

A novel NADH kinase is the mitochondrial source of NADPH in *Saccharomyces cerevisiae*

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Mitochondria require NADPH for anti-oxidant protection and for specific biosynthetic pathways. However, the sources of mitochondrial NADPH and the mechanisms of maintaining mitochondrial redox balance are not well understood. We show here that in *Saccharomyces cerevisiae*, mitochondrial NADPH is largely provided by the product of the *POS5* gene. We identified *POS5* in a *S. cerevisiae* genetic screen for hyperoxia-sensitive mutants, or cells that cannot survive in 100% oxygen. *POS5* encodes a protein that is homologous to NAD⁺ and NADH kinases, and we show here that recombinant Pos5p has NADH kinase activity. Pos5p is localized to the mitochondrial matrix of yeast and appears to be important for several NADPH-requiring processes in the mitochondria, including resistance to a broad range of oxidative stress conditions, arginine biosynthesis and mitochondrial iron homeostasis. Pos5p represents the first member of the NAD(H) kinase family that has been identified as an important anti-oxidant factor and key source of the cellular reductant NADPH.

Keywords: iron homeostasis/hyperoxia/mitochondria/NADPH/reactive oxygen species

Introduction

As a consequence of oxidative metabolism, aerobic organisms are continuously bombarded with reactive oxygen species (ROS), such as superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot\text{OH}$), which can damage DNA, proteins and lipids. Most of these highly reactive molecules are generated in the mitochondria as by-products from the reduction of O_2 to H_2O during oxidative phosphorylation (Boveris and Cadenas, 1982). Cells can also be exposed to exogenous sources of oxidative stress such as hyperoxia.

Hyperoxia is a state in which individual cells or organisms are exposed to oxygen concentrations well above that of atmospheric oxygen. In laboratory animals, hyperoxia exposure (>90% O_2) leads to death within 3–7 days (O'Reilly, 2001). Specific hyperoxia-induced disorders in humans include bronchopulmonary dysplasia (Northway *et al.*, 1967) and adult respiratory distress syndrome (Halliwell *et al.*, 1992). In spite of the importance of hyperoxia in health-related issues, little is known regarding the mechanisms by which high concentrations of oxygen can cause damage or the defense systems that guard against hyperoxia damage.

All cells possess numerous anti-oxidant defense systems. Included in this list are the superoxide dismutase enzymes, which disproportionate $\cdot\text{O}_2^-$ into H_2O_2 and O_2 , and peroxidases, which catalyze the reduction of hydroperoxides. Non-enzymatic defense systems include the tripeptide glutathione (GSH) and the small protein thioredoxin (TRX), which can serve either as reductants themselves or as cofactors for anti-oxidant enzymes such as GSH peroxidases, glutaredoxins, TRX peroxidases and methionine sulfoxide reductases (Jamieson, 1998; Carmel-Harel and Storz, 2000; Weissbach *et al.*, 2002). All of these enzymes rely on the reduced forms of GSH and TRX, which are regenerated through the action of NADPH-requiring GSH and TRX reductases. As such, NADPH lies at the heart of many anti-oxidant defenses of the cell.

In the cytosol, NADPH is provided primarily by enzymes in the pentose phosphate pathway, including glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which catalyze the reduction of NADP⁺. Accordingly, these enzymes have been shown to play an important role in protection from oxidative stress in both yeast and mammals (Pandolfi *et al.*, 1995; Juhnke *et al.*, 1996; Slekar *et al.*, 1996). The sources of NADPH in the mitochondria, however, where the bulk of ROS are generated, are less clear. In mammalian cells, mitochondrial NADP⁺-dependent isocitrate dehydrogenase (NADP-IDHm) has been reported to be an important source of mitochondrial NADPH (Jo *et al.*, 2001). However, a deletion of the corresponding gene in the bakers' yeast *Saccharomyces cerevisiae* (*IDP1*) had no effect on oxidative stress sensitivity or cell growth (Minard *et al.*, 1998). Therefore, NADP-IDHm cannot be the only means by which eukaryotic mitochondria produce NADPH. The other sources have not yet been identified.

In this study, we provide evidence that in bakers' yeast, the *POS5* gene product is a major source of mitochondrial NADPH. *Saccharomyces cerevisiae POS5* was identified in a screen for yeast genes that protect against hyperoxia damage. By sequence analysis, the *POS5* gene encodes a member of the NAD(H) kinase family. We demonstrate that Pos5p has NADH kinase activity and localizes to the yeast mitochondrial matrix, where it appears to provide the NADPH needed for oxidative stress protection and for specific mitochondrial biogenesis reactions. This is the first demonstration of an NAD(H) kinase acting as a key source of NADPH.

Results

The pos5Δ mutant is sensitive to several types of oxidative stress

In order to identify anti-oxidant factors that provide protection against hyperoxia-related damage, we devel-

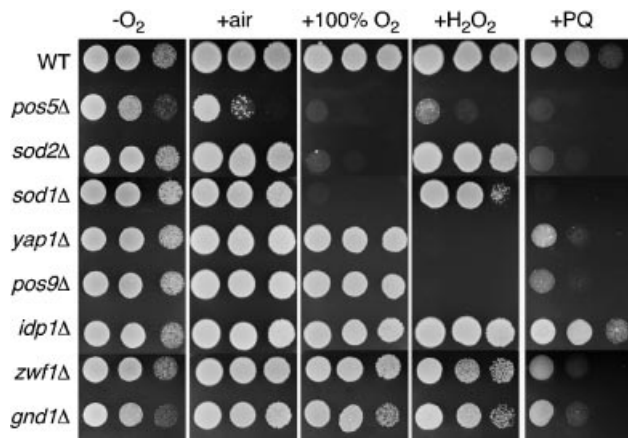


Fig. 1. Comparison of oxidative stress sensitivity of *pos5Δ* and other mutants with anti-oxidant function. The indicated yeast strains were tested for growth by plating 5 μ l of solution at 2.0, 0.2 and 0.02 OD₆₀₀ units onto YPD plates. $-O_2$ = growth in anaerobic culture jars; +air = aerobic growth; +100% O_2 = growth in chambers flushed with 100% O_2 ; + H_2O_2 = aerobic growth on YPD plates containing 2 mM H_2O_2 ; +PQ = aerobic growth on YPD plates containing 1 mM paraquat. Strains utilized: wild-type, BY4741; *pos5Δ*, CO205; *sod1Δ*, LJ284; *sod2Δ*, *yap1Δ*, *pos9Δ*, *idp1Δ*, *zwf1Δ* and *gnd1Δ* were obtained from ResGen as *kanMX4* deletions in parental strain BY4741.

oped a genetic screen for yeast mutants that are sensitive to high oxygen conditions. The Research Genetics BY4741 haploid knockout collection was screened for mutants that fail to grow under hyperoxia (100% O_2) conditions, but grow well in an oxygen-depleted environment. One of the hyperoxia-sensitive mutants identified in this screen was *pos5Δ*. To confirm that *POS5* alone was responsible, we engineered a *pos5Δ* gene deletion in strain BY4741 and found that the resultant mutant is likewise hyperoxia sensitive (Figure 1). Along with high oxygen, the *pos5Δ* mutant is also sensitive to H_2O_2 (as has been shown previously; Krems *et al.*, 1995) and paraquat, a superoxide-generating agent (Figure 1). Two other mutants isolated from the hyperoxia sensitivity screen were *sod1Δ* and *sod2Δ*, encoding the two superoxide dismutases in yeast. As shown in Figure 1, the *sod* mutants show sensitivity to hyperoxia and paraquat, but are not markedly sensitive to H_2O_2 . We also tested deletion mutants for the two principal oxidative stress transcription factors in yeast, Yap1p and Pos9p/Skn7p, which control induction of the oxidative stress response (Lee *et al.*, 1999). These mutants show hypersensitivity to H_2O_2 and paraquat, but not to hyperoxia. The strong sensitivity of *pos5Δ* mutants to all three oxidative stress conditions appears to be unique.

Pos5p is a mitochondrial matrix protein required for proper mitochondrial function

The function of *S.cerevisiae* Pos5p has not been determined previously. However, the mutant grows poorly on glycerol (Figure 2A), as has been reported previously (Dimmer *et al.*, 2002), suggesting a role in mitochondrial function. In order to determine the subcellular localization of Pos5p, a Pos5-green fluorescent protein (GFP) expression plasmid was constructed with GFP fused to the C-terminus of Pos5p. This fusion protein, under the control of the *POS5* promoter, is functional since the plasmid fully complements both the hyperoxia sensitivity and glycerol

growth defects of the *pos5Δ* strain (Figure 2A). The fact that the plasmid complements the slow growth on glycerol of *pos5Δ* mutants suggests that the apparent mitochondrial defect does not result from secondary mitochondrial DNA mutations. However, when the experiment is repeated with older stocks of *pos5Δ* mutants that have undergone several generations, only the hyperoxia, but not poor growth on glycerol, is complemented by episomal *POS5* (data not shown). This suggests that the *pos5Δ* yeast mutant does have the propensity to accumulate mitochondrial DNA mutations over time.

Cells expressing Pos5-GFP were stained with 4',6-diamidino-2-phenylindole (DAPI) and examined by fluorescence microscopy. DAPI staining for DNA highlights both the mitochondria (string-like structures) and the nucleus (large, rounded structure) as exemplified in Figure 2B. Pos5-GFP specifically co-localized with DAPI staining of the mitochondrial, but not nuclear DNA, indicating that Pos5p is localized to the mitochondria (Figure 2B). To confirm this result, cellular fractionation experiments were conducted in which crude mitochondria were isolated from cells expressing Pos5-GFP, followed by western blotting with an anti-GFP antibody. As seen in Figure 2C, Pos5-GFP co-localized with mitochondria and was not detected in the post-mitochondrial supernatant (PMS), largely cytosolic fraction. Furthermore, additional separation of the mitochondria into intermembrane space (IMS) and matrix fractions indicated that Pos5p is targeted specifically to the mitochondrial matrix as determined by co-localization with the mitochondrial processing protease Mas2p (Figure 2C).

Deletion of *COQ1* partially rescues the hyperoxia sensitivity phenotype of *pos5Δ* strains

Since Pos5p is targeted to the mitochondria, we wanted to determine whether the respiratory chain may be contributing to the hyperoxia sensitivity of the *pos5Δ* mutant. Two sites in the respiratory chain are known to contribute to ROS production: the NADH dehydrogenase and the ubiquinone anion derivative of co-enzyme Q (Boveris *et al.*, 1976; Turrens and Boveris, 1980; Turrens *et al.*, 1985; Fang and Beattie, 2003). *Saccharomyces cerevisiae* expresses three mitochondrial NADH dehydrogenases (encoded by *NDE1*, *NDE2* and *NDI1*; De Vries *et al.*, 1992; Luttk *et al.*, 1998). Although these are single subunit enzymes that do not form complex I, they still contain a flavin that can reduce oxygen (Fang and Beattie, 2003). However, single and combined deletions in these genes did not suppress the hyperoxia sensitivity of a *pos5Δ* mutant (data not shown). By comparison, a deletion in *COQ1* affecting co-enzyme Q synthesis partially suppressed the hyperoxia sensitivity of *pos5Δ* mutants (Figure 2D), suggesting that the ubiquinone anion of the respiratory chain may help contribute to high oxygen damage. Together with our mitochondrial localization of the protein, it appears that Pos5p functions in the mitochondria to protect against oxidative damage derived from component(s) in the respiratory chain.

Pos5p is an NADH-specific kinase

The amino acid sequence of Pos5p is shown in Figure 3A. Pos5p is predicted to be a 46.3 kDa protein with homology

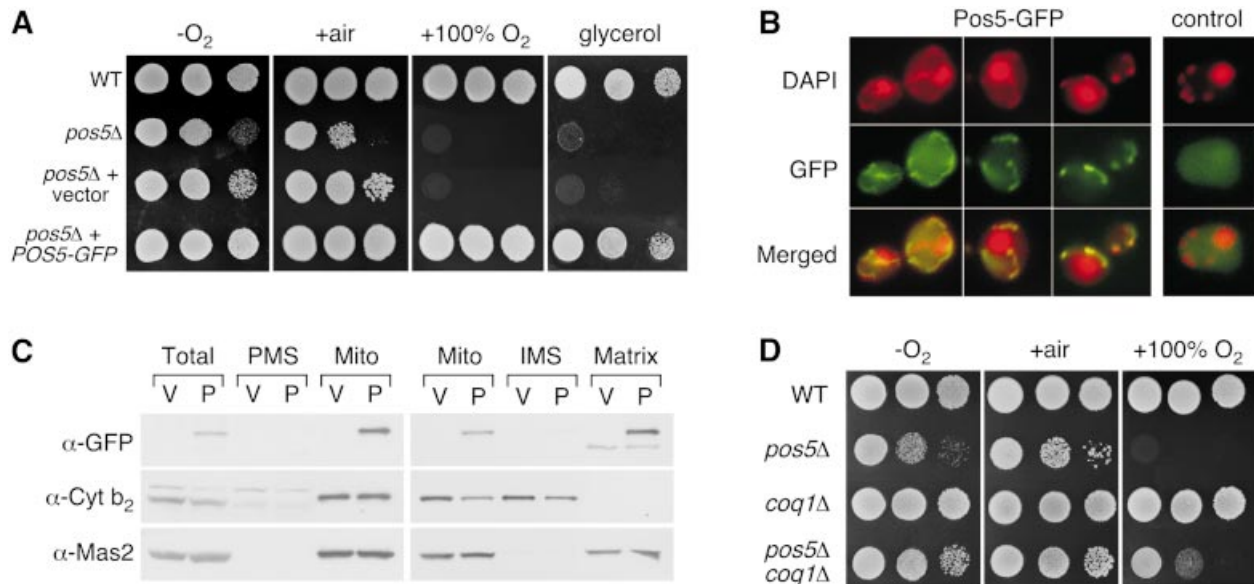


Fig. 2. Pos5p is a mitochondrial matrix protein that is needed for proper mitochondrial function. (A) The indicated strains were tested for growth by plating cell dilutions as in Figure 1 onto YPD ($-O_2$, +air, +100% O_2) or YPG (glycerol) plates in air. $-O_2$, +air, +100% O_2 = same as in Figure 1. Strains utilized: wild-type, BY4741; *pos5Δ*, CO205; *pos5Δ* + vector, strain CO205 transformed with vector pAA1; *pos5Δ* + *POS5-GFP*, strain CO205 transformed with plasmid pCO101. (B) Strain BY4741 transformed with pCO101 (Pos5-GFP) or pAA1 (control) was prepared for fluorescence microscopy as described in Materials and methods. Merged = merged images of DAPI and GFP fluorescence. (C) Cell lysates were prepared from strain BY4741 transformed with the Pos5-GFP plasmid pCO101 (P) or the vector control pAA1 (V), and 140 μ g of total cell protein was fractionated into post-mitochondrial supernatant (PMS) and crude mitochondria (Mito) (left). Where indicated, mitochondria (25 μ g of protein) were fractionated further into intermembrane space (IMS) and matrix components (right). All samples were subject to SDS-PAGE and immunoblotting using antibodies directed against either GFP (marking Pos5p), cytochrome b_2 (mitochondrial IMS) or Mas2 (mitochondrial matrix). (D) The indicated strains were tested for growth as in (A). Strains utilized: wild-type, BY4741; *pos5Δ*, BY4741 *pos5Δ::kanMX4*; *coq1Δ*, CO217; *pos5Δ coq1Δ*, CO200.

to NAD^+ and NADH kinases, which are enzymes that catalyze the phosphorylation of NAD^+ or NADH, respectively. This family of enzymes is present in organisms from bacteria to humans, including *Escherichia coli*, *Caenorhabditis elegans*, *Drosophila melanogaster*, plants and mice. In *S.cerevisiae*, NAD^+ kinase activity has been reported in the cytosol, mitochondria and microsomes, while NADH kinase activity is found solely in the mitochondria (Bernofsky and Utter, 1968; Apps, 1970; Griffiths and Bernofsky, 1972; Iwahashi *et al.*, 1989). Pos5p has two other homologs identified in the yeast genome, namely Utr1p and Yel041p. Utr1p has recently been identified as an NAD^+ kinase (Kawai *et al.*, 2001); however, the function of Yel041p is unknown. An amino acid sequence comparison of Pos5p, Utr1p and Yel041p with their human homolog PPNK [also known as FLJ13052 (accession No. NP_075394), encoding an NAD^+ kinase (Lerner *et al.*, 2001)] is given in Figure 3A. Despite the strong sequence similarities between Pos5p and its yeast homologs, only Pos5p is required for protection from hyperoxia and for proper respiratory function. As shown in Figure 3B, deletion of *UTR1* or *YEL041W* does not result in hypersensitivity to high O_2 or growth defects on a non-fermentable carbon source.

In order to determine if Pos5p has NAD(H) kinase activity, the recombinant protein was overexpressed and purified from *E.coli* (Figure 4A). The protein was tested for both NAD^+ and NADH kinase activity (see Materials and methods) using ATP as a phosphate source. The results, shown in Figure 4B, indicate that recombinant Pos5p is an NADH kinase. The recombinant enzyme also

exhibits weak NAD^+ kinase activity; however, this activity is ~50-fold lower than the NADH kinase activity. In comparison, chicken liver NAD^+ kinase has the opposite activity profile, with NAD^+ kinase activity ~150-fold higher than NADH kinase activity (Figure 4B). These results demonstrate that Pos5p can phosphorylate NADH using ATP as a phosphate donor and is therefore predicted to catalyze the production of NADPH within yeast mitochondria.

NADH and NAD^+ kinase assays were also performed on mitochondrial extracts from various yeast strains. As shown in Figure 4C, mitochondrial NADH kinase activity was greatly reduced in the *pos5Δ* mutant, while NAD^+ kinase activity was largely unaffected. Furthermore, *utr1Δ* and *yel041wΔ* gene deletions had little effect on mitochondrial NADH and NAD^+ kinase activities. These results indicate that of the three NAD(H) kinase homologs in yeast, Pos5p is the primary NADH kinase in the mitochondria.

Comparison of Pos5p with other potential sources of NADPH

There are a number of enzymes that have been predicted to contribute to cellular production of NADPH in yeast, including mitochondrial $NADP^+$ -specific isocitrate dehydrogenase (Idp1p) (Haselbeck and McAlister-Henn, 1991). However, an *idp1Δ* yeast mutant is not sensitive to hyperoxia, paraquat or H_2O_2 (Figure 1; Minard *et al.*, 1998). Two other NADPH sources are *ZWF1* and *GND1*, which encode cytosolic enzymes that produce NADPH as part of the pentose phosphate pathway (Juhnke *et al.*,

A

Pos5p	-----	DKWNEEDGR	NDHHNNNNNL	MKKAMMNNNQ	IDRTQDIDNA	KEMLRKISSE	SSSRSSLLN	KDSSLVNGNA	44	
Utr1p	MKENDMNNGV	DKWNEEDGR	NDHHNNNNNL	MKKAMMNNNQ	IDRTQDIDNA	KEMLRKISSE	SSSRSSLLN	KDSSLVNGNA	80	
Yel1041p	-----	MKTDRLL	INASPETCTK	GDAEMDTMDT	IDRMTSVKVL	AEGKVLNSFE	EPGLMRCGYH	DAKNWVR---	64	
PPNK	-----	MEMEQEK	MTMNKELSPD	AAAYCCSACH	GDETWSYNHP	IRGRAKRSRL	SASPALGSTK	EFRTRTS---	64	
Pos5p	-----	--SS--SADF	VSPNPKLQS	LIWQNPLONV	YITKKPWTPS	TREAMVFEIT	HLHESYPEVN	1		
Utr1p	NSGGGTSING	TRGSSKSNNT	HFQYASTAYG	VRMLSKDISN	TKVELDVENL	MLVTKLNDVS	LYFLTRREVE	WVLVHFPRVT	160	
Yel1041p	---RLS--SE	TIVGEDTSNL	YPFYVDTAYD	VRRLRKDLIN	AKVDLQVENL	IITCNINDIS	TVFLMREJVE	WILRNPHSIT	139	
PPNK	---LHG---	PCPVTTFGPK	ACVLQNPQTI	MHIQDPASQR	LTWNKSPKSV	LVIKKMRDAS	LLQPFKEICT	HLMEEN--MI	135	
Pos5p	VIVQPDVAEE	ISQDFKS---	PLENDPNRPH	ILYTGPEQDI	VNRTDLVVTL	GGDGTLLHGV	SMPGNTQMPF	VLAFAALGILG	177	
Utr1p	VIVQSELKNS	KKFAAGELCE	DSKCRESRK	YWTKDFIREH	DVFFDLVVTL	GGDGTLLFVS	SIFQR-HVPP	VMSFLGSLG	239	
Yel1041p	VIVQDIFKKS	TQFAVGLDCK	DSNCSKNRVK	YWSKEFVKKH	DSFFDLVITL	GGDGTLLFAS	SIFTK-DVPP	IVPFALGSLG	218	
PPNK	VIVQEKVLED	PAIAS----	ESFGAVKKKF	CTFREDYDDI	SNQIDFTIQL	GGDGTLLFAS	SIFQGSVPP	VMAFELGSLG	210	
Pos5p	FLSPDFKEH	KKVFQEVISS	RAKCLHRRL	ECHLKKKDS-	-----	-----	-----NSSIVT	HAMNDFLHR	232	
Utr1p	FLINRFKEHF	REDLPRIMNH	KIKTNLRRL	ECTLYRRHR-	-----	-----	PEVDPNTGKK	ICVVEKLSH	HILNEVITDR	308
Yel1041p	FLINRFKEHF	KETLKHLLTD	EVRLNLRRL	QCKLYRRNK-	-----	-----	PEIDAATGRK	ICYIDFISEH	HVLNEVITDR	287
PPNK	FLIPESENF	OSQVTOVIEG	NAAVVLRRL	KVRVVKELRG	KKTAVHNGLG	ENGSQAAGLD	MDVGKQAMQY	QVLENEVITDR	290	
Pos5p	GNSPHLNL	IPIDGFLTR	TTADGVALAT	PTGSTAYSLS	AGSSLVSPLV	PAILMTPICP	RSLFRPIL	PHSSHIRIKI	312	
Utr1p	GPSPPFLSME	LYGDGSLMTV	AQADGTLAAT	PTGSTAYSLS	AGSSLVCPVW	NATAITPICP	HALSFRPIL	PESINLKVKV	388	
Yel1041p	GPAPCSLE	LYGNDLSLTK	VQDGLIVAT	PTGSTAYSLS	AGSSLSPSV	NATAVTPICP	HTLSFRPIL	DSMELKVRV	367	
PPNK	GPSSYLSNVD	VYLDGHLIT	VQDGLIVST	PTGSTAYAAA	AGASMLHPNV	PALMITPICP	HSLSFRPILV	PAGVELKIML	370	
Pos5p	GSKLNQKPVN	SVKLSVQGI	POQDLVQDE	IYVIN---EV	GTYIDG--T	QLPTTRKTEN	DFNSKKPKR	SGIYCVAKTE	387	
Utr1p	SMKSR----	APAWAAFQK	DRIELQKGF	ITICASPFA	PTVEASP--D	EFINSISRQL	NWVREOQKS	FTHILSQNKQ	461	
Yel1041p	DMNSR----	GTSWVNFQK	DRVELKQGDY	VVITASPYSV	PTTESSA--S	EFFESISKNL	NWVREOQKP	FAHILSPKNQ	440	
PPNK	SPEAR----	NTAWVSFDGR	KROEIRHSDS	LSITTSCTYPL	PSICVRDPVS	DFWESLAQCL	HWVRRKQAH	FEESEEEEEE	445	
Pos5p	NDWIRGIN--	-----E	-----LLG	FNSSFRLTKR	QTDND--	-----	-----	414		
Utr1p	EKYAHEANKV	RNQAEPLEVI	RDKYSLEADA	TKENNGSD	ESDDESVCNE	ACKLKPSSVP	KPSQARFSV	530		
Yel1041p	EKYRLDS---	-----S	--KNGNDTIS	NPLESSCISS	DAQDEERKSV	TETETEIVVE	RTRQAHFAI	495		
PPNK	G-----	-----	-----	-----	-----	-----	-----	446		

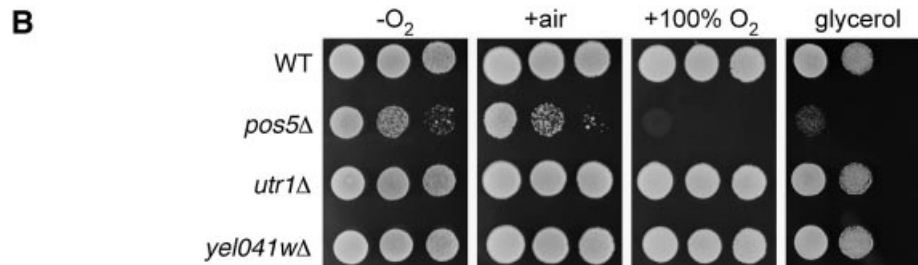


Fig. 3. Pos5p yeast homologs are not required for protection from hyperoxia or growth on a non-fermentable carbon source. (A) The amino acid sequences of *S.cerevisiae* Pos5p, Utr1p and Yel1041p and human PPNK (accession No. NP_075394) were aligned using Clustal_W 1.5. Identical residues are highlighted in black, and similar residues are outlined in gray. (B) The indicated strains were tested for growth as in Figures 1 and 2A. Strains utilized: wild-type, BY4741; *pos5Δ*, CO205; *utr1Δ*, BY4742 *utr1Δ::kanMX4*; *yel1041wΔ*, BY4741 *yel1041wΔ::kanMX4*.

1996). Deletion of either of these genes also did not produce strong hyperoxia sensitivity, and effects on paraquat and H_2O_2 resistance were mild in comparison with *pos5Δ* mutations (Figure 1). Therefore, of all the known potential sources of cellular NADPH, Pos5p seems the most crucial for protection against oxidative damage in yeast.

Mitochondrial iron defects in the *pos5Δ* mutant

Since defects in metal homeostasis have been linked to oxidative stress (Jamieson, 1998), we examined metal levels in the cytosol and crude mitochondria of the *pos5Δ* mutant. Surprisingly, the mitochondrial fraction of *pos5Δ* shows a dramatic accumulation of iron relative to wild-type cells, with an ~14-fold increase (Figure 5A). The iron levels were also elevated in the PMS fraction, with an ~6-fold increase. In contrast, the levels of manganese and copper were not considerably affected in *pos5Δ* cells (data not shown). Since hyperaccumulation of iron in the mitochondria is a hallmark of defects in iron-sulfur cluster biogenesis (for a review see Muhlenhoff and Lill, 2000), we examined the activity of mitochondrial Fe-S

enzymes in the *pos5Δ* mutant. Succinate dehydrogenase (SDH) and aconitase activities were greatly reduced in the *pos5Δ* mutant relative to wild-type (Figure 5B and C). In fact, the aconitase activity was virtually undetectable under these assay conditions (Figure 5C). Addition of iron to the growth medium did not rescue this defect (data not shown). It is noteworthy that the *pos5Δ* defects in Fe-S enzyme activity and iron accumulation are comparable to that seen with a *S.cerevisiae isa2Δ* mutant, known to be defective in iron-sulfur cluster biogenesis (Figure 5A-C) (Jensen and Culotta, 2000; Pelzer *et al.*, 2000).

We addressed whether the *pos5Δ* defects in iron homeostasis contribute to the hyperoxia sensitivity of this mutant. First, the elevated iron of *pos5Δ* mutants was reduced by deleting the *FET3* gene needed for high affinity iron uptake (Figure 5A). This reduction in mitochondrial iron levels, however, did not reverse the hyperoxia sensitivity of *pos5Δ* mutants (Figure 5D). Secondly, we noted that the *isa2Δ* mutant, which accumulates similarly high levels of mitochondrial iron (Figure 5A), is not hyperoxia sensitive (Figure 5D). Evidently, the defects in mitochondrial iron homeostasis of *pos5Δ* mutants are not

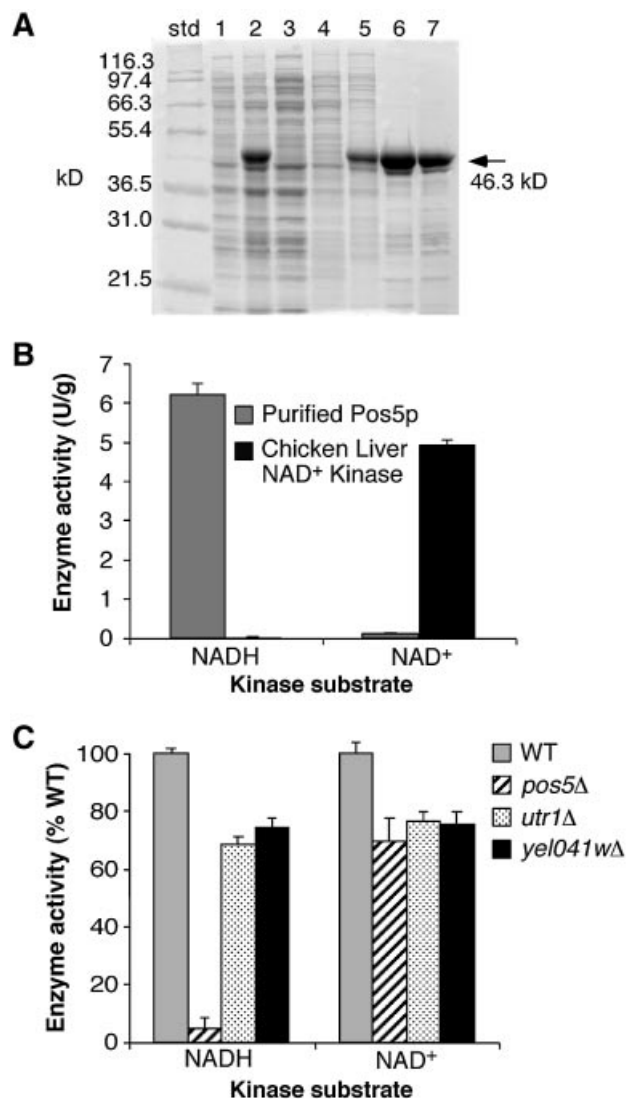


Fig. 4. Recombinant Pos5p is an NADH kinase. (A) SDS-polyacrylamide gel from recombinant Pos5p purification procedures. std, molecular weight standards; lane 1, uninduced cells; lane 2, induced cells; lane 3, sonication supernatant; lane 4, MES/urea extract; lane 5, Tris/urea extract; lane 6, DEAE flow-through; lane 7, 15 μ g of refolded Pos5p (see Materials and methods). (B) and (C) NADH and NAD⁺ kinase activities were assayed as described in Materials and methods using either (B) purified recombinant Pos5p (left hand histogram for both NADH and NAD⁺) and chicken liver NAD⁺ kinase (Sigma; right hand histogram for both NADH and NAD⁺) or (C) crude mitochondria obtained from cells grown to mid-log phase in YPD. Results are presented either in the form of enzyme units (B) where one unit = 1.0 μ mol NADPH or NADP⁺ produced per min, or as a percentage of the total activity of wild-type mitochondria (C). Yeast strains utilized in (C) are the same as in Figure 3B. The reported values in (B) and (C) are the mean of three independent experiments; error bars are standard deviations.

connected to the hyperoxia sensitivity of this strain and appear to represent a separate outcome of mitochondrial NADPH depletion (see Discussion).

The requirements for mitochondrial and cytosolic NADPH in amino acid biosynthesis

While optimizing growth conditions for the *pos5Δ* mutant, we discovered that it is auxotrophic for arginine (Figure 6).

This observation may be explained by an NADPH-requiring step in arginine biosynthesis that takes place in the mitochondria, specifically the third step in the conversion of glutamate to arginine catalyzed by *N*-acetylglutamyl phosphate reductase (Jauniaux *et al.*, 1978). While mitochondrial NADPH is required for arginine biosynthesis, cytosolic NADPH is needed for methionine biosynthesis (Thomas *et al.*, 1991; Slekár *et al.*, 1996), and low cytosolic NADPH caused by deletion of *ZWF1* results in methionine auxotrophy (Figure 6). The mitochondrial and cytosolic pools of NADPH are kept quite separate, because *pos5Δ* mutants exhibit no defect in methionine biosynthesis and *zwf1Δ* mutants are not auxotrophic for arginine. This result is consistent with the fact that the mitochondrial inner membrane is impermeable to pyridine nucleotides, prohibiting exchange of these molecules with the cytosol (von Jagow and Klingenberg, 1970).

Discussion

Due to the constant generation of respiratory chain ROS, the mitochondria may be particularly prone to oxidative damage. This organelle has therefore evolved with a set of anti-oxidant defense factors that are distinct from that of the cytosol, including a separate system for the generation of the key reductant, NADPH. Until recently, the mitochondrial source for NADPH has been poorly understood. We provide strong evidence herein that in bakers' yeast, mitochondrial NADPH is generated through the action of an NADH kinase encoded by the *POSS5* gene.

Mitochondrial NADPH and oxidative stress

In contrast to other anti-oxidant factors in yeast (e.g. superoxide dismutases and oxidative stress transcriptional regulators), Pos5p provides protection from a wide range of oxidative stress insults, including hyperoxia, hydrogen peroxide and superoxide-generating agents. The effect of *pos5Δ* mutations is consistent with the widespread requirement for NADPH in anti-oxidant defense systems. Both TRX and GSH are maintained in their reduced state through the action of NADPH-requiring reductases. TRX, in turn, serves as cofactor for thioredoxin peroxidases (peroxiredoxins) and methionine sulfoxide reductases. GSH itself can act as a reductant, but is also the cofactor for GSH peroxidases and glutaredoxins (Jamieson, 1998). Yeast express mitochondrial forms of thioredoxin reductase (Trr2p), thioredoxin (Trx3p) and peroxiredoxin (Prx1p) (Pedrajas *et al.*, 1999, 2000), as well as two mitochondrial glutaredoxins, Grx2p and Grx5p (Pedrajas *et al.*, 2002; Rodríguez-Manzanique *et al.*, 2002). Although the subcellular localization of GSH reductase has not yet been determined for yeast, oxidized glutathione (GSSG) will not cross the mitochondrial membrane (Olafsdottir and Reed, 1988), which necessitates a mitochondrial form of GSH reductase, as has been identified in mammalian cells (Mbemba *et al.*, 1985; Taniguchi *et al.*, 1986). In any case, the numerous GSH- and TRX-requiring systems in the mitochondria may account for the widespread impact of *pos5* mutations on oxidative stress resistance.

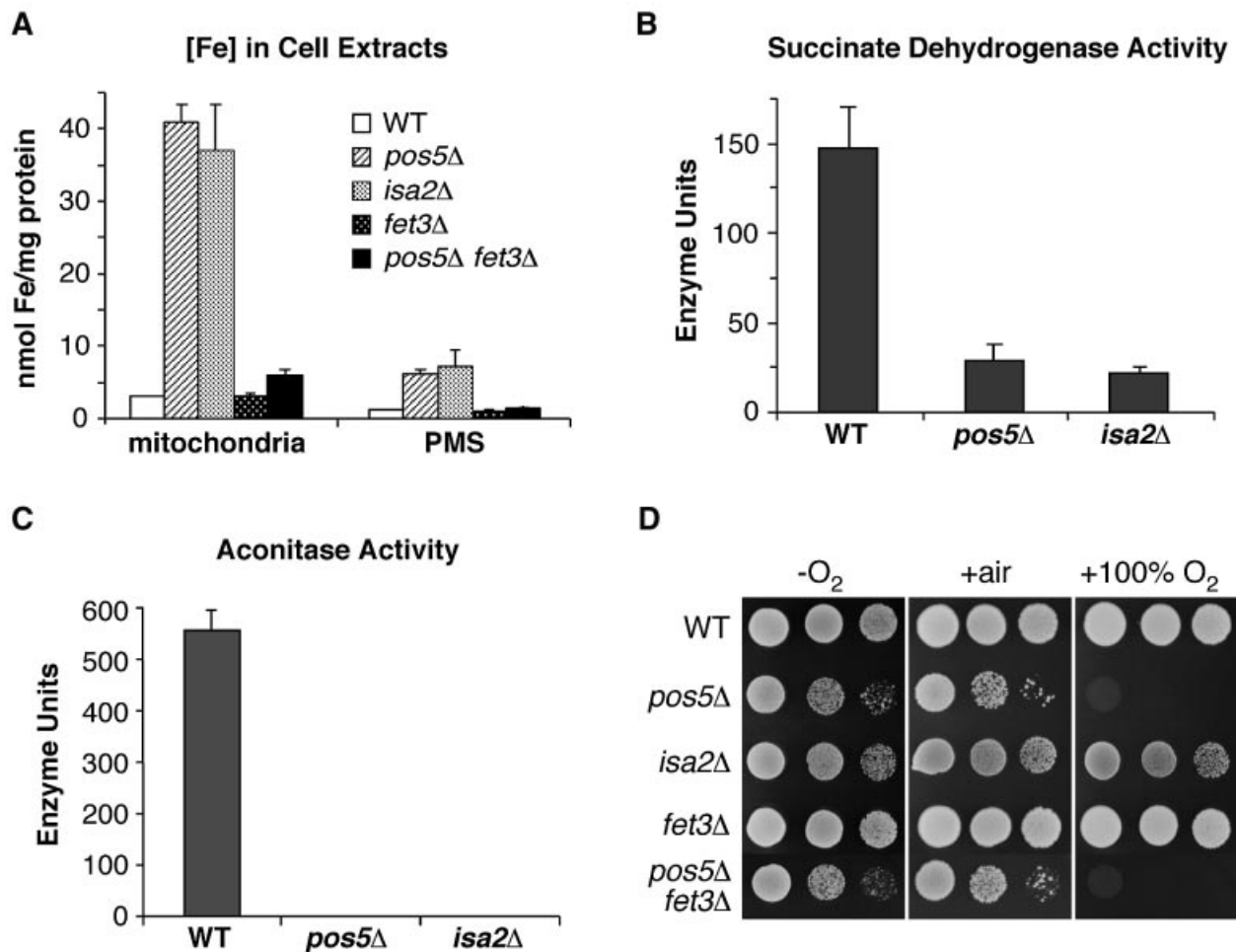


Fig. 5. Mitochondrial iron homeostasis defects in *pos5Δ*. (A) The indicated strains were grown to mid-log phase and the iron content measured in mitochondria and post-mitochondrial supernatant fractions by atomic absorption spectroscopy. (B) and (C) Cell lysates were prepared for the indicated yeast strains and tested for succinate dehydrogenase (B) and aconitase (C) activity. One unit of enzyme activity is equivalent to 1.0 nmol of substrate converted/min/mg protein. Substrates were dichlorophenol indophenol (SDH) and *cis*-aconitate (aconitase). In (A–C), the reported values are the mean of three independent experiments; error bars are standard deviations. (D) The indicated strains were tested for growth as in Figure 2A. Strains utilized: wild-type, BY4741; *pos5Δ*, CO205; *isa2Δ*, LJ102; *fet3Δ*, BY4741 *fet3Δ::kanMX4*; *pos5Δ fet3Δ*, CO207.

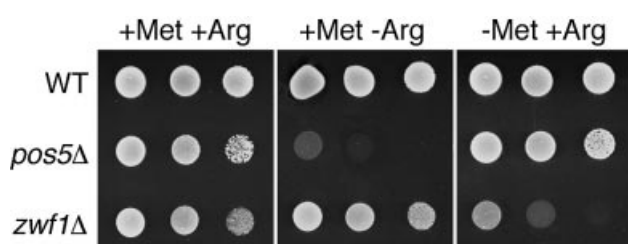


Fig. 6. Amino acid auxotrophies of *pos5Δ* and *zwf1Δ* strains. The indicated strains were tested for growth by spotting 10 μ l of solution at 2.0, 0.2 and 0.02 OD₆₀₀ units onto complete SD plates with arginine, or SD plates lacking methionine or arginine. Strains utilized: wild-type, BY4742; *pos5Δ*, BY4742 *pos5Δ::kanMX4*; *zwf1Δ*, BY4742 *zwf1Δ::kanMX4*.

Mitochondrial NADPH and iron homeostasis

In addition to a strong sensitivity towards oxidative stress, we find that *pos5Δ* mutants also exhibit defects in iron homeostasis that are suggestive of a deficiency in Fe–S cluster biogenesis. As with all known mutants for Fe–S cluster synthesis (Muhlenhoff and Lill, 2000), *pos5Δ* cells accumulate high mitochondrial iron and are defective in

mitochondrial Fe–S cluster-containing enzymes. Why would NADPH be required for Fe–S cluster biogenesis? As one possibility, NADPH may be required indirectly (via GSH) to maintain the activity of the mitochondrial Grx5p glutaredoxin, which is known to be required for Fe–S cluster assembly (Rodriguez-Manzanique *et al.*, 2002). Another possibility is that NADPH may be needed for the function of *S.cerevisiae* Arh1p, an adrenodoxin reductase homolog that can utilize either NADH or NADPH and is needed for Fe–S cluster assembly (Lacour *et al.*, 1998; Li *et al.*, 2001; Muhlenhoff *et al.*, 2002). The possibility also exists that the *pos5Δ* defect in iron homeostasis reflects an unknown requirement for NADPH in mitochondrial iron metabolism. Finally, we cannot rule out the alternative theory that the loss of Fe–S enzyme activity is not due solely to changes in iron homeostasis, but to elevated levels of mitochondrial ROS in *pos5Δ* mutants that could directly damage the Fe–S cluster proteins. In any case, our studies show that this defect in Fe–S enzyme activity is not the cause of *pos5Δ* hyperoxia sensitivity. Clearly, separate NADPH-requiring processes are involved.

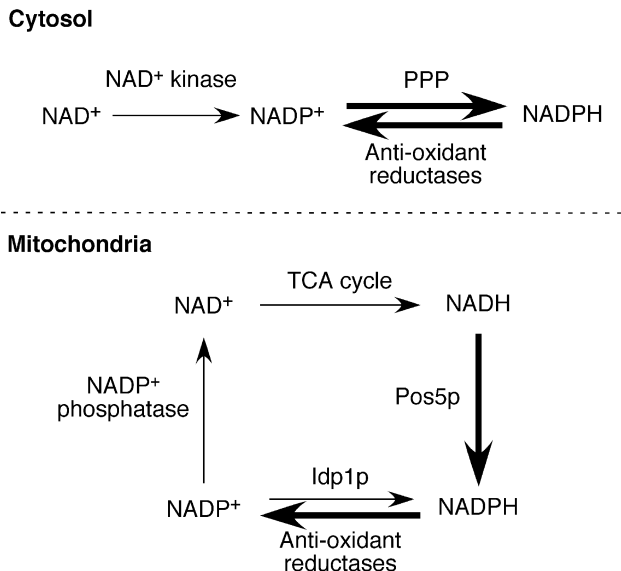


Fig. 7. Proposed model for the generation of NADPH in the cytoplasm and mitochondria of yeast. In the cytosol, NAD⁺ may be phosphorylated to NADP⁺ by an NAD⁺ kinase (e.g. *S.cerevisiae* Utr1p). The resultant NADP⁺ is then converted to NADPH by enzymes of the pentose phosphate pathway (PPP). NADPH-requiring reductases (e.g. TRX and GSH reductases) can then use NADPH as a cofactor, thereby regenerating NADP⁺ for the PPP. In the mitochondria, a different pathway exists for the primary generation of NADPH. The NADH generated in the tricarboxylic acid (TCA) cycle apparently can be phosphorylated by the NADH kinase Pos5p, generating NADPH. Following consumption of NADPH via anti-oxidant reductases, an NADP⁺ phosphatase presumably regenerates NAD⁺ for recycling back into the TCA cycle. NADP⁺-IDHm (Idp1p) may also contribute to NADPH regeneration, although to a minimal extent in *S.cerevisiae*. Arrows shown in bold indicate enzymatic reactions that are critical for oxidative stress resistance.

Cytosolic versus mitochondrial NADPH

Based on our results, we propose a model depicting two different pathways for the production of NADPH in the cytosol versus the mitochondria of yeast (Figure 7). In the cytosol, NADP⁺ is converted to NADPH by the pentose phosphate pathway. However, in the mitochondria of *S.cerevisiae*, NADH, and not NADP⁺, serves as the substrate for NADPH generation, and this is made possible through the NADH kinase activity of Pos5p. At first glance, it would appear that this method of NADPH generation would deplete mitochondrial pools of NADH. However, NADP⁺ phosphatase activity has been detected in yeast mitochondria (Bernofsky and Utter, 1968), providing a means of recycling NAD⁺ back into the citric acid cycle (Figure 7).

In the case of mammals, an NADP⁺-specific isocitrate dehydrogenase has been reported to be a key source of mitochondrial NADPH (Jo *et al.*, 2001). Although it remains possible that mammalian mitochondria also employ an NADH kinase to generate NADPH, no such enzyme has been identified to date. At this point, it is not clear why yeast mitochondria would employ NADH preferentially over NADP⁺ for the generation of NADPH. This may reflect the oxidation state of NADH. Although mammalian cells are reported to have 10–20% of their total cellular NAD(H) in the reduced form, in yeast cells ~50% is in the reduced form, and this may also be

true of mitochondrial pools (Jacobson and Jacobson, 1976; Ting *et al.*, 1977). Such availability of reduced NADH could help explain the utilization of an NADH kinase for generating NADPH.

Mitochondria and hyperoxia

Finally, our findings highlight the important role that mitochondria play in protection from hyperoxia toxicity. Although a high oxygen level has long been known to cause cellular damage, the pro-oxidant and anti-oxidant mechanisms at play have been poorly understood. Two major mitochondrial anti-oxidant factors, Pos5p and Sod2p, are absolutely required for hyperoxia resistance (Figure 1; and van Loon *et al.*, 1986; Gralla and Kosman, 1992). Sod1p was also found to be important (Gralla and Kosman, 1992), and although this enzyme is largely cytosolic, a fraction of it is known to reside in mitochondria as well (Sturtz *et al.*, 2001). Moreover, a deletion of *COQ1* needed for mitochondrial respiration partially alleviated the hyperoxia sensitivity of a *pos5Δ* mutant, directly implicating the respiratory chain in hyperoxia toxicity. This result is consistent with the observation that hyperoxia conditions increase the rate of ROS generation in mitochondria compared with aerobic conditions (Boveris and Chance, 1973). It is likely that the mitochondria play a key role in hyperoxia damage in mammalian cells as well.

Materials and methods

Yeast strains, media and growth conditions

Yeast strains used in this study were derived from the parental strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (Brachmann *et al.*, 1998). The BY4741 knockout library, a collection of yeast deletion strains created with the *kanMX4* cassette, was obtained from Research Genetics (ResGen). Details of this library can be found at ftp://ftp.resgen.com/pub/deletions/mat_a_041902.txt. Construction of LJ102 (*isa2Δ::HIS3*) was described previously (Jensen and Culotta, 2000). The strain LJ284 (*sod1Δ::LEU2*) was constructed by introducing the *SOD1* gene replacement plasmid pKS1 (Culotta *et al.*, 1995) into the strain BY4741. Strains CO205 (*pos5Δ::URA3*) and CO207 (*pos5Δ::URA3 fet3Δ*) were constructed by deleting the *POS5* gene of strains BY4741 and BY4741 *fet3Δ::kanMX4*, respectively, using the *POS5* replacement plasmid pCO105. Strains CO217 (*coq1Δ::LEU2*) and CO200 (*pos5Δ coq1Δ::LEU2*) were obtained by disruption of the *COQ1* gene in the strains BY4741 and BY4741 *pos5Δ::kanMX4*, respectively, with the *coq1Δ::LEU2* plasmid pSG108 (S.Garland, unpublished data). Yeast transformations were performed by electroporation (Becker and Guarente, 1991) or the lithium acetate procedure (Gietz and Schiestl, 1991). All gene deletions, including those from the original ResGen collection, were verified by PCR analysis.

Strains were maintained at 30°C on either enriched yeast extract-peptone-based medium supplemented with 2% glucose (YPD) or 3% glycerol (YPG), or on minimal synthetic defined medium (SD) supplemented with the appropriate amino acids (Sherman *et al.*, 1978). Anaerobic cultures were maintained by growth in an O₂-depleted culture jar (BBL Gas Pak) (Liu *et al.*, 1992). For growth in 100% O₂, cultures were placed in a modular incubator chamber (Billups-Rothenburg, Inc., Del Mar, CA) and flushed with 100% O₂ for 10–30 min before sealing.

Plasmids

To construct the *POS5* deletion plasmid pCO105, *POS5* sequences from –902 to +71 and +736 to +1293 were amplified by PCR using primers that introduced an *EcoRI* site at –893, a *BamHI* site at +64 and a *SaII* site at +726. The PCR products were digested at these sites and at an internal *EcoRI* site at +1281, and ligated in a trimolecular reaction to the *SaII* and *BamHI* sites of the *URA3* integrating vector pRS306 (Sikorski and Hieter, 1989). The resulting plasmid, pCO105, was linearized with *EcoRI* and used to delete the chromosomal *POS5* gene sequences +72 to +735. The Pos5–GFP plasmid pCO101, which contains one copy of GFP fused to the

C-terminus of Pos5p, was constructed as follows. The *POS5* gene was amplified from –894 to the stop codon with primers that introduced a *Pst*I site at –888 and a *Not*I site at +1243. The PCR fragment was digested with these enzymes and inserted into the *Pst*I and *Not*I sites of pAA1 (*LEU2 CEN*), producing an in-frame fusion with the GFP sequence already present in this vector (Hobbs *et al.*, 2001). To create pCO100, a bacterial overexpression vector for recombinant Pos5p, the *POS5* gene was amplified using primers that introduced an *Nde*I site at the start codon, and a *Sal*I site 16 bp after the stop codon. The fragment was digested with these enzymes and inserted into the *Nde*I and *Sal*I sites of pET21a (Novagen). The sequence integrity of all plasmids was confirmed by double-stranded DNA sequencing (Synthesis and Sequencing Facility, Johns Hopkins University School of Medicine). Upon sequencing, a polymorphism was detected in *POS5* that changed amino acid 180 from serine to leucine (TCA→TTA). The *POS5* gene with this single amino acid change fully complemented the hyperoxia sensitivity, arginine auxotrophy and respiratory deficiency phenotypes of the *pos5Δ* strain.

Hyperoxia sensitivity screen

The fifty-three 96-well plates comprising the ResGen *MATa* haploid knock-out collection were completely thawed and 3 μ l of cells from each well were spotted onto four sets of YPD plates. Two sets were placed in an anaerobic chamber while two sets were placed in a chamber flushed with 100% oxygen. After incubation at 30°C for 48 h, growth under the anaerobic and hyperoxic conditions was compared in order to identify deletion strains that are hyperoxia sensitive.

Fluorescence and immunodetection techniques

For fluorescence studies, the BY4741 parental strain transformed with pCO101 (Pos5–GFP *CEN* plasmid) or pAA1 (vector control) were grown aerobically to an OD₆₀₀ of 2.0–2.5 in selecting SD medium. Cells were fixed with formaldehyde then the DNA was stained by incubation with 1 μ g/ml DAPI for 5 min. GFP and DAPI were monitored by fluorescence microscopy on a Zeiss Axiovert 135TV microscope (Microscopy Facility, Johns Hopkins Medical Institutions) at a magnification of 1000 \times .

For western blot analysis, BY4741 strains transformed with pCO101 (Pos5–GFP) or pAA1 (vector control) were grown aerobically to an OD₆₀₀ of 1.0 in selecting SD medium with 2% galactose. To obtain mitochondrial and PMS fractions, cells were converted to spheroplasts prior to gentle lysis by Dounce homogenization as described previously (Daum *et al.*, 1982; Jensen and Culotta, 2000). Mitochondrial intermembrane and matrix fractions were prepared as previously described by resuspending the isolated mitochondria in a hypotonic buffer (Jensen and Culotta, 2000). All samples were separated by electrophoresis on a 12% SDS–polyacrylamide gel and analyzed by western blotting using an anti-GFP antibody (Molecular Probes) diluted to 1:5000 and a secondary anti-rabbit IgG (Amersham) diluted to 1:12 500. Mitochondrial fractions were monitored by using antibodies (diluted to 1:10 000) directed against cytochrome *b*₂ in the intermembrane space and Mas2 in the matrix (kind gifts of R.Jensen). Detection employed the enhanced chemiluminescence (ECL) kit (Amersham) used according to the manufacturer's specifications. Protein concentrations were determined using the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as the calibration standard.

Recombinant Pos5p purification

For production of recombinant Pos5p, the bacterial overexpression plasmid pCO100 was transformed into the *E.coli* strain BL21(DE3) (Novagen). The transformants were grown in 8 l of LB media with shaking at 37°C and induced with 400 μ M isopropyl- β -D-thiogalactopyranoside when OD₆₀₀ = 0.6. The cells were harvested by centrifugation 2.5 h after induction and then stored at –80°C. A portion of this frozen cell paste (equal to ~400 ml of cell culture) was thawed and sonicated in 20 ml of 20 mM Tris–HCl pH 8.0. After centrifugation and an additional wash in the sonication buffer, the cell pellet was then extracted twice in 20 ml of MES extraction buffer (20 mM MES–Na, pH 6.0, 6 M urea, 2% Triton X-100) in order to remove contaminants. The cell pellet was then resuspended in 10 ml of Tris extraction buffer [20 mM Tris–HCl pH 8.0, 6 M urea, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM dithiothreitol (DTT)] and incubated on ice for 1.5–2 h. After centrifugation, the supernatant containing the solubilized protein was loaded onto a DEAE–cellulose (Whatman) column equilibrated with 20 mM Tris–HCl pH 8.0, 6 M urea, 1 mM EDTA and 5 mM DTT. Recombinant Pos5p did not bind to this column and was collected in the flow-through fraction (~15 ml). The protein solution was concentrated 5-fold to 1 mg/ml and dialyzed in 500 ml of 20 mM MES–Na pH 6.0. After removal of precipitated protein by centrifugation, the solubilized,

refolded protein was concentrated to ~1–2 mg/ml and stored at –80°C. A 400 ml cell culture typically yielded 0.5 mg of purified protein. Despite the addition of protease inhibitors, ~10–20% of the purified protein is partially proteolyzed during the purification process as evidenced by the presence of several lower molecular weight bands in the SDS–polyacrylamide gel (see Figure 4A, lane 7). However, the protein appeared to be stable upon freezing, since there was no reduction in activity after storage for 2 months at –80°C.

NAD⁺ and NADH kinase assays

The two-step procedure for the NAD⁺ and NADH kinase assays was adapted from previous methods (Griffiths and Bernofsky, 1972; Jacobson and Jacobson, 1976; Iwahashi *et al.*, 1989) with some modifications as follows. For the first step, the reaction mixture included 90 mM Tris–HCl pH 7.8, 2 mM NADH (or NAD⁺), 3 mM ATP, 10 mM MgCl₂, 20 mM sodium acetate, 1 mg/ml BSA and 10–15 μ g of purified Pos5p or 5–10 μ g of mitochondrial extract in a final volume of 100 μ l. Purified chicken liver NAD⁺ kinase (Sigma) was utilized in a parallel reaction as a control. Reactions were initiated by the addition of enzyme and were allowed to proceed for 20 min at 30°C. The NADH and NAD⁺ kinase assays were terminated by alkalination and acidification, respectively, followed by neutralization as described previously (Iwahashi *et al.*, 1989).

For the second step in the kinase assays, the amount of NADP⁺ produced from the first step was determined using a cycling assay system. Each assay mixture contained 100 mM Tris–HCl pH 8.0, 10 mM MgCl₂, 3.3 mM glucose-6-phosphate, 0.42 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium (MTT), 1.66 mM phenazine ethosulfate (PES), 0.83 mg/ml BSA and 100 μ l of the NADH assay mixture or 50 μ l of the NAD⁺ assay mixture from step 1 to a final volume of 1.0 ml. The reaction was initiated by the addition of 2 U of glucose-6-phosphate dehydrogenase from yeast (ICN Biomedicals) and monitored at 570 nm for 5 min to determine the rate of reduction of MTT. This rate was proportional to the amount of NADP(H) present in solution in step 2. NADPH and NADP⁺ standards were also subjected to the same treatments throughout the two-step procedure in order to develop a standard curve.

Iron accumulation and Fe–S enzyme analyses

For iron accumulation analyses, yeast strains were grown to mid-log phase in YPD. Mitochondrial and PMS fractions were prepared as described above for the western blot. Iron analysis of these fractions was performed on a Perkin-Elmer AAnalyst 600 graphite furnace atomic absorption spectrometer according to the manufacturer's specifications. For SDH assays, cells were grown to stationary phase in YPD with 0.6% glucose and fractionated into mitochondria and PMS. Aconitase and SDH assays were conducted essentially as described previously (Strain *et al.*, 1998).

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