the leakage of cytochrome b2 from the intermembrane space. The membrane potential was monitored using the membrane potential sensitive fluorescent probe diSC2(5).

It was demonstrated that the presence of phosphate is essential for keeping the mitochondrial outer membrane intact and for the generation of a stable membrane potential upon respiration in vitro. In the absence of phosphate only a short-lived membrane potential is generated, and under these conditions, disruption of the mitochondrial outer membrane occurs. The ΔpH component of the pmf appears to be responsible for the compromised integrity of the outer membrane, since uncoupling by FCCP inhibited the rupture of the outer membrane, while dissipation of the ΔΨ component by valinomycin did not. This supports the notion that the inhibitory action of phosphate can be explained by matrix acidification [Velours, Rigoulet and Guérin, 1977; Manon et al., 1998]. It has been reported before that extensive swelling of mitochondria occurs upon energization in the absence of phosphate, due to changes in the permeability of the inner membrane [Velours, Rigoulet and Guérin, 1977]. In the present study, it is directly shown that the integrity of the outer membrane is compromised under these conditions in such a way that it no longer functions as a barrier for proteins up to 57 kDa. This suggests strongly that swelling has progressed beyond the physical limit where the outer membrane can still accommodate the matrix volume.

Magnesium alone is also able to prevent the rupture of the outer membrane but does not prevent the collapse of the membrane potential, indicating that the collapse of the membrane potential and the rupture of the outer membrane are related but distinct phenomena. In the absence of phosphate, irreversible changes in the permeability of the inner membrane and/or in the systems generating the membrane potential have occurred after some time. The changes in permeability cause swelling beyond the physical limit inducing rupture of the outer membrane in the absence of magnesium. The presence of magnesium may prevent excessive swelling, as judged from the maintenance of the intactness of the outer membrane under this condition. However, Mg2+ is unable to prevent irreversible dissipation of the membrane potential. Alternatively, the presence of Mg2+ may delay the mitochondrial swelling and concomitant rupture of the outer membrane in such a way that rupture is not detected after 10 min.

A stable membrane potential could only be regained when phosphate was added shortly after the collapse of the membrane potential. The reason why a time limit to the
recovery went unnoticed in a previous study is probably that in those experiments, phosphate was added within 30 s after the addition of ethanol [Castrejón et al., 1997].

The intent of the present study is to provide clarity regarding the experimental conditions required for in vitro experiments with yeast mitochondria. For a more detailed description of the underlying mechanisms of changes in permeability upon respiration, see [Manon et al., 1998]. It can be concluded that phosphate is required to maintain the integrity of the outer membrane of respiring yeast mitochondria, and also for the generation of a stable membrane potential. This knowledge is relevant to a wide variety of in vitro studies on mitochondria, e.g. related to bioenergetics, the yeast mitochondria permeability channel, and protein or lipid import.

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