

## Multiple Genes, Including a Member of the AAA Family, Are Essential for Degradation of Unassembled Subunit 2 of Cytochrome *c* Oxidase in Yeast Mitochondria

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**Cytochrome *c* oxidase consists of three mitochondrion- and several nucleus-encoded subunits. We previously found that in a mutant of *Saccharomyces cerevisiae* lacking nucleus-encoded subunit 4 of this enzyme (CoxIV), subunits 2 and 3 (CoxII and CoxIII), both encoded by the mitochondrial DNA, were unstable and rapidly degraded in mitochondria, presumably because the subunits cannot assemble normally. To analyze the molecular machinery involved in this proteolytic pathway, we obtained four mutants defective in the degradation of unassembled CoxII (*osd* mutants) by screening CoxIV-deficient cells for the accumulation of CoxII. All of the mutants were recessive and were classified into three different complementation groups. Tetrad analyses revealed that the phenotype of each mutant was caused by a single nuclear mutation. These results suggest strongly that at least three nuclear genes (the *OSD* genes) are required for this degradation system. Interestingly, degradation of CoxIII was not affected in the mutants, implying that the two subunits are degraded by distinct pathways. We also cloned the *OSD1* gene by complementation of the temperature sensitivity of *osd1-1* mutants with a *COXIV*<sup>+</sup> genetic background on a nonfermentable glycerol medium. We found it to encode a member of a family (the AAA family) of putative ATPases, which proved to be identical to recently described *YME1* and *YTA11*. Immunological analyses revealed that Osd1 protein is localized to the mitochondrial inner membrane. Disruption of the predicted ATP-binding cassette by site-directed mutagenesis eliminated biological activities, thereby underscoring the importance of ATP for function.**

Mitochondria have an intrinsic protein degradation system for turnover of constituent proteins, especially for abnormal or unassembled ones (22). Rapid turnover of unassembled polypeptides is important for mitochondria, because the respiratory chain enzyme complexes, each of which is composed of several heterologous subunits, assemble and function simultaneously there. Most of these enzyme complexes contain subunits encoded by nuclear and mitochondrial DNAs (4, 23). To avoid accumulation of excess amounts of unassembled subunits or immature intermediates, the levels of the subunits, especially those between mitochondrially and nuclearly encoded ones, need to be regulated closely. Although such regulation can be partly achieved by controlling the rates of synthesis, finer regulation can be achieved by removing excessive amounts of unassembled or misassembled subunits by degradation. Thus, such a protein degradation system may play a significant physiological role in the biogenesis of mitochondria.

Supporting this idea, several studies have revealed that isolated mammalian (12) or budding yeast (*Saccharomyces cerevisiae*) (29, 67) mitochondria have ATP-dependent proteolytic activities. In search of factors involved in mitochondrial proteolytic systems, ATP-dependent soluble proteases were isolated from the mitochondrial matrix fraction of rat liver (11), bovine adrenal cortex (63, 64), or yeast cells (33), by using exogenous substrates such as modified casein. The gene for the

budding yeast (54, 61) or human (62) homolog of this type of protease has been cloned and, in the case of *Saccharomyces cerevisiae*, has been shown to be required for mitochondrial respiratory function (54, 61). Involvement of this protease in the degradation of mitochondrial translation products has been suggested (11, 20), but no direct evidence has been provided. Furthermore, the existence of ATP-dependent proteolytic activity for mitochondrial translation products in yeast submitochondrial particles (29, 67) raises the possibility that, other than or in addition to the matrix protease, an ATP-dependent factor bound to mitochondrial inner membrane is involved.

As an alternative approach for identifying factors involved in mitochondrial degradation of unassembled translation products, we used a genetic approach in a yeast strain in which one of the subunits of cytochrome *c* oxidase is lacking. Yeast cytochrome *c* oxidase consists of at least 11 subunits (45, 55). The largest three, CoxI, CoxII, and CoxIII, are encoded by mitochondrial DNA. The remaining smaller subunits, encoded by nuclear DNA, are translated in the cytosol and then imported into mitochondria, where they assemble to form a mature complex with mitochondrially encoded subunits (4, 23). It has been reported that in a CoxIV-deficient strain, other subunits cannot assemble normally and intramitochondrial levels of CoxII and CoxIII are significantly decreased (13). In pulse-chase labeling experiments with whole cells, we earlier found that the stability of those subunits in the CoxIV-deficient strain was dramatically decreased, thereby suggesting that the reduced intramitochondrial levels were due, at least in part, to an enhanced degradation of CoxII and CoxIII in the mitochondria of the strain (40). This mitochondrial degradation was inhibited by *o*-phenanthroline, a membrane-permeating chelator for divalent metal ions, and this inhibitory effect was reversed by the addition of an excess amount of Zn<sup>2+</sup>, Mn<sup>2+</sup>, or

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
DL-1	<i>MAT<math>\alpha</math> his3 leu2 ura3</i>	13
WD-1	<i>MAT<math>\alpha</math> his3 leu2 ura3 cox4::LEU2</i>	13
WS-2	<i>MAT<math>\alpha</math> his3 leu2 ura3 cox4::LEU2 osd1-1</i>	This study
WS-3	<i>MAT<math>\alpha</math> his3 leu2 ura3 cox4::LEU2 osd2-1</i>	This study
WS-5	<i>MAT<math>\alpha</math> his3 leu2 ura3 cox4::LEU2 osd3-1</i>	This study
WS-8	<i>MAT<math>\alpha</math> his3 leu2 ura3 cox4::LEU2 osd3-2</i>	This study
H11-1B	<i>MAT<math>\alpha</math> his3 leu2 lys2</i>	Our laboratory
H41-5D	<i>MAT<math>\alpha</math> his3 leu2 lys2 cox4::LEU2</i>	This study
H35-1C	<i>MAT<math>\alpha</math> his3 leu2 lys2 cox4::LEU2 osd1-1</i>	This study
H37-3A	<i>MAT<math>\alpha</math> his3 leu2 lys2 cox4::LEU2 osd2-1</i>	This study
H38-2B	<i>MAT<math>\alpha</math> his3 leu2 lys2 cox4::LEU2 osd3-1</i>	This study
H34-2C	<i>MAT<math>\alpha</math> his3 leu2 lys2 cox4::LEU2 osd3-2</i>	This study
D273-10B	<i>MAT<math>\alpha</math></i>	American Type Culture Collection
H92-1B	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3</i>	This study
H92-6C	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 osd1-1</i>	This study
H92-2A	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 osd1-1</i>	This study
H92-5D	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 coxIV::LEU2 osd1-1</i>	This study
H92-4B	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 coxIV::LEU2</i>	This study
H92-1B $\Delta$ 1	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 osd1::HIS3</i>	This study
H92-4B $\Delta$ 1	<i>MAT<math>\alpha</math> his3 leu2 lys2 ura3 coxIV::LEU2 osd1::HIS3</i>	This study

Co<sup>2+</sup>, but not by Ca<sup>2+</sup> or Mg<sup>2+</sup>, thereby suggesting the involvement of a divalent metal ion-dependent factor. Furthermore, the degradation was significantly suppressed by treatments inhibiting the glycolysis pathway, which would reduce the intramitochondrial ATP concentration in a respiratory-deficient strain. Hence, an energy-dependent mechanism is likely to be involved (40).

We describe here the isolation and genetic characterization of a set of mutants (*osd* mutants) defective in the degradation of unassembled CoxII in a CoxIV-deficient genetic background. Our results suggest that multiple genes (putatively designated as *OSD1*, *OSD2*, and *OSD3*) are required for this degradation pathway. We also show that each of these *osd* mutations caused temperature-sensitive growth on a nonfermentable glycerol medium when introduced into a *COXIV*<sup>+</sup> genetic background, suggesting an important physiological role of the *OSD* genes in mitochondrial functions.

We cloned the *OSD1* gene on the basis of its potential to complement this temperature sensitivity. Gene disruption analysis revealed that *OSD1* is required, in addition to the degradation of unassembled CoxII, to maintain normal levels of respiratory chain enzymes. Osd1 protein is bound to the mitochondrial membrane. Sequence analysis of the *OSD1* gene revealed that it belongs to a member of a recently identified novel family of putative ATPase genes. More recently, it was seen to be identical to a gene known as *YME1* or *YTA11* (50, 57). Mutational analysis of the ATP-binding cassette in the *OSD1* gene product suggests the importance of ATP for function.

## MATERIALS AND METHODS

**Strains and plasmids.** The *S. cerevisiae* strains used for this study are listed in Table 1. DL 1 and WD 1 (13) were a generous gift from G. Schatz (Basel, Switzerland). H11-1B is congeneric with D273-10B (ATCC 25657), except for the presence of *leu2*, *lys2*, and *his3* auxotroph markers. To obtain *osd* mutant strains of the opposite mating type, each of the original *osd* mutant strains was crossed with H11-1B, a haploid strain having the wild-type *COXIV* gene, and the resultant diploid cells were sporulated. The segregants with the disrupted *coxIV::LEU2* gene (as judged by the leucine prototroph) were further examined for the presence of the *osd* mutations and for the  $\alpha$  mating type. The presence of *osd* mutations in *coxIV::LEU2* segregants was identified by examination of the accumulation of CoxII by Western blotting (immunoblotting) after acquisition of the mitochondrial fraction (described below). *coxIV::LEU2* segregants of an appropriate mating type accumulating CoxII were used for complementation tests and

for further backcrossing. After backcrossing to H11-1B was repeated at least six times, segregants with both the wild-type *COXIV* allele and one of the *osd* mutations were used for further experiments as isogenic strains with H11-1B. The presence of the *osd* mutations in strains of the *COXIV*<sup>+</sup> genetic background was examined as described in the legend to Fig. 3.

pFL-1 (9) was kindly provided by T. Hase (Osaka University, Osaka, Japan). pUCE is a derivative of pUC18 in which the multicloning site was replaced with a *SmaI-EcoRI-SmaI* site (18). The other plasmids used are described below.

**Yeast media and growth conditions.** YPD, YPR, YPGal, and YPG contained 1% yeast extract (Difco), 2% Bacto Peptone (Difco), and either 2% glucose, raffinose, galactose, or glycerol, respectively. SD (6.7 g of yeast nitrogen base [Difco] per liter, 2% glucose), SG (6.7 g of yeast nitrogen base per liter, 2% glycerol), or SR (6.7 g of yeast nitrogen base per liter, 2% raffinose) was used for auxotroph selections after supplementation with the appropriate amino acids or uracil. Solid medium contained 2% agar (Difco). Yeast cells were grown at 30°C, unless otherwise specified.

**Isolation of *osd* mutants.** WD 1 cells were mutagenized with ethyl methane-sulfonate, recovered in YPD overnight at 23°C, and plated on YPR. The plates were incubated for 3 days at 23°C and replica transferred onto nitrocellulose membranes (BA 85; Schleicher & Schuell, Dassel, Germany). The membranes were placed face up on YPR plates, further incubated at 23°C overnight, and then incubated at 37°C for 6 h. Such a scheme for cell growth was designed to allow temperature-sensitive conditional mutants to be isolated, because we did not know in advance whether a defect in the degradation of unassembled CoxII would be lethal for the cell. Cells on the membranes were lysed according to the published method (35), except that the sodium dodecyl sulfate (SDS) concentration in the lysis buffer was raised to 2%. The membranes were blocked overnight with TBS (20 mM Tris-Cl [pH 7.5], 150 mM NaCl) containing 10% skim milk and treated with affinity-purified rabbit anti-CoxII polyclonal antibody for 2 h at room temperature. They were further washed three times with TBS and treated with goat anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Bio-Rad). The membranes were washed three times with TBS, and CoxII on the membranes was visualized with 4-chloro-1-naphthol (Bio-Rad). Candidate colonies on the master plates corresponding to relatively intense signals on the membrane were recovered, and mitochondrial accumulation of CoxII was examined by Western blotting after the mitochondria were isolated.

**Pulse-chase experiments with mitochondrial translation products.** Yeast cells were grown in YPD to a late logarithmic phase (about 260 to 280 Klett units) and washed twice with distilled water. These cells were resuspended in the original volume of sulfur-free salts medium (2.9 mM MgCl<sub>2</sub>, 7.3 mM KH<sub>2</sub>PO<sub>4</sub>, 8.6 mM NaCl, 3.6 mM CaCl<sub>2</sub>, 17.9 mM NH<sub>4</sub>Cl) supplemented with 0.2% glucose. After incubation for 5 min at 30°C with vigorous shaking, cycloheximide was added to a final concentration of 1 mg/ml. After a further 5 min of incubation, labeling of mitochondrial translation products was initiated by addition of either [<sup>35</sup>S]methionine (>1,000 Ci/mmol [Amersham]) or EXPRE<sup>35S</sup> (NEN), a mixture of [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine, to a concentration of 50  $\mu$ Ci/ml. After incubation at 30°C for 30 min, the suspension was cooled on ice and centrifuged in 14,000  $\times$  g at 4°C for 5 min. The supernatant was discarded, and the pelleted cells were resuspended in the original volume of chase medium (YPD supplemented with 10 mM nonisotopic methionine, 10 mM cysteine, and 10 mg of Na<sub>2</sub>SO<sub>4</sub> per ml) and incubated at 30°C with vigorous shaking. At the times indicated, 250  $\mu$ l

of the aliquots was transferred to tubes containing 1 ml of 10% trichloroacetic acid and incubated on ice for at least 30 min. After centrifugation, the cells were washed twice with 5% trichloroacetic acid and resuspended in 60  $\mu$ l of distilled water. Cell walls were disrupted by vortexing the cell suspension with 0.2 ml of glass beads at maximum speed for 1 min. After SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer was added, the disrupted cell suspension was neutralized with Tris base, if necessary, and total proteins were extracted by incubation for 1 h at room temperature. After cell debris was removed by centrifugation, solubilized proteins were separated by SDS-PAGE (34), and the labeled mitochondrial translation products were visualized fluorographically (36). Quantitative analysis of the bands on the fluorograph was carried out with an UltraScan XL laser densitometer (Pharmacia-LKB). Alternatively, the labeled proteins were analyzed with a BAS2000 Bio-Image Analyzer (Fuji Photo Film, Tokyo, Japan).

**Isolation of mitochondria.** Yeast cells were grown to mid-logarithmic phase and collected by centrifugation. After two washings with TSB (10 mM Tris-HCl [pH 7.4], 0.6 M sorbitol), the cells were resuspended in the same buffer supplemented with 5 mM phenylmethylsulfonyl fluoride and vortexed at a maximum speed with 1.5 ml of glass beads (diameter of 0.45 to 0.5 mm) for 30 s four times with intervals of cooling on ice. Disrupted cell suspension was recovered with a pipette, avoiding contamination of glass beads, and centrifuged at  $3,500 \times g$  for 5 min. The supernatant was centrifuged at  $12,000 \times g$  for 10 min. The pellet was carefully resuspended in TSB and recentrifuged at  $3,500 \times g$  for 5 min. The supernatant was recentrifuged at  $12,000 \times g$  for 10 min, and the final pellet was washed twice with TSB and resuspended in TSB.

**Molecular cloning of the *OSD1* gene.** The *OSD1* gene was cloned by complementation of the temperature-sensitive phenotype of the *osd1-1 COXIV*<sup>+</sup> cells on glycerol medium. H92-6C (*osd1-1 COXIV*<sup>+</sup>) cells were transformed with a yeast genomic library constructed from *Sau3A* partially digested total DNA of a [*rho*<sup>-</sup>] subclone of D273-10B (ATCC 25657) in a multicopy shuttle vector, pFL-1 (9). Approximately 20,000 Ura<sup>+</sup> transformants were screened for growth at 37°C on SG plates supplemented with leucine, lysine, and histidine for 5 days. Colonies were reexamined for growth at 37°C on the same medium. Finally, six independent clones were obtained. Plasmids were recovered by transformation of the *Escherichia coli* RR1 strain with the extracted DNA and selection for ampicillin resistance. Each plasmid recovered was reintroduced into H92-6C to examine the potential to rescue the cell from growth suppression at 37°C on the glycerol medium. Each of these plasmids was also introduced into H92-5D (*osd1-1 coxIV::LEU2*) to examine the potential to complement the accumulation of unassembled CoxII in the mitochondria of *osd1* mutants.

**Sequence analysis.** The 3.5-kb *BglII-ScaI* fragments of pSG3, which are sufficient for the complementation of both of the two phenotypes of the *osd1* mutants, were ligated with *EcoRI* linkers after being blunt ended with the Klenow fragment and were subcloned into the *EcoRI* site of Bluescript KS+ plasmid (Stratagene) in both orientations. Sets of nested deletion mutants from each of the ends were obtained by the exonuclease III method (25) by using Kilo-sequence deletion kits (Takara). Some of the fragments derived from this region were also subcloned into an M13mp19 vector. All of the nucleotide sequencing was performed by the dideoxy chain termination method (49) with Sequenase (U.S. Biochemicals). Custom-made oligonucleotides were used as primers for sequencing regions not accessible with the constructions described above. Nucleotide sequences of both DNA strands were determined.

**Gene disruption of *OSD1*.** The *EcoRI* linker-attached 3.5-kb *BglII-ScaI* fragment containing an entire *OSD1* gene was subcloned into the unique *EcoRI* site of pUCE vector to generate pEOS1. The 2.2-kb *EcoRV-HindIII* region of the insert in pEOS1, containing about the NH<sub>3</sub>-terminal 90% of the *OSD1* coding region and 0.3 kb of its downstream region, was removed by digestion with *EcoRV* and *HindIII* and replaced with a *BamHI* linker after being blunt ended to generate pDOS1. The 1.8-kb *BamHI* fragment of the *HIS3* marker gene was inserted into the unique *BamHI* site of pDOS1 to generate pDOS1-HIS3. This plasmid was digested with *EcoRI* to excise the insert containing the disrupted *OSD1* gene and used to transform yeast strains H92-1B (*COXIV*<sup>+</sup>) and H92-4B (*coxIV::LEU2*). Candidate *OSD1*-disrupted cells were obtained by selection for His<sup>+</sup> colonies. Colonies grown on histidine-lacking plates from each of the two strains were recovered and examined for replacement of the *OSD1* gene by Southern blotting with the 3.1-kb *NcoI* fragment encoding the whole *OSD1*-coding region as a probe. One of the desired *OSD1*-disrupted strains derived from either H92-1B or H92-4B was chosen for further analysis (H92-1BΔ1 or H92-4BΔ1, respectively).

**Site-directed mutagenesis.** For mutagenesis of the nucleotide-binding consensus sequence, a 0.9-kb *PstI-BamHI* fragment of the *OSD1* gene containing the ATP-binding consensus sequence was subcloned into the multicloning site of M13tv19 phagemid vector (Takara), and mutations were introduced with Mutan G mutagenesis kits (Takara) and the following synthetic oligonucleotides: ONB-1, 5'-CCAACAAGTTTCACCGGTACCAGGAGGTC-3'; ONB-2, 5'-C CAACAAGTGATACCGGTACCAGGAGGTC-3'; ONB-3, 5'-CCAACAAGTTTGACCGGTACCAGGAGGTC-3'; and ONB-4, 5'-CCAACAAGTTCTACCGGTACCAGGAGGTC-3'. Sequences corresponding to the conserved lysine residue in the wild-type *OSD1* gene are underlined. For convenience, a *KpnI* site was introduced without affecting the coding amino acid. All mutations introduced were checked by sequencing with an appropriate synthetic oligonucleotide as a primer. The mutagenized fragments were excised and replaced with

the 0.9-kb *PstI-BamHI* region in the 4.2-kb *BglII* fragment of the wild-type *OSD1* gene, which had been subcloned into the *EcoRI* site of pUCE after attachment of *EcoRI-SmaI* adapters. Each of the fragments containing the full-length mutagenized *OSD1* genes was excised with *SmaI* and subcloned into the unique *PvuII* site of pFL-1, generating pFL-ONB-1, -2, -3, and -4, respectively.

**Enzyme assays.** Published methods were used for enzyme assays of cytochrome *c* oxidase (37), NADH-cytochrome *c* reductase (24), succinate-cytochrome *c* reductase (58), kinurenine hydroxylase (5), and NADPH-cytochrome *c* reductase (38).

**Subcellular fractionation.** To examine CoxII accumulation, mitochondria were prepared with glass beads as described above. To examine subcellular localization of Osd1 protein, mitochondria were prepared as follows. Yeast cells were grown in YPGal to mid-logarithmic phase (150 Klett units). Spheroplasts, prepared according to a previously published method (10), were disrupted in a Dounce homogenizer (pestle A) and centrifuged at  $3,500 \times g$  for 5 min. The pellet containing both the nucleus and undisturbed cells was discarded, and the postnuclear supernatant fraction was further subfractionated by centrifugation at  $12,000 \times g$  for 10 min. The pellet was resuspended in TSB and centrifuged at  $3,500 \times g$  for 5 min, and the supernatant was centrifuged at  $12,000 \times g$  for 10 min for separation into the pellet mitochondrial and postmitochondrial supernatant fractions. Ratios of enzymatic activities per protein for the pellet mitochondrial and postmitochondrial supernatant fractions to those of the postnuclear supernatant fraction were 6.54 and 0.11, respectively, for cytochrome *c* oxidase and 2.12 and 0.88, respectively, for NADPH-cytochrome *c* reductase.

For subfractionation of the mitochondrial membrane, mitochondria were further purified from the mitochondrial fraction by 20 to 40% stepwise Percoll (Pharmacia) density gradient centrifugation in a buffer containing 0.6 M sorbitol, 20 mM tricine-KOH (pH 8.0), 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and Percoll was removed by dilution and washing with the buffer. Purified mitochondria were subfractionated as described previously (10).

**Whole-cell spectrum.** Yeast cells were grown in YPGal medium to mid-logarithmic phase at the temperatures indicated, collected by centrifugation, and resuspended in 50% glycerol. After a few grains of sodium dithionite were added and the samples were well mixed by inversion of the cuvette several times, the whole-cell spectrum was measured at room temperature with an MP-100 multi-purpose spectrophotometer (Shimadzu, Tokyo, Japan).

**Immunological methods.** CoxII was purified electrophoretically from yeast cytochrome *c* oxidase, prepared according to a previously published method (48). Antiserum for CoxII was prepared by injecting rabbits subcutaneously once with CoxII with Freund's complete adjuvant and, subsequently, injecting them four times with Freund's incomplete adjuvant, with intervals of 2 weeks before bleeding. A CoxII affinity column was prepared by coupling of electrophoretically purified CoxII to Affi-Gel 10 (Bio-Rad) according to the manufacturer's instructions, and it was used for affinity purification of anti-CoxII antibody. The  $\beta$ -galactosidase-Osd1 fusion protein was prepared as follows. The 1.6-kb *BamHI-EcoRI* fragment containing the coding region for the C-terminal one-third of the Osd1 protein and its downstream region was excised from pUCE-*OSD1-ScaI* and subcloned into *BamHI-EcoRI* double-digested pEX2 (53) in frame with the  $\beta$ -galactosidase coding sequence. Fusion protein was expressed in bacteria and purified electrophoretically after extraction by intensive sonication and boiling for 5 min in SDS-PAGE sample buffer. After proteins were separated by SDS-PAGE, they were blotted electrophoretically onto nitrocellulose membranes. Portions of the membrane corresponding to the fusion protein were excised and dissolved in dimethyl sulfoxide and, after being mixed with complete or incomplete Freund's adjuvant, were used to immunize rabbits, as described above.

**Other methods.** Yeast manipulations were carried out basically according to the method of Rose et al. (46). Yeast transformation was done by the lithium acetate method (27). Published methods were used for SDS-PAGE (34) and electrophoretic transfer of proteins from polyacrylamide gels onto Zeta-Probe membranes (Bio-Rad) or polyvinylidene difluoride membranes (Bio-Rad) (60). Detection of the antibody-recognized proteins was carried out with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G and 2-chloro-1-naphthol (Bio-Rad) according to the manufacturer's instructions. Protein determination was carried out by the Coomassie-binding method, with Bio-Rad protein assay kits.

**Nucleotide sequence accession number.** The sequence data for *OSD1* are available from the EMBL, GenBank, and DDBJ databases under accession number D16332.

## RESULTS

**Isolation of mutants accumulating unassembled CoxII.** We expected that mutants defective for the mitochondrial protein degradation system would accumulate CoxII to a higher level than that observed in the parent strain. Thus, to obtain mutants defective in degradation of unassembled CoxII, we isolated mutants accumulating this subunit. Wild-type strains are disadvantageous for such a purpose because they would also accumulate normally assembled CoxII, which is relatively sta-

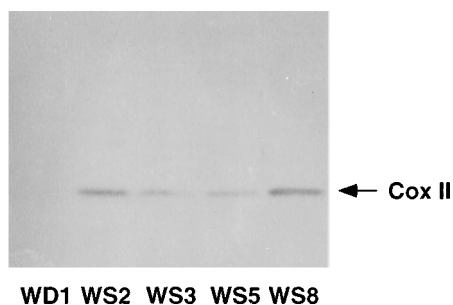


FIG. 1. Accumulation of CoxII in mitochondria of the *osd* mutants. Mitochondrial proteins (35  $\mu$ g) from the YPR-grown cells of *osd* mutants, WS-2, WS-3, WS-5, and WS-8, and their parent strain, WD 1, were separated by SDS-PAGE on 12.5% acrylamide gels and transferred onto a Zeta-Probe membrane (Bio-Rad). CoxII was detected with affinity-purified anti-CoxII polyclonal antibody.

ble and would accumulate to a higher degree than the unassembled subunit, even though degradation of the latter is defective. For this reason, we used as a parent strain a CoxIV-deficient strain (WD 1), in which CoxII cannot assemble (13) and is degraded rapidly (40).

After primary screening of mutagenized WD 1 cells on nitrocellulose membranes for CoxII accumulation, with affinity-purified anti-CoxII antibody as a probe, mitochondria were prepared from the candidate mutants grown on YPR and examined for accumulation of CoxII by Western blotting with the same antibody. Finally, after a screening of 12,000 colonies, we obtained four *osd* mutants (for cytochrome oxidase subunit degradation) that accumulated significant amounts of CoxII in mitochondria compared with the parent strain (Fig. 1). These mutant strains were designated WS-2, -3, -5, and -8, respectively.

**Dominance analysis of *osd* mutants.** To examine the dominance of the mutations, we crossed each of the isolated mutants, as well as the parent, WD 1, with a *coxIV::LEU2* haploid strain of the opposite mating type (H41-5D [*MATa*]). Diploid strains heterozygous for one of the *osd* mutations and homozygous for the *coxIV::LEU2* alleles were analyzed for mitochondrial levels of CoxII by Western blotting with anti-CoxII antibody. As shown on the first row of the panel in Fig. 2, steady-state intramitochondrial levels of CoxII in the diploids heterozygous for any of the *osd* mutations were similar to those seen in *OSD/OSD* diploids. Thus, each of the *osd* mutations is recessive.

**Complementation analysis of *osd* mutants.** To assess allelic relationships among the four mutants, complementation analysis was performed. For each of the original *osd* mutants, WS-2, WS-3, WS-5, and WS-8, a corresponding segregant of the opposite mating type (*MATa*), H35-1C, H37-3A, H38-2B, and H34-2C, respectively, was constructed (see Materials and Methods) and used for complementation analysis. The diploid strains of all of the possible combinations were analyzed for mitochondrial accumulation of CoxII by Western blotting (Fig. 2). Such analysis revealed that WS-5 and WS-8 harbor mutations on the same complementation group and that WS-2, WS-3, and WS-5 (or WS-8) are members of different complementation groups (Fig. 2). Thus, the *osd* mutants obtained were classified into three complementation groups.

**Tetrad analysis of the *osd* mutants.** One possible explanation for the CoxII-accumulating phenotype of the *osd* mutants is that the structural gene of CoxII on mitochondrial DNA was mutated so that the encoded CoxII became resistant to proteolysis. Such a mutation should segregate in a non-Mendelian

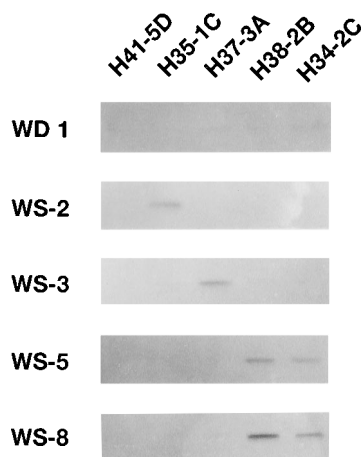


FIG. 2. Complementation analysis of the *osd* mutants. H41-5D, H35-1C, H37-3A, H38-2B, and H34-2C were derived from the crossings of WD 1, WS-2, WS-3, WS-5, and WS-8, respectively, with the wild-type *COXIV* strain, H11-1B. Diploid cells from each combination were examined for mitochondrial accumulation of CoxII, as described in the legend to Fig. 1.

fashion. We examined this possibility by analyzing the diploid cells derived from H11-1B (*COXIV*<sup>+</sup>) and each of the original *osd* mutants (*coxIV*). The presence of the *osd* mutation(s) in the two *coxIV::LEU2* segregants could be assessed directly by examination of the accumulation of CoxII (as observed in the two right lanes on the top gel in Fig. 3). The *COXIV*<sup>+</sup> segregants were analyzed for the presence of the *osd* mutations as described in the legend to Fig. 3. Although some fluctuation of the intensity of bands for CoxII between *COXIV*<sup>+</sup> strains was observed, the intensity did not always correlate with whether or not there was an *osd* mutation. We have no precise explanation, but this may be due to experimental error or to differences in genetic backgrounds.

We examined six asci for each of the *osd* mutations derived from either WS-2, WS-3, or WS-8, and in every case, the mutation segregated in a Mendelian 2:2 fashion and was unlinked to the *COXIV* locus (data not shown). The results suggest that the accumulation of CoxII in each of the three *osd* mutants of a *coxIV::LEU2* genetic background was caused by inactivation of a single nuclear gene and exclude the possibility that the stabilization of CoxII was caused by a mutation(s) in its structural gene. We designated the putative wild-type locus for each of the responsible genes corresponding to WS-2, WS-3, and WS-8 (or WS-5) *OSD1*, *OSD2*, and *OSD3*, respectively. In addition, the mutated alleles in WS-2, -3, -5, and -8 were designated *osd1-1*, *osd2-1*, *osd3-1*, and *osd3-2*, respectively.

**Suppression of degradation of CoxII in the *osd* mutants.** Accumulation of unassembled CoxII in the *osd* mutants can be caused by either a defect of the degradation of this subunit, an increase of its synthesis rate, or both. To determine whether the degradation of unassembled CoxII was suppressed in the *osd* mutants, we compared the half-lives of CoxII and CoxIII in the mutant strains with those in the parent WD 1 cells by pulse-chase experiments in the presence of cycloheximide, which specifically inhibits cytoplasmic protein synthesis (Fig. 4A). In the parent WD 1 cells, radiolabeled CoxII decreased rapidly with a half-life of about 30 min, consistent with our earlier observations (40). On the other hand, in all three of the *osd* mutants (we did not examine WS-5 because it belongs to the same complementation group as WS-8), reduction of the labeled CoxII was much suppressed. Only 10 to 15% of the

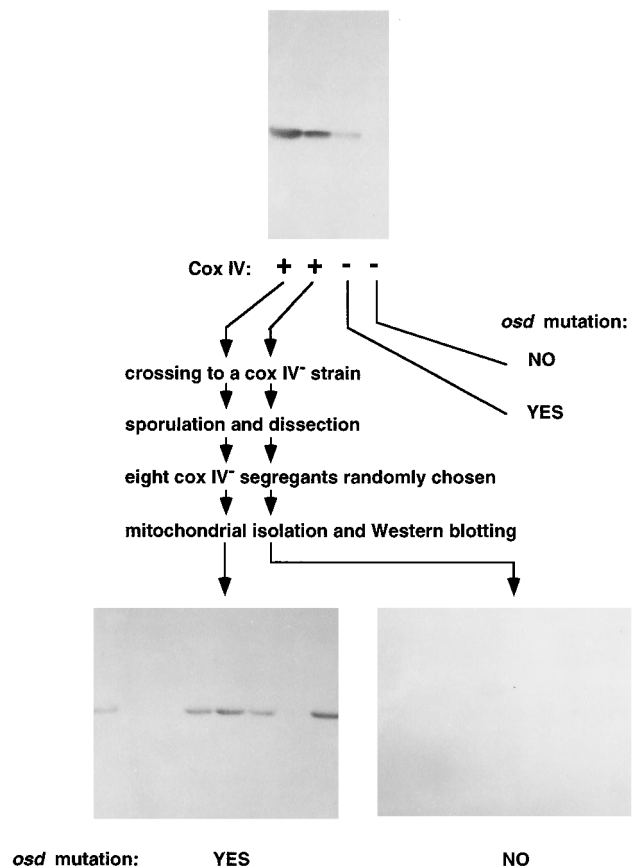


FIG. 3. Strategy for tetrad analysis. One example of the analysis of a set of four segregants derived from a crossing of WS-2 and H11-1B is given. Mitochondria (30  $\mu$ g) isolated from the four segregants, two *COXIV*<sup>+</sup> (top, left two lanes) and two *coxIV::LEU2* (top, right two lanes) were analyzed, as described in the legend to Fig. 2. Each of the two *COXIV*<sup>+</sup> segregants was further crossed with a *coxIV::LEU2* strain of the opposite mating type and sporulated. Eight *coxIV::LEU2* segregants resulting from each of the crossings were randomly chosen, and CoxII accumulation was assessed as described above (bottom). *COXIV*<sup>+</sup> segregants producing three to five CoxII-accumulating segregants (top, left lane in this example) were regarded as carrying the *osd* mutation, and those producing none (top, next to the left lane in this example) were regarded as being without mutation.

radiolabeled CoxII disappeared during 1 h of the chase in those mutants. These results suggest that the *osd* mutant cells are defective in the degradation of unassembled CoxII and that the accumulation of CoxII in the *osd* mutants was due, at least in part, to suppression of degradation in the mitochondria. The bands for CoxIII were poorly separated from that for F<sub>0</sub>F<sub>1</sub>-ATPase subunit 6. However, the instability of the bands observed in WD 1 did not seem to be affected significantly in any of the *osd* mutants.

**Growth properties of the *osd* mutants on a nonfermentable glycerol medium.** Mitochondrial respiratory function can be assessed by testing growth ability on a nonfermentable medium such as glycerol-containing YPG. To examine the effects of *osd* mutations on mitochondrial function in cells with normal CoxIV, *COXIV*<sup>+</sup> congenic strains, with or without *osd* mutations, were constructed by backcrossing each of the original *coxIV::LEU2 osd* mutants (WS-2, WS-3, and WS-8) with *COXIV*<sup>+</sup> H11-1B at least six times. Two such *COXIV*<sup>+</sup> *osd* mutant strains for each of the three *osd* mutations (*osd1-1*, *osd2-1*, and *osd3-2*), as well as the congenic wild-type strains, H92-1B and H92-6D, were examined for growth on nonfermentable YPG plates at different temperatures.

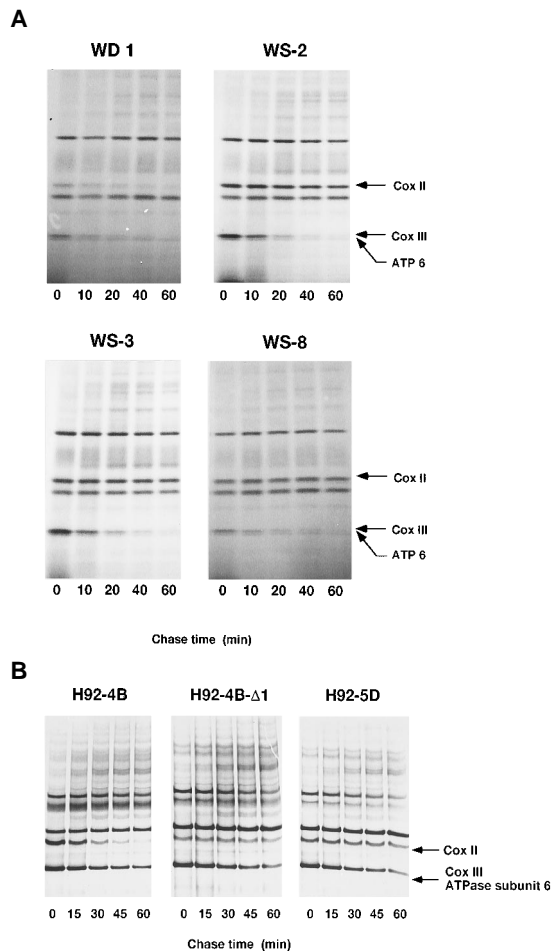


FIG. 4. Suppression of the degradation of CoxII. (A) Mutant cells WS-2, WS-3, and WS-8 and the parent WD 1 cells were pulse-labeled with [<sup>35</sup>S]methionine and chased for the period of time indicated as described in Materials and Methods. Whole-cell proteins were analyzed by SDS-PAGE on 10 to 12% polyacrylamide gradient gels followed by fluorography. The positions of CoxII, CoxIII, and F<sub>0</sub>F<sub>1</sub>-ATPase subunit 6 (ATP 6) are indicated on the right. (B) Stability of CoxII in *OSD1*-disrupted and *osd1-1* cells. Pulse-chase experiments were performed as in panel A, except that EXPRE<sup>35S</sup>S (NEN) was used instead of [<sup>35</sup>S]methionine, and proteins were separated with MULTI GEL 10/20 (Daiichi Pure Chemicals, Tokyo, Japan). Detection of radiolabeled proteins was carried out with a BAS2000 Bio-Image Analyzer (Fuji Photo Film). Note that the mobilities of CoxII and apocytochrome *b* between the two gel systems were in reverse order. Genotypes: H92-4B, *OSD1*<sup>+</sup> *coxIV::LEU2*; H92-4BΔ1, *osd1::HIS3 coxIV::LEU2*; H92-5D, *osd1-1 coxIV::LEU2*.

As shown in Fig. 5, all of the mutant strains examined grew as well as the wild-type strains at 23 and 30°C. However, at 37°C, growth of all of the *osd* mutants was reduced, compared with that of the wild-type cells, suggesting that the *OSD1*, *OSD2*, or *OSD3* genes are required for normal mitochondrial respiration at 37°C.

**Molecular cloning of the *OSD1* gene.** We used this temperature sensitivity for cloning the *OSD1* gene by functional complementation. Each of six plasmids that complemented the temperature sensitivity could also complement the mitochondrial accumulation of unassembled CoxII of *osd1-1 coxIV::LEU2* cells (data not shown). Restriction map analysis revealed that the six plasmids have a common 4.2-kb overlapping region in their insert, derived from yeast genomic DNA, and deletion analysis revealed that the 3.5-kb *BglIII-ScaI* fragment contained in the common region is sufficient to rescue both of the two phenotypes of *osd1-1* mutants (data not shown).

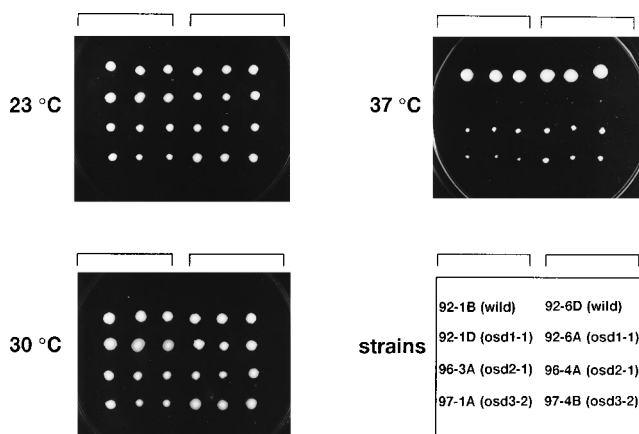


FIG. 5. Growth of the *osd* mutants of the *COXIV*<sup>+</sup> genetic background on nonfermentable YPG plates at different temperatures. Each strain was spotted in triplicate on three YPG plates. After incubation for 5 days at the temperatures indicated, photographs were taken. Identification of the strains and their genotypes are shown at bottom right.

**Deduced sequence of the *OSD1* gene.** Sequence analysis of the 3.5-kb *BglIII-ScaI* region of the cloned DNA revealed that this region can code for a protein consisting of 747 amino acid residues with a molecular mass of 81.7 kDa (Fig. 6).

Disruption of the frame downstream of the *EcoRV* site located between the second and the third ATG codons by insertion of 8- or 10-mer *HindIII* linkers inactivated the gene, while insertion of a frame-conserving 12-mer *HindIII* linker conserved its activities (data not shown), suggesting that this gene is translated from either the first or the second ATG codon. When translated from the first ATG codon, the N-terminal region of the putative translation product has known properties of the mitochondrial presequences, but not when translated from the second ATG codon, suggesting that the *OSD1* gene product is a mitochondrial protein and that it is translated from the first ATG codon. In support of this idea, cell fractionation analysis revealed that Osd1p is localized to mitochondrial membrane (described below).

The putative Osd1 protein contains an ATP-binding cassette and a zinc-binding HEXXH motif (Fig. 6). In searches of DNA (GenBank and EMBL) and protein (SWISS-PROT and PIR) databases with the FASTA program (44) for sequences homologous to the *OSD1* gene or Osd1 protein, this gene proved to be identical to *YME1*, the loss of which causes a frequent escape of the mitochondrial DNA to the nucleus (56, 57). *YME1* is also known as *YTA11* (50), which belongs to a large family of genes coding for putative ATPases known as the AAA family (32), consisting of proteins of diverse intracellular functions. Among the members of this family, this gene has a significantly higher homology to *E. coli ftsH* (59) and to yeast *YTA10* and *YTA12* (50).

**Gene disruption of *OSD1*.** To examine the null phenotypes of *OSD1*, we constructed disruptants with both *COXIV*<sup>+</sup> (H92-1B) and *coxIV::LEU2* (H92-4B) haploid strains by replacing most of the coding region with the *HIS3* marker gene fragment (Fig. 7A). Proper gene replacement on the chromosomal DNA of the disruptant strains (H92-1BΔ1 and H92-4BΔ1, respectively) was confirmed by Southern blotting (data not shown).

We first examined the growth potential of H92-1BΔ1 (*COXIV*<sup>+</sup>) on YPG at different temperatures. As shown in Fig. 7C, H92-1BΔ1 cells could also grow at 30°C, but not at 37°C, confirming the observation of Thorsness et al. (57). Like *osd1-1* strains, the disruptants were more temperature sensitive than

	10	20	30	40	
	MNVSKILVSPVTVTNVLRI	FAPRLPQIGASLLVQK	KWALR		40
	SNNFYRFYSENNSGEMPPK	KEADSSGKASNKSTI	SSIDNS		80
	QPPPPSNTNDKTKQANVA	VSHAMLATREQEANKDL	TSPDA		120
	QAIFYKLLQSNYPQYVVS	RFETPGIASSPECMELY	MEAL		160
	QRIGRHSEADAVRQNL	LTTASSAGAVNPSLASS	SSNSQSGYH		200
	GNFPMSYSPLYGSRKEPL	HVVVSESTFTVSRWVK	WLLVF		240
	GILTYSFSEGFKYITENT	TLKSSSEVADKSDVAK	TNVKF		280
	DDVCGCDEARAELEEIV	DFLKDPTKYESLGGK	LPGVLLT		320
	<u>GPPGTGKTL</u> LARATAGEAG	VDFFFMSGSEFDEVY	VGVGAK		360
	RIRD <sup>•</sup> LFAQARSRAPI	<u>IFIDELDA</u> IGGKR	PKDQAYAKQT		400
	LNQLLVELDGFSGTSGI	IIIGATNFPEALDKAL	TRPGRFD		440
	KVVNVLDLDPVGRADIL	KHHMKKITLADNVD	PTIARGTP		480
	GLSGAELANLVNQAAVY	ACQKNVAVSDMSHF	EWAKDKILM		520
	GAERKTMVLTDAARKATA	<u>FEAGH</u> IMAKYTN	GATPLYKA		560
	TTLPRGRALGITFQLPE	MDKVDIAKRECQARLD	VCMGGKI		600
	AEELIYKDNNTTSCGGS	DLQASATGTARAMV	TQYGMSSDDVG		640
	PVNLSENWESWSNKR	IDIADNEVIELLKDSE	ERARLLTK		680
	KNVELHRLAQGLIEYET	LDAHEIEQVCKGEKLD	KLKTSTN		720
	TVVEGPDSDERKDIGD	DKPKIPTMLNA			

FIG. 6. Amino acid sequence of the predicted Osd1 protein. The predicted membrane-spanning region is underlined. A set of ATP-binding consensus sequences are double underlined. The conserved lysine residue replaced for mutagenesis analysis (see Fig. 9) is marked by a dot. The putative Zn<sup>2+</sup>-binding consensus sequence is boxed and contains two conserved histidine residues and one conserved glutamic acid residue, which are marked by open circles.

*COXIV*<sup>+</sup> *osd2-1* or *COXIV*<sup>+</sup> *osd3-2* cells (data not shown). Next, H92-4BΔ1, an *OSD1* disruptant derived from a *coxIV::LEU2* strain, was examined for accumulation and stability of CoxII. The results of Western blotting with an anti-CoxII polyclonal antibody showed that H92-4BΔ1 accumulated significant amounts of CoxII in mitochondria, compared with its parent strain, H92-4B (Fig. 7B). The degree of accumulation of CoxII was comparable to that observed in the cells with the *osd1-1* mutant allele.

We also compared the stabilities of the mitochondrial translation products in H92-4BΔ1 cells and the parent H92-4B cells in pulse-chase experiments. As shown in Fig. 4B, CoxII, which is unstable in the parent, H92-4B cells was stable in the disruptant H92-4BΔ1 cells, thereby confirming the requirement of this gene for degradation of unassembled CoxII. The degree of the stabilization was similar to that observed in the *osd1-1 coxIV::LEU2* strain (H92-5D). Although CoxIII and F<sub>0</sub>F<sub>1</sub>-ATPase subunit 6 could not be separated in the gel system employed, the unstable nature of the corresponding bands observed in the parent *coxIV::LEU2* strain was not affected by disruption of the *OSD1* gene. The stability of the Var1 protein was apparently decreased in this set of experiments, perhaps because of differences in genetic backgrounds between the strains used. Together with the inability of the disruptant strain and an *osd1-1* strain to reciprocally complement their defects (data not shown), the results presented above support the notion that the cloned gene is the authentic *OSD1* and not a suppressor.

**Mitochondrial respiratory chain enzymes in *osd1* mutants.** To confirm the defect of mitochondrial function in the *osd1*

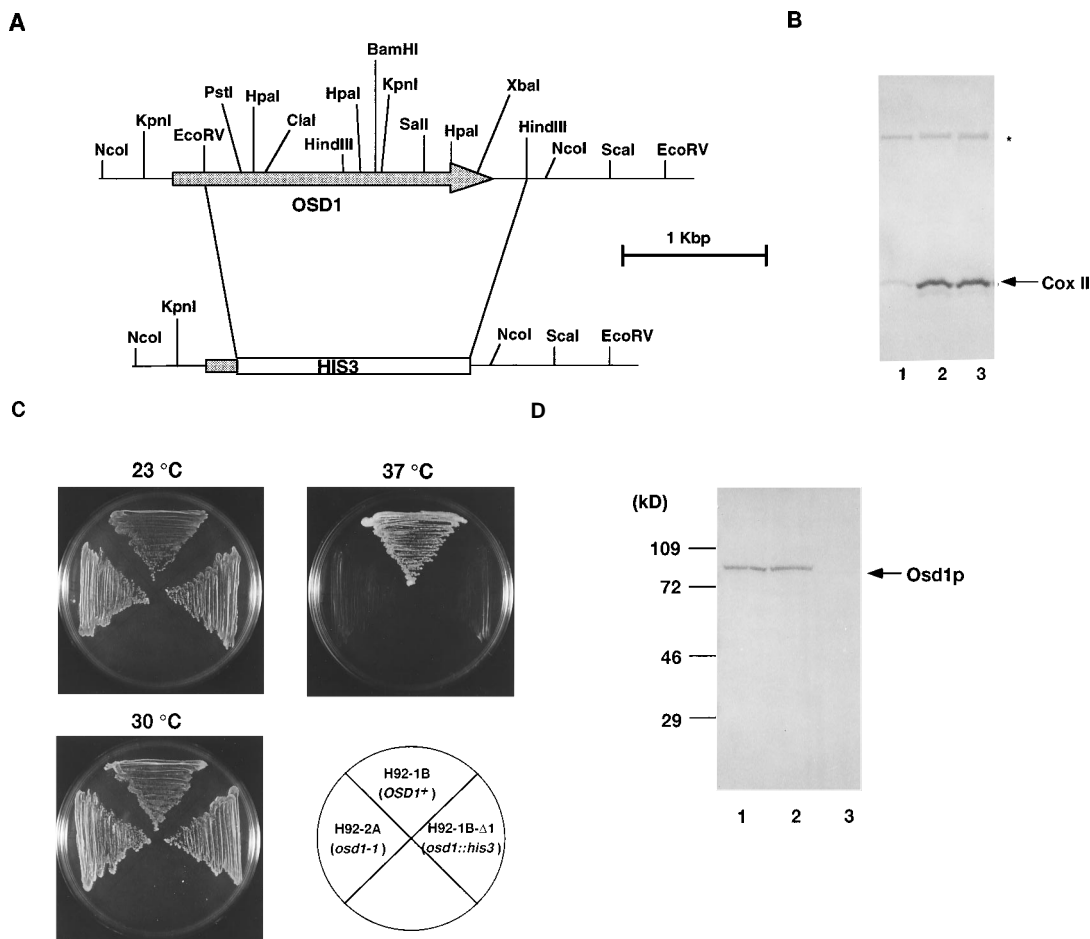


FIG. 7. Disruption of the *OSD1* gene. (A) The thick arrow represents the region and orientation of the *OSD1* open reading frame. The *EcoRV-HindIII* region (2.4 kb), encoding the C-terminal approximately 90% of Osd1 protein and 0.3 kb of its downstream sequence, of H92-4B (*coxIV::LEU2*) and H92-1B (*COXIV*<sup>+</sup>) was replaced with a 1.8-kb *BamHI* fragment containing *HIS3* as indicated. (B) H92-4B (*OSD1*<sup>+</sup> *coxIV::LEU2* [lane 1]), H92-2A (*osd1-1 coxIV::LEU2* [lane 2]), and H92-4BΔ1 (*osd1::HIS3 coxIV* [lane 3]) were examined for accumulation of unassembled CoxII as in Fig. 1, except the cells were grown in YPR and polyvinylidene difluoride membrane was used instead of the Zeta-Probe membrane. An asterisk indicates the position of an unidentified protein cross-reacting with the antibody used. (C) H92-1B (*OSD1*<sup>+</sup> *COXIV*<sup>+</sup>), H92-2A (*osd1-1 COXIV*<sup>+</sup>), and H92-1BΔ1 (*osd1::HIS3 COXIV*<sup>+</sup>) were examined for growth on YPG plates at the temperatures indicated above. Strains and their genotypes are identified on the lower right. (D) Mitochondrial proteins (30 μg) from YPGal-grown cells were probed with anti-β-galactosidase-Osd1 antiserum. Lanes: 1, H92-1B; 2, H92-2A; 3, H92-1BΔ1.

mutants, as deduced by temperature sensitivity on a nonfermentable medium, activities for the mitochondrial respiratory chain enzymes in the mitochondria were measured. As shown in Table 2, the specific activity of cytochrome *c* oxidase was

reduced in *osd1-1* or *osd1::HIS3* mutant cells, even at 30°C, a temperature at which growth on a nonfermentable medium is apparently normal. At 37°C, the differences in activities between mitochondria of the wild-type and mutant cells were

TABLE 2. Activities of mitochondrial respiratory chain enzymes in *osd1* mutants

Growth temp	Strain	Genotype	Sp act (%) <sup>a</sup>		
			NADH-cytochrome <i>c</i> reductase <sup>b</sup>	Succinate-cytochrome <i>c</i> reductase <sup>c</sup>	Cytochrome <i>c</i> oxidase <sup>d</sup>
30°C	92-1B	<i>OSD1</i> <sup>+</sup>	0.247 (100)	29.4 (100)	8.51 (100)
	92-1BΔ1	<i>osd1::HIS3</i>	0.122 (49)	20.0 (68)	2.47 (29)
	92-2A	<i>osd1-1</i>	0.189 (77)	31.6 (107)	2.90 (34)
37°C	92-1B	<i>OSD1</i> <sup>+</sup>	0.137 (100)	38.0 (100)	3.42 (100)
	92-1BΔ1	<i>osd1::HIS3</i>	0.023 (17)	12.7 (33)	0.48 (14)
	92-2A	<i>osd1-1</i>	0.017 (12)	11.7 (31)	0.33 (10)

<sup>a</sup> The values indicated are averages of duplicate measurements. The values in parentheses are percent relative activities compared with that of the wild type, 92-1B.

<sup>b</sup> Specific activity is indicated as micromoles of cytochrome *c* reduced per minute per milligram of mitochondrial proteins.

<sup>c</sup> Specific activity is indicated as picomoles of cytochrome *c* reduced per minute per milligram of mitochondrial proteins.

<sup>d</sup> Specific activity is indicated as pseudo-first-order reaction constant (per minute) per milligram of mitochondrial proteins.

more apparent. In *osd1::HIS3* and *osd1-1* strains, only 14 and 10% of the activity, respectively, of the wild-type cells could be observed. The activities of succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase were also reduced in the *osd1* mutants, indicating that respiratory chain enzymes other than cytochrome *c* oxidase are also defective. Reduction of the peak of cytochrome *aa<sub>3</sub>* and cytochrome *b* in the *OSD1*<sup>+</sup>*COXIV*<sup>+</sup> and *osd1::HIS3 COXIV*<sup>+</sup> disruptant cells grown at both 30 and 37°C, as assessed by measurement of their whole-cell spectra, (data not shown) is consistent with the results presented above.

**Localization of Osd1 protein.** We examined subcellular localization of Osd1 by using serum raised against the β-galactosidase–Osd1 fusion protein. Western blotting analysis of subfractionated yeast cells revealed a 75-kDa protein in a fraction enriched in the mitochondria (Fig. 7D and 8A), findings consistent with the results seen with Yme1 protein (57). A lower apparent molecular mass of this band than that expected from its sequence is probably due to processing of its N-terminal mitochondrial presequence. This band was not detected when mitochondria were prepared from *CoxIV osd1::HIS3* cells (Fig. 7D), confirming that it is the authentic Osd1 protein. The 75-kDa band was also detected in the mitochondria of the *osd1-1* mutant, suggesting that a mutant protein with similar stability to the wild-type protein was synthesized in this mutant.

To determine whether this protein is bound to the membrane, mitochondria from D273-10B cells were further purified by Percoll density gradient centrifugation and separated into soluble and membrane fractions by ultracentrifugation after sonication (Fig. 8B, top left). The protein was pelleted to the membrane and did not remain in the soluble fraction. When isolated mitochondria were treated with 0.1 M Na<sub>2</sub>CO<sub>3</sub>, which is known to extract peripherally bound membrane proteins but not those inserted into the membrane (17), this protein remained in the membrane fraction (Fig. 8B, top right). Urea treatment, which depletes membranes of some peripheral membrane proteins (66), did not lead to extraction of this protein (Fig. 8B, bottom). These results suggest that the *OSD1* gene product exists as an integral membrane protein.

We also assessed the submitochondrial localization of this membrane-bound protein by subfractionating mitochondrial membrane by sucrose density gradient centrifugation into two fractions—one enriched in cytochrome *c* oxidase localized to the inner membrane and the other enriched in kinurenine-hydroxidase localized to the outer membrane. As a result, the Osd1 protein showed a distribution pattern similar to that of cytochrome *c* oxidase, suggesting that the inner membrane is the area of localization (Fig. 8C).

**Importance of the ATP-binding consensus motif of Osd1 (Yme1 or Yta11) protein for its functions.** We constructed several modified *OSD1* genes by replacing the Lys-327 located in the ATP-binding cassette (G-X-X-X-X-G-K-T/S, where X can be any amino acid residue), with other ones (Fig. 9A). In either of the mutated genes, substitution of Lys-327 led to an inability to complement both the accumulation of un-assembled CoxII in *osd1::HIS3 coxIV::LEU2* cells and temperature sensitivity on a nonfermentable carbon source in *osd1::HIS3 COXIV*<sup>+</sup> cells (Fig. 9B and C). To determine if loss of complementation is due to instability of the mutant proteins, we examined the steady-state intramitochondrial levels of the mutated proteins in *COXIV*<sup>+</sup> cells. Intramitochondrial levels of the mutated Osd1 proteins increased for each of the mutated *OSD1* genes, thereby ruling out the possibility of destabilization of those proteins by the mutations

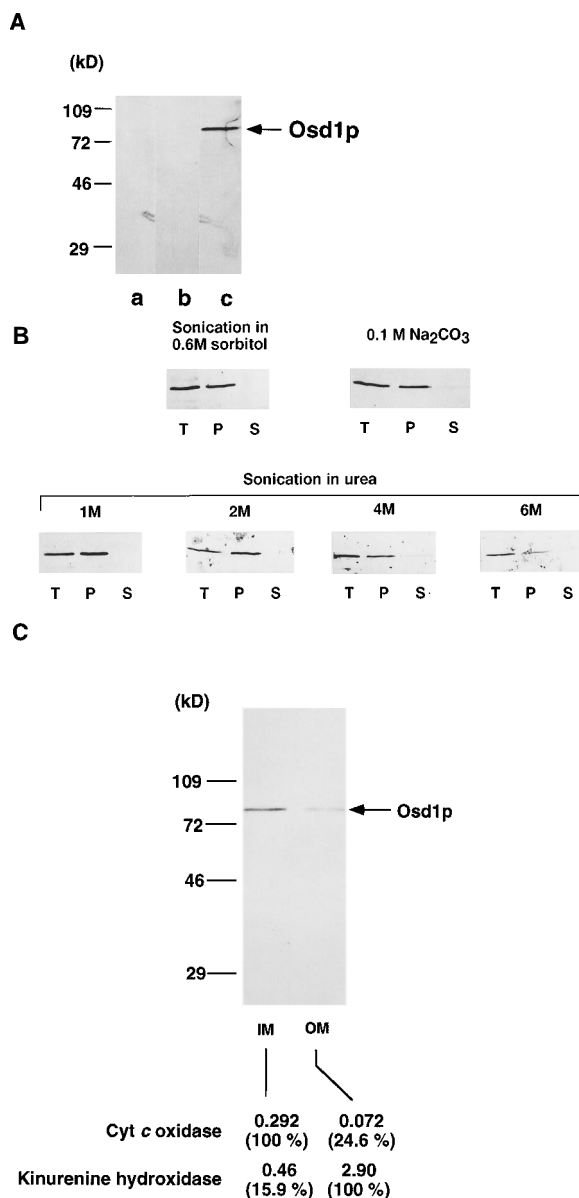
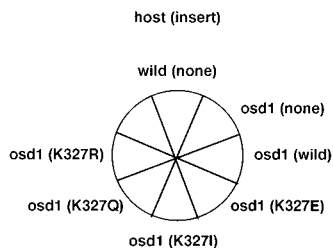
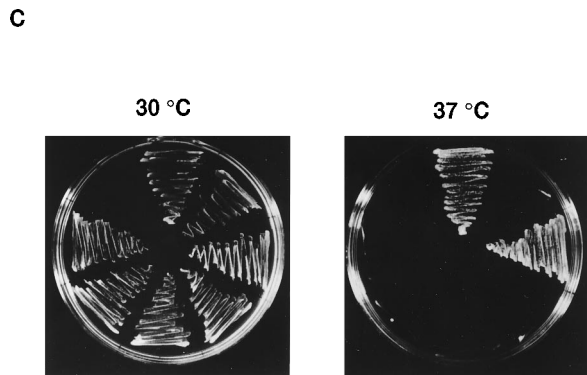
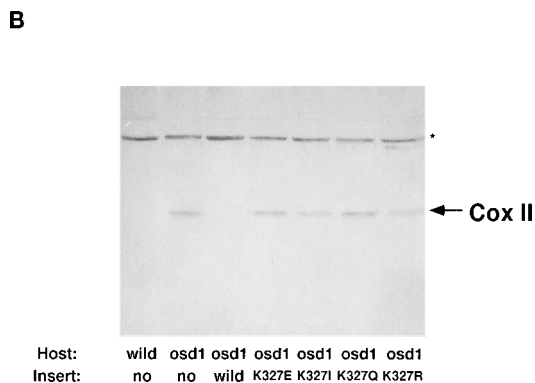
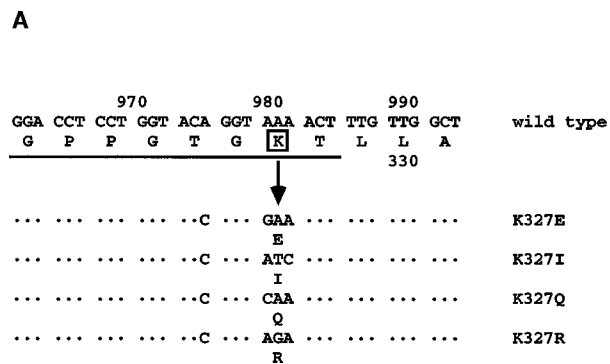


FIG. 8. Subcellular localization of Osd1 protein. (A) Postnuclear fraction (lane a), postmitochondrial fraction (lane b), and mitochondrial fraction (lane c) were prepared from D273-10B haploid cells grown on YPGal as described in Materials and Methods. Equal amounts (25 μg) of proteins from each fraction were analyzed by immunoblotting with anti-β-galactosidase–Osd1 antiserum. The positions of standard marker proteins are indicated on the left. (B) Percoll-purified mitochondria of D273-10B (300 μg) were either treated by sonication in sorbitol buffer (10 mM Tris-Cl [pH 7.4], 0.6 M sorbitol) or urea or with 0.1 M Na<sub>2</sub>CO<sub>3</sub>. After incubation on ice for 1 h, each of the treated mitochondria was divided into two identical portions: one half was used as the total (T) fraction, and the other half was further fractionated by centrifugation at 100,000 × *g* for 1 h into the pellet (P) and supernatant (S) fractions. After resuspension of the pellet fractions in the original volume of the treated solutions, the same volume of each fraction was analyzed as in panel A. (C) Mitochondria from D273-10B were further subfractionated into inner membrane (IM) and outer membrane (OM) fractions. The same amount (30 μg) of proteins from each fraction was analyzed as in panel A. Enzyme activities are shown in arbitrary units. Cyt *c*, cytochrome *c*.

(data not shown). All of these findings taken together support the idea that the conserved lysine residue, and thus most likely ATP binding, is essential for the function of Osd1 protein.





**DISCUSSION**

In the present study, we genetically identified three *OSD* genes that are required for the mitochondrial degradation of unassembled CoxII by using a CoxIV-deficient strain, and we cloned one of the genes, *OSD1*.

Isolation and characterization of the *osd* mutants revealed several important properties of the mitochondrial protein degradation system. First, identification of three complementation groups for *osd* mutants indicates the requirement of multiple nuclear gene products for degradation of improperly assembled CoxII. The only mitochondrial protease thus far characterized, except for the processing proteases, is the matrix ATP-dependent protease purified from various species, a protease similar in properties to the bacterial *lon* gene product (11, 33, 63, 64). However, it is not clear whether these soluble proteases are also involved in degradation of mitochondrial translation products, most of which are integral inner membrane proteins. Our genetic evidence for the involvement of multiple factors for the degradation of unassembled CoxII in yeasts clearly indicated that new factors (at least two) which are different from the matrix ATP-dependent protease are required for the mitochondrial proteolytic system.

**FIG. 9.** Mutational analysis of the ATP-binding cassette. (A) Nucleotide and amino acid sequences of *OSD1* around the ATP-binding cassette (underlined). Lys-270 (boxed) was mutated as indicated. (B) Inability of the mutants to complement CoxII accumulation in a CoxIV-defective *osd1* mutant strain. Wild-type or mutated *OSD1* genes were subcloned into the pFL-1 shuttle vector and introduced into H92-4BΔ1 (*osd1::HIS3 coxIV::LEU2*). For control experiments, pFL-1 vector without insert was introduced into H92-4B (*coxIV::LEU2*) or H92-4BΔ1 (the left two lanes). Accumulation of CoxII was analyzed by immunoblotting, with anti-CoxII antibody after the mitochondrial fraction was prepared, as described in the legend to Fig. 7B. An asterisk indicates the position of the bands of an unidentified protein cross-reacting with the antibody used. (C) Inability of the mutant *OSD1* genes to complement the temperature sensitivity of an *osd1::HIS3 COXIV<sup>+</sup>* strain. Wild-type and mutated *OSD1* genes were introduced into H92-1B (*OSD1<sup>+</sup> COXIV<sup>+</sup>*) or H92-1BΔ1 (*osd1::HIS3 COXIV<sup>+</sup>*). Strains and inserts of the harboring vector are identified below. Growth of the transformed cells on SG plates was examined.

Second, results of pulse-chase experiments showed that the stability of CoxIII, another subunit destabilized in a CoxIV-deficient strain, was not affected in *osd* mutants or in the *OSD1* disruptants, indicating that the three *OSD* genes are not required for degradation of CoxIII. Such selective loss of degradation in the *osd* mutants is surprising because both CoxII and CoxIII are integral inner membrane proteins (8). One explanation for such selective degradation is that by mutation, CoxII itself acquired resistance to proteolysis without a defect in the proteolytic machinery. However, this is unlikely because tetrad analysis suggested that each of the *osd* mutations is located on the nuclear genome, thereby eliminating the possibility that each of the mutations lies in the mitochondrial structural gene for CoxII. Another possibility is that selective degradation is due to some defect of posttranslational maturation of CoxII, such as attachment of heme or copper ions in the *osd* mutants, but this too seems unlikely because apoproteins without prosthetic groups are generally unstable in the cell (21). Thus, we entertain the notion that at least two distinctive pathways for the degradation of mitochondrial inner membrane proteins, one for CoxII and the other for CoxIII, are probably present in yeasts. Our previous pulse-chase experiments suggested that the degradation of CoxIII is also energy- and divalent metal ion-dependent (40). Although it is possible that a factor involved in the degradation of both CoxII and CoxIII is responsible for such properties, no such factor has been identified. We prefer the notion that a factor similar to Osd1 protein, which is also ATP and divalent metal ion dependent, is involved in the degradation of unassembled CoxIII, because involvement of Osd1 protein can explain the properties of the CoxII degradation pathway without the need for another factor. Two additional genes, *YTA10* and *YTA12*, which are highly homologous to *OSD1* (*YME1* or *YTA11*), are present in yeasts (50). Those genes also encode proteins with an ATP-binding cassette and an HEXXH motif. It seems likely that either or both may be involved in the degradation of CoxIII. In addition,

factors homologous to the *OSD2* or *OSD3* gene product may also be involved in the degradation of this subunit. At present, it is unclear whether the three *OSD* genes are involved in the degradation of proteins other than unassembled CoxII. Although only CoxII and CoxIII are destabilized in the CoxIV-deficient strains we used, it was reported that in several mutant strains, mitochondrially encoded polypeptides other than CoxII and CoxIII (for example, apocytochrome *b* or CoxI) are unstable, probably because they cannot assemble correctly (31, 51). We plan to examine the effect of *osd* mutations on the stability of those polypeptides. Examination of the stability of nuclearly encoded subunits of this enzyme in CoxIV-deficient strain and the effects of the *osd* mutations on it will also be informative.

Third, temperature sensitivity on a nonfermentable medium of *osd* mutants having normal CoxIV suggests that their mitochondrial respiratory function is disturbed. The accumulation of CoxII or other abnormal proteins could be responsible. Alternatively, each of the three *OSD* gene products may also be required for the biogenesis or maintenance of components of the mitochondrial respiratory chain.

**Sequence properties of *OSD1*.** Cloning and characterization of the *OSD1* gene revealed that it encodes a member of a novel family of putative ATPases, known as the AAA family (15), and that it is identical to a gene known as *YME1*, or *YTA11* (50, 57). This putative ATPase family includes proteins with a variety of intracellular functions, such as cell cycle regulation (VCP [30] and *CDC48* [16]), vesicular transport and fusion (*SEC18* [14], NSF [6, 65]), transcriptional regulation (TBP-1 [41], TBP-7 [43], and *MSS1* [52]), protein localization or assembly (*FtsH* [59], *BCS1* [42], and *MSP1* [39]), or organelle biogenesis (*PAS1* [15]). The existence of the ATP-binding cassette in this protein and other members of this putative ATPase family suggests that ATP plays an important role in their functions. However, the biochemical functions of ATP remain obscure. Mutational analysis of the ATP-binding cassette of Osd1 protein also confirmed the importance of ATP for function of the Osd1 protein.

Next, the presence in the Osd1 protein of the HEXXH motif, also noted by Campbell et al. (7), is intriguing. This motif serves as a part of the zinc-binding site observed in a large superfamily of metalloproteinases, including the thermolysin, astacin, serratin, snake venom, and matrixin families (28). X-ray crystallographic analysis of thermolysin revealed that, in this protease, the two histidine residues and one glutamate residue in this motif coordinate  $Zn^{2+}$ , which is required for proteolytic activity (3).

We reported that the degradation pathway of unassembled CoxII in vivo is significantly suppressed either by inhibiting the glycolysis pathway through glucose depletion or 2-deoxy-D-glucose or by bongkreik acid, an inhibitor of the mitochondrial ATP-ADP exchanger (40). Both components are expected to reduce the intramitochondrial ATP concentration in respiratory-deficient strains by eliminating the supply of ATP from outside the mitochondria (19), thereby suggesting that the degradation pathway is energy dependent. In addition, this pathway was inhibited by *o*-phenanthroline, a membrane-permeable metal chelator, and this inhibition was reversed by addition of an excess molar amount of  $Zn^{2+}$ ,  $Mn^{2+}$ , or  $Co^{2+}$  (40), suggesting its metal requirement. That Osd1 protein has both the ATP-binding cassette and the HEXXH motif is consistent with the properties described above.

**Possible roles of Osd1 protein.** The Osd1 protein itself can function as a protease, or some part of it, for a direct degradation of unassembled CoxII. The presence of the HEXXH motif in a wide variety of metalloproteinases supports this idea.

We favor the view that Osd1 protein is a novel type of metalloproteinase with ATP requirements. ATP may be required for unfolding of substrates, as discussed below, or for assembly of the Osd1 protein with other subunits, which is also required for proteolysis.

Other factors such as molecular chaperones or chemical modifiers of substrates may also be involved in proteolysis. The idea that Osd1 protein is a molecular chaperone, functioning by physically interacting with a substrate protein such as unassembled CoxII, is favored by our observation that the levels of mature, assembled cytochrome *c* oxidase and other respiratory chain enzymes were reduced significantly in the *osd1* mutants, compared with those of wild-type cells. However, it is also possible that accumulation of unassembled CoxII or other unidentified abnormal proteins caused by a defect in the proteolytic pathway may interfere with efficient assembly of CoxII or other subunits of mitochondrial respiratory chain enzymes, for example, by perturbing the mitochondrial membrane structure. The presence of the ATP-binding cassette in this protein is consistent with the idea that Osd1 has a chaperone function, because binding and hydrolysis of ATP are essential for the function of molecular chaperones such as Hsp60 or Hsp70 (for review, see reference 47). Several members of the putative ATPase family to which Osd1 protein belongs may also possess molecular chaperone-like activity (39, 42, 59). Among them, the bacterial *ftsH* gene product (59) is especially interesting, considering the function of the Osd1 protein. This protein and the Osd1 protein, together with yeast *YTA10* and *YTA12* gene products, constitute a subfamily on the basis of the existence of a higher homology between them than to the other members of the family. Interestingly, these proteins also possess the HEXXH motif observed in Osd1 protein. FtsH protein is required for the assembly of some proteins into and through plasma membrane in a correct membrane topology (1, 2). This protein is also required for destabilization of the *cII* gene product of the lambda bacteriophage, the activity of which is required for lysogenization (26). It is difficult to explain the former function only by its proteolytic activity, whereas, molecular chaperone activity can well explain both of the functions described above. For example, it may interact with cII protein to facilitate degradation (26) or with several plasma membrane proteins (1) to enhance their correct insertion into or through the plasma membrane. Similarly, Osd1 protein can have a molecular chaperone-like activity for unassembled CoxII. In this regard, it will be interesting to examine whether Osd1 protein and CoxII can physically interact and, if so, whether the process is ATP dependent.

The two hypothetical functions of Osd1 protein as a protease and a molecular chaperone presented above are compatible. We propose that Osd1 (*Yme1* or *Yta11*) protein is a novel type of metalloproteinase, or some part of it, which also possesses a molecular chaperone function. In this model, Osd1 protein, with or without other subunits, physically interacts with unassembled CoxII. Such interaction induces or maintains an assembly- and degradation-competent structure of CoxII by preventing folding or aggregation, and if not assembled, proteolytic activity of Osd1 protein itself or of other subunits further degrades it. Such close coupling of the two activities is advantageous for efficient and prompt elimination of unassembled subunits and for prevention of their untoward accumulation.

Finally, inactivation of *YME1*, which is identical to *OSD1*, causes frequent escape of mitochondrial DNA into the nucleus (57). The simplest interpretation would be that the accumulation of unassembled CoxII and/or unidentified proteins with an abnormal conformation in mitochondrial inner membrane, by

a defect in their degradation and/or proper assembly, directly causes leakiness of the membrane for DNA, for example, by perturbing membrane structures. Alternatively, the Osd1 (Yme1) protein may be required for correct localization or function of a factor needed for containment of mitochondrial DNA to this organelle.

In conclusion, this work has revealed the involvement of multiple factors and the existence of multiple substrate-specific pathways for the degradative device of mitochondrial inner membrane proteins, thereby suggesting its intricate nature, but the overall picture of its molecular organization is far from complete. Genetic approaches as described here are expected to lead to further elucidation.

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