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Characterization of the Respiration-Induced Yeast Mitochondrial Permeability Transition Pore

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Abstract

When isolated mitochondria from the yeast *Saccharomyces cerevisiae* oxidize respiratory substrates in the absence of phosphate and ADP, the yeast mitochondrial unselective channel, also called the yeast permeability transition pore (yPTP), opens in the inner membrane dissipating the electrochemical gradient. ATP also induces yPTP opening. yPTP opening allows mannitol transport into isolated mitochondria of laboratory yeast strains, but mannitol is not readily permeable through the yPTP in an industrial yeast strain, Yeast Foam. The presence of oligomycin, an inhibitor of ATP synthase, allowed for respiration-induced mannitol permeability in mitochondria from this strain. Potassium (K⁺) had varied effects on the respiration-induced yPTP depending on the concentration of the respiratory substrate added. At low respiratory substrate concentrations K⁺ inhibited respiration-induced yPTP opening, while at high substrate concentrations this effect diminished. However, at the high respiratory substrate concentrations, the presence of K⁺ partially prevented phosphate inhibition of yPTP opening. Phosphate was found to inhibit respiration-induced yPTP opening by binding a site on the matrix space side of the inner membrane in addition to its known inhibitory effect of donating protons to the matrix space to prevent the pH change necessary for yPTP opening. The respiration-induced yPTP was also inhibited by NAD, Mg²⁺, NH₄⁺, or the oxyanion vanadate polymerized to decavanadate. The results demonstrate similar effectors of the respiration-induced yPTP as those previously described for the ATP-induced yPTP and reconcile previous strain-dependent differences in yPTP solute selectivity.

Keywords

Saccharomyces

Introduction

The chemiosmotic theory states that a driving force for ATP synthesis is the maintenance of an electrochemical gradient across a membrane (Mitchell and Moyle, 1967). Mitochondria produce such a gradient across the inner mitochondrial membrane by pumping protons out of the matrix space. However, the electrical gradient formed also creates a driving force for the movement of other cations into the matrix space that could dissipate the electrochemical gradient or osmotically alter the size of the mitochondrion. Mitochondria contain many regulated transporters to maintain proper volume control, dissipate unwanted gradients, and

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dissipate cation concentrations that build up in the negatively charged matrix space (Chateaubodeau, et al., 1976). Mitochondrial K^+/H^+ , Na^+/H^+ , and Ca^{2+}/H^+ exchangers utilize the proton motive force to accomplish this task. K^+ is the primary cation responsible for organelle volume homeostasis.

Several mitochondrial cation transporters have been molecularly identified in yeast through intense efforts from Rudolf Schweyen's laboratory. These include Mg^{2+} transporters, Mrs2 (Bui, et al., 1999; Kolisek, et al., 2003) and Lpe10 (Gegan, et al., 2001); a K^+/H^+ exchange protein, Mdm38/Letm1 (Froschauer, et al., 2005; Nowikovsky, et al., 2004); and Fe^{2+} transporters, Mrs3 and Mrs4 (Froschauer, et al., 2009). Ca^{2+}/H^+ exchange activities have also been characterized in yeast mitochondria (Bradshaw, et al., 2001; Bradshaw and Pfeiffer, 2006b). A mitochondrial Ca^{2+}/H^+ exchange activity may be mediated by the K^+/H^+ exchange protein, Mdm38/Letm1 (Jiang, et al., 2009). Published electrophysiological studies of the yeast inner mitochondrial membrane ion currents have yielded varied results (Ballarin and Sorgato, 1995; Lohret and Kinnally, 1995). Much of what is known about yeast mitochondrial ion transporters comes from the pioneering research of Stephen Manon, Martine Guerin, and colleagues. These authors were the first to characterize mitochondrial K^+/H^+ exchange activity (Manon and Guerin, 1992). They also identified and characterized K^+ uniport activities in yeast mitochondria (Manon and Guerin, 1993). Since K^+ uniport in both yeast and mammalian mitochondria is inhibited by quinine, Mg^{2+} , and mersalyl, the process may have been conserved throughout evolution (Castrejon, et al., 1997; Castrejon, et al., 2002). In the presence of a physiological P_i concentration, the addition of K^+ to respiring yeast mitochondria causes a protonophoric effect resulting from K^+ entering mitochondria electrophoretically and exiting by the K^+/H^+ exchange (Castrejon, et al., 1997; Manon, et al., 1995). This process lowers the electrochemical gradient ($\Delta\Psi$) and increases the respiratory rate (Manon and Guerin, 1997).

Yeast mitochondria contain many ATP induced permeabilities, including a K^+ transport pathway (Roucou, et al., 1995), a H^+ transport pathway (Prieto, et al., 1992), and the yPTP (Jung, et al., 1997), also call the yeast mitochondrial unselective channel (YMUC) (Guerin, et al., 1994), an unspecific channel activated by respiration or cytosolic ATP in the absence of phosphate (P_i) or ADP. All these transport activities are likely mediated by the same molecular entity, the yPTP. ATP-induced opening of the yPTP in mitochondria isolated from an industrial yeast strain called Yeast Foam allowed for transport of gluconate, glutamate, chloride, and acetate, but not mannitol into the matrix space (Guerin, et al., 1994). In contrast, when characterizing mitochondrial transport properties from laboratory yeast strains, mannitol was found to be permeable through the yPTP (Jung, et al., 1997). ATP also stimulated respiration in mitochondria from laboratory strains in the absence of K^+ (Prieto, et al., 1992), but this did not occur in mitochondria from the Yeast Foam strain (Roucou, et al., 1995).

In the absence of P_i (or ADP) the addition of a respiratory substrate such as ethanol to mitochondria opens the yPTP (Jung, et al., 1997). This was first identified as irreversible structural damage of mitochondria (Velours, et al., 1977). The opening of the yPTP is mediated by a rise in the matrix space pH, because the addition of P_i or uncoupler prevents channel opening. In the Yeast Foam strain, in the presence of 10 mM KCl, the addition of either valinomycin or oligomycin increased the rate of yPTP opening, most likely by increasing the matrix space pH. The addition of NH_4^+ or weak acids such as P_i , arsenate, propionate, or acetate that donate protons to the matrix space also inhibited yPTP opening (Velours, et al., 1977).

Due to the incomplete understanding of the respiration-induced yPTP, we investigated its regulation. We also sought to further characterize the solute selectivity and inhibitor

sensitivity of the yPTP in the W303-1A and Yeast Foam strains previously studied. By using different experimental conditions, we sought to better understand the regulation of the yPTP in the different yeast strains to better resolve previous condition or strain-specific results. Lastly, we discuss the implications of these results in regards to data indicating that dimers of mitochondrial ATP synthase constitute the mitochondrial permeability transition pore in mammals (Giorgio, et al., 2013).

Materials and Methods

Reagents

All reagents, except when otherwise noted, were from Sigma Chemical Company (St. Louis, MO, USA) and were of reagent grade. Decavanadate was made by lowering the pH of a stock solution of 0.1 M Na⁺ orthovanadate to pH 6.0 which gives an orange color and then further diluted (Roucou, et al., 1997a).

Yeast culture

The yeast strains Yeast Foam and W303-1A were grown aerobically in a media containing 2% lactate, 1% yeast extract, 2% peptone, 0.05% dextrose, and 0.01% adenine at pH 5.0. 50 ml of media in a 250 mL flask was inoculated with a colony from a YPD plate and incubated for 48 hours in an orbital shaker (180 rpm) at 30°C. The culture was emptied into 450 mL of culture media in a 2 L baffled flask and shaken for another 24 hours until A₆₀₀= 1.8 - 2.0 and a 15-20 gram pellet of yeast was obtained.

Mitochondrial isolation

Mitochondria were isolated from spheroplasts, created by a 90 minute treatment with 40 mg Zymolyase 20T (MP Biomedicals), by differential centrifugation following 15-20 strokes with a tight-fitting pestle of a Dounce homogenizer as previously described (Daum, et al., 1982; Jung, et al., 1997), except 0.6 M sucrose was used in the homogenization buffer instead of 0.6 M mannitol. The isolated yeast mitochondria were suspended in 0.6 M mannitol 20 mM HEPES (K⁺), and 0.1 mM EGTA, pH 6.8 at 4°C. Protein concentration was determined by the A₂₈₀ of SDS solubilized mitochondria.

Mitochondrial swelling, oxygen consumption, and membrane potential assays

Unless otherwise indicated in the figure legends, mitochondria were suspended in our standard assay medium containing 0.6 M mannitol, 10 mM HEPES tetraethylammonium⁺ (TEA⁺), pH 7.20 for analysis. Mitochondria were suspended at 1 mg protein/ml in a 1 mL cuvette at 22°C in an SLM-Aminco DW-2C spectrophotometer in split beam mode. Solute permeability was monitored by following light scattering at A₅₄₀, which is inversely proportional to mitochondrial swelling (Tedeschi and Harris, 1958). Oxygen uptake was measured using a Clark oxygen electrode (YSI Incorporated, Yellow Springs, OH USA) and a chart recorder with mitochondria suspended at 1 mg protein/ml at 25°C. 474 ng O/ml was taken as 100 % oxygen saturation at 25°C (Lessler and Brierley 1969). Mitochondrial membrane potential changes were followed with 12 μM safranin O (ΔA₅₁₁₋₅₃₃) using an SLM-Aminco DW-2C spectrophotometer in dual beam mode at 22°C with mitochondria suspended at a concentration of 1 mg/ml as previously described (Bradshaw, et al., 2001). In most assays mitochondria were suspended in 0.6 osM media, which is physiological given the 300 mM K⁺ concentration found intracellularly in yeast (Olz, et al., 1993; Sunder, et al., 1996), except where 0.4 osM media was used, as was first used to monitor yPTP (Guerin, et al., 1994).

Results

Oligomycin stimulates respiration-induced mitochondrial swelling in Yeast Foam

Mitochondria isolated from many laboratory strains of yeast undergo large amplitude swelling when they are suspended in a mannitol medium in the presence of ethanol (Jung, et al., 1997). In contrast to this result, only a very slow rate of ethanol-induced swelling occurred when mitochondria from the Yeast Foam strain were suspended under similar conditions (Velours, et al., 1977). These authors found that mitochondrial swelling was greatly stimulated by the addition of oligomycin in the presence of 10 mM KCl with a corresponding increase in the rate of respiration. When using Yeast Foam mitochondria, we found that respiration-induced mitochondrial yPTP opening and large amplitude swelling occur even in the absence of any added KCl when oligomycin is present (Figure 1 A). When the mitochondria were suspended in a 0.3 M tetraethylammonium (TEA) chloride (Cl) medium, respiration-induced yPTP opening caused swelling of mitochondria in the absence of oligomycin (Figure 1 B). In this salt-based medium oligomycin had little further stimulatory effect on ethanol-induced swelling when present. These results indicate that respiration-induced yPTP opening occurs in the Yeast Foam strain in a similar way as it does in laboratory strains except oligomycin needs to be present when mitochondria are suspended in a mannitol-based medium. When W303-1A mitochondria were used, TEA and all solutes tested having a size less than or equal to 180 Da (the size of a monosaccharide) were permeable through the respiration-induced yPTP and oligomycin addition gave no further stimulation of the rate of swelling, while disaccharides and other larger solutes were largely impermeable (data not shown).

Inhibitors of the yPTP

The known physiological inhibitors of the respiration (high matrix space pH)-induced yPTP are ADP and P_i . One explanation for why ATP has been shown to uncouple and stimulate respiration in mitochondria from the W303-1A strain, but not in the Yeast Foam strain, could be due to a decreased affinity of the respiration-induced yPTP for P_i , allowing yPTP opening before ATP is added. The experiments testing for ATP-induced uncoupling are challenging in that a low level of P_i (or another respiration-induced yPTP inhibitor) must be present to keep the respiration-induced yPTP closed, while that P_i concentration must be low enough to allow the ATP-induced yPTP to be opened. We therefore performed experiments to determine if there were differences in P_i affinity for the yPTP in mitochondria from the different strains. As shown in Figure 2 A, a lower concentration of P_i inhibits the Yeast Foam respiration-induced (ethanol as the respiratory substrate) yPTP than is needed for inhibition of the yPTP in W303-1A mitochondria, with IC_{50} values of 0.50 mM and 1.05 mM respectively. These results do not support the proposed hypothesis and do not indicate a role for different P_i sensitivities of the respiration-induced yPTP in the inability of ATP to stimulate respiration in Yeast Foam mitochondria.

We performed further experiments to determine if P_i could inhibit yPTP opening by binding a site on the inner mitochondrial membrane. When suspended in a mannitol medium at pH 8.2 in the presence of FCCP and no respiratory substrate, mitochondria swell by opening the yPTP. Pore opening is caused by an alkalization of the matrix space. The presence of FCCP prevents an accumulation of protons in the matrix space and should therefore prevent P_i from inhibiting the yPTP if matrix acidification is the only mechanism for P_i inhibition. As shown in Figure 2 B, P_i blocked mitochondrial swelling in the presence of FCCP indicating that matrix acidification is not the only mechanism of P_i inhibition and that a P_i binding site on the inner membrane is likely present. P_i inhibited mitochondrial swelling to a similar extent in both Yeast Foam and W303-1A mitochondria under these conditions. To determine which side of the inner membrane the P_i binding site is located, mitochondria were

suspended at pH 8.2 after they had been incubated with mersalyl, an inhibitor of the mitochondrial H^+/P_i symporter (Guerin, et al., 1990). Mersalyl greatly reduced the inhibitory effect of P_i suggesting that the P_i binding site is located on the matrix space side of the inner membrane, confirming an earlier report (Cortes, et al., 2000).

Vanadate polymerized to decavanadate is an inhibitor of the ATP-induced transport activities in yeast mitochondria. It is a potent inhibitor of the proton conducting channel in laboratory yeast strains and the K^+ channel in the Yeast Foam strain (Roucou, et al., 1997a). Decavanadate has even been shown to inhibit the (respiration-induced) yPTP stimulated by ethanol and 20 mM KCl in mitochondria from a commercial yeast strain. The inhibition may be mediated through an interaction of decavanadate with the voltage-dependent anion channel (VDAC) as inhibition was much diminished in a VDAC-deficient $\Delta por1$ yeast strain (Gutierrez-Aguilar, et al., 2007). We also found decavanadate inhibition of the ATP-induced yPTP ($IC_{50} \sim 2 \mu M$) in W303-1A mitochondria (data not shown). As shown in Figure 3 A, decavanadate also inhibited ethanol-induced opening of the yPTP of mitochondria suspended in a mannitol medium in the absence of K^+ . IC_{50} 's of approximately 16 μM and 32 μM decavanadate were obtained when using mitochondria from the W303-1A and Yeast Foam strains respectively.

The extent to which decavanadate inhibits swelling of mitochondria suspended in a mannitol medium at pH 8.2 in the presence FCCP (Figure 3 B) was also determined. Decavanadate was more effective at inhibiting this high medium pH-induced swelling than the ethanol-induced swelling. When comparing mitochondria from the different strains, a lower concentration of decavanadate was required to inhibit yPTP opening in W303-1A mitochondria compared to that in Yeast Foam mitochondria with IC_{50} 's of 4 μM and 12 μM respectively.

NH_4^+ inhibits respiration-induced swelling of yeast mitochondria (Velours, et al., 1977) and also plays a role in yeast programmed cell death (Vachova and Palkova, 2005). Therefore, we sought to examine the affinity of NH_4^+ to inhibit yPTP opening. NH_4Cl addition inhibited respiration-induced swelling of W303-1A mitochondria suspended in a mannitol medium with an IC_{50} of 0.25 mM (Figure 4 A). It is possible that NH_4^+ is transported into the matrix space where it releases a proton to prevent the pH change necessary for yPTP opening. But NH_4^+ may also bind to a site on the inner membrane to inhibit yPTP opening. To determine which of these mechanisms occur, mitochondria were suspended in a mannitol medium at pH 8.20 in the presence of FCCP. At this pH, the equilibrium between NH_3 and NH_4^+ is shifted more toward NH_3 than at the more neutral pH. NH_4^+ did not inhibit swelling at any of the concentrations tested up to 10 mM (data not shown). Therefore NH_4^+ likely inhibits respiration-induced yPTP opening by altering the matrix space pH change required for yPTP opening.

Mg^{2+} , which inhibits the ATP induced opening of the yPTP (Guerin, et al., 1994) and also inhibits yPTP opening induced by ethanol and 20 mM KCl in a commercial yeast strain (Perez-Vazquez, et al., 2003) also partially inhibited the respiration-induced opening in W303-1A mitochondria in the absence of K^+ (Figure 4 B). Mg^{2+} was reported to inhibit the ATP-induced yPTP by binding free ATP, forming a Mg-ATP complex, which does not stimulate yPTP opening (Guerin, et al., 1994). Therefore, Mg^{2+} relies on a different mechanism to inhibit respiration-induced yPTP opening. Mg^{2+} only maximally inhibited the respiration-induced opening of the yPTP by 60%. In intact yeast cells, Mg^{2+} likely plays a role inhibiting the respiration-induced yPTP since Mg^{2+} possesses a half-maximal inhibitory effect at 70 μM , below its physiological free concentration of 0.1 to 1 mM (Beeler, et al., 1997). Consistent with previous studies of ATP-induced yPTP opening, we observed Mg^{2+}

to have an IC_{50} value of 1.5 mM for preventing 2 mM ATP-induced swelling of W303-1A mitochondria suspended in a tetramethylammonium (TMA) Cl medium (data not shown).

Since ADP inhibits ATP and respiration-induced yPTP opening (Jung, et al., 1997), we sought to determine if a pyridine nucleotide could inhibit opening as well. We found that NAD inhibited both the respiration and ATP-induced yPTP opening (Figure 5). NAD had a slightly higher inhibitory effect on ethanol-induced opening ($IC_{50} \sim 1.5$ mM) than on NADH-induced opening ($IC_{50} \sim 2.5$ mM). The NAD-mediated inhibition was also stronger on the respiration-induced yPTP opening than on the ATP-induced yPTP opening ($IC_{50} \sim 4$ mM). Interestingly, concentrations of NAD greater than 3 mM inhibited respiration when ethanol was used as the respiratory substrate. 4 mM NAD, a concentration previously found intracellularly (Anderson, et al., 2003), inhibited the respiratory rate of mitochondria oxidizing 4 mM ethanol by 38 % (Figure S1).

K⁺ partially inhibits ethanol-induced yPTP opening

To determine if K⁺ inhibits respiration-induced yPTP opening, the ethanol concentration added to W303-1A or Yeast Foam mitochondria suspended in a KCl-based medium was varied and the extent of mitochondrial swelling was compared to the extent of swelling in a tetramethylammonium (TMA) Cl medium. A KCl based medium substantially inhibited mitochondrial swelling, especially at low ethanol concentrations. These rates of swelling were then compared to those obtained in a KCl medium in the presence of both 10 mM P_i and 0.1 mM decavanadate (Figure 6), which keep the yPTP closed as judged by the maintenance of a membrane potential (see Figure 8). A complex, somewhat oscillating pattern of maximal swelling was obtained as the ethanol concentration increased when mitochondria were suspended in the KCl medium. The presence of the combination of P_i and decavanadate decreased the swelling by nearly 50% at 10 mM ethanol, while the extent of swelling inhibition decreased at higher ethanol concentrations until almost no inhibition of swelling was observed at 32 mM ethanol.

When mitochondria are suspended in a KCl medium, they swell spontaneously at a slow rate by matrix space K⁺ entry through the K/H⁺ exchange and Cl⁻ entry through an anion channel. Addition of less than 0.5 mM ethanol decreased the amount of swelling, likely due to efflux of K⁺ through the K⁺/H⁺ exchange. The yPTP is inhibited by proton entry into the matrix space mediated by the K⁺/H⁺ exchange, which prevents a rise in matrix space pH. The increased swelling at higher ethanol concentrations could be caused by two different mechanisms. The rate of the K⁺/H⁺ exchange may not keep pace with the increased proton pumping at high respiratory substrate concentrations; therefore, a pH gradient could form, the yPTP would then open, and swelling would occur. Alternatively the rate of the K⁺/H⁺ exchange may not keep up with the rate of electrophoretic K⁺ entry accompanied by Cl⁻ entry through the anion channel at high respiratory substrate concentrations (Castrejon, et al., 2002), resulting in swelling without yPTP opening. Since the combination of both P_i and decavanadate, which keep the yPTP closed, partially inhibited swelling, both of these mechanisms likely contribute to the total extent of swelling under these conditions.

K⁺ antagonizes P_i inhibition at high respiratory substrate concentrations

Experiments were performed to determine the ability of the yPTP inhibitors P_i or decavanadate to inhibit respiration-induced swelling of mitochondria suspended in a KCl medium. Either mannitol, a yPTP-permeable sugar (Jung, et al., 1997), or sucrose, a yPTP-impermeable sugar (Bradshaw and Pfeiffer, 2006a), were added to a suspension of swollen mitochondria. Mitochondria were first swollen either by the addition of 32 mM ethanol in the presence or absence of decavanadate, or by valinomycin, a K⁺ ionophore, with the yPTP inhibited with decavanadate. Following mannitol addition to ethanol-swollen mitochondria,

a further swelling occurred after an initial contraction as mannitol entered the matrix space demonstrating that the yPTP was open (Figure 7 A). Therefore K^+ by itself was not an effective respiration-induced yPTP inhibitor at this high ethanol concentration used. Mannitol addition to valinomycin-swollen mitochondria resulted in a slight mitochondrial contraction due to the mannitol-impermeability under this condition. As expected, the addition of mannitol to ethanol-swollen mitochondria in the presence of decavanadate caused a contraction similar to the valinomycin-swollen mitochondria indicating a closed yPTP.

To further confirm the open or closed state of the yPTP, sucrose, a yPTP impermeable substrate was added to mitochondria (Figure 7 B) swollen under the same conditions as when mannitol was added. The addition of sucrose to ethanol-swollen mitochondria in the absence of decavanadate caused a large osmotic contraction, much greater than that of sucrose added to mitochondria swollen by valinomycin with the yPTP closed. When sucrose was added to the ethanol-swollen mitochondria in the presence of decavanadate, the mitochondria only contracted as quickly as those given valinomycin with the yPTP closed. Therefore, when mitochondria are suspended in a KCl medium in the presence of high ethanol concentrations, decavanadate is able to keep the yPTP closed confirming the previous observation.

Further experiments were performed under identical conditions in the KCl medium to check the efficacy of the yPTP inhibitor P_i . 10 mM P_i was used, which completely inhibits the respiration-induced yPTP in a mannitol medium (Jung, et al., 1997), even at high ethanol concentrations. Surprisingly, when mitochondria were swollen with ethanol in the presence of 10 mM P_i , a slight swelling occurred following mannitol addition (Figure 7 C). This result was similar to the swelling result in the absence of P_i , and not at all like the result with decavanadate present and the yPTP closed. Therefore, 10 mM P_i was unable to keep the respiration-induced yPTP closed in a majority of the mitochondria. Adding sucrose to ethanol-swollen mitochondria in the presence of P_i (Figure 7 D) yielded an intermediate extent of contraction between the conditions where the yPTP was completely open or completely closed. Therefore, the respiration-induced yPTP remained largely open in the presence of 10 mM P_i when mitochondria were suspended in a KCl medium oxidizing high amounts of ethanol.

To verify the previous results, the W303-1A mitochondrial membrane potential ($\Delta\Psi$) was monitored following mitochondrial suspension in different concentrations of KCl isoosmotically balanced with mannitol in the presence of 10 mM P_i . No decrease in the $\Delta\Psi$ of mitochondria oxidizing 32 mM ethanol was observed in the presence of 70 mM K^+ and 10 mM P_i (Figure 8) indicating the yPTP remained closed. This is consistent with previous results indicating that the presence of 70 mM K^+ does not alter the ATP/O ratio of mitochondria (Manon, et al., 1995). But the addition of 150 mM K^+ led to a partial collapse of the $\Delta\Psi$ and the presence of 200 mM KCl led to a total loss of $\Delta\Psi$, which was completely prevented by the presence of decavanadate.

Similar results were obtained for mitochondria from the Yeast Foam strain in the presence of oligomycin (Figure S2). Once again, 0.1 mM decavanadate with P_i completely blocked respiration-induced $\Delta\Psi$ loss. The combination of P_i and NAD also blocked the loss of $\Delta\Psi$. However, 10 mM NH_4^+ together with P_i did not maintain the $\Delta\Psi$. Therefore, NAD (and most likely also ADP) together with P_i play an important role in maintaining the respiration-induced yPTP in the closed state under high, physiological KCl conditions when mitochondria are oxidizing high levels of a respiratory substrate such as ethanol.

Discussion

The major novel findings in this report are that NAD and NH_4^+ inhibited respiration-induced yPTP opening. K^+ addition also yielded a partial inhibition of yPTP opening, while high K^+ concentrations largely prevented P_i -mediated yPTP inhibition when mitochondria were rapidly respiring. Oligomycin also sensitized to respiration-induced yPTP opening in the Yeast Foam strain. Although the molecular identity of the mitochondrial permeability transition pore in yeast has not definitively been determined and the experiments performed in this report were on isolated mitochondria removed from their physiological environment, several important insights into yPTP regulation were discovered.

The mechanism of how K^+ affects respiration-induced yPTP opening

Addition of K^+ to respiring yeast mitochondria causes a protonophoric effect (Manon, et al., 1995), which partially dampens the increase in matrix space pH when isolated mitochondria respire. Due to this effect, respiration-induced yPTP opening is partially inhibited. The inhibition is strongest at low respiratory substrate concentrations. At high respiratory substrate concentrations K^+ only partially blocked respiration-induced yPTP opening and also partially prevented P_i inhibition of the yPTP. In respiring mitochondria in the presence of P_i and K^+ , the H^+/P_i symporter and K^+/H^+ exchange compete for the use of the proton gradient (Manon, et al., 1995). At high respiratory substrate and K^+ concentrations, when entry of K^+ into the matrix is rapid, the K^+/H^+ exchange may out-compete the H^+/P_i symporter for protons. Therefore, less P_i may be transported into the matrix space and the yPTP may open. However, this failure of P_i to inhibit respiration-induced yPTP opening only occurs in mitochondria suspended in a high KCl-containing medium (see 150 mM and 200 mM KCl concentrations in Figure 8). Concentrations of K^+ less than 70 mM do not stimulate respiration-induced yPTP opening and have actually been shown to increase P_i transport into the matrix space (Manon, et al., 1995). Consistent with this data, concentrations of K^+ up to 70 mM did not cause any significant effect on the ΔpH (Manon, et al., 1995) or $\Delta\Psi$ (see Figure 8) of mitochondria in the presence of high (10 mM) P_i concentrations. However, at low (0.4 mM) P_i concentrations, the addition of (20 mM) K^+ sharply decreased the $\Delta\Psi$ and ΔpH . This effect was blocked by NH_4^+ (Castrejon, et al., 1997), which inhibits the respiration-induced yPTP. Therefore, the presence of low K^+ concentrations (20 mM) at low P_i concentrations (0.4 mM) likely opens the yPTP, while at high P_i concentrations (10 mM), greater K^+ concentrations (150 mM) are required (see Figure 8).

The mechanism of P_i inhibition of respiration-induced yPTP opening

In addition to inhibiting respiration-induced yPTP opening by donating a proton to the matrix space to prevent matrix alkalization when transported into mitochondria (Jung, et al., 1997; Velours, et al., 1977), P_i also binds a site on the matrix side of the inner membrane to inhibit yPTP opening. Therefore, P_i possesses a dual role in inhibiting respiration-induced yPTP opening. Previous reports also determined that P_i enters the matrix space to inhibit respiration-induced K^+ transport (Roucou, et al., 1997a) and that P_i enters the matrix space to inhibit the respiration-induced depletion of the membrane potential in the presence of 40 mM K^+ , because inhibition was blocked by the presence of mersalyl (Cortes, et al., 2000). Strikingly, P_i binds the cytoplasmic side of the inner membrane to inhibit ATP-induced K^+ transport (Roucou, et al., 1997b), gluconate (Guerin, et al., 1994), and mannitol (Jung, et al., 1997) transport. Therefore two separate sites for P_i -mediated inhibition of the yPTP exist.

The cation specificity of the Ca^{2+} -induced yPTP in yeast mitochondria

Under optimized experimental conditions containing ~ 2 mM P_i , the addition of Ca^{2+} and the electrophoretic Ca^{2+} ionophore ETH-129 to yeast mitochondria led to an initial uptake

of Ca^{2+} followed by Ca^{2+} release and mitochondrial swelling, indicative of a Ca^{2+} -induced PTP (Yamada, et al., 2009). The mitochondria in that study were suspended in a mannitol medium and were oxidizing NADH. The data appear to conflict with the known ability of Ca^{2+} to inhibit the yPTP (Perez-Vazquez, et al., 2003), which may occur through an interaction with VDAC (Gutierrez-Aguilar, et al., 2007). In our experiments we found that adding high concentrations of K^+ to rapidly respiring mitochondria even in the presence of 10 mM P_i led to opening of the respiration-induced yPTP. A similar mechanism may lead to respiration-induced yPTP opening in the presence of Ca^{2+} , ETH-129, and 2 mM P_i as we have found in media containing 200 mM K^+ and 10 mM P_i . Since the P_i binding site for inhibition of the respiration-induced yPTP is present on the matrix side of the inner membrane, fast influx of either Ca^{2+} or K^+ across the inner mitochondrial membrane could decrease the rate of P_i transport into the matrix space leading to respiration-induced yPTP opening. Alternatively, the decreased $\Delta\Psi$ caused by rapid cation uptake could stimulate the respiratory chain to increase proton pumping, transiently increasing ΔpH to induce yPTP opening without decreasing matrix space P_i levels. Increased matrix space Ca^{2+} could have also bound and precipitated P_i , lowering P_i levels allowing yPTP opening. In any case, more studies are needed to confirm the presence of a yPTP that is specifically opened by matrix space Ca^{2+} and to verify that rapid electrophoretic ionophore-mediated mitochondrial Ca^{2+} uptake does not just non-specifically stimulate respiration-induced yPTP opening in the presence of a low P_i concentration.

Selectivity of the yPTP

There are likely two slightly different open conformations of the yPTP in mitochondria from the Yeast Foam strain. Respiration in the presence of oligomycin allows mannitol transport into the matrix space while ATP addition or respiration in the absence of oligomycin does not (Guerin, et al., 1994). These latter conditions may open a smaller conformation of the yPTP allowing TEA^+ and K^+ transport, but not mannitol. We observed that mannitol is permeable through the ATP-induced yPTP in Yeast Foam mitochondria, but for this to occur the mitochondria need to be swollen first by yPTP opening in a salt-based medium (data not shown). Therefore mitochondrial swelling is likely another inducer of the larger, mannitol-permeable form of the yPTP in Yeast Foam mitochondria.

ATP-induced yPTP proton permeability in Yeast Foam mitochondria

It was difficult to reconcile previous results that various large cations and anions (Guerin, et al., 1994), but not protons (Roucou, et al., 1995), were permeable through the ATP-induced yPTP in the Yeast Foam strain. We therefore sought to determine conditions that allow ATP to stimulate respiration in this strain. We found that ATP could stimulate respiration of mitochondria suspended in a sucrose-containing medium (Figure S3 A). ATP decreased the membrane potential under these conditions as well (Figure S3 B) consistent with the opening of the yPTP and uncoupling. ATP also stimulated respiration in a mannitol medium if NAD instead of P_i was present to keep the respiration-induced yPTP closed (Figure S1). Therefore protons and all solutes that we have tested of equal or smaller size than monosaccharides (~ 180 Da) are permeable through the ATP-induced yPTP in both laboratory strains as well as in the Yeast Foam strain, at least under specific experimental conditions. However, we could not detect substantial release of Mg^{2+} (24 Da) from yeast mitochondria following respiration-induced yPTP opening (Bradshaw and Pfeiffer, 2006a). The reason for this observation requires further study.

Comparing the yPTP to the mammalian and fruit fly PTPs

Is the yPTP a homologue of the mammalian mitochondrial PTP? This question has been extensively reviewed (Azzolin, et al., 2010; Manon, et al., 1998; Uribe-Carvajal, et al., 2011). The opening of either proton-permeable structure for an extended period of time

could lead to cellular ATP depletion and cell death. Therefore, these structures likely only normally flicker in the cell to prevent ATP depletion. yPTP opening has been demonstrated in permeabilized spheroplasts (Manon and Guerin, 1998), so it is not an artifact caused by the mitochondrial isolation procedure. The mammalian PTP also opens physiologically, albeit at a low conductance state (Fall and Bennett, 1999; Huser and Blatter, 1999), when cell death is not induced. The low conductance, sucrose impermeable sub-state of the mammalian PTP is largely controlled by matrix pH (Ichas, et al., 1997). The regulation of this sub-state is very similar to that of the respiration-induced yPTP. While the mammalian PTP functions in a low conductance mode as a Ca^{2+} release channel, the yeast PTP may function as a K^{+} release mechanism to maintain mitochondrial volume homeostasis.

A Ca^{2+} -induced PTP has been described in permeabilized S2R+ cells from *Drosophila melanogaster* (von Stockum, et al., 2011). PTP opening was inhibited by P_i similar to the yPTP, but PTP opening did not cause mitochondrial swelling even when the mitochondria were suspended in KCl-containing media. The high solute selectivity and inhibition by P_i led the authors to conclude that the fruit fly PTP may be an evolutionary intermediate between the yPTP and the mammalian PTP.

A role for the NAD/NADH ratio in regulating yPTP opening

Under fermentative conditions in yeast when the pyruvate from glycolysis is converted into acetaldehyde and then into ethanol, NADH is oxidized to NAD, which contributes to keeping the yPTP closed. Under these conditions the NAD concentration has been measured to be around 4 mM while NADH was less than 0.2 mM (Anderson, et al., 2003). So the NAD/NADH ratio was greater than 20. Others have calculated the free NAD/NADH ratio to be as high as 320 during similar conditions (André, et al., 2008). This ratio may be especially important in keeping the yPTP closed because the ATP/ADP ratio may be high under this condition, which would favor yPTP opening. However under non-fermentative conditions when glucose levels are exhausted and yeast use the ethanol for oxidative metabolism the NAD/NADH ratio is much lower due to the reduction of NAD to NADH by ethanol dehydrogenase. The NAD/NADH ratio has been measured to be around 0.7 under these conditions (Hall and Wills, 1987). This low NAD/NADH ratio would favor opening of the yPTP, which could function to rid the cell of excess reducing equivalents through yPTP-mediated mitochondrial uncoupling. However, yPTP opening ultimately relies on the integration of many different signals including ATP, ADP, other nucleotide di- and triphosphates, Mg^{2+} , NH_4^{+} , P_i , SO_4 , matrix space pH, and other unknown factors. This complex regulation would best be studied by monitoring yPTP function in intact yeast cells.

A role for the mitochondrial F_1F_0 -ATP synthase in PTP formation

Our findings that the ATP synthase inhibitor oligomycin sensitizes respiration-induced yPTP opening of Yeast Foam mitochondria when mitochondria are suspended in a mannitol medium is intriguing in that dimers of mammalian mitochondrial ATP synthase (Giorgio, et al., 2013), and the F_0 ATP synthase subunit c (Bonora, et al., 2013) have recently been implicated in mammalian PTP formation. In this regard, the soluble matrix space cyclophilin D protein, a potent activator of mammalian PTP and target of the PTP inhibitor cyclosporin A, was found to bind the oligomycin sensitivity-conferring protein (OSCP) in the F_1 stalk of the ATP synthase to sensitize to PTP opening. Bz-423, a small compound inducer of apoptosis, was shown to bind OSCP (Johnson, et al., 2005) and induce PTP channel formation in reconstituted ATP synthase dimers in the presence of Ca^{2+} (Giorgio, et al., 2013). Oligomycin may bind OSCP in Yeast Foam mitochondria to sensitize to high matrix space pH-induced yPTP opening in a similar way as Bz-423 binds to OSCP to sensitize to Ca^{2+} -induced PTP opening in mammalian mitochondria. Further data in support of a role for ATP synthase comprising the yPTP is that both entities have nearly the same anion

specificity for inhibition. Both are inhibited by arsenate and sulfate while many other similar anions have no effect on either. The only difference found was the inhibition of the yPTP by P_i , while P_i binds as a substrate to the ATP synthase (Cortes, et al., 2000). Future studies aim to identify which, if any, of the subunits of the ATP synthase are required for yPTP activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Phosphate binds to an internal site to inhibit the respiration-induced yPTP. (A) Standard assay medium was used with the addition of 1 mM ethanol, 15 $\mu\text{g/ml}$ oligomycin, and the indicated amount of $\text{TEA}^+ \text{P}_i$. Mitochondria from the strain indicated were used (W303-1A—open circles, Yeast Foam—filled circles). The extents of swelling at $t=240$ sec. were compared. (B) Conditions were the same as in panel A except, the medium contained 0.6 M mannitol, 10 mM tricine (TEA^+), pH 8.20, 15 $\mu\text{g/ml}$ oligomycin, and 4 μM FCCP. Mitochondria were pre-incubated for 10 minutes with 30 nmol/mg mersalyl in mitochondrial isolation buffer at 4°C where shown (filled squares).

Figure 3.

Decavanadate inhibits the respiration-induced yPTP. (A) Standard assay medium was used with the addition of 1 mM ethanol, 15 $\mu\text{g/ml}$ oligomycin, and the amount of decavanadate (Na^+) indicated. Mitochondria from the W303-1A strain (open circles) or Yeast Foam strain (filled circles) were used as indicated. (B) The medium contained 0.6 M mannitol, 10 mM tricine (TEA^+), pH 8.2, 15 $\mu\text{g/ml}$ oligomycin, 4 μM FCCP, and the amount of decavanadate (Na^+) indicated. For both panels the extents of swelling at 240 sec. were compared.

Figure 4.

NH_4^+ and Mg^{2+} inhibit the respiration-induced yPTP. (A) Standard assay medium was used with the addition of 2 mM ethanol. The indicated amount of NH_4Cl was present. (B) Standard assay medium pH 7.40 was used with the addition of 1 mM ethanol. The indicated amount of MgCl_2 was present. For both panels mitochondria from strain W303-1A were used and the extents of swelling at 240 sec. were compared.

Figure 5.

NAD inhibits the respiration and ATP-induced yPTP. Standard assay medium was used. 1 mM ethanol, 1 mM NADH, or 2 mM ATP (Na^+) was added to induce swelling. When ATP was added 4 μM FCCP was present. The extents of swelling at 240 sec. were compared. Mitochondria from strain W303-1A were used.

Figure 6.

K^+ inhibition of respiration-induced mitochondrial swelling depends on respiratory substrate concentration. The medium contained either 0.2 M KCl, 10 mM HEPES, pH 7.20 (traces a, b, e, and f) or 0.2 M TMA Cl, 10 mM HEPES (TEA^+), pH 7.20 (traces c and d).

Mitochondria from the Yeast Foam strain (traces e and f) or W303-1A (traces a-d) were used as indicated. 10 mM KP_i and 0.1 mM decavanadate (Na^+) were present as indicated (traces b, d, and f). The indicated amount of ethanol was present. The extents of swelling at 180 sec. were compared. The maximal amount of swelling in 0.2 M TMA Cl was taken to be 100 % swelling.

Figure 7.

Loss of yPTP phosphate sensitivity when mitochondria are suspended in a KCl medium at a high ethanol concentrations. Mitochondria from strain W303-1A were used. (A) The medium contained 0.2 M KCl, 10 mM HEPES, pH 7.20. Where indicated 32 mM ethanol (etOH) ($t=60$ sec.) (traces a and b) and 40 mM mannitol ($t=240$ sec.) (all traces) were added. 0.1 mM decavanadate (Na^+) (indicated as dv) (traces b and c) or 0.5 μ g/ml valinomycin (indicated as val) (trace c) were present as shown. (B) The media composition was the same as in panel A except 40 mM sucrose was added at 240 sec. instead of mannitol. (C) The medium composition was the same as in panel A, except 10 mM KP_i was present where indicated instead of decavanadate. Where indicated 32 mM ethanol (etOH) ($t=60$ sec.) (traces d and e) or 40 mM mannitol ($t=240$ sec.) (all traces) were added. 10 mM KP_i (traces e and f) or 0.5 μ g/ml valinomycin (indicated as val) (trace f) were present as shown. (D) The medium composition was the same as in panel C except 40 mM sucrose was added at 240 sec. instead of mannitol.

Figure 8.

Increasing KCl concentrations prevents the maintenance of a mitochondrial membrane potential at a high ethanol concentration in the presence of phosphate. The medium contained 0.2 M KCl, 10 mM HEPES, pH 7.20 (traces d and e), 0.4 M mannitol, 10 mM HEPES (TEA⁺), pH 7.20, (trace a) or a 0.4 osM combination of the 0.2 M KCl and 0.4 M mannitol media listed above (traces b and c). The concentration of KCl present is shown. The medium also contained 10 mM KP_i, and 12 μM safranin O. 0.1 mM decavanadate (dv) was present in trace e. W303-1A mitochondria (t=30 sec.), 32 mM ethanol (t=60 sec.) and 4 μM FCCP (t=360 sec.) were added to all traces as shown. Oxygen was depleted from the suspension between t=230-270 sec. in different traces causing a loss in membrane potential.