Mutations in *Saccharomyces cerevisiae* Iron-Sulfur Cluster Assembly Genes and Oxidative Stress Relevant to Cu,Zn Superoxide Dismutase*

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Laran T. Jensen‡§, Raylene J. Sanchez‡, Chandra Srinivasan¶, Joan Selverstone Valentine, and Valeria Cizewski Culotta‡

From the ‡Department of Environmental Health Sciences, Johns Hopkins University, Bloomberg School of Public Health, Baltimore, Maryland 21205 and ¶Department of Chemistry and Biochemistry, UCLA, Los Angeles, California 90095-1569

*Saccharomyces cerevisiae* lacking Cu,Zn superoxide dismutase (*SOD1*) show several metabolic defects including aerobic blockages in methionine and lysine biosynthesis. We have previously shown that mutations in genes implicated in the formation of iron-sulfur clusters, designated *seo* (suppressors of endogenous oxidation), reverse the oxygen-dependent methionine and lysine auxotrophies of a *sod1*Δ strain. We now report the surprising finding that *seo* mutants do not reduce oxidative damage as shown by the lack of reduction of EPR-detectable “free” iron, which is characteristic of *sod1*Δ mutants. In fact, they exhibit increased oxidative damage as evidenced by increased accumulation of protein carbonyls. The *seo* class of mutants overaccumulates mitochondrial iron, and this iron accumulation is critical for suppression of the *sod1*Δ biosynthetic defects. Blocking overaccumulation of mitochondrial iron abolished the ability of the *seo* mutants to suppress the *sod1*Δ auxotrophies. By contrast, increasing the mitochondrial iron content of *sod1*Δ yeast using high copy *MMT1*, which encodes a mitochondrial iron transporter, was sufficient to mimic the *seo* mutants. Our studies indicated that suppression of the *sod1*Δ methionine auxotrophy was dependent on the pentose phosphate pathway, which is a major source of NADPH production. By comparison, the *sod1*Δ lysine auxotrophy appears to be reversed in the *seo* mutants by increased expression of genes in the lysine biosynthetic pathway, perhaps through sensing of mitochondrial damage by the retrograde response.

Superoxide dismutases catalyze the disproportionation of superoxide to hydrogen peroxide and water and play a key role in protecting aerobic cells against oxidative damage (1, 2). Eukaryotes, such as the baker’s yeast *Saccharomyces cerevisiae*, contain two intracellular superoxide dismutases, Cu,Zn-Sod1p, which is predominantly localized in the cytosol (3), and Mn-Sod2p, which is localized to the matrix of the mitochondria (4). Yeast strains in which *SOD1* has been disrupted (*sod1*Δ) exhibit several oxygen-dependent growth defects including the inability to grow on medium lacking the amino acids methionine or lysine (5, 6). These amino acid biosynthetic defects do not occur under anaerobic conditions and appear to be the result of endogenously generated reactive oxygen species (5, 7–10). The disruption of methionine biosynthesis in *sod1*Δ strains appears to be caused by the depletion of NADPH by oxidative stress, because the biosynthesis of methionine requires large quantities of NADPH (11–13). The *sod1*Δ methionine auxotrophy can be bypassed by increasing NADPH production through the pentose phosphate pathway (PPP) (11). By comparison, the nature of the lysine biosynthetic defect in *sod1*Δ strains remains elusive.

Several genetic suppressors of *sod1*Δ that are involved in metal ion metabolism have been isolated. Mutations in two genes in metal ion trafficking, *PMR1* and *BSD2*, have been identified as *sod1*Δ suppressors (14, 15). These mutations result in the overaccumulation of intracellular manganese in *pmr1* and copper and manganese in *bsd2*. In addition, two multicopy suppressors of *sod1*Δ, *ATX1* and *ATX2*, cause the overaccumulation of copper and manganese, respectively (16, 17). In each case, these suppressors not only rescued the methionine and lysine auxotrophies of *sod1*Δ mutants, they also increased resistance to superoxide generating agents, such as paraquat. The overaccumulation of redox-active manganese or copper ions in these suppressors is believed to replace the need for *SOD1* through the formation of metal-based superoxide dismutase mimics that directly detoxify superoxide radicals (14–17).

Six additional *sod1*Δ suppressors that also participate in metal ion metabolism, in this case iron were isolated previously. These mutations were found in a screen for genes that contribute to endogenous sources of oxidative stress and were designated *seo* (suppressors of endogenous oxidation) (18). Interestingly, all of the *seo* mutants were found to contain defects in the assembly or repair of iron-sulfur clusters. The four *seo* suppressors identified so far are mutations in *SSQ1*, *JAC1*, *NFS1*, and *ISU1*, all of which encode mitochondrial proteins (18, 19). The mechanism by which mutations in iron-sulfur cluster assembly proteins bypassed the need for *SOD1* appears distinct from suppressors that involve copper and manganese metabolism. Unlike *pmr1* and *bsd2* mutants, the *seo* suppressors only reversed a subset of the *sod1*Δ phenotypes, namely restoring growth of a *sod1*Δ strain on medium lacking methionine or lysine; however, the *seo* mutants did not reverse the sensitivity of *sod1*Δ strains to the superoxide-generating agent paraquat (18). This suggested that the *seo* suppressors were

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| Current address: Dept. of Chemistry and Biochemistry, California State University, Fullerton, CA 92834.
| ¶ To whom correspondence should be addressed: Johns Hopkins University, 615 North Wolfe St., Rm. 7032, Baltimore, MD 21205. Tel.: 410-955-9643; Fax: 410-955-0116; E-mail: ljensen@jhsph.edu.

1 The abbreviations used are: PPP, pentose phosphate pathway; WT, wild type.
not functioning by increasing resistance to oxidative stress, as is the case for pmr1 and bsd2 mutants, but rather they may be reducing the endogenous production of reactive oxygen species.

The goal of this study was to uncover how defects in iron-sulfur cluster assembly were effective in alleviating the need for SOD1 in methionine and lysine biosynthesis. Unexpectedly, we found that mutations in iron-sulfur cluster assembly genes do not reduce oxidative damage under aerobic growth but actually increase oxidative damage and oxidative stress. Surprisingly, we found that overaccumulation of mitochondrial iron, the likely cause of the increased oxidative damage in the seo mutants, is critical for the suppression of the sod1Δ blocks in methionine and lysine biosynthesis. Suppression of the sod1Δ methionine auxotrophy was dependent on a functional PFP and may be operating through increased NAPDH production. Attenuation of the sod1Δ lysine defect appears to involve the elevated expression of lysine biosynthetic genes, perhaps through the retrograde response, which alters nuclear gene expression in response to mitochondrial damage.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Strains used in this study were derived from either the wild type strain 1783 (Mata, leu3-112, ura3-52, trp1-1, his3, and can1Δ) or the isogenic sod1Δ strain KS105. FET3 and ZWF1 were disrupted using plasmids pΔFET3 (20) and pBM2720 (13). Disruptions of MMT1, MMT2, and MKS1 were generated with plasmids pLJ186, pLJ188, and pLJ244, respectively. Yeast transformations were performed using the lithium acetate procedure (21). Cells were propagated either in an enriched yeast extract, peptone-based medium supplemented with 2% glucose (YPD) or in minimal synthetic dextrose (22). Cells for Fe(III) EPR spectroscopy experiments were grown in synthetic dextrose medium supplemented with four times the normal amount of leucine, histidine, tryptophan, methionine, adenine, and uracil (23).

Plasmids—A high copy plasmid containing MMT1 (pLJ183) was generated by PCR amplification of MMT1 (~681 to +2176) with the introduction of BamHI and HindIII sites at the termini followed by digestion and ligation into pRS425 (LEU2 2μ) (24). The MMT1 disruption plasmid pLJ186 was generated by mobilizing the upstream (~619 to -213) and downstream (~1378 to +2025) sequences of MMT1 from plasmid pLJ183 by digestion with EcoRI and XhoI (upstream) or Smal and EcoRI (downstream) and ligation into pRS306 (URA3) (24) digested with SmaI and XhoI.

Disruption plasmids for MMT2 and MKS1 were constructed by first amplifying upstream and downstream regions of these genes by PCR. The PCR products were digested with the appropriate enzymes, and each pair of PCR products was ligated in a trimeric restriction into either pRS305 (LEU2) (24) digested with SpeI and NotI for MMT2 or into pRS306 (URA3) digested with NotI and EcoRI for MKS1. The MMT2 disruption plasmid pLJ188 contained upstream sequences (~418 to +69 (BglIII and SpeI) and downstream sequences +718 to +1145 (EaeI and BglII). The MKS1 disruption plasmid pLJ244 was constructed with upstream sequences ~920 to +184 (BamHI and EcoRI) and downstream sequences +1728 to +2308 (NotI and BamHI).

Transformation of yeast strains with pLJ186, pLJ188, or pLJ244 digested with EcoRI, BglIII, or BamHI, respectively, resulted in the deletion of MMT1 sequences ~214 to +1377, MMT2 sequences +70 to +717, and MKS1 sequences +185 to +1727. All gene deletions were verified by in vivo PCR.

Detection of Carboxylated Proteins—Carboxylated proteins were detected using the OxyBlot protein oxidation detection kit (Intergen). Mitochondrial and post-mitochondrial supernatant fractions were isolated from cells grown aerobically in YPD medium as described previously (25). 10 μg of protein from each fraction was reacted with 2,4-dinitrophenylhydrazine for 15 min at 25 °C. Samples were resolved on 12% denaturing polyacrylamide gels, and 2,4-dinitrophenol-derivatized proteins were identified by immunoblot assays using an anti-2,4-dinitrophenol antibody. To monitor sample loading, the cytosolic protein Pgk1 and the mitochondrial protein Mss2 were also detected by immunoblotting with the corresponding antibodies.

EPR-detectable Iron—Samples for EPR spectroscopic analyses were prepared as described previously (26). EPR spectra were recorded on a Bruker X-band spectrometer. Samples were maintained below 95 K during the recording of the spectra by using either a finger Dewar flask filled with liquid nitrogen or a variable temperature gas-cooled cavity. Parameters for low temperature Fe(III) EPR spectroscopy using the finger Dewar flask were reported previously (23). Parameters using the variable temperature cavity were: center field 1560 G, sweep width 500 G, frequency 9.45 GHz, microwave power 31 milliwatts, attenuation 10 dB, modulation amplitude 20 G, modulation frequency 100 kHz, receiver gain 2 × 10^4, sweep time 20.97 s, time constant 81.92 ms, resolution 2048 points, and number of scans 16. Quantitation and calculation of EPR-detectable iron levels were carried out as described previously (26). Iron Measurements on Isolated Mitochondria—Yeast cells grown aerobically to mid-log phase in YPD medium were centrifuged to spheroplasts, and mitochondria were isolated as described previously (27).

Iron analysis of isolated mitochondria was carried out on a PerkinElmer Lambda graphite furnace atomic absorption spectrophotometer according to the manufacturer’s specifications. Results are from at least two independent experiments.

Quantitation of mRNA Levels by Reverse Transcription PCR—Cells were grown in synthetic dextrose medium to A600 ~ 1, and RNA was isolated using the RNeasy kit (Qiagen) with the optional DNase digestion. The Superscript First-strand Synthesis System for reverse transcription PCR (Invitrogen) was used to prepare single-stranded cDNA. Amplification of the target cDNA was performed using specific primers to LYS9, LYS12, and LYS20 according to the manufacturer’s specifications. Control primers for ACT1 were also included in each PCR reaction. PCR products were separated on 2.5% agarose gels and visualized with ethidium bromide staining.

RESULTS
Mutants to suppress the sod1 by ssq1—We observed that limiting iron accumulation by disruption of the iron uptake gene still capable of reversing the methionine auxotrophy (Fig. 4). This clearly demonstrated that high affinity iron uptake is needed for suppression of the sod1 amino acid auxotrophies in the seo mutants.

Fig. 2. EPR-detectable iron is not reduced in the seo mutants compared with the sod1Δ strain. A. low temperature iron EPR spectra of WT, sod1Δ, ssq1 sod1Δ, and jac1 sod1Δ strains. Spectra are normalized to represent equivalent number of cells. The major signal appears at 4.3 G. B, elevation of EPR-detectable iron compared with wild type cells. EPR-detectable 4.3 G iron was measured in WT, sod1Δ, and the seo mutants, and iron measurements were divided by the value for the wild type strain. Data are the average of at least three different colonies.

MMT1 Is a High Copy Suppressor of sod1Δ Methionine and Lysine Auxotrophies—MMT1 and MMT2 encode putative mitochondrial iron transporters and were identified by their ability to increase mitochondrial iron by 5-fold when present on high copy plasmids (40). If overaccumulation of mitochondrial iron (23). Therefore, the seo mutants do not behave as antioxidant suppressors of sod1Δ and appear to rescue only a subset of sod1Δ defects, e.g. methionine and lysine auxotrophy.

seo Class of Mutants Overaccumulates Mitochondrial Iron—Four of the seo mutants represent genes that are known to function in an iron-sulfur cluster assembly (ssq1, jac1, nfs1, and isu1) (18, 19, 29, 30; two others (seo3 and seo6) remain unidentified but may also participate in the assembly of iron-sulfur clusters (18). Mutations in yeast genes that function in an iron-sulfur cluster assembly typically result in the overaccumulation of mitochondrial iron (19, 27, 30–35). We therefore tested whether this also occurs in the background of a sod1Δ mutant by measuring the mitochondrial iron content of the four known and two unidentified seo mutant strains. As seen in Fig. 3, we found that ssq1, jac1, nfs1, and isu1 mutations in conjunction with a sod1Δ mutation continue to overaccumulate mitochondrial iron. Additionally, strains containing seo3 and seo6 mutations also share the mitochondrial iron overaccumulation phenotype. Overaccumulation of iron has been proposed to lead to increased oxidative damage (36, 37), and this may account for the increased oxidative damage associated with the seo mutants. It is noteworthy that the two strains that exhibit the least accumulation of mitochondrial iron (isu1 and seo6) also show the least effect on oxidative damage to mitochondrial protein (Fig. 1B). Yeast containing yfh1Δ and atm1Δ mutations are also known to overaccumulate mitochondrial iron (36, 38); however, we were unable to test these mutants for suppression of sod1Δ because they grew poorly on a medium lacking methionine or lysine in air, even when wild type for SOD1 (not shown).

Suppression of sod1Δ by seo Mutations Requires High Affinity Iron Uptake—It is curious that the seo mutants exhibit both an increase in mitochondrial iron and an increase in oxidative damage while suppressing sod1Δ defects in amino acid biosynthesis. To address whether there is a connection between the high iron uptake and suppression of sod1Δ, we tested whether reducing cellular iron would alter the ability of seo mutants to rescue the methionine and lysine auxotrophies of a sod1Δ mutant. Although the suppression of the sod1Δ lysine auxotrophy by ssq1 and jac1 mutations was very weak, these mutants were still capable of reversing the methionine auxotrophy (Fig. 4). We observed that limiting iron accumulation by disruption of the iron uptake gene FET3 (39) abolished the ability of the seo mutants to suppress the sod1Δ amino acid biosynthetic defects (not shown).
The anaerobic control shown is medium lacking methionine; however, indicated medium and grown for 3 days in air or anaerobic chambers.

iron is required for the seo suppression of the sod1Δ methionine and lysine defects, then increasing mitochondrial iron with high copy MMT1 and MMT2 may mimic the seo mutations and should also suppress these defects. As seen in Fig. 5, MMT1 is a new high copy suppressor of the sod1Δ methionine and lysine auxotrophies. High copy MMT2 was also capable of suppressing the sod1Δ metabolic defects (not shown). Consistent with the results from the seo mutants, disruption of high affinity iron uptake through fet3Δ mutations eliminated the suppression of sod1Δ methionine and lysine auxotrophies obtained with high copy MMT1 (Fig. 5), clearly demonstrating a role for mitochondrial iron in the suppression of sod1Δ biosynthetic defects.

Disruption of the MMT Genes Limits Mitochondrial Iron in seo3 and seo6 Strains—The transporters responsible for overloading the mitochondria with iron in the seo mutants have not been identified, but increased expression of MMT1 was capable of causing overaccumulation of mitochondrial iron (40), similar to that seen in the seo mutants. In an attempt to limit mitochondrial iron, double disruptions of both MMT1 and MMT2 were generated in the seo mutants. Monitoring mitochondrial iron accumulation in these strains revealed that seo3 and seo6 lacking the MMT genes now had mitochondrial iron levels that were similar to the sod1Δ strain (Fig. 6A). Surprisingly, this effect of MMT deletions was not observed with the other seo mutants encoding known members of the iron-sulfur cluster machinery, e.g. ssq1, jac1, nfs1, and isu1 (Fig. 6A). The high mitochondrial iron in these mutants must involve a separate metal transport system. Nevertheless, the reversal of high mitochondrial iron in seo3 and seo6 mutants allowed us to test the role of MMT-driven mitochondrial iron in suppression of sod1Δ. As expected, loss of the MMT genes eliminated the ability of seo3 and seo6 mutants to suppress the sod1Δ aerobic auxotrophies for methionine and lysine (Fig. 6B). This effect indeed correlated with lowered iron because the remaining seo mutants that were unaffected by mmt mutants still suppressed the sod1Δ growth defect on medium lacking methionine or lysine. Together, these results indicate that overaccumulation of mitochondrial iron is necessary for the seo suppression. In the case of seo3 and seo6 mutants, this elevated level of iron is contributed by MMT, whereas yet unknown transporters contribute to high mitochondrial iron in the case of ssq1, jac1, nfs1, and isu1 mutants.

seo Suppression of sod1Δ Methionine Auxotrophy Requires the Pentose Phosphate Pathway—The sod1Δ defect in methionine biosynthesis has been attributed to depletion of NADPH, a critical co-factor for two methionine biosynthetic enzymes (11). Slekar et al. (11) found that the methionine defect is reversed by overexpression of transketolase (TKL1), which
serves to activate NADPH production by the PPP. Suppression of the sod1Δ methionine defect by high copy TKL1 required the presence of ZWF1, encoding the glucose-6-phosphate dehydrogenase. We therefore tested whether the seo suppression of sod1Δ methionine auxotrophy was likewise dependent on the PPP and ZWF1. As seen in Fig. 7, disruption of ZWF1 in the seo mutants eliminated the ability of these strains to grow on medium lacking methionine in air. Although aerobic growth was also reduced somewhat by zwf1Δ mutations on medium lacking lysine, this lack of growth did not represent sod1Δ lysine auxotrophy because it was also seen in WT SOD1 strains (Fig. 7). Hence, zwf1 mutations appear to preferentially block the seo suppression of sod1Δ methionine auxotrophy. The requirement for Zwf1 suggests that the PPP may be activated in the seo mutants. How the PPP might be activated in the seo mutants is still unknown, but a possibility is that the cell could induce PPP in response to oxidative damage.

Lysine Biosynthetic Genes Are Induced in the seo Mutants—The molecular defect responsible for the lysine auxotrophy of sod1Δ strains remains elusive; however, lysine biosynthesis in yeast lacking the copper chaperone for SOD1, CCS1 (also known as LYS7) (41) appears to be blocked prior to the formation of α-amino adipate (42). In an attempt to better understand this lysine biosynthetic defect, we searched for mutations that suppressed the lysine but not the methionine auxotrophy of sod1Δ yeast. As seen in Fig. 8A, a deletion mutation in MKS1 (also known as LYS80) was capable of suppressing the sod1Δ lysine biosynthetic defect but did not reverse the methionine auxotrophy. As such, mks1Δ is the first suppressor known to reverse specifically the sod1Δ lysine auxotrophy. Interestingly, MKS1 has been shown to be a negative regulator of retrograde regulation in which nuclear gene expression is altered in response to mitochondrial damage by the Rtg1-3 proteins (43, 44).

A result of the disruption of MKS1 and the loss of its negative regulation of the retrograde response is the elevated expression of genes in lysine biosynthesis (44, 45), but the loss of MKS1 was not found to alter the expression of genes in the PPP or methionine biosynthesis (44). To test whether genes in the lysine biosynthetic pathway were indeed induced in the seo mutants, we used reverse transcriptase PCR to monitor the expression of LYS9, LYS12, and LYS20, which was strongly induced by mks1Δ mutations (44). An increase in expression of the three LYS genes tested was observed in each of the seo mutants, compared with the sod1Δ control (Fig. 8B). We propose that the suppression of the sod1Δ lysine auxotrophy represents a retrograde stress response of the cell triggered by mitochondrial damage, which in turn increases expression of lysine biosynthetic genes and lysine content.

**DISCUSSION**

The goal of this study was to understand how the seo class of suppressors alleviates the metabolic defects of sod1Δ strains. Unlike other suppressors of sod1Δ mutants, such as pmr1, the seo mutants do not show general antioxidant behavior and fail to reduce sensitivity to environmental oxidants (18). Although it was initially speculated that the seo suppressors function by reducing the production of endogenous sources of oxidative damage, this is not the case. Our results suggest that the seo mutations do not reduce the burden of endogenous reactive oxygen species as shown by their failure to reduce the high amount of EPR-detectable “free” iron characteristic of sod1Δ mutants. Instead, they appear actually to increase oxidative damage as evidenced by increased protein carbonyl levels. Interestingly, the increased oxidative damage closely correlated with overaccumulation of mitochondrial iron, and this hyperaccumulation of iron was found to be critical for suppression of the sod1Δ methionine and lysine auxotrophies in the seo mutants. Limiting iron uptake with fet3 mutations or blocking mitochondrial iron overaccumulation with mmt deletions as in the case of seo3 and seo6 mutants abolished the suppression of the sod1Δ metabolic defects. The form of the iron that is elevated in the mitochondria of the seo mutants is not EPR-detectable “free” iron. Mitochondria isolated from sod1Δ yeast do not show an increase in EPR-detectable iron, indicating that this iron is not localized to the mitochondria.2

Although the seo mutants all overaccumulate mitochondrial iron, the molecular pathways by which this iron accumulation is mediated are distinct. The seo mutants appear to fall into at
least two classes, one of which includes the seo3 and seo6 mutants that rely on the MMT genes for mitochondrial iron accumulation. By contrast, the route by which mitochondrial iron is accumulated in the remaining seo mutants (seo1, jac1, nfx1, and issu1) remains unclear. The mitochondrial carrier proteins Msr3 and Msr4 have been proposed to contribute to mitochondrial iron in yfh1Δ strains (46) and may play a role in iron accumulation due to seo1, jac1, nfx1, and issu1 mutations. Alternatively, other yet uncharacterized mitochondrial iron transporters may be involved in the overaccumulation of iron in these mutants.

Having eliminated a reduction in oxidative stress as the cause of seo suppression, we investigated how overaccumulation of mitochondrial iron could reverse the sod1Δ metabolic defects. Our results indicate that the seo suppression of the sod1Δ methionine and lysine auxotrophies is mediated through the sensing of mitochondrial stress, but the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct.

Our studies suggest that reversal of the lysine defect involves activation of the retrograde response, which is controlled in part through the action of the Rtg and Mks1 proteins. The retrograde response in yeast is known to alter nuclear gene expression in reaction to changes in the functional state of mitochondria, and the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct. Having eliminated a reduction in oxidative stress as the cause of seo suppression, we investigated how overaccumulation of mitochondrial iron could reverse the sod1Δ metabolic defects. Our results indicate that the seo suppression of the sod1Δ methionine and lysine auxotrophies is mediated through the sensing of mitochondrial stress, but the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct. Having eliminated a reduction in oxidative stress as the cause of seo suppression, we investigated how overaccumulation of mitochondrial iron could reverse the sod1Δ metabolic defects. Our results indicate that the seo suppression of the sod1Δ methionine and lysine auxotrophies is mediated through the sensing of mitochondrial stress, but the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct. Having eliminated a reduction in oxidative stress as the cause of seo suppression, we investigated how overaccumulation of mitochondrial iron could reverse the sod1Δ metabolic defects. Our results indicate that the seo suppression of the sod1Δ methionine and lysine auxotrophies is mediated through the sensing of mitochondrial stress, but the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct. Having eliminated a reduction in oxidative stress as the cause of seo suppression, we investigated how overaccumulation of mitochondrial iron could reverse the sod1Δ metabolic defects. Our results indicate that the seo suppression of the sod1Δ methionine and lysine auxotrophies is mediated through the sensing of mitochondrial stress, but the pathways used for rectifying the methionine and lysine biosynthetic defects may be distinct.
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