Mitochondrial Function Is an Inducible Determinant of Osmotic Stress Adaptation in Yeast*

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Hyperosmotic stress triggers a great variety of adaptive responses in eukaryotic cells that affect many different physiological functions. Here we investigate the role of the mitochondria during osmostress adaptation in budding yeast. Mitochondrial function is generally required for proper salt and osmotic stress adaptation because mutants with defects in many different mitochondrial components show hypersensitivity to increased NaCl and KCl concentrations. Mitochondrial protein abundance rapidly increases upon osmoshock in a selective manner, because it affects Calvin cycle enzymes (Sdh2 and Cit1) and components of the electron transport chain (Cox6) but not the ATP synthase complex (Atp5). Transcription of the *SDH2*, *CIT1*, and *COX6* genes is severalfold induced within the first minutes of osmotic shock, dependent to various degree on the Hog1 and Snf1 protein kinases. Mitochondrial succinate dehydrogenase enzyme activity is stimulated upon osmostress in a Snf1-dependent manner. The osmosensitivity of mitochondrial mutants is not caused by impaired stress-activated transcription or by a general depletion of the cellular ATP pool during osmostress. We finally show that the growth defect of mitochondrial mutants in high salt medium can be partially rescued by supplementation of glutathione. Additionally, mitochondrial defects cause the hyperaccumulation of reactive oxygen species during salt stress. Our results indicate that the antioxidant function of the mitochondria might play an important role in adaptation to hyperosmotic stress.

All living cells dynamically respond to environmental stress to adapt and survive. A fundamental challenge for the cell is a change in the osmolarity of the surrounding medium. Hyperosmotic and salt stress cause the rapid loss of water from the cell, and the resulting intracellular ion imbalances interfere with many vital functions. The hyperosmotic stress response has been intensively studied in the budding yeast *Saccharomyces cerevisiae* (1). A complex picture is emerging from these studies where many diverse physiological functions of the cell have to be coordinately modulated to efficiently adapt to the stress. The major signal transduction pathway in yeast that is specifically activated upon hyperosmotic stress is the high

osmolarity glycerol MAPK[2] pathway with its terminal MAPK Hog1 (2, 3). The adaptive responses orchestrated by the high osmolarity glycerol pathway in response to osmostress are complex and involve the direct regulation of plasma membrane cation transporters (4), the modulation of translation efficiency (5, 6), the controlled cell cycle arrest during stress (7–9), the transcriptional activation of more than 150 genes in the nucleus (10, 11), and the production of osmolytes as a long term adaptation (12). Our work identifies the mitochondrial function as an important physiological determinant of the efficient adaptive response to osmostress.

Mitochondria are the main ATP-generating organelles in eukaryotic cells. In addition to their function as the cell power supply via oxidative phosphorylation, mitochondria play fundamental roles in secondary metabolism, Ca^{2+} signaling, homeostasis of redox equivalents, and apoptosis. Recent research has characterized the mitochondria as a much more dynamic organelle than anticipated, because they continuously and dynamically change their metabolic activity, biomass, and morphology (13, 14). In *S. cerevisiae*, the mitochondrial function is regulated over a large range, being repressed as long as sufficient ATP can be produced by the fermentation of sugars, and activated in the presence of nonfermentable carbon sources. This phenomenon is known as glucose repression and affects the nuclear and mitochondrial transcription of many components of the mitochondrial oxidative phosphorylation machinery (15–18). The central activator of respiratory metabolism is the evolutionarily conserved Snf1 protein kinase, which is essential for the metabolic adaptation upon glucose starvation (19, 20). Snf1 is necessary to activate the transcription of a large set of genes in response to nutrient limitation, which include many mitochondrial functions (21). Activation of glucose-repressed genes encoding respiratory functions is globally controlled by the Hap2,3,4,5 transcriptional activator complex (22–24). Additionally, Snf1 affects the tolerance to salt stress and is necessary for the salt-induced transcription of the *ENA1* gene (25); however, the reason why *snf1* mutants fail to adapt to salt stress remains elusive (26).

Apart from their up-regulation during the switch from fermentation to respiration, signal transduction pathways exist to monitor mitochondrial function and to trigger compensatory adaptation upon mitochondrial dysfunction. This is the so-called retrograde signaling pathway, which transmits signals

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[2] The abbreviations used are: MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; GFP, green fluorescent protein; SDH, succinate dehydrogenase; HPLC, high pressure liquid chromatography; TAP, tandem affinity purification.

from mitochondria to the nucleus to effect changes in nuclear gene expression to accommodate cells to defects in mitochondria (27). The key transcriptional activators of the retrograde pathway are the Rtg1 and Rtg3 proteins (28, 29). Specifically, the Hap and Rtg regulators can activate the same target genes, for example the first enzymes of the citric acid cycle, in response to diauxic shift or compromised mitochondrial function, respectively (30).

Mitochondria are considered to be the major source of reactive oxygen species (ROS) in eukaryotic cells because of leakage of electrons from their electron transport chains (31, 32). Mutants in mitochondrial functions have been shown to result in altered production of ROS in a number of eukaryotic organisms including yeast (33). Moreover, ROS production is intimately linked to aging and apoptosis (32, 34). Mitochondria are both the source and the site for the detoxification of reactive oxygen species in yeast. The importance of the antioxidant function of the mitochondria is reflected by the fact that the majority of mitochondrial mutants in yeast are hypersensitive to hydrogen peroxide (35, 36). The implication of the mitochondria in ROS production has been mainly derived by *in vitro* studies or by the use of artificial uncouplers or mutants in mitochondrial functions. However, it is crucial to understand how mitochondrial metabolism is regulated under physiological conditions upon environmental challenges that cause oxidative stress. In the present work we show that the up-regulation of the mitochondria is an essential and novel feature of the adaptation to hyperosmotic stress, which involves the reduction of the levels of reactive oxygen species.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—*S. cerevisiae* strains used in this study were: wild type BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) and the mutant alleles *aco1::KanMX4, atp5::KanMX4, atp7::KanMX4, cox6::KanMX4, rpo41::KanMX4, abf2::KanMX4, fzo1::KanMX4, snf1::KanMX4, hog1::KanMX4, rtg1::KanMX4, rtg3::KanMX4, hap2::KanMX4, hap3::KanMX4, sdh1::KanMX4* (37). All of the mutants were rho$^+$ as determined by the isolation of mitochondrial DNA as described in Ref. 38. Yeast strains expressing chromosomally tagged GFP fusion proteins were: BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) with *SDH2-GFP(S65T)-His3MX, COX6-GFP(S65T)-His3MX, CIT1-GFP(S65T)-His3MX, ATP5-GFP(S65T)-His3MX, IDP1-GFP(S65T)-His3MX* (39). Yeast strains expressing chromosomally tagged TAP fusion proteins were: BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) with *SDH1-TAP-His3MX, SDH2-TAP-His3MX,* or *ATP5-TAP-His3MX* (40). Yeast cultures were grown in yeast extract-peptone containing 2% dextrose (YPD) with or without the indicated supplementation of NaCl, KCl, or glutathione. For continuous growth experiments, yeast strains were grown in microtiter plates in a Bioscreen C system (Thermo). For osmostress-inducible β-galactosidase expression, we used plasmid pMP224 (*CYC1prom-(2xCRE$_{ENA1}$)-lacZ; 2μ; URA3*). It contains a tandem insertion of the binding site for the transcription factor Sko1 (CRE) into the *CYC1* core promoter fused to *lacZ*, conferring very low expression levels under normal growth conditions and high inducibility upon hyperosmotic stress (41).

Screening for Osmosensitive Yeast Strains—Our original screen aimed at identifying novel proteins involved in transport processes necessary for the adaptation to hyperosmotic stress. We focused at the subset of the haploid yeast deletion strain collection with an annotated function in transport. All of the strains were individually spotted in serial dilutions onto YPD plates containing 1 M NaCl or 1.5 M KCl. Growth was scored and compared with the BY4741 wild type parent. We identified nine mutant strains with significantly reduced growth at high osmolarity media. The genes involved encoded either mitochondrial Fe^{2+} or Cu^{2+} transporters/chaperones (*SCO1, MRS4, ISA1, ISA2,* and *COX19*) or Fe^{2+} transporters of the plasma membrane and their transcriptional activator (*FET3, FTR1,* and *RCS1*). Additionally, a mutant in the Ca^{2+}/Mn^{2+} ATPase Pmr1 was identified as hypersensitive to osmotic stress.

Western Blot—Total protein extracts were produced by glass bead lysis of yeast cells in buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 15 mM EDTA, 0.1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) with a Mini Beadbeater (Biospec Products). An equal total amount of protein was separated on 8% SDS-PAGE and analyzed by immunoblotting using anti-GFP antibody (Roche Applied Science) or anti-peroxidase-anti-peroxidase (anti-PAP) antibody (Sigma). The bands were visualized with ECL Plus (Amersham Biosciences).

Northern Blot—Total RNA was isolated by acid phenol extraction from YPD-grown yeast cells that were either untreated or subjected to the indicated osmotic stress conditions. Approximately 30 μg of RNA/lane were separated in formaldehyde gels and blotted onto nylon membranes (Hybond N; Amersham Biosciences). Radioactively labeled probes were hybridized in PSE buffer (300 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.2, 7% SDS, 1 mM EDTA) at 65 °C. The probes used were PCR fragments spanning nucleotides 66–740 of *SDH2*, 56–443 of *COX6*, 96–1380 of *CIT1*, and 519–1182 of *ACT1*. Signal intensities were quantified using a Fujifilm BAS-1500 phosphorimaging device.

Succinate Dehydrogenase (SDH) Assay—Mitochondria enriched extracts were prepared in TSB buffer (10 mM Tris/HCl, pH 7.5, 0.6 M sorbitol) according to Ref. 42. SDH assays were performed with *p*-iodonitrotetrazolium violet as an artificial electron acceptor for the SDH complex. Extracts were incubated in 300 μl of succinate buffer (10 mM succinic acid hexahydrate in 50 mM phosphate buffer, pH 7.4) with 100 μl of *p*-iodonitrotetrazolium violet solution (2.5 mg of *p*-iodonitrotetrazolium violet in 50 mM phosphate buffer, pH 7.4). The reactions were stopped with 1 ml of Stop solution (10 g of trichloroacetic acid in 100 ml of ethyl acetate/ethanol (1:1 v/v)), and the absorbance was measured in the supernatant at 490 nm.

β-Galactosidase Assay—Transformed yeast strains were grown selectively until saturation in synthetic dextrose-uracil medium and were then diluted in YPD. Logarithmically growing cells were treated or not with the indicated salts and permeabilized by ethanol/toluene treatment, and β-galactosidase activity was determined as described in Ref. 43.

Quantification of AMP/ATP Pools—7.5 ml of YPD grown yeast culture (OD$_{660}$ of 0.6) was rapidly quenched in 30 ml of 60% methanol-water solution at −20 °C. After centrifugation the pellet was extracted twice by the addition of 1 ml of 100%

methanol. The collected supernatant was vacuum-dried, and the samples were kept at $-20\,°C$ for HPLC analysis. Just before analysis the samples were resuspended in 100 μl of water, filtered through Millipore HV filters (0.45 μm), and injected onto a reversed phase C18 column (LiChrosphere 100, 4×250 mm, 5-μm particle size; Merck)), eluted, and detected as described (44). Detection of nucleotides was performed by HPLC (Waters 600E) coupled to a photo diode array (PDA996). ATP and AMP peaks were identified by co-injection with standards.

ROS Measurements—The yeast cells were grown in YPD to $0.5-0.8 \times 10^7$ cells/ml. Culture aliquots were incubated for 30 min with 2′,7′-dichlorodihydrofluorescein diacetate (Sigma) at a final concentration of 10 μM. The cells were washed with water and resuspended in 1 ml of 50 mM Tris/HCl, pH 7.5. After the addition of 10 μl of chloroform and 5 μl of 0.1% SDS, the cells were extracted by rigorous agitation (Mini Beadbeater). Fluorescence was quantified in the supernatant in a Tecan Genios microplate reader at 492-nm excitation and 525-nm emission wavelengths. For microscopic visualization of ROS production, the cells were incubated with dihydroethidium (Sigma) at a final concentration of 20 μM. The cells were visualized at 518-nm excitation and 605-nm emission wavelengths with a Nikon Eclipse E600 fluorescence microscope.

RESULTS

Defects in Many Mitochondrial Functions Cause Sensitivity to Hyperosmotic Stress—In an attempt to characterize new determinants involved in the tolerance to osmotic stress, we screened a subset of the yeast deletion mutant collection for strains, which showed hypersensitivity to elevated concentrations of NaCl or KCl (see "Experimental Procedures" for details). Because we found a strong bias toward mutants affecting mitochondrial and cytoplasmatic Fe^{2+} and Cu^{2+} transport ("Experimental Procedures"), we wanted to know whether defects in mitochondrial function generally cause osmosensitivity. Therefore we individually tested selected strains with nuclear mutations in mitochondrial functions for their growth under high Na^+ or K^+ conditions. As shown in Fig. 1*A*, deletion mutants in citric acid cycle enzymes (*ACO1* encoding aconitase), ATP synthase subunits (*ATP5, 7*), enzymes of the mitochondrial electron transport chain (*COX6* encoding cytochrome *c* oxidase subunit 6), the mitochondrial RNA polymerase (*RPO41*), or factors involved in mitochondrial DNA replication (*ABF2*) showed a clear hypersensitivity to osmotic stress. All of the mutants were severely affected by high Na^+ concentrations and to a minor extent by high K^+ concentrations. We next addressed the question whether mitochondrial biogenesis was needed for the efficient adaptation to osmostress. We tested a yeast *fzo1* mutant strain, which has impaired mitochondrial fusion activity because of the lack of the mitofusin function (45, 46). We found that the loss of Fzo1 function causes a severe sensitivity to both Na^+ and K^+ stress (Fig. 1*B*). The observed sensitivities are comparable with the osmostress phenotype caused by the loss of the Snf1 kinase (Fig. 1*C*), the key activator of respiratory metabolism in the absence of fermentable carbon sources.
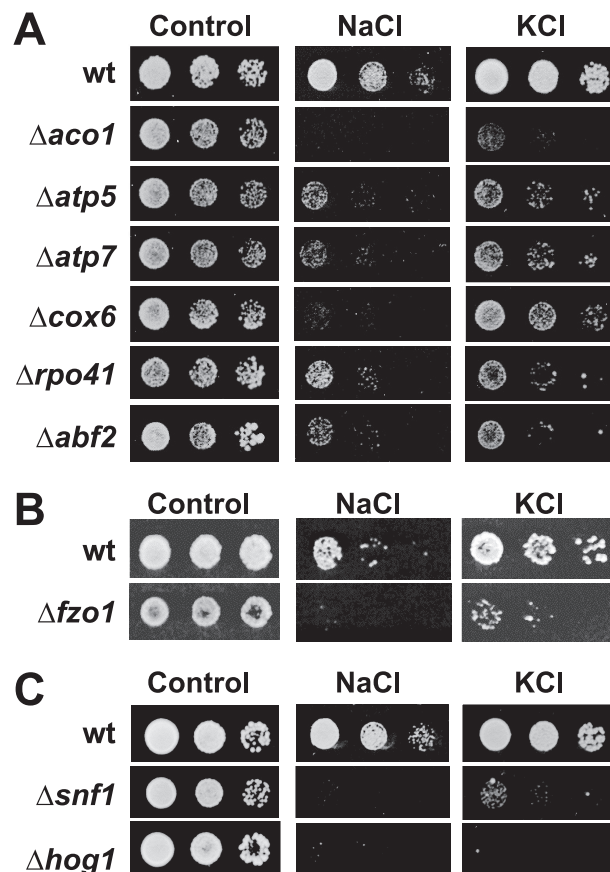
FIGURE 1. **Mutations in many mitochondrial functions lead to osmotic and salt stress sensitivity.** Isogenic yeast strains BY4741 (wild type, *wt*) and deletion mutants *aco1Δ, atp5Δ, atp7Δ, cox6Δ, rpo41Δ, abf2Δ* (*A*); *fzo1Δ* (*B*); and *snf1Δ* and *hog1Δ* (*C*) were spotted onto YPD plates (control) and YPD plates containing 1 M NaCl or 1 M KCl.

Osmotic Stress Causes the Selective Up-regulation of Mitochondrial Proteins—We next addressed the question of whether adaptation to osmostress involved the activation of the mitochondria. We tested by Western blot whether the protein abundance of several GFP-tagged mitochondrial proteins was affected by treatment of the cells with 0.4 M NaCl. As depicted in Fig. 2*A*, the protein levels of mitochondrial succinate dehydrogenase subunit 2 (Sdh2), cytochrome *c* oxidase subunit 6 (Cox6), and mitochondrial citrate synthase (Cit1) were rapidly stimulated upon NaCl shock. Two other mitochondrial components, subunit 5 of the ATP synthase complex (Atp5) and isocitrate dehydrogenase (Idp1), did not show changes along the same stress treatment (Fig. 2*A*). In contrast, the amount of all five mitochondrial proteins was stimulated when the cells were shifted from fermentative (glucose) to respiratory (ethanol) growth (Fig. 2*B*). To visualize protein changes in the early phase of osmotic induction, we employed yeast strains expressing TAP-tagged Sdh1, Sdh2, or Atp5. The more sensitive immunological detection of the TAP fusion proteins revealed that an increase of succinate dehydrogenase can be observed already at 30 min of osmotic treatment (Fig. 2*C*). Taken together, our data suggested that NaCl stress caused a partial and selective up-regulation of mitochondrial proteins, which occurs rapidly after the osmotic insult.
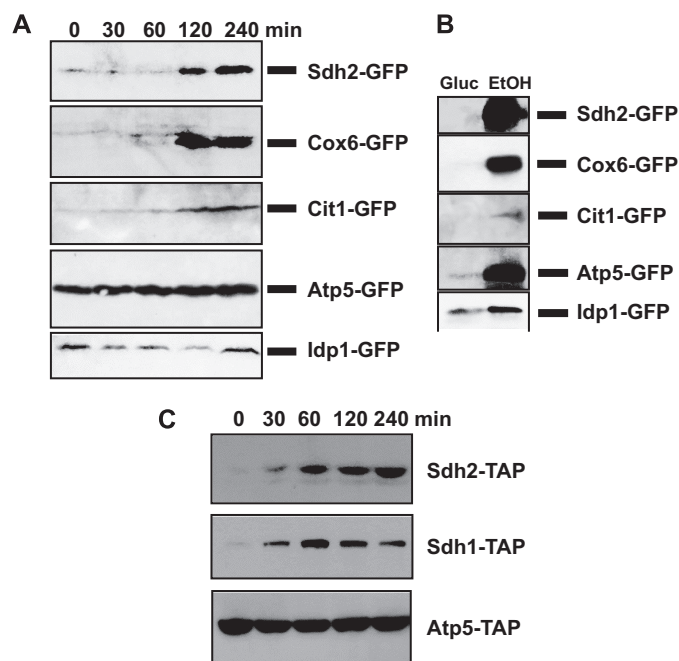
FIGURE 2. **The abundance of the mitochondrial proteins Sdh1, Sdh2, Cox6, and Cit1 is stimulated upon salt stress.** *A*, yeast strains expressing the indicated C-terminal GFP fusion proteins under control of their endogenous promoter were treated with 0.4 M NaCl for the indicated times in YPD medium. Immunoblot analysis was performed on equal amounts of total protein extract using anti-GFP antibody. *B*, the same yeast strains were grown in YPD (2% dextrose) or YPEtOH (3% ethanol) to mid-log phase, and immunodetection was performed as described above. *C*, yeast strains expressing the indicated C-terminal TAP fusion proteins under control of their natural promoter were treated as in *A*. TAP fusion proteins were detected using anti-peroxidase-anti-peroxidase (anti-PAP) antibody.

Transcriptional Control of SDH2, CIT1, and COX6 upon NaCl Stress—We next tested whether the induction of the mitochondrial Sdh2, Cox6, and Cit1 proteins was the consequence of the transcriptional activation of the corresponding nuclear genes. We quantified the expression levels of all three genes by Northern blot before and during the brief exposure to NaCl stress. We found that *SDH2*, *COX6*, and *CIT1* were rapidly and transiently activated by 0.4 M NaCl treatment in wild type cells (Fig. 3). The expression levels increased 5–12-fold in the first 10 min of the osmoshock. We next assayed for the participation of several signaling kinases and specific transcription factors in this transcriptional activation. Deletion of the Hog1 MAPK or the Snf1 kinase completely abolished the stress-activated transcription of *COX6* and partially diminished the induction of *SDH2* and *CIT1* (Fig. 3). The transcriptional activator complexes Rtg1/3 and Hap2/3/4/5 are known to stimulate transcription of genes encoding mitochondrial functions upon dysfunction of the organelle or glucose limitation, respectively. We tested whether *rtg1,3* or *hap2,3* mutants displayed an altered transcriptional response of *SDH2*, *CIT1*, and *COX6* upon NaCl stress. As shown in Fig. 3, mutations in the Hap2/3/4 complex resulted in a complete loss of *COX6* induction, whereas *CIT1* and *SDH2* expression upon salt shock was only very slightly reduced. The loss of Rtg1/3 function mainly affected the NaCl induced expression of *CIT1*, which was >2-fold reduced and much more transient as compared with wild type. *SDH2* or *COX6* transcription upon salt shock appeared to be largely independent on Rtg1/3. Taken together,

we show that a rapid transcriptional activation of mitochondrial functions occurs in response to hyperosmotic shock and that increased expression depends on multiple signaling kinases (Snf1 and Hog1) and specific transcriptional activators (Hap2/3/4 and Rtg1/3).

Regulation of Mitochondrial SDH Activity in Response to Salt Stress—Having seen that components of the citric acid cycle and the mitochondrial respiratory chain are immediately upregulated at the transcriptional level, we aimed at quantifying mitochondrial enzyme activity in response to osmotic stress. We measured succinate dehydrogenase activity in yeast wild type cells before and after osmoshock. A brief treatment with 0.4 M NaCl caused a 2.5-fold increase in SDH activity in wild type cells (Fig. 4). We compared this induction with several regulatory mutants. The loss of Hog1 MAPK function did not alter the induction of SDH activity. However, in the absence of Snf1, the induction of SDH upon salt shock was completely absent. A significant reduction of SDH stimulation was observed in *rtg1* and *rtg3* mutants, whereas mutants in the Hap2/3/4 complex showed levels of SDH induction comparable with the wild type (Fig. 4).

The Transcriptional Response to Osmostress in Mutants with Mitochondrial Defects—We have characterized the induction of mitochondrial functions as a component of the adaptive response to hyperosmotic stress. We next wanted to identify possible protective functions of the mitochondria that would be necessary to properly adapt to the stress. Because transcriptional activation of many defense genes is one of the major physiological responses of the yeast cell to osmoshock, we addressed the question of whether transcriptional stimulation was impaired by the loss of mitochondrial function. To monitor activated transcription during exposure to osmostress in a sensitive manner, we employed a highly osmoresponsive *lacZ* reporter gene (for details see "Experimental Procedures"). Exposure of yeast wild type cells to 1 M KCl leads to a rapid induction of *lacZ* expression, which reaches its maximal level within 60 min (Fig. 5). When we tested various mutants in mitochondrial functions, which cause strong hypersensitivity to osmotic stress (Fig. 1), we observed a slight delay of the induction profile as compared with wild type (Fig. 5, *30-min time point*). However, all strains tested were able to reach very similar fully induced expression levels after 60 min of KCl treatment. We performed a second set of expression analysis under more severe conditions using 1 M NaCl. The higher toxicity of Na⁺ as compared with K⁺ ions results in a slower induction profile of the *lacZ* reporter. Maximal induction occurs in wild type cells at 120 min (Fig. 5). Again, the strains with various mitochondrial defects revealed a slight delay in β-galactosidase induction (Fig. 5, *60-* and *120-min time points*) but were not defective for high expression levels at longer time points. We conclude that mitochondrial function might be necessary for the efficient transcriptional response to osmostress. However, a defect in transcriptional activation caused by the lack of mitochondrial function cannot explain the severe growth defect under hyperosmotic stress.

AMP/ATP Ratios in Mutants with Mitochondrial Defects upon Osmotic Shock—We next addressed the question of whether mitochondrial function was essential for the energy or
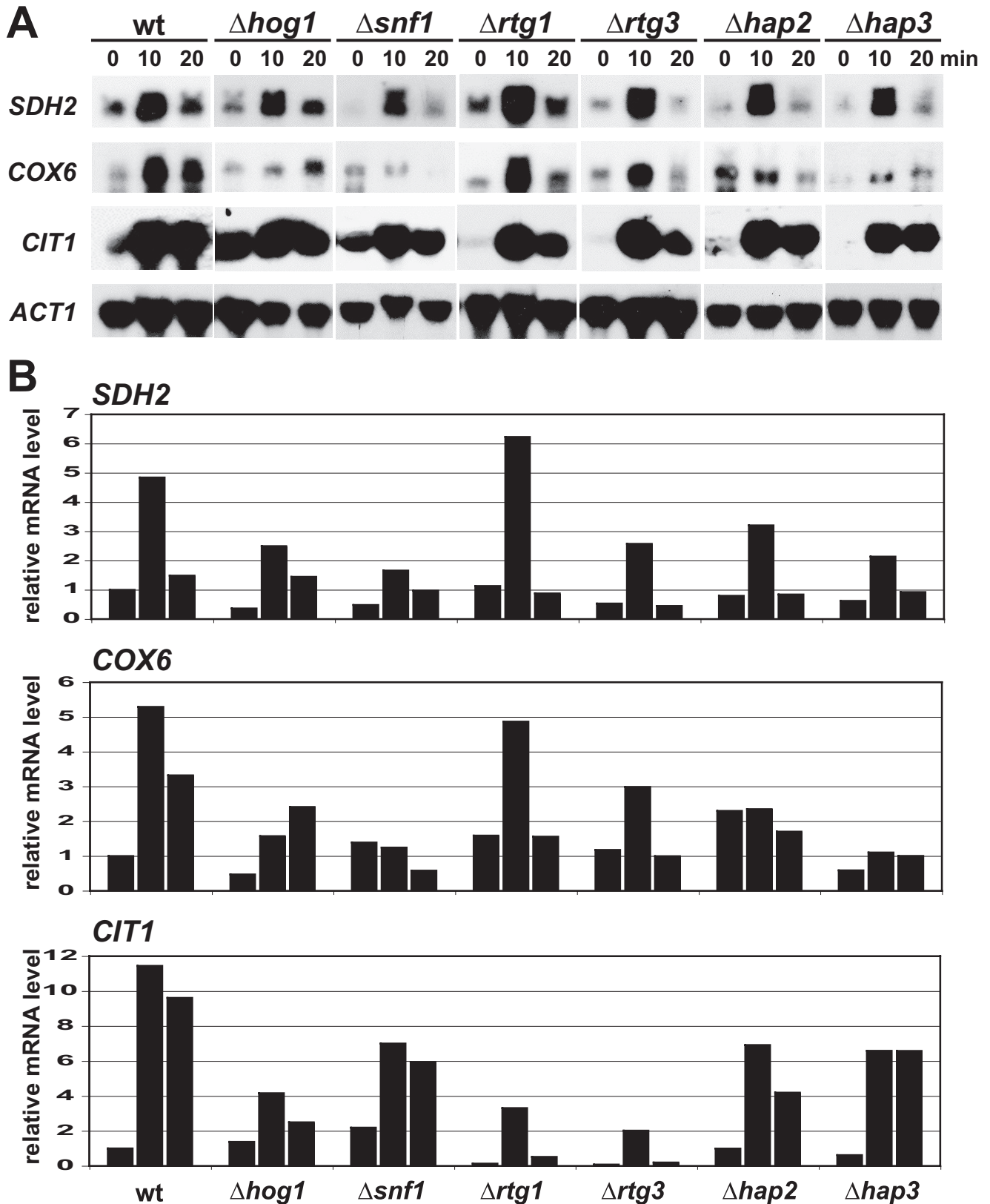
FIGURE 3. **Transcriptional regulation of *SDH2*, *COX6*, and *CIT1* in response to salt stress.** Yeast wild type (BY4741, *wt*) and the indicated isogenic deletion strains were treated for the indicated times with 0.4 M NaCl. *A*, transcript levels of the indicated genes were monitored by Northern blot. *B*, the mRNA levels were quantified, normalized for the *ACT1* loading control, and depicted in the graphs. The value obtained for the uninduced mRNA level in the wild type was arbitrarily set to 1 for each gene. The results shown come from the same original blot, which was probed with the indicated genes.
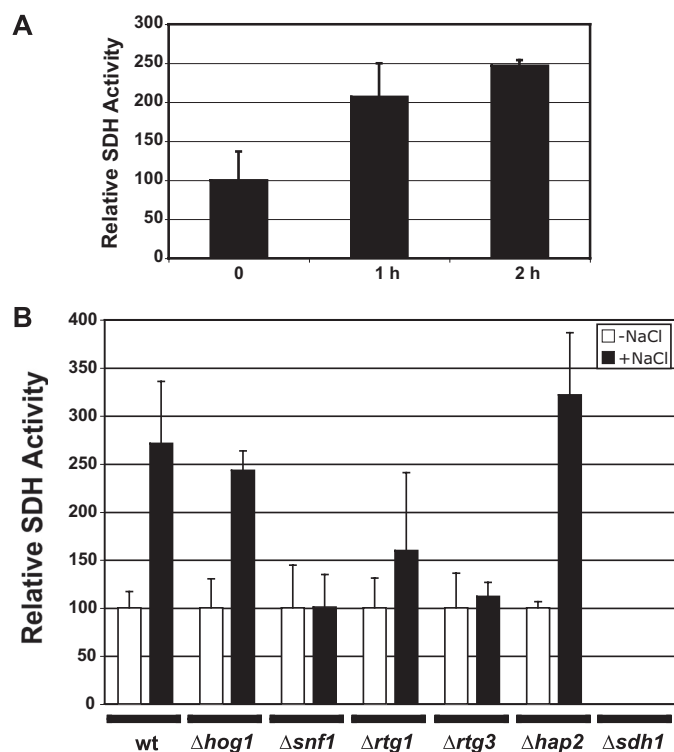
FIGURE 4. **SDH enzyme activity is activated by salt stress.** *A*, yeast wild type (BY4741) cells were grown in YPD (0) or subjected to a salt shock (0.4 M NaCl) for 1 and 2 h. The specific SDH enzyme activity was measured in mitochondria enriched extracts, and basal SDH activity was arbitrarily set to 100 for each strain. SDH activity was measured in duplicate in three independent cultures. *B*, yeast wild type (BY4741, *wt*) and the indicated deletion strains were grown in YPD (−*NaCl*) or subjected to a brief salt shock (0.4 M NaCl for 2 h). SDH activity was measured as in *A*.

ATP supply during osmostress. We therefore quantified the intracellular ATP and AMP levels by HPLC before and during osmotic stress (1 M NaCl) and compared wild type with mutants in mitochondrial enzymes (*aco1*, *cox6*, and *sdh1*), mitochondrial biogenesis (*fzo1*) and regulation (*snf1*). As shown in Fig. 6, yeast wild type cells are able to maintain low AMP to ATP ratios even under acute osmotic stress caused by 1 M NaCl. No difference was observed for the *aco1* and *cox6* mutant strains. Slightly elevated AMP/ATP ratios were detected in *snf1* and *fzo1* mutants, whereas the *sdh1* deletion strain showed the highest AMP/ATP ratio comparable with wild type cells in stationary growth phase. In none of the cases, however, was a depletion of ATP observed, and the severe osmosensitivity of the mutants in mitochondrial functions was not generally accompanied by increases in the AMP/ATP ratios.

The Growth Defect on Hyperosmotic Media of Mutants Involved in Mitochondrial Functions Is Rescued by Antioxidant Supplementation—One of the growth inhibitory effects of hyperosmotic stress is the formation of ROS. In an attempt to define the role of ROS formation in the protective function of the mitochondria under hyperosmotic growth conditions, we investigated the effect of antioxidant supplementation on the growth kinetics under osmostress of mutants with impaired mitochondrial function. As shown in Fig. 7, mutants in mitochondrial metabolism (*aco1*, *sdh1*), biogenesis (*fzo1*), or regulation (*snf1*) showed a marked delay and lower growth efficiency under high salt conditions. Mutants in the mitochon-

drial ATP synthase complex (*atp7*; Fig. 7) showed a more subtle growth defect in this continuous liquid culture assay. The addition of glutathione to the growth medium significantly improved growth of all of the mitochondrial mutants tested, almost exclusively by shortening the lag phase in response to the salt shock.

Defects in Mitochondrial Functions Increase ROS Production upon Osmotic Stress—Our supplementation experiments with antioxidants indicated that ROS detoxification was an important determinant of the adaptation to hyperosmotic stress and that it was deficient in mutants with impaired mitochondrial function. We next measured directly the levels of ROS in different mitochondrial mutant strains compared with wild type cells along the osmotic shock. We first quantified ROS by the fluorescence generated by the oxidation of dichlorodihydrofluorescein to dichlorofluorescein. Salt stress caused by 1 M NaCl resulted in an ~2-fold increase in intracellular ROS levels in wild type cells (Fig. 8A). In *aco1*, *sdh1*, and *fzo1* mutants, which are defective for mitochondrial energy metabolism and biogenesis, respectively, ROS levels were about 2-fold increased under normal growth conditions and 3–5-fold increased under salt stress conditions as compared with wild type (Fig. 8A). Additionally, the regulatory mutant *snf1* showed moderately elevated ROS levels under both normal and salt stress conditions (Fig. 8A). We finally visualized ROS production in living cells by the oxidation of hydroethidine to ethidium by fluorescence microscopy. We confirm that *snf1*, *aco1*, and *fzo1* mutants display an overproduction of ROS upon exposure to salt stress (Fig. 8B).

Growth in Nonfermentable Carbon Sources Prepares Yeast Cells for Salt Stress Adaptation—We have shown that activation of mitochondrial function is an important feature of salt stress adaptation. We reasoned that a preactivation of mitochondria prior to the salt shock could be advantageous to withstand the stress. We therefore compared growth on high salinity plates (1 M NaCl or 1.5 M KCl) of yeast wild type cells that were grown to exponential phase with glucose (repression of mitochondrial metabolism) or galactose, glycerol, or ethanol (partially or fully activating mitochondrial metabolism) as the sole carbon source. As shown in Fig. 9, the preculture in either galactose, glycerol, or ethanol conferred salt stress resistance over the glucose grown yeast cells.

DISCUSSION

Here we investigate the regulation and function of mitochondria in the adaptive response of budding yeast to hyperosmotic stress. We define a positive role for the mitochondria in the osmostress adaptation based on the sensitivity phenotype of mutants in diverse mitochondrial functions, the selective up-regulation of mitochondrial proteins, and the stimulated transcription of genes encoding mitochondrial enzymes upon exposure to hyperosmotic conditions. To understand osmostress tolerance, mitochondrial metabolism has therefore to be considered as an important physiological parameter that is modulated in a stress-dependent fashion.

Activation of Mitochondrial Functions as a Strategy for Osmostress Adaptation—A broad range of mitochondrial defects leads to sensitivity to hyperosmotic stress. This phe-
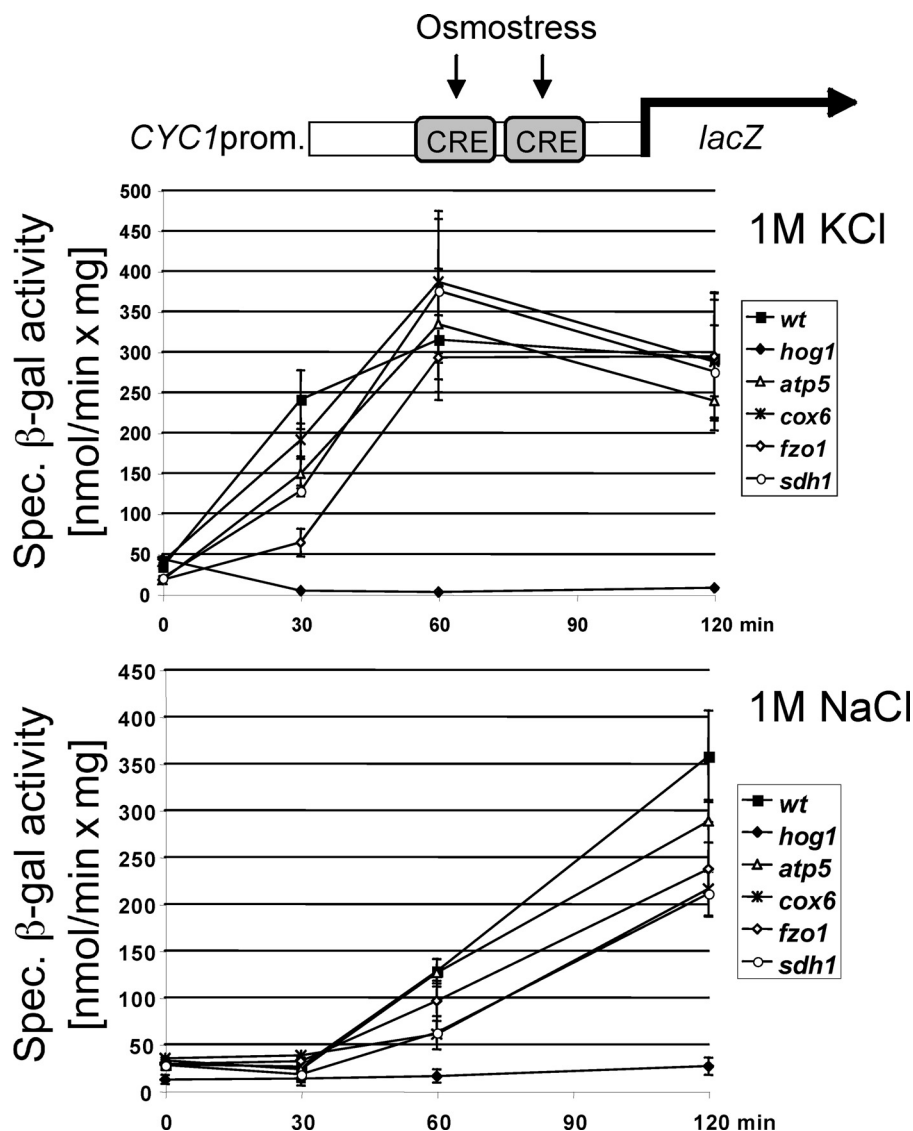
FIGURE 5. **The transcriptional response to osmoshock is only minimally affected in mitochondrial mutants.** A reporter gene (*CYC1-(2xCRE)-lacZ*; pMP224) was used to monitor transcriptional induction by KCl or NaCl treatment. Yeast wild type (BY4741) and the indicated null mutant strains, transformed with pMP224, were subjected to osmotic stress caused by 1 M KCl or 1 M NaCl, and the specific β-galactosidase activity was determined. The values were obtained by duplicate measurements of three independent transformants.

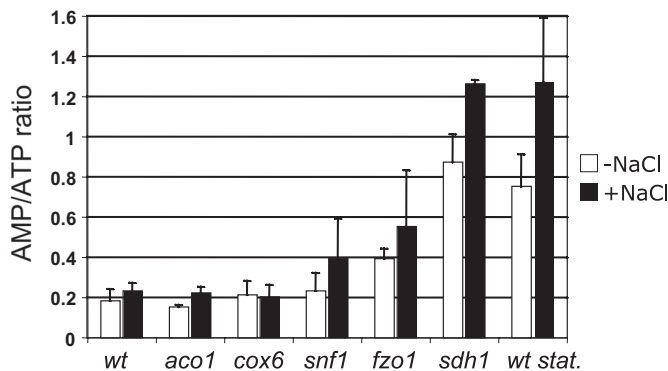FIGURE 6. **AMP/ATP ratios in yeast wild type and mutants with mitochondrial defects.** Yeast wild type (BY4741, *wt*) and the indicated mutant strains were grown in YPD and subjected or not to a brief osmotic shock with 1 M NaCl for 2 h. The cells were immediately quenched, and the extracted nucleotides were analyzed by HPLC. The AMP/ATP ratio was calculated for each strain in triplicate. A stationary wild type yeast culture (*wt stat.*) was grown for 2 days in YPD medium.

nomenon is not restricted to specific enzymatic activities like citric acid cycle enzymes, proteins involved in the mitochondrial electron transport chain, or ATP synthase subunits (Fig. 1*A*) but also applies for a defect in mitochondrial biogenesis caused by the deletion of the yeast mitofusin Fzo1 (Fig. 1*B*). These phenotypes suggest that yeast cells employ an up-regulation of the organelle in general to efficiently cope with hyperosmolarity. This activation has an important transcriptional component because we find genes like *SDH2*, *COX6*, or *CIT1* to be induced by acute osmo-shock. Transcriptional activation (5–12-fold) occurs rapidly upon mild osmostress (0.4 M NaCl), and its transient kinetics resembles the one typically found for other stress defense genes (10, 11). Thus mitochondrial activation at the level of the expression of nuclear genes is targeted immediately during the adaptation to hyperosmotic insult. However, mitochondrial function has not been identified as a functional category by several transcription profiling experiments performed under the same osmotic stress conditions (10, 11, 47). This might be due to the moderate induction rate observed for osmostress for the mitochondrial markers shown here. Also, the fully induced mitochondrial protein levels observed for nonfermentative growth are significantly higher than for salt induction (Fig. 2, *A* and *B*).

This leads to the conclusion that although necessary for the proper adaptation to the stress, the up-regulation of mitochondria upon osmotic stress is weaker as compared with its massive activation during the diauxic shift. This interpretation is confirmed by our unpublished observation that mitochondrial morphology only moderately changes upon acute or chronic salt stress, whereas a much more extensive tubular network is found in cells growing with a nonfermentable carbon source.

Here we have tested the function of two signaling kinases, Hog1 and Snf1, for osmostress-activated transcription of genes encoding mitochondrial functions. Hog1 MAPK is the dominant regulator of the yeast transcriptional osmostress program (2), and Snf1 is the master activator of cellular functions needed for the switch from fermentative to respiratory metabolism (19, 48). Both kinases contribute to the activated transcription of the *SDH2*, *COX6*, and *CIT1* genes upon osmoshock (Fig. 3). Specifically, the activated transcription
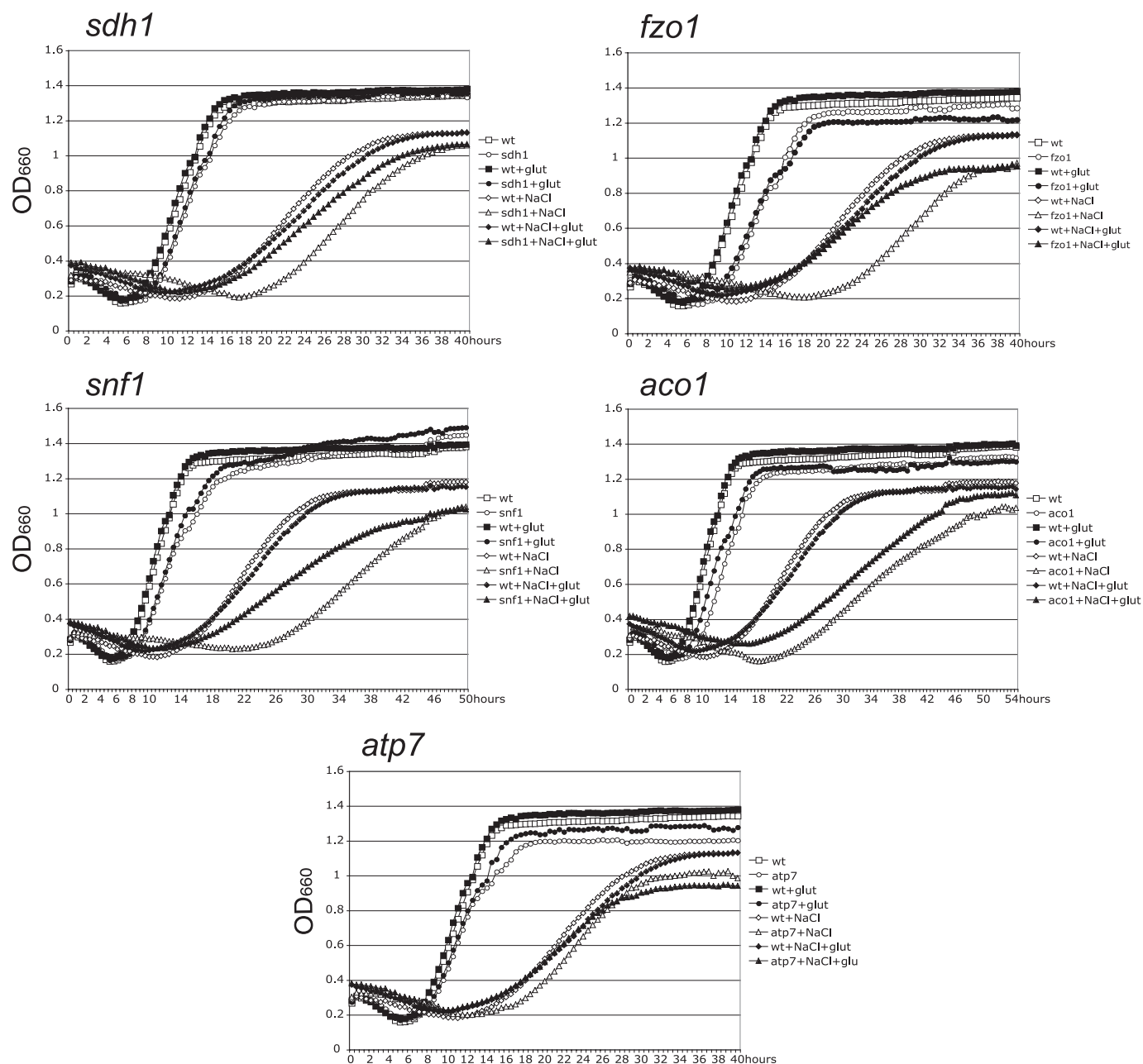
FIGURE 7. **Growth in high salinity of mutants with mitochondrial defects is rescued by addition of glutathione.** The growth of the indicated wild type (BY4741, *wt*) and mutant strains was monitored. The cells were grown in YPD, YPD + 5 mM glutathione, YPD + 1 M NaCl, or YPD + 5 mM glutathione + 1 M NaCl. The growth curves are derived from the mean values of three independent cultures for each time point.

of *COX6* was completely absent in *snf1*, *hap2*, and *hap3* mutants. *COX6* is known to be positively regulated by Snf1 and the Hap2,3,4,5 transcriptional activator upon glucose starvation (49, 50), which suggests that similarly upon osmostress the Snf1 kinase activates *COX6* expression via the Hap2,3,4,5 transcription factor complex.

The Snf1 Protein Kinase Is a Potential Functional Link between Mitochondrial Up-regulation and Salt Stress Resistance—Here we present evidence for a much broader implication of the Snf1 kinase in the adaptation to salt stress via the activation of mitochondrial metabolism. Deletion of Snf1 results in reduced resistance to elevated Na^+ and K^+ concentrations (Fig. 1), impaired transcriptional control of mitochondrial markers

(Fig. 3), and absent activation of mitochondrial SDH enzyme activity upon salt stress (Fig. 4). The sensitivity of *snf1* mutants to salt stress has been previously reported (25, 51). However, the only functional link between Snf1 activity and the adaptive response to salt stress has been identified with the carbon source-regulated *ENA1* gene (25, 52, 53), which encodes a Na^+ extrusion ATPase of yeast (54). We propose that a major contribution of Snf1 to salt stress adaptation is the activation of mitochondrial function. It has been shown that salt stress indeed rapidly activates the catalytic activity of Snf1 but does not promote its accumulation in the nucleus (55, 56). The role of Snf1 in transcriptional activation upon salt stress might therefore be less pronounced and not reflected by a massive

A

B
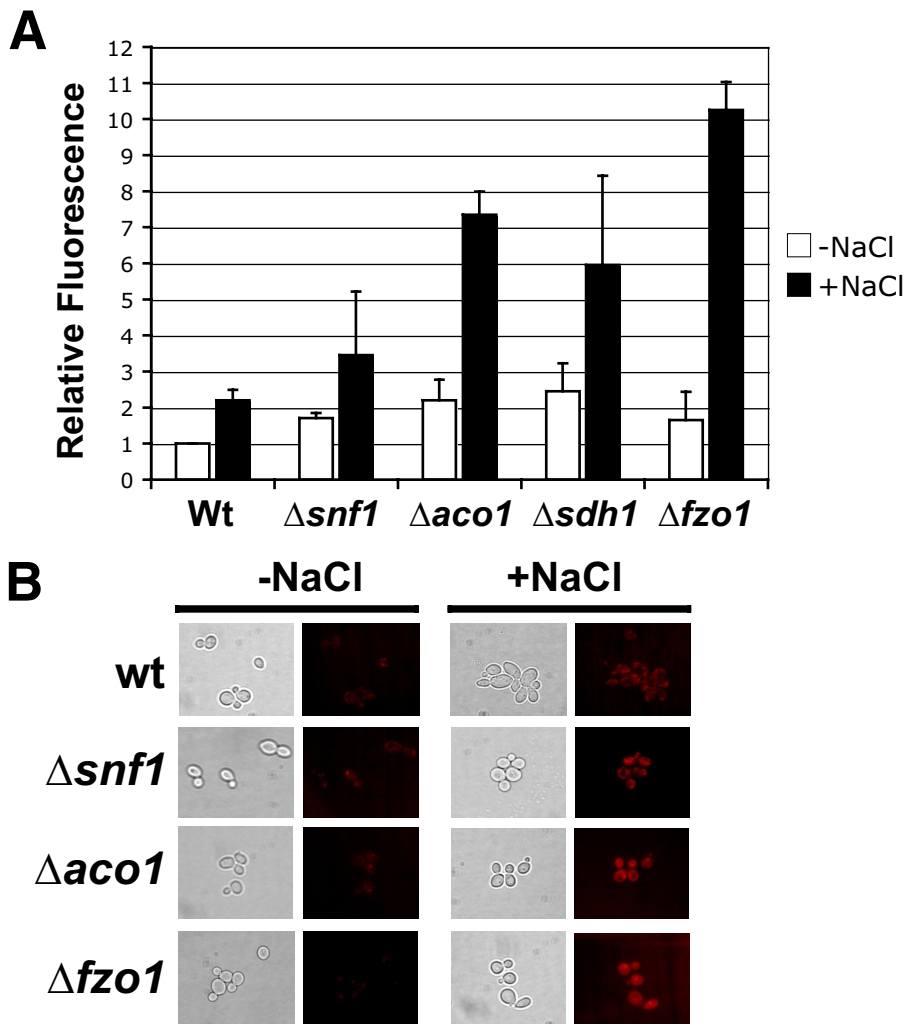
FIGURE 8. **Mitochondrial defects and loss of Snf1 function cause hyperaccumulation of ROS upon salt stress.** Yeast wild type (BY4741, *Wt*) and isogenic Δ*snf1*, Δ*aco1*, Δ*sdh1*, and Δ*fzo1* strains were grown in YPD and subjected or not to a brief osmotic shock with 1 M NaCl for 2 h. *A*, ROS was quantified by the oxidation of dichlorodihydrofluorescein as described under "Experimental Procedures." Fluorescence of the wild type before stress was arbitrarily set to 1. Fluorescence was determined for three independent cultures in duplicate. *B*, ROS was visualized by the oxidation of hydroethidine using fluorescence microscopy as indicated under "Experimental Procedures." *wt*, wild type.
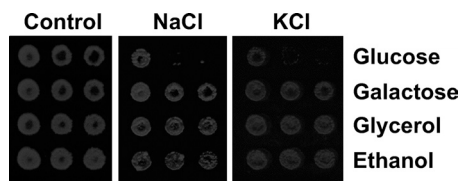
FIGURE 9. **Growth with galactose or nonfermentable carbon sources makes yeast cells more resistant to osmotic stress.** Wild type (BY4741) cells were pregrown in YPD (2% glucose), YPGal (2% galactose), YPGlyc (3% glycerol), or YPE (3% ethanol) to mid log growth phase and then spotted onto YPD plates (control) and YPD plates containing 1 M NaCl or 1.5 M KCl.

nuclear accumulation as observed under nutrient starvation (57).

Mitochondria Do Not Serve as ATP Supply under Osmostress—We further tested whether a higher ATP demand during the adaptation to salt stress was the reason for the partial up-regulation of the mitochondria. Specifically, the involvement of the Snf1 kinase, which is activated generally by nutrient and energy depletion, prompted us to test this hypothesis. Salt stress does not appear to increase the cellular AMP/ATP ratio in wild type yeast (Fig. 6). Also *cox6* and *aco1* mutants, which display severe growth defects on high salt media, maintain very low AMP/ATP ratios identical to wild type. Although other defects linked to mitochondrial function or activation, like *sdh1*, *fzo1*, or *snf1* mutants, seem to partially increase the AMP/ATP ratio, these results exclude ATP depletion as the driving force for the observed mitochondrial stimulation upon salt stress. Consistent with this interpretation, it was found that yeast cells actually adapt more quickly to salt stress under anaerobic conditions, *i.e.* in the absence of ATP generation via oxidative phosphorylation (58). Accordingly we find that the efficiency of transcriptional activation upon salt stress, one of the major energy consuming processes instigated by salt stress, is only minimally affected by mitochondrial defects. We additionally found that intracellular accumulation of glycerol, the main osmolyte produced by budding yeast in response to salt shock, is not affected by mitochondrial defects.[3]

Amino acid starvation is another potential link between salt stress and mitochondrial function. Mitochondrial metabolism is essential for the biosynthesis of the majority of amino acids, and amino acid starvation can be caused by salt stress (59–61). Specifically, the supplementation with an excess of amino acids improves the adaptation of yeast cells to saline stress (60). However, external feeding of amino acids to mitochondria-deficient strains did not rescue the salt-sensitive growth.[3]

Mitochondrial Function Is Necessary to Keep Low Intracellular ROS Levels upon Salt Stress—Here we present experimental evidence that supports a model in which mitochondrial activation is needed to counteract ROS production upon salt stress. External addition of the antioxidant glutathione generally rescues the growth defect of mitochondrial mutants in the presence of high NaCl concentrations (Fig. 7), and mitochondrial deficiency results in higher intracellular ROS concentrations, especially upon exposure to Na^{+} shock (Fig. 8). Although it is well documented that saline and osmotic stress cause increases in toxic ROS production (62) and transcriptional activation of

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[3] M. Martínez Pastor, M. Proft, and A. Pascual-Ahuir, unpublished results.

oxidative stress defense genes (10, 11, 63, 64), it is not known how ROS is formed under those conditions. Electron leakage from the mitochondrial electron transport chain upon salt challenge is one candidate but most probably not the only explanation (34). Mitochondrial dysfunction can result in both an increase and a decrease in ROS production (reviewed in Ref. 33). In *S. cerevisiae*, mitochondrial function is generally required for oxidative stress resistance (36), and a very recent report shows that in *Schizosaccharomyces pombe* mitochondrial dysfunction causes intrinsic oxidative stress (65). On the other hand, although mitochondrial respiration is the major source of ROS under physiological conditions (66, 67), a controlled stimulation of the mitochondrial respiratory capacity by calorie restriction or mild uncouplers has been found to promote decreases in ROS and increases in life span both in yeast and in human fibroblasts (68, 69). Thus it seems likely that in *S. cerevisiae* signaling pathways that operate upon salt stress (high osmolarity glycerol and glucose repression pathways) stimulate mitochondrial function. The enhanced respiratory capacity is needed to more efficiently eliminate ROS after the exposure to saline stress. This is of special relevance in *S. cerevisiae* where mitochondrial biogenesis and metabolism are generally repressed by fermentative carbon sources.

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