博士論文

Genetic Analysis of Protein Degradation System in Yeast Mitochondria Responsible for Eliminating Unassembled Subunit 2 of Cytochrome c Oxidase

酵母ミトコンドリアにおいて未会合のチトクローム酸化酵素サブユニット2の分解除去に関与する蛋白分解系の遺伝学的解析

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Notre nature est dans la movement; le repos entier est la mort.

Pascal

CONTENTS:

Abbreviations:

osd:	*oxidase subunit degradation*
cox:	*cytochrome c oxidase*
HRP:	*horse radish peroxidase*
orf:	*open reading frame*

Chapter 1 Introduction

One of the roles of intracellular protein degradation is to maintain stoichiometry among different subunits of a heterooligomeric enzyme by eliminating excess unassembled subunits (Goldberg and St. John 1976). In mitochondria, rapid turnover of unassembled subunits is especially important because 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 (Attardi and Schatz 1988; Hartl et al. 1989). 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. Though such regulation can be partly achieved by controlling rates of synthesis, finer regulations can be achieved by removing excessive amounts of unassembled or mis-assembled subunits by degradation. Thus, such protein degradation system appears to play a significant physiological role in the biogenesis of mitochondria.

A common feature of intracellular protein degradation is ATP-requirement (Goldberg and St. John 1976; Hershko and Ciechanover 1982). Studies of cell-free systems for ATP-dependent protein degradation from extracts of bacterial (Murakami et al. 1979) and higher eukaryotic(Etlinger and Goldberg 1977) cells have successfully identified several factors including ATP-dependent proteases (for recent reviews, see references (Goldberg 1992; Hershko and Ciechanover 1992)). For example, in bacteria, two different types of soluble ATP-dependent proteases have been identified. One is protease La, the product of the *lon* gene in *E. coli*, consisting of homo-tetramer (Charette et al. 1981; Chung and Goldberg 1981), and, the other is protease Ti (Clp) consisting of both catalytic and ATP-binding regulatory subunits (Hwang et al. 1987; Katayama-Fujimura et al. 1987). In eukaryotic cells, most intensively analyzed ATP-dependent proteolytic system is the ubiquitin- and ATP-dependent pathway (Hershko and Ciechanover 1992). Biochemical and genetic analyses revealed strikingly intricate nature of this system in which interplays of a large number of factors are required. In this pathway, substrate proteins are first conjugated with a small

protein named ubiquitin in an ATP-dependent way (Hershko 1988), and these ubiquitin-tagged conjugates are then recognized and degraded, also in an ATP-dependent manner, by a protease known as the 26 S protease complex (Hough et al. 1987; Waxmann et al. 1987) formed by assembly of proteasome and other components which requires ATP (Driscoll and Goldberg 1990; Eytan et al. 1989; Orino et al. 1991).

Ubiquitin-dependent system is not the sole intracellular pathway for ATP-dependent protein degradation in eukaryotic cells. Mitochondria (Desautels and Goldberg 1982b; Kalnov et al. 1979) and chloroplasts in plant cells (Malek et al. 1984) also have energy-dependent proteolytic activities.

Existence of an ATP-dependent protein degradation pathway(s) in mitochondria has been shown by several lines of experiments. First, isolated mitochondria from yeast (Kalnov et al. 1979; Yasuhara et al. 1994) or rat liver (Desautels and Goldberg 1982b) are capable of degrading their radiolabeled translation products to TCA-soluble peptides in the presence of ATP or ATP-regenerating system *in vitro*. In the case of rat liver, the degradation was stimulated when abnormally truncated polypeptides were generated by incorporation of puromycin (Desautels and Goldberg 1982b), suggesting that it serves for eliminating abnormal or mutated proteins.

Those experimental systems using isolated mitochondria are not suited for assay system to purify the involved factors. Thus, exogenous proteinous substrates such as modified casein were used to purify those ATP-dependent factors, and an ATP-dependent soluble protease was isolated from mitochondrial matrix fraction of rat liver (Desautels and Goldberg 1982a), bovine adrenal cortex (Watabe and Kimura 1985a; Watabe and Kimura 1985b) or yeast (Kutejová et al. 1993). Some candidate substrates which can be degraded *in vitro* by this protease from at least one of the above sources have been suggested (Watabe et al. 1993). Moreover, the gene for the yeast homolog of this type of protease, *PIM1/LON*, has been cloned and shown to be required for the mitochondrial respiratory function (Dyck et al. 1994; Suzuki et al. 1994). Though this soluble matrix protease is also suggested to be also responsible for degradation of the mitochondrial translation products (Desautels and Goldberg 1982a; Goldberg 1992), most of which are intrinsic inner membrane proteins (Attardi and Schatz 1988), direct evidence for that seems to be still missing. Furthermore, existence of an

ATP-dependent proteolytic activity for mitochondrial translation products in yeast submitochondrial particle (Kalnov et al. 1979; Yasuhara et al. 1994) raises a possibility that, other than or in addition to the matrix protease, factor(s) bound to the mitochondrial inner membrane is involved in this degradation pathway.

In this study, I used a genetic approach to analyze the degradation of unassembled mitochondrially encoded subunits of cytochrome *c* oxidase in yeast mitochondria. Such an approach have advantageous because, first, it is possible to study a degradation system for known physiological substrates, which is difficult by using artificial substrates, and second, it is suitable for identification of factors involved in complex biological processes in a molecular level. Yeast cytochrome *c* oxidase consists of at least eleven subunits (Power et al. 1984; Taanman and Capaldi 1992). The larger three of them, Cox I, II, III, are encoded by mitochondrial DNA. The remaining smaller subunits encoded by nuclear DNA are translated in cytosol, imported into mitochondria where they assemble to form a mature complex with mitochondrially-encoded subunits (Attardi and Schatz 1988; Hartl et al. 1989). It was reported that in a Cox IV-deficient strain (WD 1) other subunits can not assemble normally and intramitochondrial levels of Cox II and Cox III are significantly decreased (Dowhan et al. 1985). Considering that those subunits are unstable in this strain and such instability would be suitable for isolating degradation-defective mutants by examining accumulation of them, I chose this strain for the present study.

I first investigated the stability of Cox II and Cox III in this strain using whole cell pulse-chase labeling experiments (chapter 3). The results showed that those subunits are degraded rapidly and suggested that this protein degradation pathway is ATP- and metal-dependent. Next, isolation and characterization of mutants defective in this pathway is described (chapter 4). These genetic analyses showed that at least three *OSD* (for cytochrome *c* Oxidase Subunit Degradation) genes are involved in it and implied that degradation pathways for Cox II and Cox III are at least in part distinct. Molecular cloning and characterization of the three OSD genes is also described (chapters 5 and 6). Osd1 protein has an ATP-binding cassette and a sequence motif for zinc binding, and is localized to mitochondrial membrane. *OSD2* and *OSD3* genes code for new types of proteins, of which at least one is probably mitochondrial protein

Chapter 2 Materials and Methods

2. 1 Strains and plasmids

Yeast strains used in this study are listed in Table 2-1. *Saccharomyces cerevisiae* haploid strain DL 1 (*Mat* α *his3 leu2*$^-$ *ura3*$^-$) and its Cox IV-deficient derivative WD 1 (*Mat* α *his3 leu2*$^-$ *ura3*$^-$ *cox IV*$^-$*::LEU2*) (Dowhan et al. 1985) were gifted from G. Schatz (Basel). H11-1B is congenic with D273-10B (ATCC, 25657) except the presence of *leu2, lys2, and his3* auxotroph markers.

For obtaining *osd* mutant strains of the opposite mating type, each of the original *osd* mutant strains were crossed to H11-1B, a haploid strain having wild-type *COX IV* gene, and the resultant diploid cells were sporulated. The segregants with the disrupted *cox IV::LEU2* gene (as judged by leucine prototroph due to the *LEU2* gene inserted into the *COX IV* locus) were chosen and further examined for the presence of the *osd* mutations and for a mating type. Presence of the *osd* mutations in *cox IV::LEU2* segregants was identified by examining accumulation of Cox II using Western blotting after mitochondrial fraction was prepared from those cells (see later sections). *cox IV::LEU2* segregants of an appropriate mating type accumulating Cox II were used for complementation test and for further back-crossing. After back-crossing to H11-1B was repeated at least six times in a similar way as above, segregants with both the wild-type *COX IV* allele and one of the *osd* mutations were used for further experiments as isogenic strains with H11-1B. Presence of the *osd* mutations in strains of the *COX IV*$^+$ genetic background was assessed as described in the text.

pFL-1 (Chevallier et al. 1980) was kindly provided by Toshiharu Hase (Osaka university, Japan). pUCE is a derivative of pUC18 in which multicloning site has been replaced with a SmaI-EcoR1-SmaI site (Fujiwara et al. 1990). Other plasmids were described below where relevant.

2. 2 Yeast media and growth conditions

YPD, YPR, YPGal or YPG contained 1% yeast extracts, 2% peptone, and either 2% of glucose, raffinose, galactose, or glycerol, respectively. SD (6.7 g/l yeast nitrogen base

Table 2-1. Yeast strains used in this study.

Strain	Genotype	Source
DL-1	*Mat* α *his3 leu2 ura3*	Dawhan et al., 1985
WD-1	*Mat* α *his3 leu2 ura3 cox4::LEU2*	Dawhan et al., 1985
WS-2	*Mat* α *his3 leu2 ura3 cox4::LEU2 osd1-1*	this study
WS-3	*Mat* α *his3 leu2 ura3 cox4::LEU2 osd2-1*	this study
WS-5	*Mat* α *his3 leu2 ura3 cox4::LEU2 osd3-1*	this study
WS-8	*Mat* α *his3 leu2 ura3 cox4::LEU2 osd3-2*	this study
H11-1B	*Mat* **a** *his3 leu2 lys2*	our laboratory
H41-5D	*Mat* **a** *his3 leu2 lys2 cox4::LEU2*	this study
H35-1C	*Mat* **a** *his3 leu2 lys2 cox4::LEU2 osd1-1*	this study
H37-3A	*Mat* **a** *his3 leu2 lys2 cox4::LEU2 osd2-1*	this study
H38-2B	*Mat* **a** *his3 leu2 lys2 cox4::LEU2 osd3-1*	this study
H34-2C	*Mat* **a** *his3 leu2 lys2 cox4::LEU2 osd3-2*	this study
D273-10B	*Mat* α	ATCC
H92-1B	*Mat* α *his3 leu2 lys2 ura3*	this study
H92-6D	*Mat* **a** *his3 leu2 lys2 ura3*	this study
H92-6C	*Mat* **a** *his3 leu2 lys2 ura3 osd1-1*	this study
H92-2A	*Mat* **a** *his3 leu2 lys2 ura3 osd1-1*	this tudy
H92-5D	*Mat* **a** *his3 leu2 lys2 ura3 cox IV::LEU2 osd1-1*	this study
H92-4B	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2*	this study
H96-3A	*Mat* α *his3 leu2 lys2 ura3 osd2-1*	this tudy
H96-1B	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 osd2-1*	this study
H97-4B	*Mat* α *his3 leu2 lys2 ura3 osd3-2*	this tudy
H97-3B	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 osd3-2*	this study
H92-1BΔ1	*Mat* α *his3 leu2 lys2 ura3 osd1::HIS3*	this study
H92-1BΔ2-N1[a]	*Mat* α *his3 leu2 lys2 ura3 osd2::HIS3*	this study
H92-1BΔ2-X1[a]	*Mat* α *his3 leu2 lys2 ura3 orf-d::HIS3*	this study
H92-1BΔ3	*Mat* α *his3 leu2 lys2 ura3 osd3::HIS3*	this study
H92-4BΔ1	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 osd1::HIS3*	this study
H92-4BΔ2-N1[b]	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 osd2::HIS3*	this study
H92-4BΔ2-X1[b]	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 orf-b::HIS3*	this study
H92-4BΔ3	*Mat* α *his3 leu2 lys2 ura3 cox IV::LEU2 osd3::HIS3*	this study

[a]Genotypes of H92-1BΔ2-N2, -N3, and -N4 are same as H92-1BΔ2-N2. Genotypes of H92-1BΔ2-X2, -X3, and -X4 are same as H92-1BΔ2-X2.

[b]Genotypes of H92-4BΔ2-N2 and H92-4BΔ2-X2 are same as those of H92-4BΔ2-N1 and H92-4BΔ2-X1, respectively.

(Difco), 2% glucose), SG (6.7 g/l yeast nitrogen base, 2% glycerol), or SR (6.7 g/l yeast nitrogen base, 2% raffinose) supplemented with appropriate amino acids or uracil, was used for auxotroph selections. Solid medium contained 2% agar (Difco) in addition to the corresponding liquid medium. Yeast cells were usually grown at 30°C if not specified.

2. 3 Pulse-chase experiments of mitochondrial translation products

Yeast cells were grown in YPD to a late logarithmic phase (about 260 - 280 Klett units) and washed with distilled water. The cells were resuspended in the original volume of sulfur-free salts (2.9 mM $MgCl_2$, 7.3 mM KH_2PO_4, 8.6 mM NaCl, 3.6 mM $CaCl_2$, 17.9 mM NH_4Cl), supplemented with 2% or 0.2% glucose. After an incubation of 5 min at 30°C with vigorous shaking, cycloheximide was added to a final concentration of 1mg/ml. After further 5 min incubation, labeling of mitochondrial translation products was started by adding [^{35}S]methionine (>1000 Ci/mmol, Amersham) or EXPRE^{35}S^{35}S (a mixture of [^{35}S]methionine and [^{35}S]cysteine, NEN) to a concentration of 50 μCi/ml. After incubating at 30°C for 30 min, the suspension was cooled on ice and centrifuged in 14,000 x *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 medium supplemented with 10 mM non-isotopic methionine, 10 mM cysteine and 10 mg/ml Na_2SO_4) and incubated at 30°C with vigorously shaking for chasing. At times indicated, aliquots were taken and then transferred to tubes containing 10% TCA and incubated on ice for at least 30 min. After centrifugation, the cells were washed twice with 5% TCA and resuspended in distilled water. Cell walls were disrupted by vortexing the cell suspension with glass beads at a maximum speed for 1 min. After SDS-PAGE sample buffer was added, the disrupted cell suspension was neutralized with Tris base, if necessary, and total proteins were extracted by incubating for 1hr at room temperature. After cell debris was removed by centrifugation, solubilized proteins in the supernatants were separated by SDS-PAGE (Laemmli 1970), and the labeled mitochondrial translation products were visualized either fluorographically (Maccecchini et al. 1979) or using a BAS2000 Bio-Image Analyzer (Fuji Photo Film, Japan). The bands on fluorograph were analyzed quantitatively either with an UltroScan XL laser densitometer (Pharmacia-LKB) or with the NIH *Image* program for Macintosh computers (by W. Rasband) after the images of the fluorograms were captured in

the computer by using a GT-8000 (EPSON, Japan).

2. 4 ATP assay

A portion of cell culture was taken and 100% TCA stock solution was added immediately to a final concentration of 10 %. Cell debris was removed by centrifugation, then the supernatant was extracted three times with diethyl ether to remove TCA and diluted with 40mM HEPES (pH 7.6), and ATP was estimated with a luciferin-luciferase system kit (Boehringer Mannheim) using a Biocounter M2010 (Lumac BV).

2. 5 Isolation of *osd* mutants

WD 1 cells were mutagenized with ethyl methanesulfate, incubated in YPD at 23°C overnight and plated on YPR. The plates were incubated for three days at 23°C. Replica were transferred onto nitrocellulose membranes (BA 85, Schleicher & Schuell, Germany). The membranes were placed on YPR plates with the face attaching cells upward, further incubated at 23°C overnight, and then at 37°C for 6 h. Such scheme for cell growth was designed in order to also allow temperature-sensitive conditional mutants to be isolated theoretically, because we did not know in advance whether defect of the degradation of unassembled Cox II is lethal for the cell or not. Cells on the membranes were lysed basically according to the published method (Lyons and Nelson 1984) except that SDS concentration in the lysis buffer was raised to 2%. The membranes were blocked with TBS (20 mM Tris Cl, pH 7.5, 150 mM NaCl) containing 10% skimmed milk overnight and treated with affinity-purified rabbit anti-Cox II polyclonal antibody for 2 h at room temperature. They were further washed three times with TBS and then treated with goat anti-rabbit IgG antibody conjugated with horse-radish peroxidase (Bio-Rad). The membranes were washed three times with TBS and Cox II on the membranes was visualized using 4-chloro-1-naphthol (Bio-Rad). Candidate colonies on the master plates were recovered and mitochondrial accumulation of Cox II was examined by Western blotting after mitochondria were isolated.

2. 6 Isolation of mitochondrial fraction

Yeast cells were grown to a mid-logarithmic phase and collected by centrifugation.

After washed twice with TSB (10 mM TrisHCl, pH 7.4, 0.6 M sorbitol), cells were resuspended in the same buffer supplemented with 5 mM PMSF, and vortexed at a maximum speed with 1.5 ml of glass beads (diameter of 0.45-0.5mm) for 30 s four times with intervals of cooling on ice. Disrupted cell suspension was recovered with a pipette, and then centrifuged at 3,500 x *g* for 5 min. The supernatant was centrifuged at 12,000 x *g* for 10 min. The pellet was carefully resuspended in TSB and recentrifuged at 3,500 x *g* for 5 min. The supernatant was recentrifuged at 12,000 x *g* for 10 min, and the final pellet was washed twice with TSB and resuspended in TSB.

2. 7 Molecular cloning of the *OSD* genes

The *OSD1* gene was cloned using the temperature sensitive phenotype of the *osd1-1 COX IV*$^+$ cells at 37°C on a medium containing glycerol as a carbon source. H92-6C (*osd1-1 COX IV*$^+$) were transformed with the yeast genomic library constructed from Sau3A-partially digested total DNA of a ρ^- subclone of D273-10B (ATCC 25657) on a multi-copy shuttle vector pFL-1 (Chevallier et al. 1980). Approximately 20,000 Ura$^+$ transformants were screened for growth at 37°C on SG plates supplemented with leucine, lysine, and histidine for five days. Colonies formed in this condition were re-examined for growth at 37°C on the same medium. Finally, six independent clones were obtained. Harbouring plasmids were recovered by transforming *E.coli* RR1 strain with the extracted DNA and selecting for ampicilin-resistance. Each plasmid recovered was re-introduced into H92-6C to examine its ability to release the cell from the growth suppression at 37°C on a glycerol medium. Each of these plasmids was also introduced into H92-5D (*osd1-1 cox IV::LEU2*) in order to examine its ability to cure the cells of mitochondrial accumulation of unassembled Cox II in the absence of Cox IV.

The *OSD2* and *OSD3* genes were cloned in a similar way as in the cloning of the *OSD1* gene except that in these cases, H96-3A (*osd2-1 COX IV*$^+$) or H97-4B (*osd3-2 COX IV*$^+$) were used, respectively, for transformation of the yeast genomic library. The resulting colonies which were larger than those around them were isolated and plasmids were recovered from those strains using JM109 bacterial strain. Candidate plasmids for the *OSD2* or *OSD3 were* checked for their complementing ability of the temperature sensitivity by re-introducing them into H96-3A, or H97-4B, respectively. They were also introduced into H96-1B (*osd2-1*,

cox IV::LEU2) or H97-3B (*osd3-2, cox IV::LEU2*) for examining their ability to complement mitochondrial Cox II accumulation of *osd2-1, cox IV::LEU2* cells.

2. 8 Sequence analysis

For sequencing the *OSD1* gene, 3.8 kb BglII-ScaI fragment of pSG3, which is sufficient for the complementation of both of the two phenotypes of the *osd1* mutants (see Fig. 5-2), were ligated with EcoRI linkers after blunt-ended with Klenow fragment, and then subcloned into the EcoRI site of Bluescript KS+ plasmid (Stratagene) in both orientations. For the *OSD2* gene, 2.6 kb SalI fragment of pOS2-1 containing the entire overlapping region derived from yeast genome (Fig. 6-2) and a portion of the vector sequence was subcloned into the SalI site of Bluescript KS+ plasmid in both orientations. For the *OSD3* gene, 3.5 kb XbaI fragment of pOS3-3 covering most of the overlapping region (Fig. 6-7) was subcloned into the XbaI site of Bluescript KS+ plasmid in both orientations. Sets of nested deletion mutants of each of the above inserts from each of the ends were obtained by the exonuclease III method (Henikoff 1984) using Kilo-sequence deletion kit (Takara). Some of the fragments derived from this region were also subcloned into a M13mp19 phagemid vector. All of the nucleotide sequencing was performed using the dideoxy chain termination method (Sanger et al. 1977), using Sequenase (U. S. Biochemicals). Custom-made oligonucleotides were used as primers for sequencing regions not accessible with the above constructions. Both DNA strands were sequenced.

2. 9 Gene disruption

For disrupting the *OSD1* gene, EcoRI linker-attached 3.5 kb BglII-ScaI fragment containing the whole *OSD1* gene was subcloned into the unique EcoRI site of pUCE vector to generate pEOS1. 2.2 kb EcoRV-HindIII region of the insert in pEOS1 containing about NH_3-terminal 90% of the *OSD1* coding region and 0.3 kb of its downstream region was removed by digestion with EcoRV and HindIII, and then replaced with a BamH1 linker after blunt-ended to generate pDOS1. 1.8 kb BamHI fragment of the *HIS3* marker gene was inserted into the unique BamHI site of pDOS1 to generate pDOS1-HIS3.

In the case of the *OSD2*, two different coding regions, of which one is full and the

other is truncated, were found in the overlapping portion. So, two different constructs in which either of the two coding regions is disrupted were made. 2.6 kb SalI fragment of pOS2-1 was blunt-ended and EcoRI linkers were attached to the both ends. This fragment was ligated into the EcoRI site of pUCE to obtain pUC-OS2. For disruption of the former orf, 0.7 kb NcoI region containing most part of pUC-OS2 was replaced with 1.8 kb BamHI fragment of the *HIS3* marker gene by blunt-end ligation after treatment with Klenow fragment to generate pDOS2-N. For disruption of the latter truncated orf, BamHI fragment of the *HIS3* was inserted into the XhoI site of pUC-OS2 by blunt-end ligation after treatment with Klenow fragment to generate pDOS2-X.

For disruption of the *OSD3*, 3.8 kb XbaI fragment of pOS3-3 was ligated with EcoRI linker and subcloned into the EcoRI site of pUCE to generate pUC-OS3, and BamHI fragment of the *HIS3* was inserted into the BamHI site of pUC-OS3 situated in the coding region.

Each of these four plasmids containing one of the above disrupted genes were digested with EcoRI and used for transforming yeast haploid strains H92-1B (*COX IV* $^{+}$) or H92-4B (*cox IV::LEU2*). Candidate disruptant cells were obtained by selection for His^{+} colonies. Colonies grown on histidine-lacking plates from each transformation were recovered and then examined for desired replacement on the chromosomal DNA by Southern blotting. For probes, 3.1 kb NcoI fragment encoding the whole OSD1-coding region, 1.5 kb EcoRV-XhoI fragment of pUC-OS2 and 3.8 kb XbaI fragment of pUC-OS3 were used, respectively, for analyzing the *OSD1*, *OSD2*, and *OSD3* disruptants. One or several of the desired disrupted strains derived from either H92-1B or H92-4B for each of the disrupted genes were used for further analysis.

2. 10 Site-directed mutagenesis

For mutagenesis of the nucleotide-binding cassette, 0.9 kb PstI-BamH1 fragment of the *OSD1* gene was subcloned into the multicloning site of M13tv19 phagemid vector (Takara), and mutations were introduced using Mutan G mutagenesis kit (Takara) and the following synthetic oligonucleotides; 5'-CCAACAAAGTTTCACCGGTACCAGGAGGTC-3' (ONB-1), 5'-CCAACAAAGTGATACCGGTACCAGGAGGTC-3' (ONB-2), 5'-CCAACAAAGTTTG-ACCGGTACCAGGAGGTC-3' (ONB-3), 5'-CCAACAAAGTTCTACCGGTACCAGGAG-

GTC-3' (ONB-4). Sequences corresponding to the conserved lysine residue in the wild type *OSD1* gene are underlined. For convenience for monitoring the introduction of the mutations, a KpnI site was introduced without affecting the coding amino acid. All of the mutations introduced were checked by sequencing using 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 were excised with SmaI and subcloned into the unique PvuII site of pFL1 generating pFL-ONB-1, -2, -3, and -4, respectively.

2. 11 Enzyme assays

Published methods were used for enzyme assays of cytochrome *c* oxidase (Mason et al. 1973), NADH-cytochrome *c* reductase (Hatefi and Rieske 1967), succinate-cytochrome *c* reductase (Tisdale 1967), kinurenine hydroxidase (Bandlow 1972), and NADPH-cytochrome *c* reductase (Masters et al. 1967).

2. 12 Cellular subfractionations

For examining subcellular localization of Osd1 protein, mitochondria were prepared using the spheroplast method as follows: Yeast cells were grown in YPGal to a mid-logarithmic phase (Klett 150). Spheroplasts were prepared according to the published method (Daum et al. 1982), disrupted by Dounce homogenizer (pestle A) and centrifuged at 3,500 x *g* for 5 min. The pellet containing both the nucleus and undisrupted cells was discarded and the supernatant "PNS" (post nuclear supernatant) fraction was further subfractionated by centrifugation at 12,000 x *g* for 10 min. The pellet was resuspended in TSB, centrifuged at 3,500 x g for 5 min, and the supernatant was centrifuged at 12,000 x *g* for 10 min to separate into the pellet "Mit" (mitochondria) and the supernatant "PMS" (post mitochondrial supernatant) fractions. Ratios of the enzymatic activities per proteins for the "Mit" and "PMS" fractions to those of the "PNS" 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 was further purified

from the "Mit" fraction by 20-40% 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 DTT, 1 mM PMSF, and Percoll was removed by diluting and washing with the buffer. Purified mitochondria were subfractionated as described (Daum et al. 1982).

2. 13 Whole cell spectrum

Yeast cells were grown in a YPGal medium to a 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 inverting a cuvette several times, whole cell spectrum was measured at room temperature using MP-100 multipurpose spectrophotometer (Shimadzu).

2. 14 Immunological methods

Cox II and Cox III were purified electrophoretically from yeast cytochrome *c* oxidase prepared according to the published method (Rubin and Tzagoloff 1978). Antiserum against Cox II was prepared by injecting rabbits subcutaneously with the purified subunit once with Freund's complete adjuvant and, subsequently, four times with Freund's incomplete adjuvant with the intervals of two weeks before bleeding. Anti-Cox III antisera was also prepared similarly. Cox II-affinity column was prepared by coupling electrophoretically purified Cox II to Affigel 10 (Bio-Rad) according to the manufacturer's instructions, and it was used for affinity-purification of anti-Cox II antibody. Immunoprecipitation was carried out according to the published method (Maccecchini et al., 1979).

β-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 (Stanley and Luzio 1984) in frame with the b-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 electrophoresis, the proteins were electroblotted onto nitrocellulose membranes. Portions of the membrane corresponding to the fusion protein were excised and dissolved in DMSO and,

after mixed with complete or incomplete Freund's adjuvant, used for immunizing rabbits.

2. 15 Other methods

Total cellular proteins were estimated according to the published method (Lowry et al. 1951) after proteins had been extracted by disrupting the cells with glass beads and incubating them in the presence of 1.3 % SDS. Cell debris was removed by centrifugation before measurement. In other cases, protein determination was carried out by the Coomassie-binding method using Protein Assay kit (Bio-Rad).

Published methods were used for SDS-PAGE (Laemmli 1970) and electrophoretic transfer of proteins from polyacrylamide gels onto Zeta-Probe or PVDF membranes (Bio-Rad) (Towbin et al. 1979). Detection of the antibody-recognized proteins on membrane was carried out using HRP-conjugated goat anti-rabbit IgG (Bio-Rad) using 4-chloro-1-naphtol (Bio-Rad) according to the manufacturer's instructions.

Yeast manipulations were carried out basically according to (Rose et al. 1990). Yeast transformation was performed with the lithium acetate method (Itoh et al. 1983). Published methods were used for standard DNA manipulations (Sambrook et al. 1989).

Chapter 3 Whole Cell Analysis of the Degradation of Unassembled Subunits 2 of Cytochrome *c* Oxidase

In a Cox IV-lacking *Saccharomyces cerevisiae* WD 1 strain, other subunits of cytochrome *c* oxidase are incapable of assembling the active enzyme (Dowhan et al. 1985). This chapter describes that, in this strain, mitochondrial-encoded Cox II and Cox III are degraded rapidly as compared to those in a strain possessing normal Cox IV *in vivo*. This *in vivo* degradation is significantly suppressed by treatments reducing intracellular ATP level, suggesting an ATP-dependence of this protein degradation system. Moreover, the results of experiments using *o*-phenanthroline suggested a novel divalent-metal ion requirement of this *in vivo* degradation which has not been observed for the matrix ATP-dependent proteases reported previously, implying that some factor(s) other than or in addition to the matrix ATP-dependent protease is involved in the initial step of the degradation of unassembled Cox II and Cox III.

3. 1 Instability of mitochondrially encoded Cox II and Cox III in a Cox IV-deficient strain

First, stability of the mitochondrially encoded subunits of cytochrome *c* oxidase *in vivo* was examined by pulse-chase experiments. Mitochondrial translation products were specifically labeled with [^{35}S]methionine in the presence of cycloheximide for inhibiting cytosolic protein translation, and chased with an excess of non-isotopic methionine for one hour. As shown in Fig. 3-1, Cox II in WD 1 cells was apparently reduced to about 20 % after a 60 min chase, while about 70 % of that in DL 1 cells still remained. The apparent half-life of Cox II was about 30 min in WD 1. Though Cox III and F_0F_1-ATPase subunit 6 co-migrated, the corresponding band also decreased to 10 % of the initial intensity after a 60 min chase in WD 1 strain. In order to examine the stability of Cox III alone, we further analyzed the radio-labeled mitochondrial translation products by immunoprecipitating Cox III with rabbit anti-Cox III polyclonal antiserum (Fig. 3-2). The half-life of Cox III in WD 1 was estimated

to be about 20 min, while in DL 1 cells 75 % of the initial amount of this subunit still remained after chase for an hour. In contrast, Cox I was very stable in the presence or absence of Cox IV (Fig. 3-1*A*). Further, var1 and apo-cytochrome *b* were only slightly degraded and no significant difference between the degradation rates of DL 1 and WD 1 was observed (Fig. 3-1*A*).

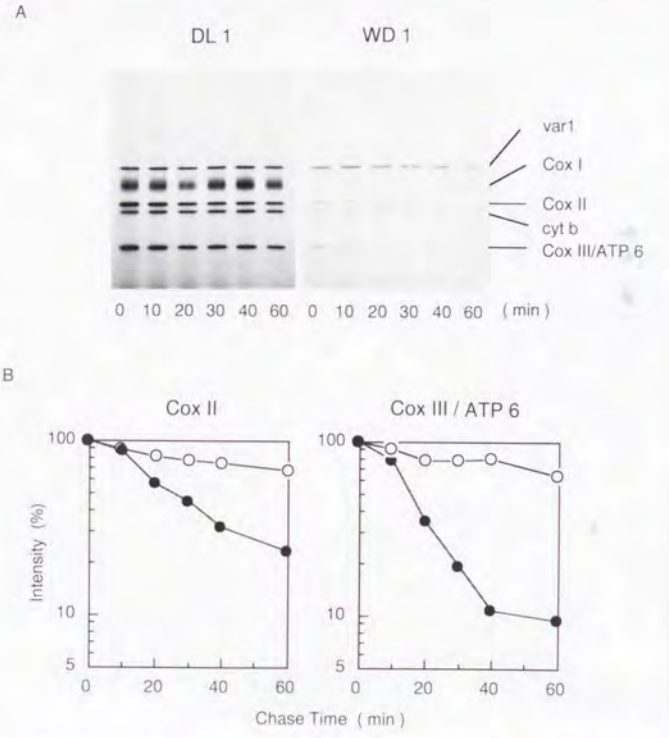


Fig. 3-1. Degradation of mitochondrial translation products in DL1 and WD1 cells. DL 1 and WD 1 cells were separately pulse-labeled with [^{35}S]methionine in the presence of cycloheximide, chased in non-radioactive medium, YPD supplemented with 10 mM cold methionine, 10 mM cysteine and 10 mg/ml Na_2SO_4, at 30°C, and aliquots were taken for protein extraction at the times indicated during the chase. Cell proteins were extracted and separated on 10-15% SDS-PAGE. (*A*), fluorograms, exposed for 2 days, of the radio-labeled mitochondrial translation products from DL 1 (left) and WD 1 (right). Positions of Cox II, Cox III, apo-cytochrome *b* (Cyb), and F_0F_1-ATPase subunit 6 (ATP6) are indicated on the right. (*B*), quantitative analysis of Cox II (left) and Cox III (right); DL1 (○), WD1 (●) expressed as a percentage of the intensity at 0 min.

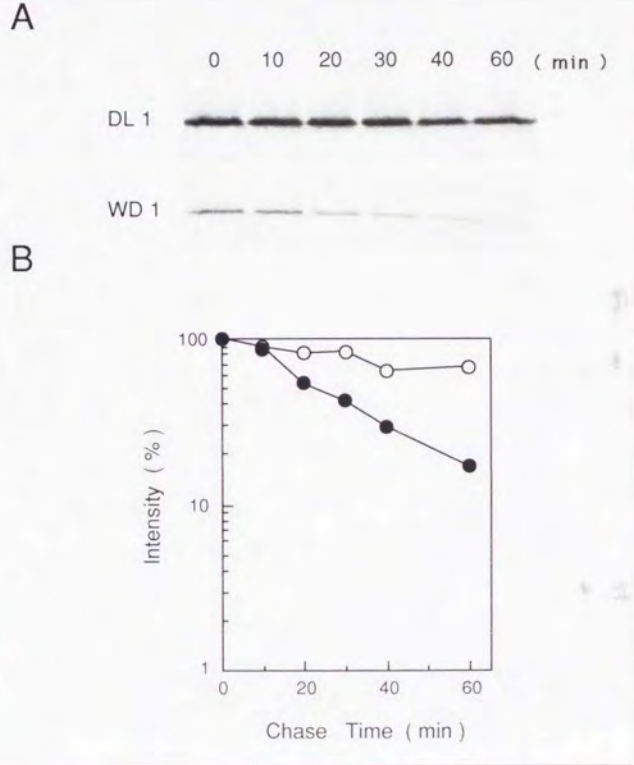


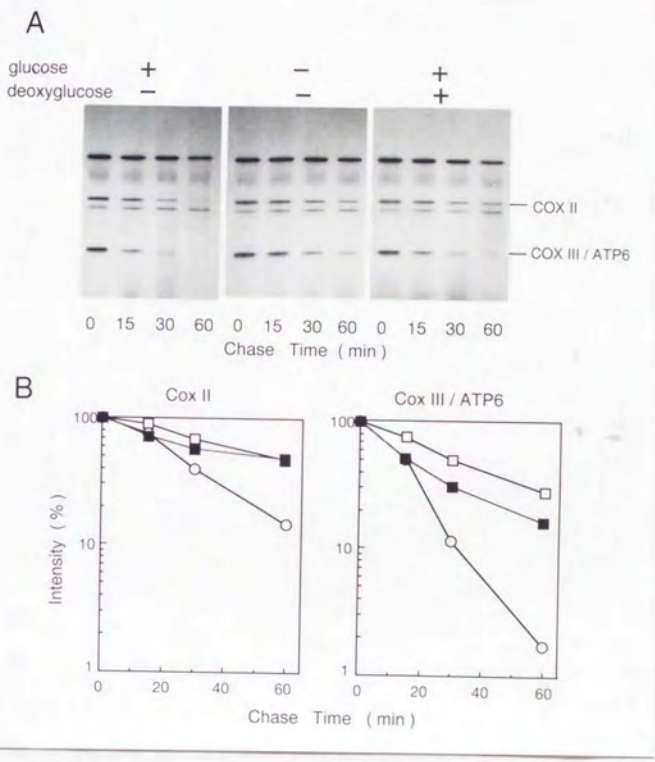
Fig. 3-2. Turnover of Cox III in DL 1 and WD 1 cells. Cells were pulse-chased and analyzed as in Fig. 1 except that Cox III was immunoprecipitated with rabbit anti-yeast Cox III antiserum. SDS-PAGE was done using 12.5 % polyacrylamide gel followed by analysis for radioactivity with a BIO-Image Analyzer. (A), visualization of radiolabeled Cox III; (B), quantitative analysis of Cox III; DL 1 (○), WD 1 (●).

3. 2 Effects of treatments reducing intramitochondrial ATP level

Isolated mitochondria can degrade translation products in an ATP-dependent way (Desautels and Goldberg 1982b; Kalnov et al. 1979). Thus, a possibility of ATP requirement for the degradation of unassembled Cox II and Cox III in WD 1 cells was examined. Either addition of 2-deoxy-D-glucose, a competitive inhibitor of the glycolysis pathway, in addition to glucose, or, depletion of glucose in the medium during the chase is expected to decrease generation of ATP by inhibiting the glycolytic pathway. First, effects of those treatments on the intracellular ATP level were examined. As shown in Fig. 3-3*C*, in the presence of glucose, ATP level per cellular proteins increased sharply and remained high for an hour. On the other hand, when 2-deoxy-D-glucose co-existed, or when the medium was depleted of glucose, such an increase did not occur and ATP levels remained low during the incubation. So, such treatments can keep the intracellular ATP level low during the chase relative to that without the treatments. Using the same experimental conditions as above, stability of the mitochondrial translation products was examined by pulse-chase experiments. As shown in Fig. 3-3*A* and *B*, 50 % of the radioactivity of Cox II and 20 to 30 % of that of Cox III/F_0F_1-ATPase subunit 6 remained after 1 h of chase in the presence of either of the above treatments, while in the control experiment without such treatments, only 15 % and 2% of the initial radioactivity was detected, respectively.

Second, to eliminate a possibility that decrease of the intracellular level of a metabolic product of the glycolytic pathway other than ATP is responsible for the suppression of the degradation, and to confirm that mitochondrial ATP, not cytosolic ATP, is required for the degradation, the effect of bongkrekic acid was also examined. This reagent is known to inhibit specifically the mitochondrial ATP-ADP exchanger which is required for the transport of ATP across the inner membrane of mitochondria. So, it is expected that in the presence of this reagent, cytosolically synthesized ATP, which is the sole source of ATP in respiratory-deficient cells such as WD 1, can not enter the mitochondria, and, consequently, those cells become depleted of intramitochondrial ATP (Gbelska et al. 1983). As shown in Fig. 3-4, degradation of Cox II during an hour of chase was suppressed from 87 % to 49 % when bongkrekic acid (75 μM) was present during the chase. Similarly, degradation of Cox

III/F_0F_1-ATPase subunit 6 was suppressed from 86 % to 56 % during 1 h of the chase.



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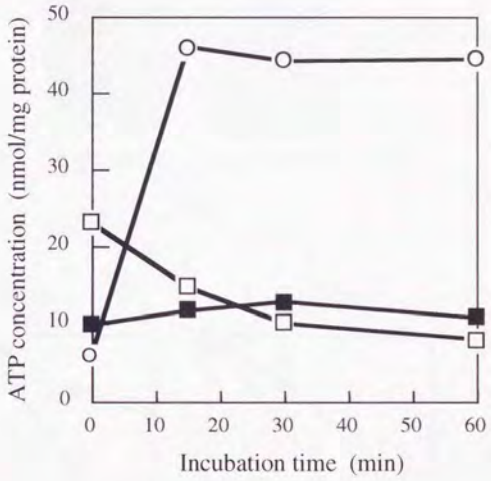


Fig. 3-3. Effects of glucose depletion or deoxyglucose. (*A*) WD 1 cells were pulse-chased and analyzed as in Fig. 1 except that chasing was carried out in a medium (0.1 % yeast extract, SFS, 10 mM cold methionine, 10 mM cysteine and 10 mg/ml Na_2SO_4) containing 2 % glucose, no glucose, or 2 % glucose plus 2 % 2-deoxy-D-glucose. (*B*) Radioactivity of Cox II (left) and Cox III (right) was determined as in Fig. 1; (○), + glucose; (□), - glucose; (■), + glucose plus 2-deoxy-D-glucose. (*C*) Effects of glucose depletion or 2-deoxy-D-glucose on the intracellular ATP level were examined by treating cells as in (*A*) except that non-isotopic methionine was used instead of [^{35}S]methionine. At the same time points as in (*A*), aliquots were withdrawn and used both for measurements of ATP levels and for protein determination as described in the Materials and Methods section; (○), + glucose; (□), - glucose; (■), + glucose plus 2-deoxy-D-glucose.

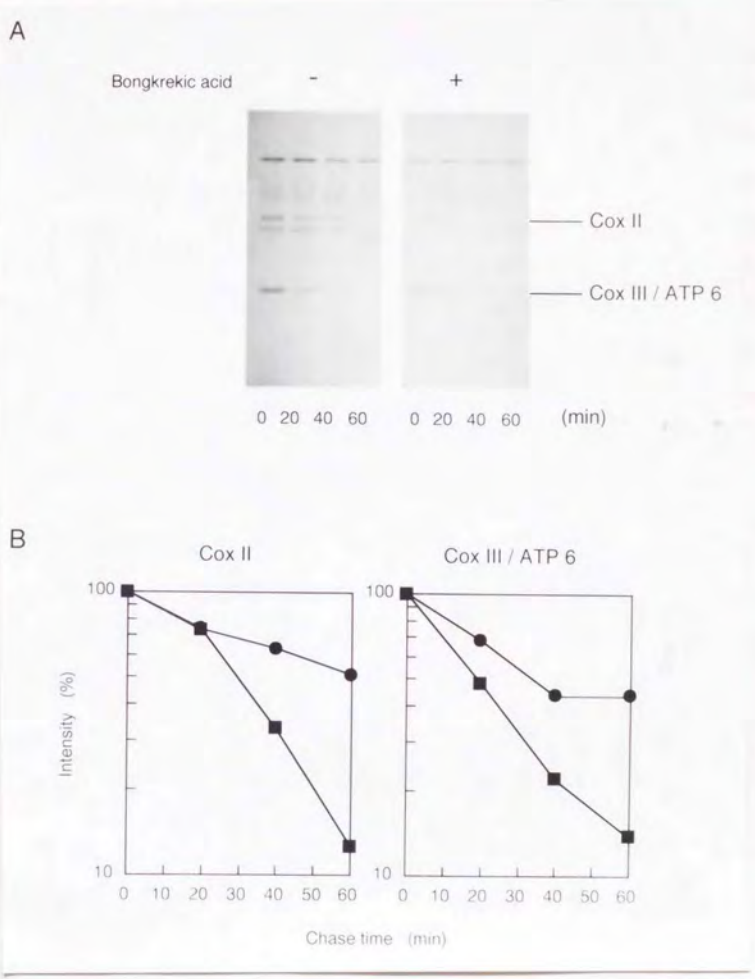


Fig. 3-4. Effects of bongkrekic acid. (*A*) WD 1 cells were pulse-chased and analyzed as in Fig. 1 except that the chasing medium contained no (left) or 75 μM (right) bongkrekic acid. (*B*) Radioactivity of Cox II (left) and Cox III /F_0F_1-ATPase subunit 6 (right) was determined as in Fig. 1; absence (■), or presence (●) of bongkrekic acid.

3. 3 Effects of *o*-phenanthroline, a divalent metal ion chelator

The effects of divalent metal ions on the degradation of unassembled Cox II and Cox III/F_0F_1-ATPase subunit 6 were investigated. *o*-Phenanthroline was used for chelating divalent metal ions because of its membrane-permeable nature. As shown in Fig. 3-5, *o*-phenanthroline caused almost complete inhibition of the degradation of both Cox II and Cox III/F_0F_1-ATPase subunit 6 during chase. It is known that yeast cells possess transport machinery for divalent metal ions such as Mn^{2+}, Co^{2+}, Zn^{2+}, Mg^{2+}, or Ca^{2+} (Fuhrmann and Rothstein 1968). So, effects of the addition of a 10-fold molar excess of either of those metal ions on the inhibition of the degradation by *o*-phenanthroline were examined. When either Mn^{2+}, Co^{2+} or Zn^{2+} was added after 5 min incubation in the presence of the chelator, the inhibitory effect of *o*-phenanthroline was suppressed (Fig.3-5*A* and *B*). In contrast, addition of an excess of either Ca^{2+} or Mg^{2+} was not effective.

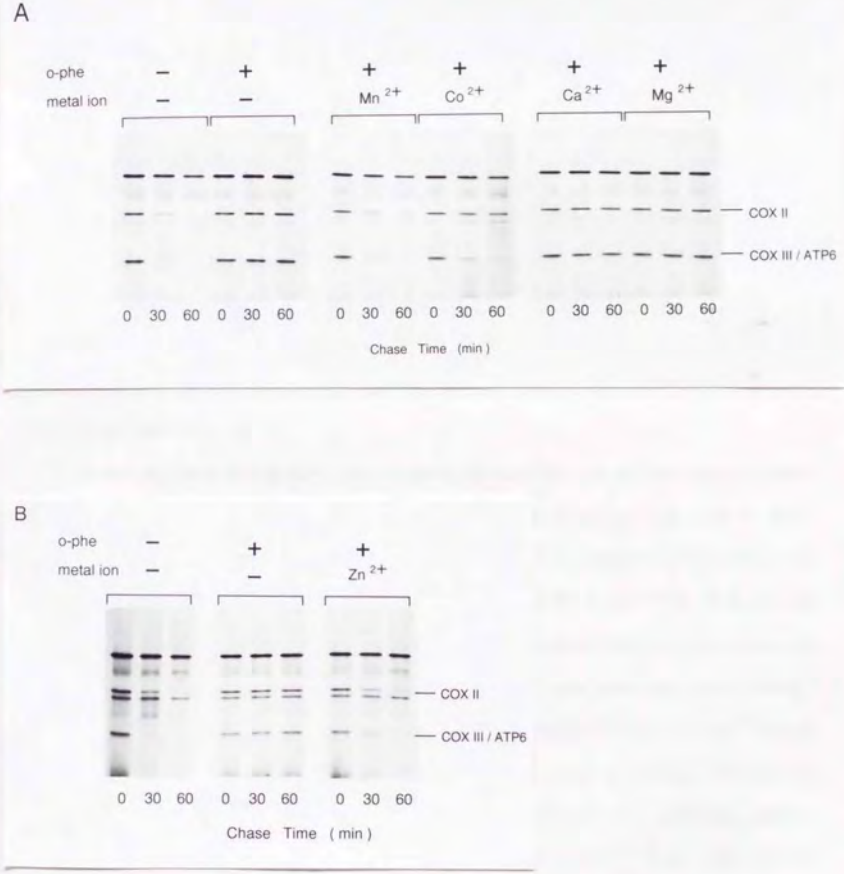


Fig. 3-5. Effects of *o*-phenanthroline and divalent metal ions. Cells were pulse-labeled and chased at 30 °C as in Fig. 1 except that the samples were incubated for 5 min on ice in the presence (+) or absence (-) of 2.5 mM *o*-phenanthroline, and, subsequently, they were supplemented with the indicated divalent metal ions to a final concentration of 10 mM prior to chasing. Experiments A and B were separately performed.

3. 4 Discussion for chapter 3

In this chapter, I described the results of pulse-chase experiments showing that unassembled Cox II and Cox III were degraded rapidly in a Cox IV-deficient strain (WD 1). It explains, at least partially, the reduced intramitochondrial levels of those subunits in WD 1 cells (Dowhan et al. 1985), though decreased synthesis rates, as suggested by the less intense bands observed for Cox II and Cox III in the fluorography for WD 1 cells (Fig. 3-1*A*), may also contribute.

Another finding of pulse-chase experiments is that Cox I, which also can not be assembled into the mature complex in WD 1, is relatively stable with a half-life of longer than an hour in both wild-type and Cox IV-deficient cells. This stability is not intrinsic to Cox I because it has been reported that this subunit is unstable in a mutant (Krummeck and Rödel 1990). Probably, formation of a complex with subunit(s) other than Cox IV, or some post-translational modification of this subunit before complex formation with Cox IV is sufficient to stabilize Cox I.

It was reported that isolated mitochondria from yeast and rat liver possess ATP-dependent proteolytic activities for mitochondrial translation products (Kalnov et al. 1979; Desautels and Goldberg 1982b). In addition, ATP-dependent protease was purified from mammalian (Desautels and Goldberg 1982a; Watabe and Kimura 1985a; Watabe and Kimura 1985b) and yeast (Kutejová et al. 1993) mitochondrial matrix fractions using exogenous proteinous substrates. Thus, it is important to know whether the degradation of unassembled Cox II and Cox III observed in Cox IV-deficient WD 1 cells is also ATP-dependent. Though the use of isolated mitochondria would be suitable for such analysis, isolated mitochondria from Cox IV-deficient WD 1 cells incorporated radio-labeled amino acid only very poorly. As it was not possible to examine the effects of ATP directly in whole-cell experimental system because of the difficulty of eliminating the effect of endogenous ATP and of the impermeability of the cell membrane to ATP, ATP-dependence was assessed by the use of several treatments which reduce intramitochondrial ATP concentration in WD 1 cells. In this strain, ATP supplied from cytosol is the sole source of it due to its respiratory incompetence caused by absence of functional cytochrome *c* oxidase. So, decrease in cytosolic ATP via inhibition of glycolysis by 2-deoxy-D-glucose or glucose depletion, or, inhibition of the

transportation of ATP into mitochondria by bongkrekic acid should cause decrease of intramitochondrial ATP concentration. Suppressive effect of those treatments on the degradation of unassembled Cox II and Cox III in WD 1 indicates probable requirement of intramitochondrial ATP for this degradation pathway.

Next, it was shown that the degradation was inhibited by *o*-phenanthroline and that excessive Co^{2+}, Mn^{2+} or Zn^{2+}, but not Ca^{2+} or Mg^{2+}, was effective for recovery from this inhibition (Fig. 3-5). This metal dependence is very similar to that for the mitochondrial processing protease which is required for removing the N-terminal presequence from a precursor of a mitochondrial protein encoded by nuclear DNA (Böhni et al. 1980). However, involvement of the processing protease in the degradation of unassembled Cox II and Cox III is unlikely because the processing protease is known to be highly specific for cleaving presequence portion of the mitochondrial precursor proteins (Böhni et al. 1983). Though metal dependence of the proteolytic activities of isolated mitochondria for translation products has not been reported, the ATP-dependent matrix protease purified from rat liver mitochondria is sensitive to EDTA, and, Mg^{2+} is effective for recovery from this inhibition (Desautels and Goldberg 1982a). However, the effect of *o*-phenanthroline on its activity has not been reported, nor has that of Co^{2+}, Mn^{2+} or Zn^{2+}. In this study, it was first shown that at least some of the mitochondrial metabolic protein degradation requires a metal ion(s) other than Mg^{2+}. Though at present the physiologically required metal ion is still unknown, and whether a protease with such metal dependence is directly involved in the degradation of unassembled Cox II and Cox III remains unclear, those observations support the idea that factor(s) requiring such metal ions, other than the ATP-dependent matrix protease, is involved, directly or indirectly, in the degradation of unassembled Cox II and Cox III. It can be a protease or other factors such as a regulatory factor for a protease, molecular chaperone or a factor modifying the substrates for degradation.

This Cox IV-deficient strain has advantage compared to wild-type cells for isolating mutants defective in the degradation of unassembled Cox II or Cox III by examining their accumulation, because inability of those subunits to assemble normally eliminates the accumulation of them as parts of mature enzyme, which is stable and would interfere the detection of the accumulation of unassembled subunits. In the next chapter, I describe the

isolation and characterization of such mutants.

Chapter 4 Isolation and Characterization of *osd* Mutants

This chapter describes the isolation and genetic characterization of a set of mutants (*osd* mutants) defective in the degradaton of unassembled Cox II in Cox IV-deficient genetic background. The results suggest that multiple genes (*OSD* genes), at least three, are required for this degradation pathway, and that the pathways for the degradation of Cox II and Cox III are, at least partly, distinct. It is also shown that these mutations caused temperature sensitive growth on a non-fermentable glycerol medium when introduced into a strain of the wild-type *COX IV*$^{+}$ genetic background, suggesting an important physiological role of the *OSD* genes for mitochondrial function.

4. 1 Isolation of mutants accumulating unassembled Cox II.

It is expected that defects in degradation of a protein cause its accumulation at a higher level than observed in the parent strain. Thus, to obtain mutants defective in degradation of unassembled Cox II, mutants accumulating this subunit were isolated. Wild type strains are disadvantageous for such a purpose because they would also accumulate normally assembled Cox II which is relatively stable and would accumulate to a higher degree than unassembled ones, even though degradation of the latter is defective. For that reason, as a parent strain, a Cox IV-deficient strain (WD 1), in which Cox II cannot assemble and is degraded rapidly, was chosen.

After primary screening of mutagenized WD-1 cells on nitrocellulose membranes for Cox II-accumulation, using affinity-purified anti-Cox II antibody as a probe (Fig. 4-1), mitochondria were prepared from the candidate cells grown on YPR and were examined for accumulation of Cox II by Western blotting, using the same antibody. Finally, after screening 12,000 colonies, four *osd* mutants (for cytochrome *o*xidase *s*ubunit *d*egradation) accumulating Cox II significantly in mitochondria compared to the parent strain were obtained (Fig. 4-2). These mutant strains were designated as WS-2, 3, 5, and 8, respectively.

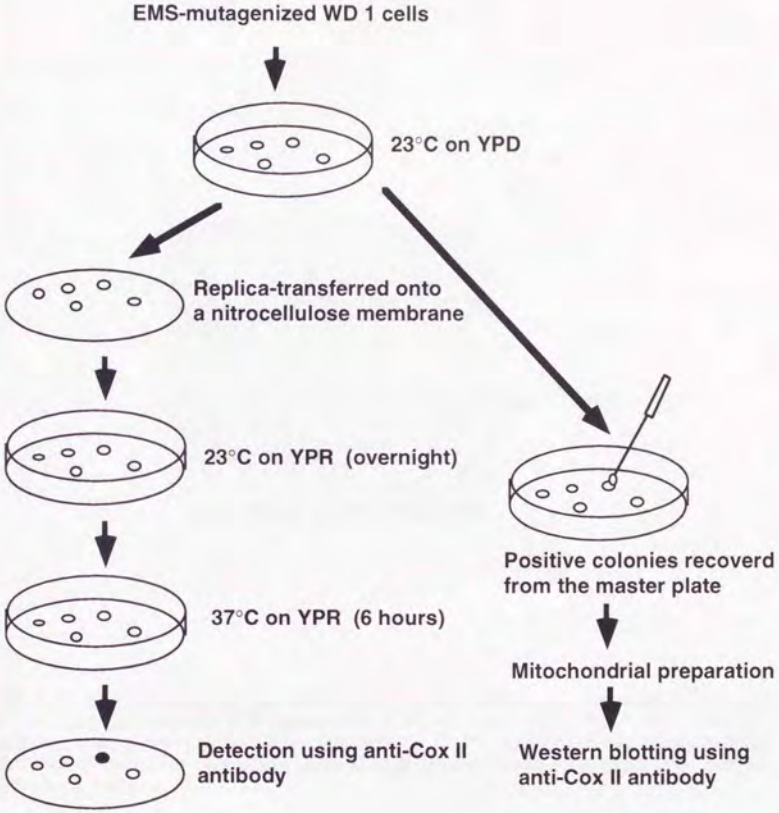


Fig. 4-1. Schematic diagram for isolating mutants defective in the degradation of unassembled Cox II. Cox IV-deficient WD 1 cells mutagenized with ethyl methanesulfonate were incubated on YPD plates at 23 °C and replica were transferred onto nitrocellulose membranes. The membranes were incubated on YPR plates at 23°C overnight and at 37°C for 6hr, successively. Cox II on the membrane was detected using anti-Cox II antibody and the colonies on the master plates corresponding to the intense signals on the membranes were recovered. Mitochondrial accumulation of Cox II was confirmed by Western blotting.

Fig. 4-2. Accumulation of Cox II in mitochondria of the osd mutants. Mitochondrial proteins (35 μg) 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 on 12.5% SDS-PAGE and transferred onto a Zeta-Probe membrane (Bio-Rad). Cox II was detected using affinity-purified anti-Cox II polyclonal antibody.

4. 2 Dominance analysis of *osd* mutants.

To examine the dominance of the mutations, each of the isolated mutants as well as the parent WD 1 was crossed to a *cox IV::LEU2* haploid strain of the opposite mating type (H41-5D, *Mat* **a**). Obtained diploid strains heterozygous for one of the *osd* mutations and homozygous for the *cox IV::LEU2* alleles were analyzed for mitochondrial level of Cox II, by Western blotting using anti-Cox II antibody. As shown on the first row of the panel in Fig. 4-3, steady state intramitochondrial levels of Cox II in the diploid cells derived from the crossing of either of the four *osd* mutants and H41-5D were similar to levels in the diploid strain derived from the crossing of WD 1 and H41-5D. This means that each of the *osd* mutations causing accumulation of unassembled Cox II in the cells of *cox IV::LEU2* genetic background is recessive to the wild type allele(s), and thus, of loss-of-function type.

4. 3 Complementation analysis of *osd* mutants.

To assess allelic relationships among the four mutants, complementation analysis was performed. For such an analysis, haploid *cox IV::LEU2 osd* mutant strains of the opposite mating type are required. As *cox IV::LEU2* homozygous diploid strains could not sporulate (data not shown), each of the four original *osd* mutants were first crossed to a strain having wild-type *COX IV*$^{+}$ allele (H11-1B) and the resulting diploid cells were sporulated. Half the number of segregants derived from such sporulation should have a *cox IV::LEU2* allele. Examination of several such *cox IV::LEU2* segregants for the accumulation of Cox II revealed that they either accumulated Cox II to a similar extent seen in the original mutants or have a reduced mitochondrial Cox II level comparable to that in the parent WD 1. No segregant with intermediate phenotype was observed (data not shown). In this way, for each of the original *osd* mutants, WS-2, WS-3, WS-5 and WS-8, a corresponding segregant of the opposite mating type (*Mat* **a**), H35-1C, H37-3A, H38-2B and H34-2C , respectively, was obtained.

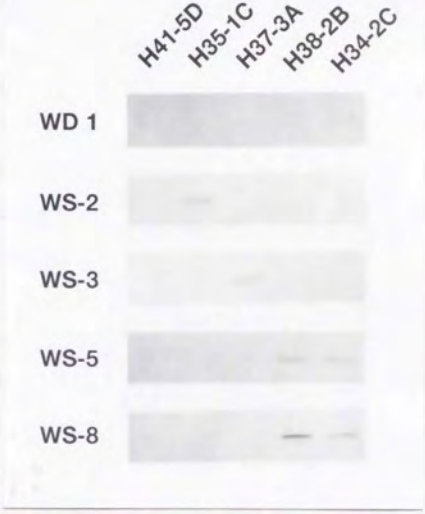


Fig. 4-3. Complementation analysis of the *osd* mutants. H41-5D, H35-1C, H37-3A, H38-2B, and H34-3C were derived from the crossings of WD 1, WS-2, WS-3, WS-5, and WS-8, respectively, to the wild type COX IV strain, H11-1B. Diploid cells from each combination were examined for mitochondrial accumulation of Cox II as described in Fig. 2.

For complementation analysis, such haploid strains as well as a *OSD*$^+$ *cox IV::LEU2* strain H41-5D (*Mat* **a**) were further crossed to the original mutants or to the parent WD 1 to generate diploid strains of all the possible combinations. The resultant diploid strains were examined for mitochondrial accumulation of Cox II. Western blots are shown in Fig. 4-3. First, cells derived from the combinations of one of the original *osd* mutants and its corresponding derivative strain (for example, WS-2 and H37-3A) accumulated Cox II. Second, a combination of WS-5 and WS-8-derived H34-2C, or, WS-8 and WS-5-derived H38-2B was also not effective for suppressing the Cox II-accumulating phenotype, suggesting that the causal mutations in WS-5 and WS-8 are allelic. Finally, all of the remaining combinations could suppress the Cox II-accumulating defect, indicating that the causal mutations in WS-2, WS-3 and WS-5 (or WS-8) are not allelic. Thus, the obtained *osd* mutants were classified into three complementation groups.

4. 4 Tetrad analysis of the *osd* mutants.

One possible explanation for the Cox II-accumulating phenotype of the *osd* mutants is that the structural gene of Cox II on mitochondrial DNA was mutated so that the encoded Cox II became resistant against proteolysis. This possibility was examined by tetrad analysis. Since *cox IV::LEU2* homozygous diploid cells were incapable of sporulating, each of the original mutants was first crossed to H11-1B carrying the wild-type *COX IV*$^+$ allele, the resultant diploid cells were sporulated and the segregants were analyzed. As expected, the *cox IV::LEU2* genotype, which could be identified by presence of the leucine auxotroph due to the inserted *LEU2* gene, segregated in 2:2 ratio. The amount of Cox II in the mitochondria of such segregants was estimated by Western blotting. Fig. 4-4 (top gel) shows an example of such analysis for the four segregants derived from a diploid cell obtained by the crossing of WS-2 and H11-1B. Presence of the *osd* mutation(s) in the two *cox IV::LEU2* segregants could be assessed directly by such analysis (as observed in the right two lanes). In the remaining two *COX IV*$^+$ segregants (the left two lanes), Cox II always accumulated to a higher level than observed in the *cox IV::LEU2* segregants. The existence of *osd* mutation(s) in such *COX IV*$^+$ segregants was examined, as follows: First, such *COX IV*$^+$ segregants were

crossed to a *cox IV::LEU2* haploid strain of the opposite mating type (WD 1 and H41-5D for *Mat* **a** and *Mat* α cells, respectively). After sporulation of the resultant diploid cells, eight *cox IV::LEU2* segregants were randomly chosen for every crossing and examined for the mitochondrial accumulation of Cox II. The results were classified into two cases; in one case, three to five of eight segregants accumulated Cox II; in the other case, not a single Cox II-accumulating segregant was observed (for example, the left and right gels, respectively, on the bottom in Fig. 4-4). Accordingly, *COX IV*$^{+}$ cells generating Cox II-accumulating *cox IV::LEU2* segregants were regarded as having the *osd* mutation. Also, those producing no such segregants were considered as having the wild-type *OSD* genes.

Six asci for each of the *osd* mutations derived from either WS-2, WS-3, or WS-8 were examined, respectively, and, in every case, the mutation was segregated in a Mendelian 2:2 fashion unlinked to the *COX IV* locus based on the above criteria. WS-5 was not examined because this mutant belongs to the same complementation group as WS-8. The results suggest that the accumulation of Cox II in each of the three *osd* mutants of a *cox IV::LEU2* genetic background was caused by inactivation of a single nuclear gene. These results exclude the possibility that the stabilization of Cox II is caused by a mutation(s) on its structural gene, for such mutations on mitochondrial DNA would not segregate in a Mendelian fashion. The wild type locus for each of the responsible genes corresponding to WS-2, WS-3 and WS-8 (or WS-5) was designated as *OSD1*, *OSD2*, and *OSD3*, respectively. In addition, the mutated alleles in WS-2, 3, 5 and 8 were designated as *osd1-1*, *osd2-1*, *osd3-1* and *osd3-2*, respectively.

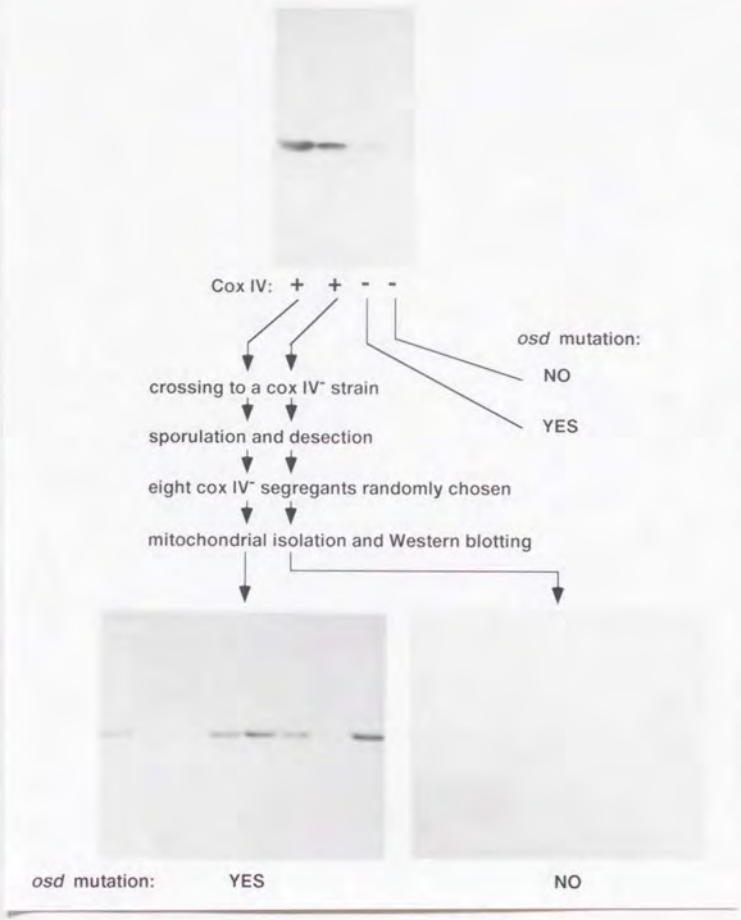


Fig. 4-4. Strategy for tetrad analysis. One example of the analysis of a set of four segregants derived from a crossing of WS-2 and H11-1B is indicated. Mitochondria (30 μg) isolated from the four segregants, two *COX IV*$^+$(top, left two lanes) and two *cox IV::LEU2* (top, right two lanes) were analyzed as in Fig.2. Each of the two *COX IV*$^+$ segregants were further crossed to a *cox IV::LEU2* strain of the opposite mating type and sporulated. Eight *cox IV::LEU2* segregants resulted from each of the crosssings were randomly chosen and their Cox II accumulation was assessed as above (bottom). *COX IV*$^+$ segregants producing three to five Cox II-accumulating segregants (top, left lane in this example) were regarded as having the *osd* mutation and those producing none (top, next to the left lane in this example) as not having.

4. 5 Suppression of degradation of Cox II in the *osd* mutants.

Accumulation of unassembled Cox II 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 Cox II was suppressed in the *osd* mutants, the half-life of mitochondrial translation products, including Cox II and Cox III, in the mutant strains was compared to those in the parent WD 1 cells, by pulse-chase experiments in the presence of cycloheximide which specifically inhibits cytoplasmic protein synthesis (Fig. 4-5). In the parent WD 1, radio-labeled Cox II decreased rapidly with a half-life of about 30 min, consistent with our earlier observations (Nakai et al. 1994). On the other hand, in all of the three *osd* mutants (we did not examine WS-5 because it belongs to the same complementation group as WS-8), reduction of the labeled Cox II was much suppressed. Only 10 to 15% of the radiolabeled Cox II 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 Cox II, and that the accumulation of Cox II in the *osd* mutants was due, at least in part, to suppression of the degradation in mitochondria.

No significant reduction of radio-labeled Cox I, apo-cytopchrome *b* and var 1 was observed in any of the *osd* mutant cells or in WD1 cells. The doublet bands corresponding to Cox III and F_0F_1-ATPase subunit 6, upper with decreasing and lower with relatively constant intensity during the chase in any of the strains examined, were so poorly separated as to be separately quantitated, densitometrically. However, stabilities of those bands did not seem to be affected significantly in any of the osd mutants

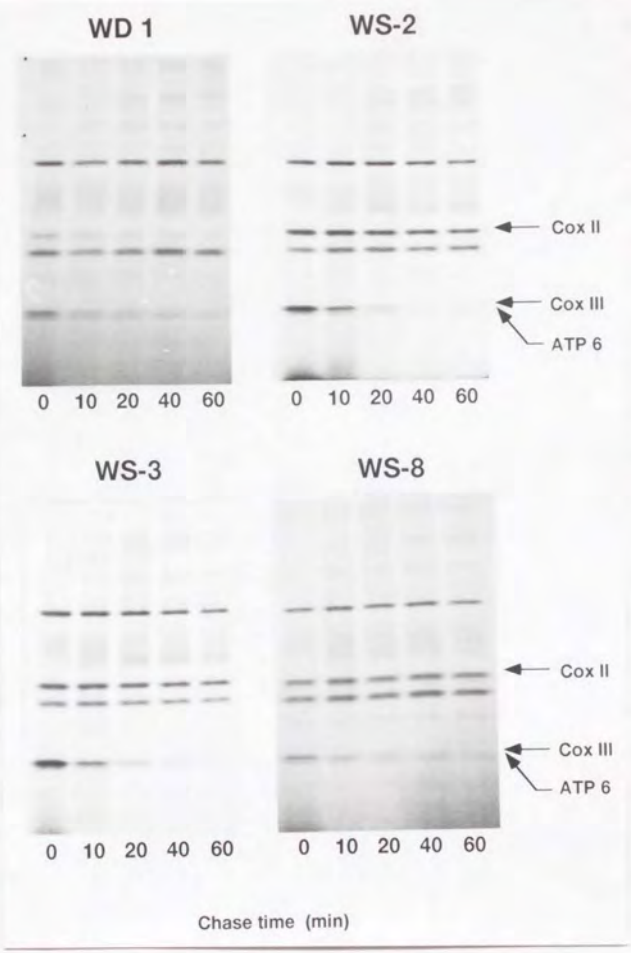


Fig. 4-5. Suppression of the degradation of Cox II. Mutants cells, WS-2, WS-3, and WS-8, and the parent WD 1 were pulse-labelled with [^{35}S]-methionine and chased for the periods of time indicated. Whole cell proteins were analyzed by SDS-PAGE on 10-12% gradient gels followed by fluorography. The positions for Cox II, Cox III, and F_0F_1-ATPase subunit 6 (ATP 6) were indicated.

4. 6 Growth properties of the *osd* mutants on a non-fermentable glycerol medium.

Competence of mitochondrial respiratory function can be assessed by testing growth ability on a non-fermentable medium such as glycerol-containing YPG. As the original *osd* mutants had been isolated from a Cox IV-deficient strain, those mutants as well as the parent WD 1 are respiratory-deficient due to the lack of functional cytochrome *c* oxidase. To examine effects of *osd* mutations on mitochondrial function in cells with normal Cox IV, *COX IV*$^+$ congenic strains, with one of the *osd* mutations or without them, were constructed by back-crossing each of the original *cox IV::LEU2 osd* mutants (WS-2, WS-3, WS-8) to a *COX IV*$^+$ H11-1B, at least six times. Two such *COX IV*$^+$ *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 non-fermentable YPG plates, at different temperatures.

As shown in Fig. 4-6, all of the mutant strains examined grew as well as the wild-type strains at 23°C and 30°C. However, at 37°C, growth of all of the *osd* mutants was suppressed, compared to findings in the wild-type cells, suggesting that *OSD1*, *OSD2*, or *OSD3* genes are required for the normal mitochondrial respiration at 37°C. The temperature sensitive phenotype always co-migrated with the ability to produce Cox II-accumulating Cox IV-deficient segregants when crossed to a *cox IV::LEU2* haploid strain and sporulated (data not shown), thereby confirming that the temperature sensitivity was caused by *osd* mutations . Severity of temperature sensitivity differed among the *osd* mutant strains with the *osd1-1* mutants showing the severest defect. At 37°C, these mutants had almost a complete inhibition of growth. In the case of *osd2-1* and *osd3-2* mutants, the temperature sensitivity was less than that observed in the *osd1-1* mutants.

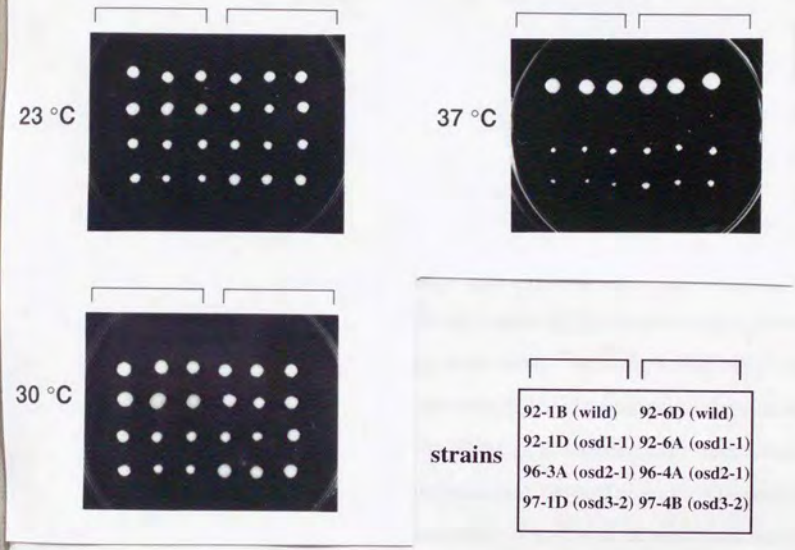


Fig. 4-6. Growth defect of the *osd* mutants on a non-fermentable medium. Each strain was spotted in triplicate on YPG plates. After incubation for five days at the temperatures indicated, the plates were photographed. Identification of the strains and genotypes are shown at the bottom right.

4. 7 Discussion for chapter 4

This chapter describes genetic analysis of the yeast mitochondrial degradation system for unassembled Cox II using a Cox IV-deficient strain. Isolation and characterization of *osd* mutants defective in this proteolytic pathway revealed that, first, at least three complementation groups exist, second, degradation of Cox III is normal in these mutants, and, finally, such mutations derange mitochondrial respiratory function, especially at a high temperature.

These findings are important when considering the properties of mitochondrial protein degradation. First, identification of three complementation groups for *osd* mutants indicates the requirement of multiple nuclear gene products for degradation of not properly assembled Cox II. The only mitochondrial protease thus far characterized, except for the processing proteases, is the matrix ATP-dependent protease purified from various species and which is similar in property to the bacterial *lon* gene product (Desautels and Goldberg 1982a; Kutejová et al. 1993; Watabe and Kimura 1985a; Watabe and Kimura 1985b). More recently, the gene for yeast homolog of this type of protease, *PIM1/LON* , has been cloned, and, using this gene, it was shown that inactivation of this protease in yeast results in the respiratory-deficient ρ^- phenotype (van Dyck et al. 1994; Suzuki et al. 1994). As described in the previous chapter, several treatments done to reduce intracellular ATP concentrations partially inhibited the degradation of unassembled Cox II and Cox III in Cox IV-deficient cells, suggesting that this degradation pathway is also ATP-dependent (Nakai et al. 1994). The matrix ATP-dependent protease may be responsible for the ATP-dependent degradation of some mitochondrial proteins, especially in the matrix, (Suzuki et al. 1994; Watabe et al. 1993; Watabe et al. 1994). 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. Though both the activity of the matrix ATP-dependent protease (Desautels and Goldberg 1982a) and the degradation of unassembled Cox II (Nakai et al. 1994) were inhibited by divalent metal chelators, the former activity was reversed by an excess of Mg^{2+}, whereas, in the latter case, the degradation was recovered by Mn^{2+}, Co^{2+} or Zn^{2+}, but Mg^{2+} was not effective. Thus, a factor, most likely dependent on Mn^{2+}, Co^{2+} or Zn^{2+} for activity, is involved in the mitochondrial degradation of unassembled Cox II or Cox III, in addition to or instead of the yeast homolog of the ATP-dependent matrix protease. This discrepancy could

be attributed to the difference of the species or chelators used (EDTA and *o*-phenanthroline, respectively). Nevertheless, genetical evidence described in this chapter for the involvement of multiple factors for the degradation of unassembled Cox II in yeast clearly indicated that new factors, at least three ones different from the matrix ATP-dependent protease as shown later in chapters 5 and 6, is required for the mitochondrial proteolytic system. Whether the matrix protease is involved in degradation of unassembled Cox II awaits further analysis.

An well-known example of the requirement of multiple factors for intracellular protein degradation is the ubiquitin-dependent pathway in eukaryotic cells (Hershko and Ciechanover 1992). In this pathway the proteins destined for degradation must first be conjugated with ubiquitin molecules, a process which requires multiple enzyme complexes, each of which consists of several subunits. These ubiquitinated proteins are recognized and degraded by the 26S protease complex consisting of 'proteasome', also known as the 20S protease complex, and additional subunits (Rechsteiner et al. 1993; Tanaka et al. 1992). Though the biological significance is not well understood, this complexity may be required for strict regulation of intracellular protein degradation. It is most likely that also in mitochondria, multiple factors or a heterooligomeric protease is needed for the degradation of at least some proteins including unassembled Cox II.

Second, the results of the pulse-chase experiments showed that stabilities of the mitochondrial translation products other than Cox II are not affected in *osd* mutants. As described in the previous chapter (Nakai et al. 1994), in addition to Cox II, Cox III was also unstable in a Cox IV-deficient strain. Though Cox III was almost co-migrating with F_0F_1-ATPase subunit 6, in the fluorogram of Fig. 4-5, they were separated to lower fainter stable and upper more intense unstable bands for the four strains examined. The upper bands were regarded as Cox III based on the observation described in chapter 3 that it was very unstable in a Cox IV-deficient strain. As degradation of Cox III was not significantly affected in the three *osd* mutants, the three *OSD* genes are not required for its degradation. Such selective suppression of the degradation in the *osd* mutants is surprising because both Cox II and Cox III are integral inner membrane proteins (Capaldi 1990).

One explanation for such selective degradation is that, by mutation, Cox II itself acquired resistance to proteolysis without a defect in the proteolytic machinery, but this is

unlikely because tetrad analysis suggested that each of the *osd* mutations is on nuclear genome, eliminating a possibility that each of the mutations locates on the mitochondrial structural gene for Cox II. Another possibility that it is due to some defect of post-translational maturation of Cox II such as attachment of heme or copper ions in the *osd* mutants seems also unlikely because apo-proteins without prosthetic groups are generally unstable in the cell (Goldberg and Dice 1974). Thus, at least two distinctive pathways for mitochondrial protein degradation, one for Cox II and the other for Cox III, are probably present in yeast.

At present, it is unclear whether the three *OSD* genes are involved in the degradation of proteins other than Unassembled Cox II. Though only Cox II and Cox III are destabilized in the Cox IV-deficient strains used in this study, it was reported that in several mutant strains mitochondrially-encoded polypeptides other than Cox II and Cox III (for example, apo cytochrome *b* or Cox I) are unstable, probably because they cannot assemble correctly (Krummeck and Rödel 1990; Schoppink et al. 1989). We plan to examine the effect of *osd* mutations on the stability of those polypeptides, using mutant strains.

Finally, temperature sensitivity on a non-fermentable medium of *osd* mutants with normal Cox IV suggests that their mitochondrial respiratory function is disordered. Accumulation of Cox II or other abnormal proteins could be responsible for that. Alternatively, each of the three *OSD* gene products may be also required more directly for the biogenesis or maintenance of the components of mitochondrial respiratory chain.

In this regard, different severity of the temperature sensitivity among the *osd* mutants is interesting. A possibility that it is due to leakiness of the *osd2-1* and *osd3-2* mutant alleles for degradation of unassembled Cox II is unlikely, because severity of the suppression of degradation, estimated by our pulse-chase experiments, is similar among the three mutants. Thus, *OSD1* gene is probably required also for a biological process, distinct from the degradation pathway of Unassembled Cox II, for which *OSD2* and *OSD3* are not involved.

Chapter 5 Molecular Cloning and Characterization of *OSD1* Gene

This chapter describes the molecular cloning and characterization of the *OSD1* gene, one of the genes involved in the degradation of unassembled Cox II. This gene is required, in addition to the degradation of unassembled Cox II, for maintaining the normal levels of respiratory chain enzymes, especially at a high temperature. Osd1 protein is bound to the mitochondrial membrane. Sequence analysis of this gene revealed that it belongs to a member of a recently identified novel family of putative ATPases, and, it was recently proved to be identical to *YME1/YTA11* gene (Schnall et al. 1994; Thorsness et al. 1993). Inactivation of the *OSD1* gene by replacement of the conserved lysine residue located in the putative ATP-binding cassette to other amino acids suggests the importance of ATP for its function.

5.1 Molecular cloning of *OSD1* gene

The *osd1* mutant was originally isolated by screening ethyl methanesulfonate-mutagenized Cox IV-deficient cells for the accumulation of unassembled Cox II. As described in the previous chapter, *osd1-1* mutant cells of *COX IV*$^{+}$ genetic background manifested temperature sensitivity on a non-fermentable glycerol-containing medium. It was used for molecular cloning of the *OSD1* gene by functional complementation. All of the obtained six plasmids which could complement the temperature sensitivity (data not shown) also could complement the mitochondrial accumulation of unassembled Cox II of *osd1-1 cox IV::LEU2* cells , the phenotype based on which original *osd* mutants were isolated (Fig. 5-1). Restriction map analysis revealed that the six plasmids have a common 4.2 kb overlapping region in their insert derived from yeast genomic DNA (Fig. 5-2*a*). Deletion analysis revealed that 3.5 kb BglII-ScaI fragment contained in the overlapping region is sufficient for rescuing both of the two phenotypes of *osd1-1* mutants (Fig. 5-2*b*).

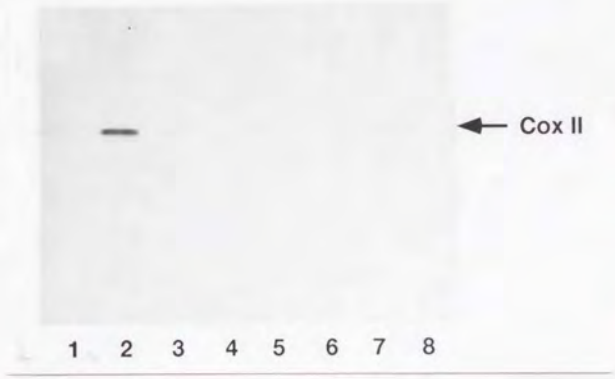


Fig. 5-1. Complementation of the Cox II-accumulating phenotype of *osd1-1* mutant by the cloned plasmids. Yeast strain H92-4B (*OSD1*$^{+}$ *cox IV::LEU2*) or H92-5D (*osd1-1 cox IV::LEU2*) were transformed either with pFL-1 or with the cloned plasmids. After cells were grown in an SR medium supplemented with lysine and histidine, mitochondria were prepared, and the same amount of the mitochondrial proteins were separated on 15% SDS-PAGE. The proteins were blotted onto Zeta-Probe membranes (Bio-Rad), and CoxII was detected with affinity-purified anti-CoxII polyclonal antibody; lane 1, H92-4B harbouring pFL-1; lanes 2-8, H92-5D harbouring pFL-1, pSG-1, 2, 3, 5, 6, and 7, respectively.

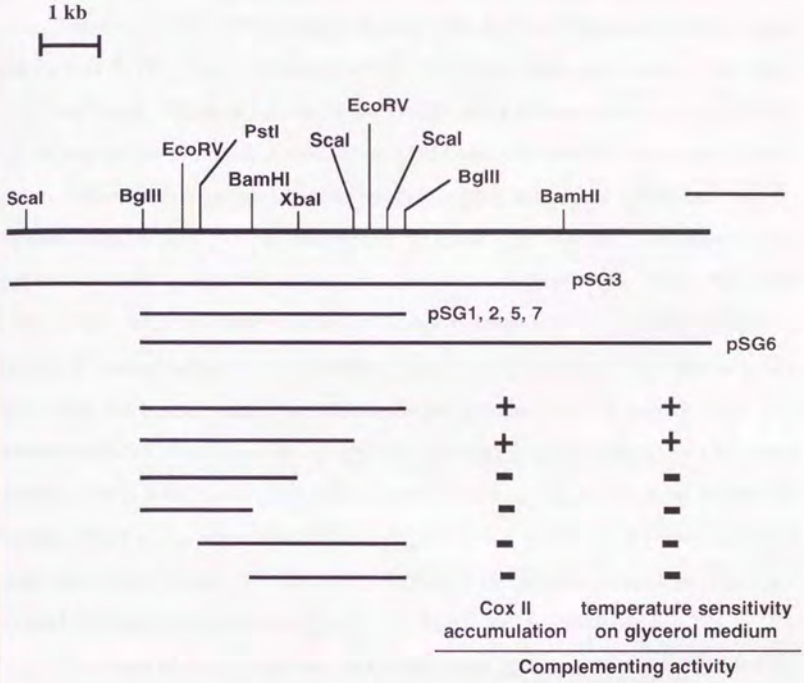


Fig. 5-2. Deletion analyses of *OSD1* gene. Top, restriction map of the cloned DNA. Spanning region of the insert in each of the cloned plasmids is indicated below. Bottom indicates a summary for the deletion mapping of the functional region. Parts of the overlapping region of the cloned fragments (indicated by bars) were subcloned into pFL-1 shuttle vector, and examined for their capability to recover the two phenotypes of osd1-1 mutants. The results are indicated on the right (+, capable; -, incapable). Accumulation of unassembled Cox II was tested as in Fig. 1. Temperature sensitivity on a glycerol medium was tested by spreading H92-6C (*osd1-1 COX IV+*) cells harbouring each of the above plasmids on SG plates supplemented with histidine, leucine and lysine, and incubated at 30 °C or 37 °C, respectively, for five days.

5. 2 Deduced sequence of *OSD1* gene

3.5 kb BglII-ScaI region of the cloned DNA contained an open-reading-frame (orf) with a significant length. This region can code for a protein consisting of 747 amino acid residues with 81.7 kD (Fig. 5-3).

Disruption of the frame downstream of the EcoRV site located between the second and the third ATG codons by inserting 8 mer or 10 mer HindIII-linkers inactivated the gene, while insertion of a frame-conserving 12 mer HindIII-linker still conserved its activities (data not shown), suggesting that this gene is translated from either the first or the second ATG codon. Mitochondrial proteins encoded by nuclear DNA are usually synthesized with N-terminal presequences with an amphiphilic structure rich in basic, hydrophobic, and hydroxylated amino acids and lacking acidic amino acids (Hartl et al. 1989; Roise and Schatz 1988). Translated from the first ATG codon, the N-terminal region of the putative translation product contains seven positively-charged residues such as arginine and lysine prior to the 50th residue where a negatively-charged glutamic acid first appears. Thus, it is consistent with the properties of the mitochondrial presequences. In contrast, when translated from the second ATG codon, negatively charged residues appear already at the 6th and 8th residues. These results suggest that *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 (see the later section).

By search of DNA (GenBank and EMBL) and protein (SWISS-PROT and PIR) databases for sequences homologous to *OSD1* gene or Osd1 protein using the FASTA program (Pearson and Lipman 1988), this gene was revealed to encode a member of a large family of putative ATPases recently described, which contains proteins of diverse intracellular functions (see Discussion). Moreover, it was recently proved to be identical to *YME1* , loss of which is known to cause a frequent escape of the mitochondrial DNA to nucleus (Thorsness and Fox 1993; Thorsness et al. 1993), and to *YTA11*, which was cloned by homology to the conserved region among the members of this ATPase family (Schnall et al. 1994). Among the members of this family, this gene showed significantly higher homology to *E. coli ftsH* (Tomoyasu et al. 1993) and yeast *YTA10* and *YTA12* (Schnall et al. 1994).

```
         10        20        30        40
MNVSKILVSPTVTTNVLRIFAPRLPQIGASLLVQKKWALR  40

SNNFYRFYSENNSGEMPPKKEADSSGKASNKSTISSIDNS  80

QPPPPSNTNDKTKQANVAVSHAMLATREQEANKDLTSPDA 120

QAAFYKLLLQSNYPQYVVSRFETPGIASSPECMELYMEAL 160

QRIGRHSEADAVRQNLLTASSAGAVNPSLASSSSNQSGYH 200

GNFPSMYSPLYGSRKEPLHVVVSESTFTVVSRWVKWLLVF 240

GILTYSFSEGFKYITENTTLLKSSEVADKSVDVAKTNVKF 280

DDVCGCDEARAELEEIVDFLKDPTKYESLGGKLPKGVLLT 320

GPPGTGKTLLARATAGEAGVDFFFMSGSEFDEVYVGVGAK 360

RIRDLFAQARSRAPAIIFIDELDAIGGKRNPKDQAYAKQT 400

LNQLLVELDGFSQTSGIIIIGATNFPEALDKALTRPGRFD 440

KVVNVDLPDVRGRADILKHHMKKITLADNVDPTIIARGTP 480

GLSGAELANLVNQAAVYACQKNAVSVDMSHFEWAKDKILM 520

GAERKTMVLTDAARKATAFHEAGHAIMAKYTNGATPLYKA 560

TILPRGRALGITFQLPEMDKVDIAKRECQARLDVCMGGKI 600

AEELIYGKDNTTSGCGSDLQSATGTARAMVTQYGMSDDVG 640

PVNLSENWESWSNKIRDIADNEVIELLKDSEERARRLLTK 680

KNVELHRLAQGLIEYETLDAHEIEQVCKGEKLDKLKTSTN 720

TVVEGPDSDERKDIGDDKPKIPTMLNA
```

Fig. 5-3. Amino acid sequence of the predicted Osd1 protein. 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 closed circle above. Putative Zn^{2+}-binding consensus motif (HEXXH motif) is boxed, in which conserved two histidine and one glutamic acid residues are marked by open circles above.

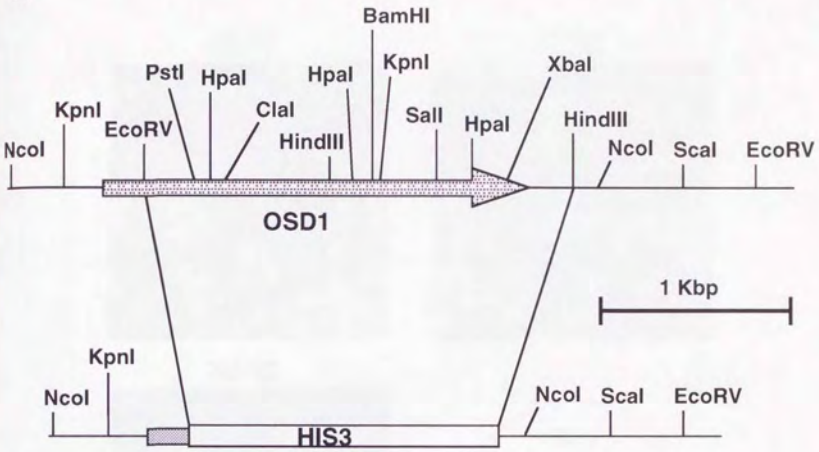
5.3 Gene disruption of *OSD1*

To examine the null phenotypes of *OSD1*, disruptant of this gene was constructed by replacing most of the coding region of *OSD1* on chromosome with the *HIS3* marker gene fragment (Fig. 5-4*a*). As the original *osd1-1* mutants showed two distinct phenotypes in *COX IV*$^+$ and *cox IV::LEU2* genetic backgrounds, respectively, disruptants were constructed using both *COX IV*$^+$ (H92-1B) and *cox IV::LEU2* (H92-4B) haploid strains.

An *osd1-1 COX IV*$^+$ cell shows temperature sensitivity on a non-fermentable YPG medium. First, to assess whether the complete inactivation of the Osd1 protein also causes such temperature sensitivity, growth ability of H92-1BΔ1 (*osd1::HIS3 COX IV*$^+$) on YPG at different temperatures was examined. As shown in Fig. 5-4*c*, H92-1BD1 cells could also grow at 23 or 30°C, but not at 37°C, which is similar to the case of the *osd1-1, COX IV*$^+$ cells, suggesting that the function of the *OSD1* gene product is dispensable for growth on a non-fermentable medium at 23 or 30°C, but essential at 37°C.

Next, H92-4BΔ1, an *OSD1*-disruptant derived from a *cox IV::LEU2* strain, was examined for accumulation and stability of Cox II. Immunoblotting analysis using anti-Cox II polyclonal antibody showed that H92-4BΔ1 accumulated Cox II in mitochondria significantly compared to its parent strain, H92-4B (Fig. 5-4*b*). The degree of the accumulation of Cox II was comparable to that observed in the cells with the *osd1-1* mutant allele. Stabilities of the mitochondrial translation products in H92-4BΔ1 and the parent H92-4B were examined by pulse-chase experiments. As shown in Fig.5- 5, Cox II, which is very unstable in the parent H92-4B, was stable in the disruptant H92-4BΔ1, confirming the requirement of this gene for the degradation of unassembled Cox II. The degree of the stabilization was similar to that observed in the *osd1-1 cox IV::LEU2* strain (H92-5D). In contrast, instability of the band corresponding to both Cox III and F_0F_1-ATPase subunit 6 observed in the parent *cox IV::LEU2* strain was not affected by the disruption of the *OSD1* gene. With the above observations, it was concluded that the cloned gene is the authentic *OSD1*, not its suppressor. Inability of the disruptant strain and an *osd1-1* strain to complement each other's defects (data not shown) further supports this conclusion.

A


BamHI
PstI
HpaI
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KpnI
XbaI
KpnI
ClaI
SalI
HpaI
HindIII
EcoRV
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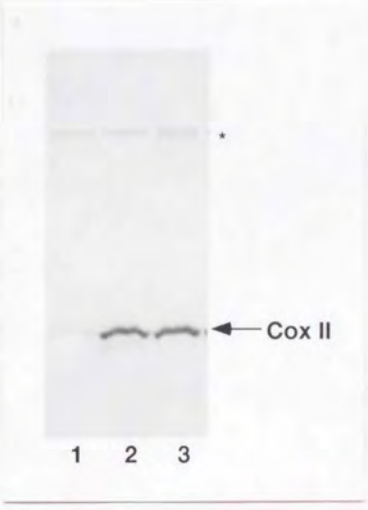
HIS3

B


*
Cox II
1
2
3

C

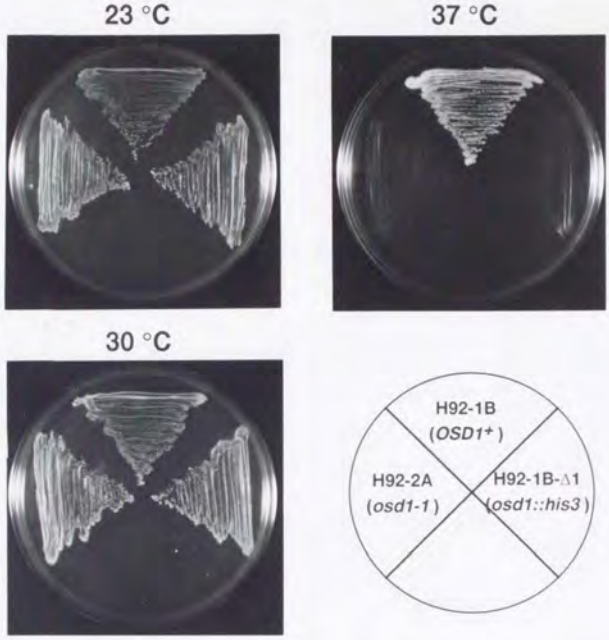


Fig. 5-4. *OSD1* gene disruption causes both suppression of the degradation of unassembled Cox II and temperature sensitivity on a glycerol medium. (*A*) Thick arrow represents the region and the orientation of *OSD1* open reading frame. 2.4 kb EcoRV-HindIII region containing the COOH-terminal approximately 90% of *OSD1* coding sequence and 0.3 kb of its downstream sequence of H92-4B (*cox IV::LEU2*) and H92-1B (*COX IV*$^+$) was replaced with 1.8 kb BamHI fragment containing *HIS3* as indicated. (*B*) H92-4B (*OSD1*$^+$ *cox IV::LEU2*, lane 1), H92-2A (*osd1-1 cox IV::LEU2*, lane 2) and H92-4BΔ1 (*osd1::HIS3 cox IV*, lane 3) were examined for their accumulation of unassembled Cox II as in Fig. 1 except that cells were grown in YPR, proteins were separated on SDS-PAGE and PVDF membrane was used instead of Zeta-Probe membrane. An asterisk indicates the position of the bands of an unidentified protein cross-reacted with the antibody used, which serves as an inner control. (*C*) H92-1B (*OSD1*$^+$ *COX IV*$^+$), H92-2A (*osd1-1 COX IV*$^+$) and H92-1BΔ1 (*osd1::HIS3 COX IV*$^+$) were examined for their growth ability on YPG plates at the temperatures indicated above. Strains and their genotypes are identified on the right below.

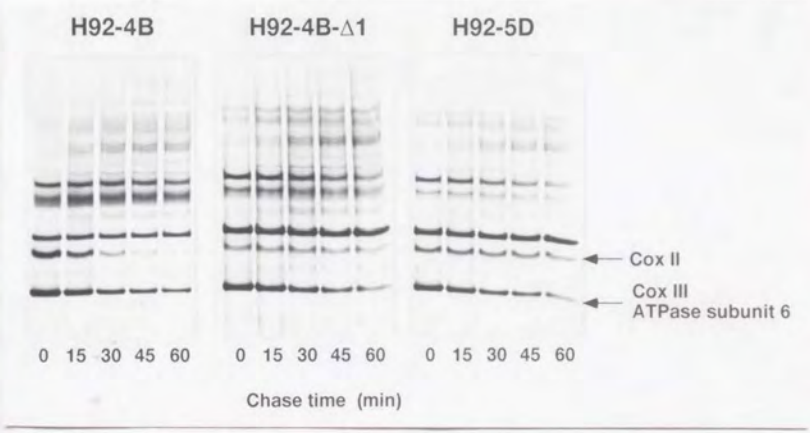


Fig. 5-5. Stabilization of Cox II in *OSD1*-disrupted and *osd1-1* cells. Pulse-chase experiments and detection of radiolabeled proteins were carried out as described in Materials and Methods; left, H92-4B (*OSD1*$^{+}$ *cox IV::LEU2*); middle, H92-4BΔ1 (*osd1::HIS3 cox IV::LEU2*); right, H92-5D (*osd1-1 cox IV::LEU2*).

5. 4 Mitochondrial respiratory chain enzymes in *osd1* mutants

The levels of intracellular cytochromes were examined by measuring the whole cell spectra. Comparison of the spectra revealed that peak of cytochrome a·a_3 reflecting normally assembled cytochrome *c* oxidase, was markedly reduced in the *OSD1*$^+$ *COX IV*$^+$ and *osd1::HIS3 COX IV*$^+$ disruptant cells grown at both 30 and 37°C. Significantly, this reduction of the peak was observed even at 30°C at which growth suppression was not apparent (Fig. 5-6, left). Though the level of cytochrome a·a_3 was significantly reduced also in the wild type cell at 37°C, the decrease of the peak in the *OSD1*-disrupted H92-1BΔ1 was more prominent (Fig. 5-6, right). Reduction of the peak corresponding to cytochrome *b* (in the complex III) was also evident at 30°C. Furthermore, at 37°C, the corresponding peak was almost disappeared.

In order to confirm the above results, activities for the mitochondrial respiratory chain enzymes in the mitochondria were also measured. As shown in Table 5-1, specific activity of cytochrome *c* oxidase was reduced in *osd1-1* or *osd1::HIS3* mutant cells even at 30°C, at which growth on a non-fermentable medium is apparently normal. At 37°C, the difference of the activities between the mitochondria of the wild type and the mutant cells were more severe. 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 compared. Though the difference between wild type and either of the *osd* mutant strains was less prominent at 30°C, their activities were also severely reduced at 37°C. Thus, activities of the respiratory chain enzymes other than cytochrome *c* oxidase also appeared to be redeuced significantly in the mitochondria of *osd1* mutants.

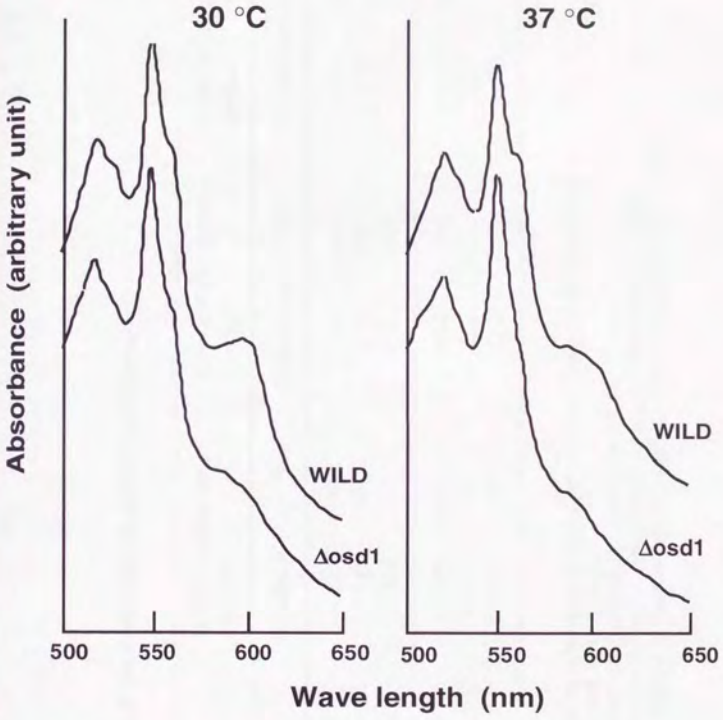


Fig. 5-6. **Whole cell spectrum of osd mutant.** H92-1B (WILD) and *OSD1*-disrupted H92-1BΔ1 (Δosd1) cells were grown on YPGal at either 30°C (left) or 37°C (right) and whole cell spectra of them were measured as described in Materials and Methods.

Table 5-1 Activities of the mitochondrial respiratory chain enzymes in *osd1* mutants

Growth temperature	Strain	Genotype	NADH-cytochrome *c* reductase		Succinate-cytochrome *c* reductase		Cyotchrome *c* oxidase	
			Specific activity* (%)		Specific activity// (%)		Specific activity¶ (%)	
30 °C	92-1B	*OSD1*⁺	0.247‡	(100)§	29.4	(100)	8.51	(100)
	92-1BΔ1	*osd1::HIS3*	0.122	(49)	20.0	(68)	2.47	(29)
	92-2A	*osd1-1*	0.189	(77)	31.6	(107)	2.90	(34)
37 °C	92-1B	*OSD1*⁺	0.137	(100)	38.0	(100)	3.42	(100)
	92-1BD1	*osd1::HIS3*	0.023	(17)	12.7	(33)	0.48	(14)
	92-2A	*osd1-1*	0.017	(12)	11.7	(31)	0.33	(10)

*Specific activities are indicated as μmole of cytochrome *c* reduced per min/mg of mitochondrial proteins.
‡Indicated values are averages of the duplicated measurements.
§The values in parentheses are percent relative activities to that of wild type 92-1B.
//Specific activities are indicated as pmole of cytochrome *c* reduced per min/mg of mitochondrial proteins.
¶Specific activities are indicated as pseudo first order reaction constant (min^{-1}) per mg of mitochondrial proteins.

5. 5 Localization of Osd1 protein

By Western blotting analysis of the total mitochondrial proteins from wild type cells, a protein of about 75 kD reacted with anti-β-galactosidase-Osd1 fusion protein (Fig. 5-7, lane *1*). This band was not evident in mitochondria from *osd1::HIS3* cells (Fig. 5-7, lane *3*), suggesting that this band represents Osd1 protein. This antiserum also recognized a mitochondrial protein from *osd1-1* cells which is indistinguishable by the electrophoretic mobility from that recognized in the wild type cells (Fig. 5-7, lane *2*), suggesting that the *osd1-1* allele encodes an unfunctional protein which is almost of the same size with wild type Osd1 protein.

Next, in order to examine subcellular localization of the Osd1 protein, wild-type D273-10B cells were subfractionated by differential centrifugation. Western blotting analysis revealed that the 75 kD protein was detected in a fraction enriched in mitochondria (Fig. 5-8*a*), consistent with the results reported for Yme1 protein (Thorsness et al. 1993). To examine whether this protein is bound to the membrane, mitochondria from D273-10B cells were further purified by Percoll density gradient centrifugation and separated into the soluble and membrane fractions by ultracentrifugation after sonication (Fig. 5-8*b*, top left). The protein was detected in the membrane but not in the soluble fraction. When isolated mitochondria was treated with 0.1 M Na_2CO_3 which extracts peripherally-bound membrane proteins but not those inserted into the membrane (Fujiki et al. 1982), this protein remained in the membrane fraction (Fig. 5-8*b*, top right). Urea treatment, which is also known to deplete membranes of some peripheral membrane proteins (Wong and Molday 1986), was also not effective to extract this protein (Fig. 5-8*b*, bottom). Thus, *OSD1* gene product is most probably an integral membrane protein. Hydropathy analysis of the predicted Osd1 protein also predicted a region relatively hydrophobic which could be inserted in the membrane (Fig. 5-3).

We also assessed the submitochondrial localization of this membrane-bound protein by subfractionating mitochondrial membrane by sucrose density gradient 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 membranes. As a result, the Osd1 protein was enriched to the fraction enriched in cytochrome *c* oxidase, suggesting that inner membrane

is its localization (Fig. 5-8*c*).

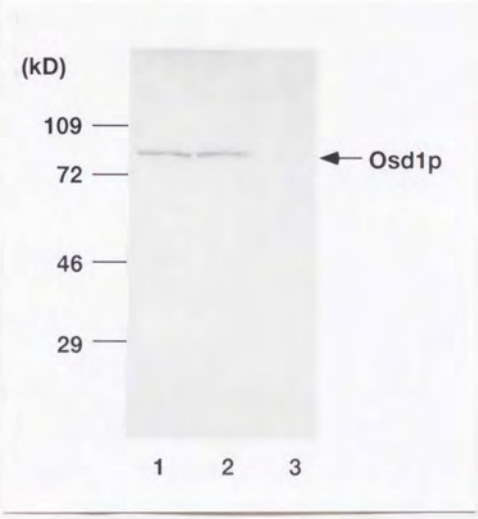
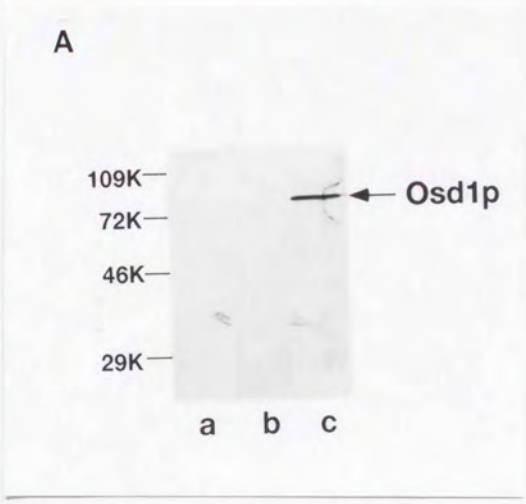
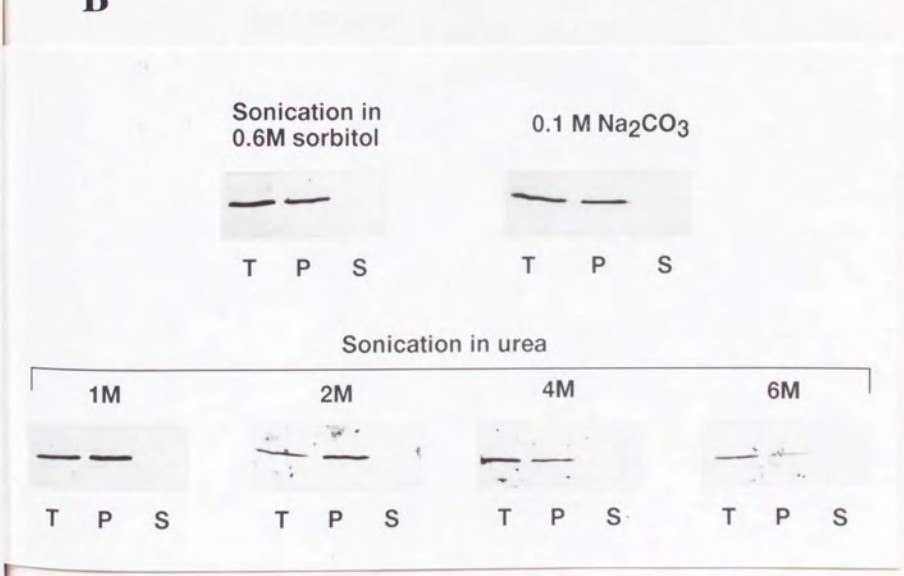


Fig. 5-7. Absence of Osd1 protein in *OSD1*- disruptant cells. Mitochondrial proteins (30 µg) prepared from YPGal-grown cells of H92-1B (lane *1*), H92-2A (lane *2*) and H92-1BΔ1(lane *3*) were separated on 10% SDS-PAGE, transferred onto a Zeta-Probe membrane (Bio-Rad), and probed using anti-Osd1 fusion protein antiserum.


A
109K
72K
46K
29K
Osd1p
a b c


B
Sonication in
0.6M sorbitol
0.1 M Na2CO3
T P S
T P S
Sonication in urea
1M
2M
4M
6M
T P S
T P S
T P S
T P S

C

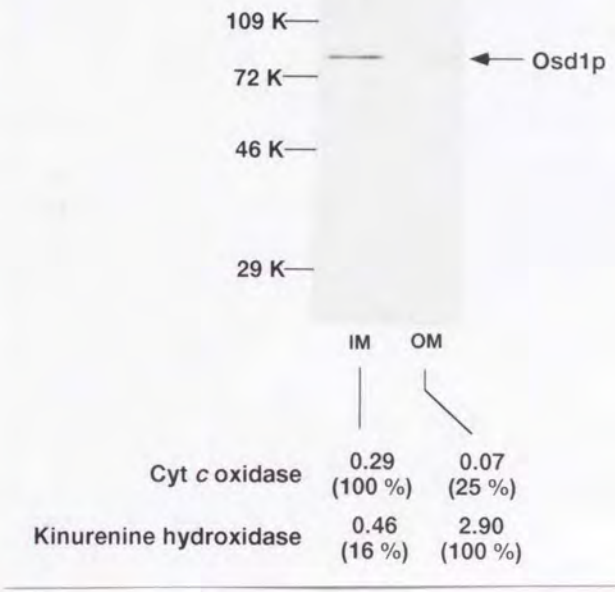


Fig. 5-8. Subcellular localization of Osd1 protein. (*A*) post-nuclear fraction (PNS) (lane *a*), post-mitochondrial fraction (PMS) (lane *b*), and mitochondrial fraction (Mit) (lane *c*) were prepared from D273-10B haploid cells grown on YPGal as described in Materials and Methods. Equal amount (25 µg) of proteins from each fraction was analyzed by immunoblot with anti-b-galactosidase-Osd1 antiserum. Standard marker proteins are on the left. (*B*) Percoll-purified mitochondria of D273-10B (300 µg) were either treated by sonication in sorbitol buffer (10 mM TrisCl, pH 7.4, 0.6 M sorbitol) or urea or with 0.1M Na_2CO_3. After an incubation on ice for one hour, 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 100,000 x g centrifugation for 1 hr into the pellet (P) and supernatant (S) fractions. After the pellet fractions were resuspended in the original volume of the treated solutions, the same volume of each fraction was analyzed as in (*A*). (*C*) Mitochondria from D273-10B were further subfractionated into the inner membrane (IM) and outer membrane (OM) fractions. The same amount (30 µg) of proteins from each fraction was analyzed as in (A). Enzyme activities were shown in arbitrary units.

5. 6 Importance of the ATP-binding cassette of Osd1 protein for its functions

For assessing the importance of ATP, *OSD1* gene was mutated so that the codon for 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) encodes another amino acids (Fig 5-9*a*). In either of the mutated genes, substitution of the Lys^{327} caused inability for complementing both the accumulation of unassembled Cox II in *osd1::HIS3 cox IV::LEU2* cells and the temperature sensitivity on a non-fermentable carbon source in *osd1::HIS3 COX IV*$^+$ cells (Fig. 5-9*b* and *c*). To evaluate whether loss of complementability is due to an instability of those mutant proteins, steady-state intramitochondrial levels of the mutated proteins in *COX IV*$^+$ cells were examined. Intramitochondrial levels of the mutated Osd1 proteins rather increased for each of the mutated *OSD1* genes, eliminating a possibility of instability of those proteins by the mutations (data not shown). The above results indicate that the conserved lysine residue, and thus most likely, ATP binding, is essential for the function of the Osd1 protein.

A

```
                970                 980                 990
GGA CCT CCT GGT ACA GGT AAA ACT TTG TTG GCT      wild
 G   P   P   G   T   G   K   T   L   L   A
                                     330

... ... ... ... ..C ... GAA ... ... ... ...      K327E
                        E
... ... ... ... ..C ... ATC ... ... ... ...      K327I
                        I
... ... ... ... ..C ... CAA ... ... ... ...      K327Q
                        Q
... ... ... ... ..C ... AGA ... ... ... ...      K327R
                        R
```

B

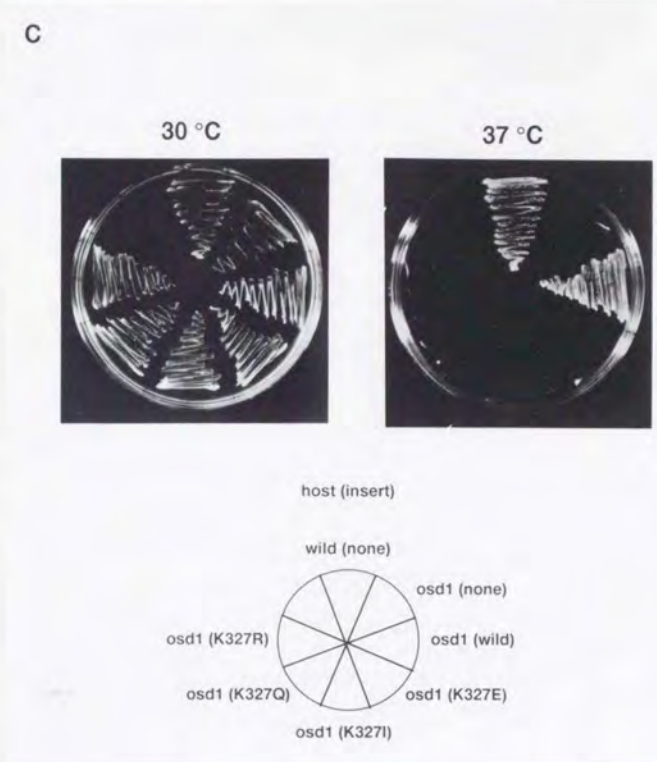


Fig.5-9. Mutational analysis of the ATP-binding cassette. (*A*) Nucleotide and amino acid sequences of *OSD1* around the ATP-binding cassette (underlined). Lys^{270} (boxed) was mutated as indicated. (*B*) Inability of the mutants to complement Cox II accumulation in a Cox IV-defective osd1 mutant strain. Wild type or mutated *OSD1* gene were subcloned into pFL1 shuttle vector and introduced into H92-4BΔ1 (*osd1::HIS3 cox IV::LEU2*). As control experiments, pFL-1 vector without insert was introduced into H92-4B (*cox IV::LEU2*) or H92-4BΔ1 (the left two lanes). Accumulation of Cox II was analyzed by immunoblotting using anti-Cox II antibody after mitochondrial fraction was prepared as in Fig. 5-4 (*B*). (*C*) Inability of the mutant *OSD1* genes to complement the temperature sensitivity of an *osd1::HIS3 COX IV*$^+$ strain. Wild type and mutated OSD1 genes were introduced into H92-1B (*OSD1*$^+$ *COX IV*$^+$) or H92-1BD1 (*osd1::HIS3 COX IV*$^+$). Strains and inserts of the vector harbouring were identified below. Growth of the transformed cells on SG plates was exaimined as in Fig. 5-2.

5. 7 Discussion for chapter 5

5. 7. 1 Sequence properties of *OSD1*

OSD1 encodes a member of a novel family of putative ATPases described recently (Erdmann et al. 1991), and it is identical to a gene known as *YME1/YTA11* (Schnall et al. 1994; Thorsness et al. 1993). This putative ATPase family includes proteins with a variety of intracellular functions such as cell cycle regulation [VCP (Koller and Brownstein 1987), *CDC48* (Fröhlich et al. 1991)], vesicle transport and fusion [*SEC18* (Eakle et al. 1988), NSF (Block et al. 1988; Wilson et al. 1989)], transcriptional regulation [TBP-1 (Nelböck et al. 1990), TBP-7 (Ohana et al. 1993), *MSS1* (Shibuya et al. 1992)], protein localization or assembly [*FtsH* (Tomoyasu et al. 1993), *BCS1* (Nobrega et al. 1992), *MSP1* (Nakai et al. 1993)], or organelle biogenesis [*PAS1* (Erdmann et al. 1991)]. As discussed later, some of the members of this family have been suggested to be subunits of the 26S protease complex (Dubiel et al. 1992; Dubiel et al. 1993), or to be involved in the intracellular protein degradation in bacteria (Herman et al. 1993).

Osd1 protein has two characteristic sequence motifs when considering its function. First, existence of ATP-binding cassette in this protein and other members of this putative ATPase family suggests that ATP plays an important role for their functions. Though role(s) of ATP is still unclear, one of its members was biochemically demonstrated to have an ATPase activity (Peters et al. 1992). Mutational analysis of the ATP-binding cassette of Osd1 protein also confirmed the importance of ATP for its function.

Second, presence in Osd1 protein of the HEXXH motif (Fig. 3), which is known to serve as a part of zinc-binding site of a large superfamily of metalloproteinases which includes the thermolysin, astacin, serratia, snake venom, or matrixin family (Jiang and Bond 1992), is intriguing. X-ray crystallographic analysis of thermolysin revealed that, in this protease, the two histidine and one glutamate residues in this motif coordinate Zn^{2+} which is required for its proteolytic activity (Argos et al. 1978).

These sequential features of Osd1 protein are consistent with the known properties of the degradation pathway of unassembled Cox II *in vivo*. It was previously observed that this pathway is significantly suppressed either by inhibiting glycolysis pathway through glucose

depletion or 2-deoxy-D-glucose, or by bongkrekic acid, inhibitor of mitochondrial ATP-ADP exchanger, (Nakai et al. 1994), both of which are expected to reduce intramitochondrial ATP concentration in respiratory-deficient strains by eliminating the supply of ATP from outside of the mitochondria (Gbelska et al. 1983). Based on such observations, its energy-dependence was proposed. Furthermore, this pathway was inhibited by *o*-phenanthroline, a membrane-permeable metal chelator, and this inhibition was reversed by adding an excess molar amount of Zn^{2+}, Mn^{2+}, or Co^{2+}, but not by Ca^{2+} or Mg^{2+} (Nakai et al. 1994), suggesting that it is metal-dependent. Most probably, Osd1 protein is responsible, if not all, for these energy- and metal-dependence of this degradation pathway, for it possesses both ATP-binding cassette and the HEXXH motif.

5. 7. 2 Implications for substrate specificity

Construction of null mutants of the *OSD1* gene confirmed our earlier observation that this gene is required for the degradation of unassembled Cox II, but not of unassembled Cox III, and eliminated a possibility that this selectivity observed in the original *osd1-1* mutant cells is due to leakiness of this mutant allele. Our previous pulse chase experiments suggested that the degradation of Cox III in Cox IV-deficient cells is also energy- and divalent metal ion-dependent (Nakai et al. 1994). Though it is possible that a factor other than Osd1 protein, which is commonly involved in the degradation of both Cox II and Cox III, is responsible for such characteristic similarity, such factor has not been identified. I prefer to think that a similar factor to Osd1 protein which is also ATP- and divalent metal ion-dependent is involved in the degradation of unassembled Cox III because, first, involvement of Osd1 protein can explain the properties of Cox II degradation pathway without hypothesizing an additional new factor with such properties, and, second, two additional genes, *YTA10* and *YTA12* which are highly homologous to *OSD1/YME1/YTA11* were also identified from yeast (Schnall et al. 1994). Those genes also encode proteins with an ATP-binding cassette and the HEXXH motif. An intriguing hypothesis is that either or both of them are involved in the degradation of Cox III.

It is currently unknown whether Osd1 protein is also involved in the degradation of other proteins. In this study, only the stability of mitochondrial translation products in Cox

IV-deficient strains was examined (Nakai et al. 1994). It will be interesting to examine the effects of *osd1* mutation on the degradation of other mitochondrially translated polypeptides such as Cox I or apo-cytochrome *b* which are reported or suggested to be unstable in some mutants (Krummeck and Rödel 1990; Schoppink et al. 1989). Moreover, the reported synthetic lethality of *yme1* mutation when combined with ρ⁻ petite mutations (Thorsness et al. 1993) suggests that Cox II is not a sole protein through which Osd1/Yme1/Yta11 protein functions because, if it is true, elimination of Cox II, such is the case in ρ⁻ mutants, should have eliminated the effect of *osd1/yme1* mutation. Thus, Osd1/Yme1/Yta11 protein may be also required for other biological process(es) in which factor(s) other than mitochondrial translation products is involved.

5. 7. 3 Mitochondrial respiratory defect and other phenotype of *osd1* mutants

Temperature-sensitive growth of the *osd1/yme1* cells on a non-fermentable glycerol medium raised a possibility that mitochondrial respiratory chain is somewhat defective at a restrictive temperature. Then, how can inactivation of *OSD1* gene causes defect of the two apparently distinct biological processes, degradation of unassembled Cox II and normal biogenesis or maintenance of mitochondrial respiratory chain enzymes? Though it is possible that Osd1/Yme1/Yta11 protein functions independently for the two process, existence of two functionally distinct domains on this protein is not likely because destruction of the ATP-binding cassette deprived it of the ability to complement both of the two phenotypes at once without reducing its intramitochondrial level. Still another possibility is that Osd1/Yme1/Yta11 protein can directly or indirectly interact with two distinct effectors required for each of the two processes, respectively. However, I prefer to a simpler explanation that the two phenotypes are intrinsically related to each other. Accumulation of unassembled Cox II or other unidentified abnormal proteins may in some way interfere with the efficient assembly of Cox II or other subunits of mitochondrial respiratory chain enzymes, for example, by perturbing the mitochondrial membrane structure. Alternatively or in addition, interaction of Osd1 protein with Cox II or other components, directly or indirectly, can be required for both of the two possible fates of them, its efficient assembly and degradative elimination. The latter possibility will be further discussed later.

Inactivation of *YME1*, which is identical to *OSD1*, is known to cause frequent escape of mitochondrial DNA to nucleus (Thorsness et al. 1993). Simplest interpretation for this phenotype would be that the accumulation of unassembled Cox II and/or unidentified proteins with abnormal conformation in mitochondrial inner membrane, through defect of their degradation and/or proper assembly, directly causes the leakiness of the membrane for DNA, for example, by perturbing membrane structure. Alternative interpretation that Osd1/Yme1/Yta11 protein is required for function of another factor which is needed for the containment of the mitochondrial DNA to this organelle cannot also be eliminated..

5. 7. 4 Role of the Osd1/Yme1/Yta11 protein

In addition to protease directly involved in the proteolysis, several other factors such as molecular chaperone or chemical modifier of substrate could be also involved in proteolytic pathways.

Among them, I first discuss the possibility that Osd1 protein is a factor with some molecular chaperone-like activity, functioning by physically interacting with a substrate protein, such as unassembled Cox II. It may enhance the degradation of its substrate by unfolding it or by preventing formation of non-specific aggregates which prevent the attack of protease. Presence of ATP-binding cassette in this protein supports this hypothesis because it is well established that binding and hydrolysis of ATP is essential for the function of molecular chaperones such as members of Hsp60 or Hsp70 family (for review, see Rothman, 1989). Until now, several members of the putative ATPase family, to which Osd1 protein belongs to, are also suggested to possess a molecular chaperone-like activity. For example, Bcs1p required for the correct assembly of cytochrome *b* and Msp1p needed for the correct localization of cytochrome c_1 into the intermembrane space, both of which are also membrane-bound mitochondrial proteins in yeast, are suggested to function as molecular chaperones by interacting with those substrate proteins (Nakai et al. 1993; Nobrega et al. 1992). Another example is bacterial *ftsH* gene product (Tomoyasu et al. 1993). This protein, together with yeast *YTA10* and *YTA12* gene products, seems to constitute a subfamily because Osd1 protein showed higher homology to those three proteins than to the other members of this family. Functions of those proteins are expected to be also similar to one another. Interestingly, these proteins

also possess the HEXXH motif observed in Osd1 protein. FtsH protein is known to be required for the assembly of some proteins into and through plasma membrane in a correct membrane topology (Akiyama et al. 1994a; Akiyama et al. 1994b). This protein is also known to be required for instabilization of *cII* gene product of the lambda bacteriophage whose activity is required for lysogenization (Herman et al. 1993). It seems unlikely that proteolysis is involved in the former function, though such possibility can not be completely eliminated. Thus, a molecular chaperone interacting with cII protein for facilitating its degradation (Herman et al. 1993), or, with several plasma membrane proteins (Akiyama et al. 1994a) for enhancing their correct insertion into or through the plasmamembrane, can well explain both of the two functions of FtsH protein. Similarly, I think that Osd1/Yme1/Yta11 protein functions by interacting with the unassembled Cox II and maintaining its degradation-competent structure or preventing it from abnormally aggregating to protease-resistant form, until it is eliminated by degradation. Supporting for it, inactivation of Osd1 protein also caused decrease of cytochrome *c* oxidase. It is also explained by such molecular chaperone activity of this protein which may also be required directly for the efficient assembly of the newly-synthesized Cox II and some of the other components of mitochondrial respiratory chain enzymes. In this regard, it will be interesting to examine whether Osd1 protein and Cox II can interact with each other, and, if so, whether it is ATP-dependent.

Second possibility is that Osd1 protein is itself a protease or a part of it. Though factor(s) other than Osd1 protein, for example, such as *OSD2*, *OSD3*, or *Lon/PIM1* gene product, can be directly involved in the hydrolysis of unassembled Cox II, presence of the HEXXH motif observed in a wide variety of metalloproteinases strongly supports this idea. It may itself possess a metal-dependent proteolytic activity or form a proteolytic catalytic site together with other subunits. Another supporting evidence for it is a recent finding that some of the subunits of the 26S protease complex, which is involved in the degradation of ubiquitinated proteins (Hough et al. 1987; Waxmann et al. 1987), are the members of the putative ATPase family to which Osd1/Yme1/Yta11 protein belongs (Dubiel et al. 1992; Dubiel et al. 1993) is suggestive. This complex is formed by assembly of the 20S protease complex, also known as proteasome, and several additional subunits (Rechsteiner et al. 1993; Tanaka et al. 1992) to acquire the ability for degrading ubiquitinated proteins (Driscoll and Goldberg 1990;

Eytan et al. 1989; Orino et al. 1991). Though precise roles of ATP is still unclear, it has been suggested that processes of both assembly of proteasome with other subunits and hydrolysis of ubiquitinated proteins require hydrolysis of ATP (Ganoth et al. 1988). Similarly, ATP is required for assembly of Osd1 protein with another subunits or for enhancing degradation of substrate protein by, for example, unfolding it.

The two possible functions of Osd1 protein described above are not necessarily exclusive to each other. I propose here a hypothesis that Osd1/Yme1/Yta11 protein is a novel type of metalloproteinases or a part of it which also possesses molecular chaperone function. In this model, Osd1 protein, with or without other subunit(s), interacts with unassembled Cox II first physically to affect its structure. Such interaction induces or maintains an assembly- and degradation-competent structure of Cox II, and if not assembled, proteolytic activity of Osd1 protein itself or of other subunit further degrades it. Such close coupling of molecular chaperone activity and proteolytic activity is probably needed for efficient and prompt elimination of unassembled subunit when it is not assembled, accumulation of which may be undesired for cells.

Another possibility is that Osd1/Yme1/Yta11 protein is a chemical modifier of substrate proteins rendering them susceptibility for degradation, as is known in ubiquitin-dependent pathway, though evidence supporting it is absent at present. Still another one is that Osd1/Yme1/Yta11 protein is required indirectly for the degradation of the unassembled Cox II. For example, it might be required for the localization or activation of other factor(s) such as protease or molecular chaperone, which is more directly involved in this process.

Chapter 6 Molecular cloning and characterization of the *OSD2* and *OSD3*

This chapter deals with molecular cloning and sequence analysis of the two genes which are required, in addition to the *OSD1* gene, for the degradation of unassembled Cox II.

6. 1 Molecular cloning of the *OSD2*.

As described in chapter 4, yeast strains of *COX IV*$^+$ genetic background carrying either *osd2-1* or *osd3-2* mutation exhibit growth defect on a glycerol medium at 37°C, though which is not so severe as that of *osd1-1* mutants. For cloning the *OSD2* gene, this temperature sensitivity was used. Yeast genomic library was screened for those which can complement this defect when introduced into *osd2-1* mutants of *COX IV*$^+$ genetic background. All of the obtained four plasmids which could complement the temperature sensitivity, pOS-2-1, 2, 3, and 4, were also able to complement the mitochondrial Cox II accumulation of an *osd2-1* mutant strain of *cox IV::LEU2* genetic background (Fig. 6-1).

Restriction map analysis of the inserts of those plasmids revealed that they have a common overlapping region (Fig 6-2). The nucleotide sequence of the 2.3 kb fragment from SalI to the end of insert covering this overlapping region was determined This region contains a complete open reading frame (shown to be the *OSD2*, see later sections) and an truncated one (*ORF-D*) downstream of the former (Fig. 6-3 and Fig. 6-4*A*). The former can code for a protein with 417 amino acid residues and the latter can code for a C-terminal truncated protein consisting of 162 amino acid residues (Fig. 6-3).

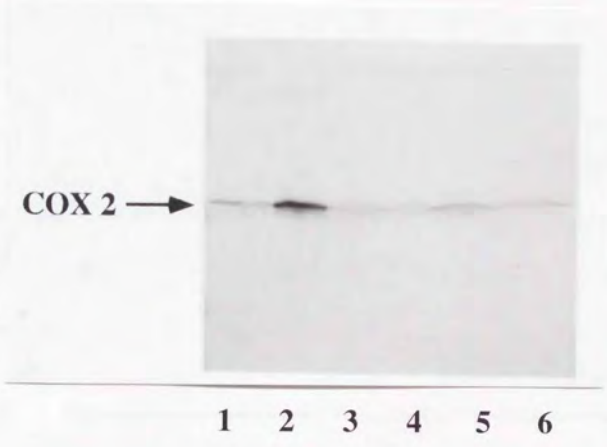


Fig. 6-1. Complementation of the cloned plasmids of accumulation of Cox II in Cox IV-deficient osd2-1 mutant. Yeast strain H92-4B (*OSD1*$^{+}$ *cox IV::LEU2*) or H96-1B (*osd2-1 cox IV::LEU2*) were transformed either with pFL-1 or with the cloned plasmids. Levels of intramitochondrial Cox II were analyzed as in Fig. 5-1. Lane *1*, H92-4B harbouring pFL-1; lanes *2-6*, H96-1B harbouring pFL-1, pOS2-1, 2, 3, and 4, respectively.

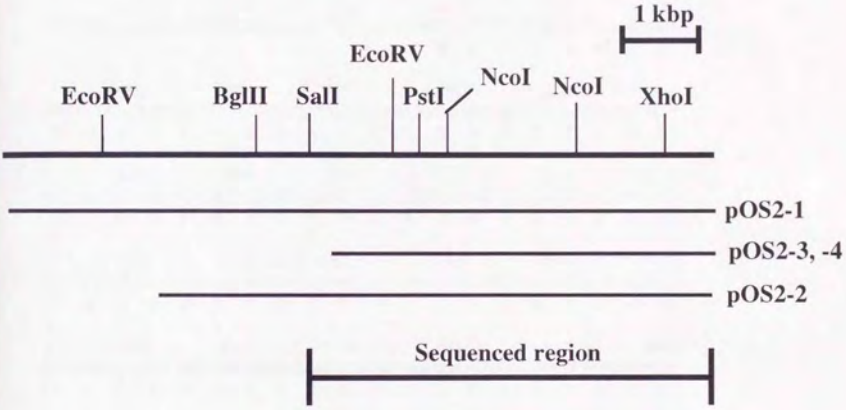


Fig.6-2. Restriction analysis of *OSD2* gene. Restriction map (above) and the spanning regions (middle) of the inserts of the cloned plasmids are indicated. Sequenced region is also shown below.

```
        10         20         30         40         50         60
GTCGACGGTAAGGCCTTGTACGAAGAAGCCCAGGAAAAAGCTGCTGAGGAAGCCGATGCT

        70         80         90        100        110        120
GACGCTGAATTGGCTGACGAAGAAGATGCCATTCACGATGAATTGTAATTCTGATCACTT

       130        140        150        160        170        180
TGGTTTTTCATTAAATAGAGATATATAAGAAATTTTCTAGGAAGTTTTTTTAAAAAAATC

       190        200        210        220        230        240
ATAAAAAGATAAACGTTAAAATTCAAACACAATAGTTGTTCGCTATATTCGTCACACTGC

       250        260        270        280        290        300
ACGAACGCCTTAGGGAAAGAGAAAATTGACCACGTAGTAATAATAAGTGCATGGCATCGT

       310        320        330        340        350        360
CTTTTACTTAAATGTGGACACTTGCTTTACTGCTTAGGAAACTACTTATCTCATCCTCCT

       370        380        390        400        410        420
CCATTCCCTCTCCTTTTCCAATTACCGTAATAAAAGATGGCTGTATTTACTCCTCCATCA
                                      M  A  V  F  T  P  P  S

       430        440        450        460        470        480
GGTAATAGCAATTCCACCGACCATACTCACACACAAGATGACCACGACAAAGATGATAAT
 G  N  S  N  S  T  D  H  T  H  T  Q  D  D  H  D  K  D  D  N

       490        500        510        520        530        540
GATATCAAGAAATTCTACATAAGGCCAAGTTTAGGCTTAAAACTGTGGGGTCCGCTCGTA
 D  I  K  K  F  Y  I  R  P  S  L  G  L  K  L  W  G  P  L  V

       550        560        570        580        590        600
CCCGCTCCTGATAACCTACCGGGACTATACACTCTAATCACTATCCAATCTGCAGTGGGT
 P  A  P  D  N  L  P  G  L  Y  T  L  I  T  I  Q  S  A  V  G

       610        620        630        640        650        660
TTCTTTGCCCTTTGGAGACTGAGAAGGCTCTACAAACTACCGCCACCGCGCCGCATTGCC
 F  F  A  L  W  R  L  R  R  L  Y  K  L  P  P  P  R  R  I  A

       670        680        690        700        710        720
ACTGGCACTCACTCGGATTTATCCTTTGGCGAACTACCCAGTGAAATGATTGTCAATGGC
 T  G  T  H  S  D  L  S  F  G  E  L  P  S  E  M  I  V  N  G

       730        740        750        760        770        780
AAGACTAAAATCAAAAAGGATATTGCTGACTTTCCAACTTTGAACCGCTTCTCCACCACC
 K  T  K  I  K  K  D  I  A  D  F  P  T  L  N  R  F  S  T  T

       790        800        810        820        830        840
CATGGTGACATTGTGCTCGCCCCTCCTCCCATCATACCTCGCCAATCTCGATTCGTCAGC
 H  G  D  I  V  L  A  P  P  P  I  I  P  R  Q  S  R  F  V  S

       850        860        870        880        890        900
GTCAGAAAGCTCTTATGGGGGTTGTTTGGCTCTTTGCTACTTTCTCAGTCACTGTTGGAG
 V  R  K  L  L  W  G  L  F  G  S  L  L  L  S  Q  S  L  L  E

       910        920        930        940        950        960
CTTACTCGCCTGAACTTTCTTAAATACGACCCCTGGTGCGACGAAATGAAATCCGTACGT
 L  T  R  L  N  F  L  K  Y  D  P  W  C  D  E  M  K  S  V  R
```

```
      970       980       990      1000      1010      1020
GACAAGAAGTTTTTCAACAATATTGTCAAATATTATCACGAGGGCATAGACCCCACCAAA
 D  K  K  F  F  N  N  I  V  K  Y  Y  H  E  G  I  D  P  T  K

      1030      1040      1050      1060      1070      1080
ATAAAAGTCAAGGATGCTATGAACGGTACTCCTCTCTCGACAAATATCCCTGAGGTCAAA
 I  K  V  K  D  A  M  N  G  T  P  L  S  T  N  I  P  E  V  K


      1090      1100      1110      1120      1130      1140
CAAAGCGTCGCTCTCGCTAGAGCGCAAGTTGAGGCGCAGAATCCCATTATTAAATGGTTC
 Q  S  V  A  L  A  R  A  Q  V  E  A  Q  N  P  I  I  K  W  F

      1150      1160      1170      1180      1190      1200
GGACCCTTGGAATACAAGCCCATGTCTTTCAACGAGTACCTCAATCGCATGGAATTTCAC
 G  P  L  E  Y  K  P  M  S  F  N  E  Y  L  N  R  M  E  F  H

      1210      1220      1230      1240      1250      1260
TTGGACATGTTCGAGTTTTTTCAAAATAAAAGAAACATTAGAGAAAATTCCATTGAACTC
 L  D  M  F  E  F  F  Q  N  K  R  N  I  R  E  N  S  I  E  L

      1270      1280      1290      1300      1310      1320
ATCAATTCCATATCCCACAATCCGCAGTCTTCTTCTACTGGCCTTGAAGGTCTTTCCGAG
 I  N  S  I  S  H  N  P  Q  S  S  S  T  G  L  E  G  L  S  E

      1330      1340      1350      1360      1370      1380
TCCAAAAAACTCCATCTACAAAATGTGGAAAAAAGACTGCATTTCTTAGCATCTTCGGGA
 S  K  K  L  H  L  Q  N  V  E  K  R  L  H  F  L  A  S  S  G

      1390      1400      1410      1420      1430      1440
GATTCCATTTCCGCACCAGTAAAGAAGAGATCCAGCACCACACTCTCCCGAGGTGTCATT
 D  S  I  S  A  P  V  K  K  R  S  S  T  T  L  S  R  G  V  I

      1450      1460      1470      1480      1490      1500
TTGCCCCATGACACGAAAGGCCCGCAAGATATTGATCTCGATACAATAAGATCGCTTTAT
 L  P  H  D  T  K  G  P  Q  D  I  D  L  D  T  I  R  S  L  Y

      1510      1520      1530      1540      1550      1560
GATCCATGGATGACTTTGGCCTTAGAAACTTCGCTAAGCATCAAATTCATACCAACTACC
 D  P  W  M  T  L  A  L  E  T  S  L  S  I  K  F  I  P  T  T

      1570      1580      1590      1600      1610      1620
ATGCCCTCCCATACCAAGACACCCACTAGCACGGACCAGCCGTTACCAGGGCCTACCCCC
 M  P  S  H  T  K  T  P  T  S  T  D  Q  P  L  P  G  P  T  P

      1630      1640      1650      1660      1670      1680
AAGGCTCTCACTAATGAAAAGACACATTAGGTCTTTGCGGATTGCTTAGTTGTACCGTGC
 K  A  L  T  N  E  K  T  H ***

      1690      1700      1710      1720      1730      1740
GTATATTAAATCCTTCTTCGTACACCGTGATTTTTCAGACGGCTACCAGAGAAAATATGG

      1750      1760      1770      1780      1790      1800
CTGGGATTTCATATTATATATGCAAAATATATAATAACATATATATATATACACTCCATT

      1810      1820      1830      1840      1850      1860
AATAACTAATACCTACATTCATATCAACACTTTCATCCTCCCCTTCCCCCATAGCAATGA
                                                          M
```

```
      1870       1880       1890       1900       1910       1920
AGATAACGTGTACAGACTTGGTGTACGTCTTCATTTTACTCTTCCTAAACACGAGTTGTG
K  I  T  C  T  D  L  V  Y  V  F  I  L  L  F  L  N  T  S  C

      1930       1940       1950       1960       1970       1980
TCCAAGCCGTTTTTTCAGATGATGCATTTATCACTGATTGGCAACTGGCTAACTTAGGTC
V  Q  A  V  F  S  D  D  A  F  I  T  D  W  Q  L  A  N  L  G

      1990       2000       2010       2020       2030       2040
CTTGGGAGAAAGTCATCCCTGATTCTCGAGACCGCAACAGGGTTCTCATCTTATCGAACC
P  W  E  K  V  I  P  D  S  R  D  R  N  R  V  L  I  L  S  N

      2050       2060       2070       2080       2090       2100
CTACCGAAACTTCCTGCTTAGTTTCTTCGTTTAACGTTTCTTCCGGACAGATTCTTTTCA
P  T  E  T  S  C  L  V  S  S  F  N  V  S  S  G  Q  I  L  F

      2110       2120       2130       2140       2150       2160
GAAACGTTTTACCCTTTACCATTGATGAGATTCAACTGGATAGTAATGACCATAACGCAA
R  N  V  L  P  F  T  I  D  E  I  Q  L  D  S  N  D  H  N  A

      2170       2180       2190       2200       2210       2220
TGGTTTGTGTGAACTCTTCAAGCAACCATTGGCAGAAATATGATTTACACGATTGGTTTT
M  V  C  V  N  S  S  S  N  H  W  Q  K  Y  D  L  H  D  W  F

      2230       2240       2250       2260       2270       2280
TACTAGAGGAAGGCGTAGATAATGCCCCTTCTACGACCATTTTACCTCAATCCTCATATT
L  L  E  E  G  V  D  N  A  P  S  T  T  I  L  P  Q  S  S  Y

      2290       2300       2310       2320       2330       2340
TAAACGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCCACAGGTGCGGTT
L  N  D  P  L  R  R  T  H  R  G  R  H  H  R  R  H  R  C  G
```

Fig.6-3. Sequence analysis of the *OSD2* gene. Portion from SalI site to the boundary between the insert and the vector covering the overlapping region was sequenced. Sequence of the predicted proteins for the two putative protein-encoding regions were also indicated below the corresponding nucleotide sequences.

6. 2 Disruption analysis of the *OSD2* gene

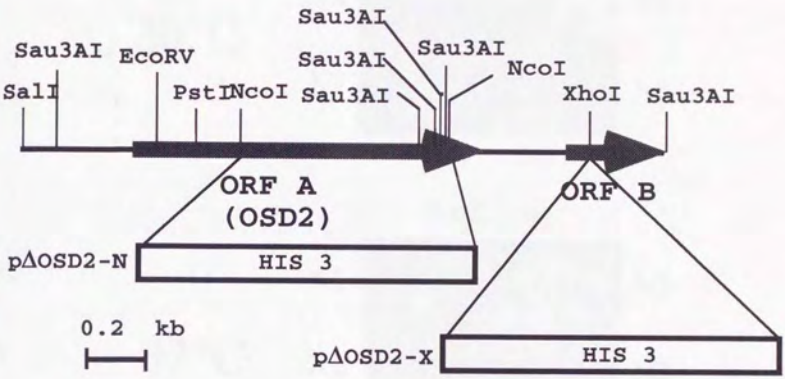
In order to determine which of the two open reading frames codes for *OSD2* gene, disruptant strains in which either of the two coding regions was disrupted were constructed. For disrupting the former orf, the 0.7 kb NcoI-NcoI region within this orf was replaced with *HIS3* fragment. For the latter, *HIS3* fragment was inserted into a unique XhoI restriction site. Such disruptants were constructed using both *cox IV::LEU2* and *COX IV*$^+$ haploid cells because two different phenotypes of *osd2* mutants corresponding to the two different genetic backgrounds are known.

First, we examined the growth ability of the COX *IV*$^+$ disruptants in a glycerol medium at 30 and 37°C. The *osd2-1* mutant cells of *COX IV*$^+$ genetic background shows growth suppression in a glycerol medium at 37°C, though which is not so severe as those of *osd1-1* or *osd1::HIS3* disruptant strains. As seen in Fig. 6-4*B*, disruption of the former orf resulted in significant suppression of the cell growth at 37°C, while disruption of the latter not. Extent of the growth suppression was similar to that of *COX IV*$^+$ *osd2-1* cells.

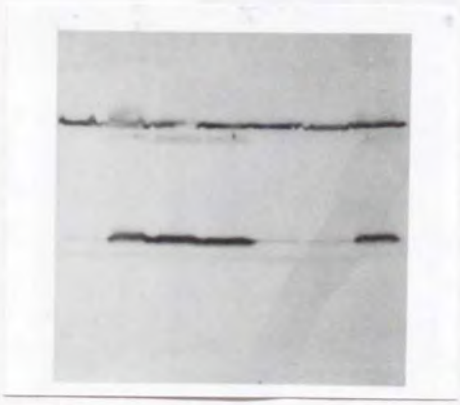
Second, disruptants from a *cox IV::LEU2* strain were examined for mitochondrial accumulation of unassembled Cox II, expecting that disruption of the orf corresponding to *OSD2* gene would result in the accumulation of intramitochondrial Cox II. As shown in Fig. 6-4*C*, disruption of the former orf resulted in the accumulation of Cox II to an extent similar to that observed for *cox IV::LEU2 osd2-1* mutant cells. Disruption of the latter orf had no effect on the intramitochondrial steady-state level of Cox II.

The above results of the gene disruption experiments showing that the two known phenotypes of *osd2-1* mutant cells were regenerated by disrupting the former orf of the clone gene but not by the disruption of the latter indicate that the former orf encodes the *OSD2* gene.

A


Sau3AI
Sau3AI
Sau3AI
Sau3AI
EcoRV
Sau3AI
NcoI
SalI
PstI
NcoI
Sau3AI
XhoI
Sau3AI
ORF A
(OSD2)
ORF B
pΔOSD2-N
HIS 3
0.2 kb
pΔOSD2-X
HIS 3

B



1 2 3 4 5 6 7

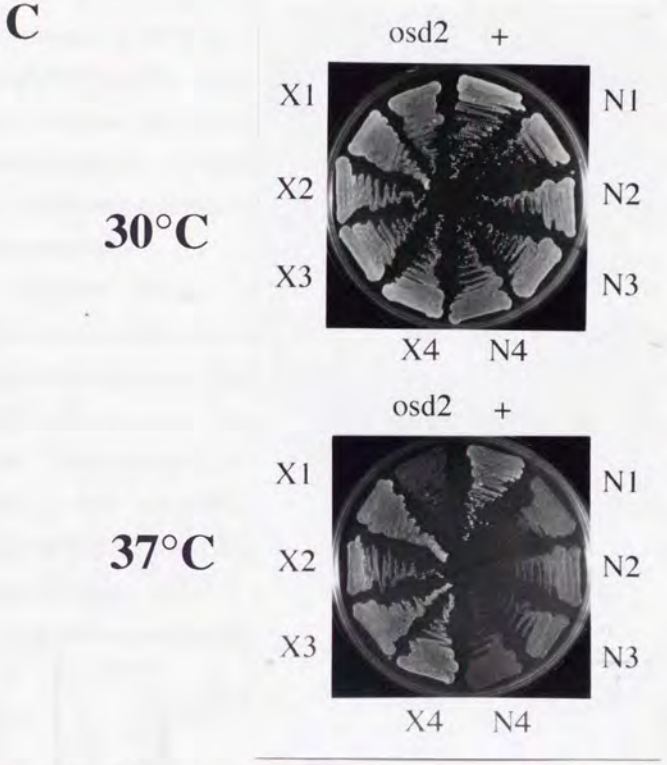


Fig. 6-4. Disruption analysis of *OSD2* gene. (*A*) Two thick arrows represent the regions and orientations of the corresponding two predicted protein-encoding regions. Each of the two coding regions was disrupted by replacement or insertion using *HIS3* fragment as indicated. (*B*) Mitochondrial accumulation of Cox II was analyzed as in Fig. 5-4 (*B*). Lane *1*, H92-4B (*cox IV::LEU2*); lane *2*, H96-1B (*osd2-1 cox IV::LEU2*); lanes *3* and *4*, H92-4BD2-N1 and -N2, two independent disruptants derived from H92-4B in which the left coding region in (*A*) was disrupted; lanse *5* and *6*, H92-4BD2-X1 and -X2, two independent disruptants derived from H92-4B in which the right coding region in (*A*) was disrupted; lane *7*, H92-4B-D1 (*osd1::HIS3 cox IV::LEU2*). (*C*) Growth on YPG plates at the indicated temperatures was examined as in Fig. 5-4(*C*). +, H92-1B(*COX IV*$^+$); osd2, H96-3A (*osd2-1 COX IV*$^+$); N1, N2, N3, and N4, four independent disruptants derived from H92-1B in which the left coding region in (A) was disrupted; X1, X2, X3, and X4, four independent disruptants derived from H92-1B in which the right coding region in (A) was disrupted.

6. 3 The *OSD2* gene encodes a new type of protein

The *OSD2* gene codes for a protein consisting of 417 amino acid residues with the molecular weight of 47,155 Da. Homology search of DNA (EMBL and NBRF) and protein (SWISS-PROT and PIR) databases revealed that this gene is identical to a hypothetical protein designated YCL44C or YCL314 with unknown function, identified through determination of the complete sequence of the left arm of chromosome III (Scherens et al., 1992). It is located downstream of the yeast *PDI* gene encoding protein disulfate isomerase in the same orientation.

Though the *OSD2* gene is required for the mitochondrial protein degradation, N-terminal primary sequence of the predicted protein has no properties characteristic for the mitochondrial localization presequences. For example, it is known that mitochondrial presequences are generally rich in basic amino acid residues such as Lys and Arg and lack acidic amino acid residues. The N-terminal region of the putative Osd2 protein is rich in acidic Asp residues and contains few basic amino acid. Hydropathy analysis of this protein (Fig 6-5) revealed that, first, N-terminal region of this protein is very hydrophilic and, second, three relatively hydorphobic portions exist.

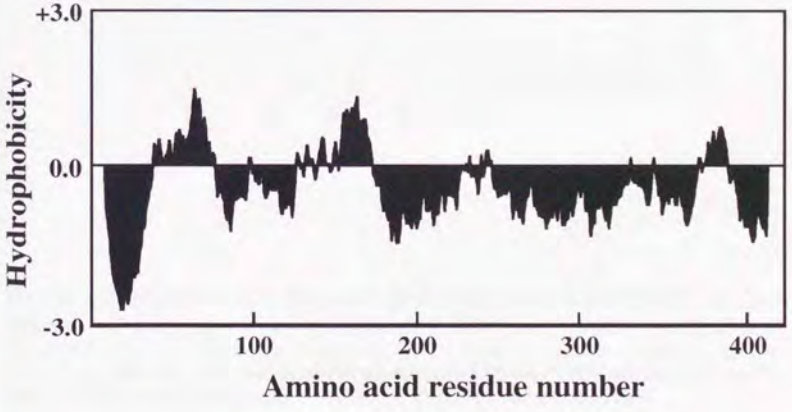


Fig. 6-5. Hydropathy analysis of predicted Osd2p. Hydropathy profile of the putative Osd2p was predicted according to the method of Kyte and Doolittle, 1982, with a scanning window of 19 amino acid residues.

6. 4 Molecular cloning of the *OSD3* gene.

The *OSD3* gene was cloned by complementation of the temperature sensitive growth of an *osd3-2 COX IV*$^+$ cells at 37°C on a glycerol medium. H97-4B haploid strain (*osd3-2 COX IV*$^+$) were used for transformation with the yeast genomic library, and, finally, four independent plasmids which can complement the temperature sensitivity, pOS3-1, 3, 14, and 19, were obtained. Those plasmids were all able to rescue *osd3-2 cox IV::LEU2* cells from the accumulation of unassembled Cox II (Fig. 6-6).

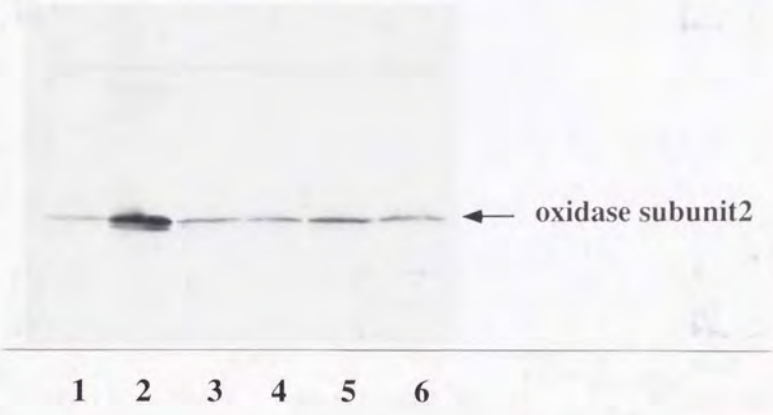


Fig. 6-6. Complementation of the cloned plasmids of accumulation of Cox II in Cox IV-deficient *osd3-2* mutant. Yeast strain H92-4B (*OSD1*$^+$ *cox IV::LEU2*) or H97-3B (*osd3-2 cox IV::LEU2*) were transformed either with pFL-1 or with the cloned plasmids. Levels of intramitochondrial Cox II were analyzed as in Fig. 5-1. Lane *1*, H92-4B harbouring pFL-1; lanes *2-6*, H97-3B harbouring pFL-1, pOS3-1, 3, 14, and 19, respectively.

6. 5 Gene disruption of the *OSD3*.

Restriction map analysis of the inserts of those plasmids identified an overlapping region of about 3.5 kb among them (Fig. 6-7). To analyze whether this region codes for the *OSD3* gene, the corresponding regions on the chromosomal DNA of H92-1B (*COX IV*$^+$) and H92-4B (*cox IV::LEU2*) were disrupted by inserting a *HIS3* fragment in the BamH1 site situated almost in the midst of the overlapping region (see Fig. 6-8*A*), and effects of the disruption were examined.

First, the effect of the disruption on growth of a *COX IV*$^+$ strain at 37°C in a glycerol medium was examined. As seen in Fig. 6-8*B*, the disruptant cells grew very poorly compared to the wild-type cells. However, similar to the case of *osd2* disruptant, growth defect was not so severe as that of the *osd1* disruptant which can almost no longer grow in the restrictive temperature. Next, the effect of the disruption on the steady-state level of intramitochondrial Cox II of a *cox IV::LEU2* strain was examined. As can be seen in Fig. 6-8*C*, mitochondria of the disruptant cells accumulated Cox II to an extent similar to that observed in the *osd1* disruptant cells of *cox IV::LEU2* genetic background. The above results of the gene disruption experiments suggest strongly that the overlapping region contains the *OSD3* gene.

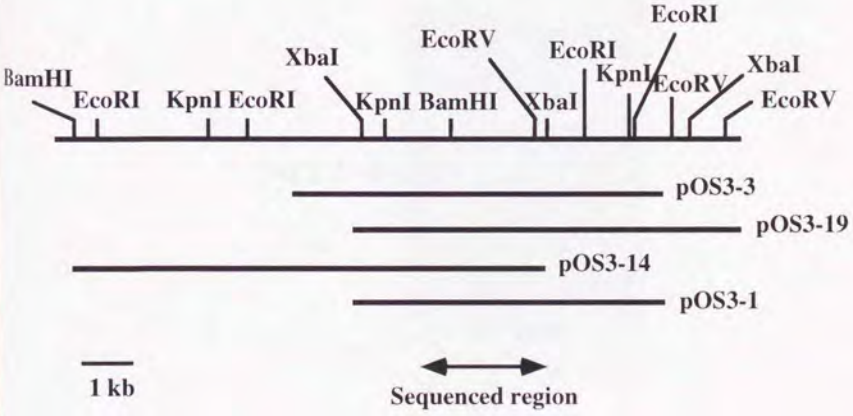
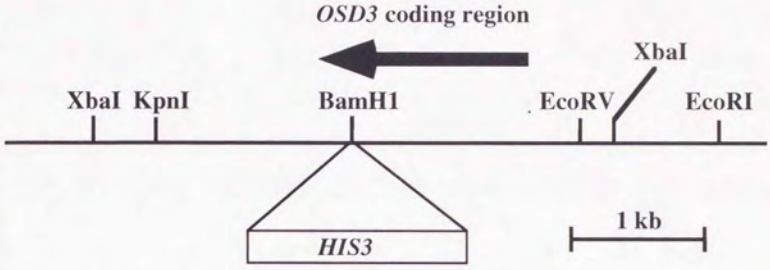
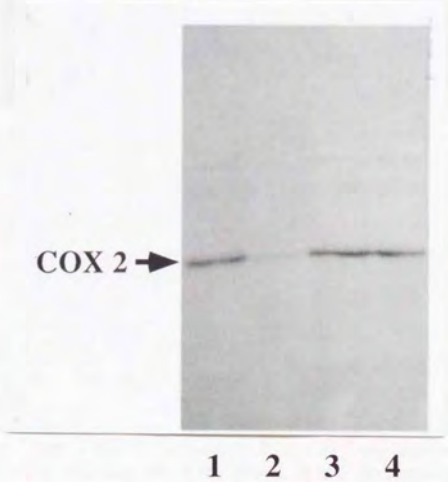


Fig. 6-7. Restriction analysis of the cloned gene. Restriction map (top), the spanning regions (middle) of the inserts of the cloned plasmids are indicated. Sequenced region is also indicated below.

A


OSD3 coding region
XbaI
XbaI KpnI
BamH1
EcoRV
EcoRI
HIS3
1 kb

B


COX 2
1 2 3 4

C

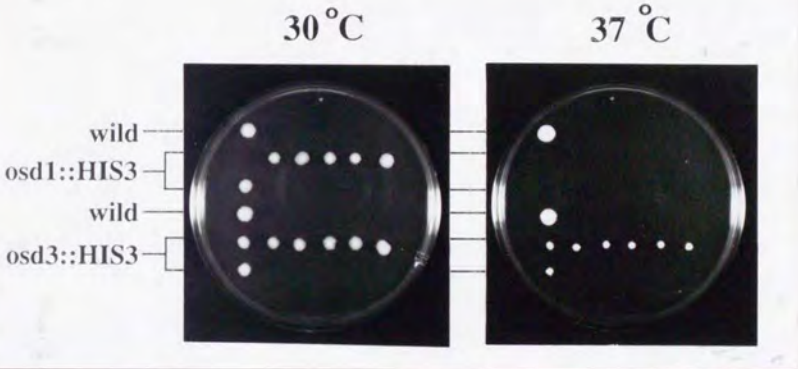


Fig. 6-8. Disruption analysis of *OSD3* gene. (*A*) Thick arrow represents the region and orientation of the predicted protein-encoding region. This coding region was disrupted by insertion of *HIS3* fragment as indicated. (*B*) Mitochondrial accumulation of Cox II was analyzed as in Fig. 5-4 (*B*). Lane *1*, H92-4B-Δ1 (*osd1::HIS3 cox IV::LEU2*); lane 2, H97-3B (*osd3-2 cox IV::LEU2*); lanes *3* and *4*, H92-4BΔ3-1 and -2, two independent disruptants derived from H92-4B. (*C*) Growth on YPG plates at the indicated temperatures was examined as in Fig. 5-4(*C*); wild, H92-1B(*COX IV*$^+$); osd1::HIS3, H92-1B-Δ1 (*osd1::HIS3 COX IV*$^+$); osd3::HIS3, H92-1B-Δ3 (*osd3::HIS3 COX IV*$^+$), an *OSD3*-disruptant derived from H92-1B.

6. 6 Sequence analysis of the *OSD3* gene

Sequence analysis of the cloned gene revealed the existence of an orf with a significant length within it. This orf could code for a protein consisting of 501 amino acid residues with the calculated molecular weight of about 58 kDa (Fig. 6-9). N-terminal region of the predicted Osd3 protein showed properties characteristic to the mitochondrial localization presequences. First, this region is rich in the basic amino acid residues such as Lys and Arg and it lacks acidic amino acid residues (Fig. 6-10*A*). Second, when the secondary structure of this region was predicted by the methods of Chou and Fasmann, 1974, it was predicted to form alpha helix (Fig.6-10*B*), and, finally, a wheel plot analysis revealed that this helix is amphiphilic (Fig. 6-10*C*). Hydropathy analysis revealed that this protein is relatively hydrophilic except the three distinct regions which are relatively hydrophobic (Fig. 6-11).

```
   1  TCTAGAAATGAAATAAGGCGTCTTCTTTTTCCCTACGGTTTTCCATTCAAAATATCCGC    59

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 180 AGAATGGTACAAGGCCCCATCTCATAAATTGTATTGCTTTAGTATCAGGACGATATACCG   239

 240 CAGAGTAGTTTGTAGGGGAAATATTGTAAGAGGCCTTGAAGATATCCTTACTAACGGTAG   299

 300 GCTGATCTTTGGTGTCTTCCTCGTCATGTGGATGCTGGCTGTTCGATGAGGCGTCCTTGG   359

 360 GTGTATTAACCGGTAAGTTCCAATCTCTTAGAAGTTGCGGCAAATCTCCGCTATCGTAAG   419

 420 CTAAGGCAAACCTACCACACATCTTGGGTTATGAAAGTCTGGTATTAGCATCAGGTATAC   479

 480 TTTAATTAAGTNTCTTGGTTCAACTGTTCATTGCTTTGTTCCCTTTTTTGATCCCTTTCA   539

 540 TTCAGATATTTTTCGTGGGGGTTCGGCCTCACAAAATTGCATTTACAAATCATCAACATT   599

 600 TTTATGCAAAAGTGGGCTACTTTCATTTACTCACAAAGGTGTAAACACAAGCAGGAAGAT   659

 660 CTCAGTTTAACAGGCTAAAAGTCCCTCCTTTTCGGTATGCTTTTACAAGGAATGCGTTTA   719
   1                                   M  L  L  Q  G  M  R  L       8

 720 TCGCAAAGGTTACATAAGAGACATCTATTTGCTTCCAAGATTTTAACGTGGACTACGAAC   779
   9  S  Q  R  L  H  K  R  H  L  F  A  S  K  I  L  T  W  T  T  N    28

 780 CCTGCTCATATACGCCACCTACATGATATAAGGCCGCCTGCATCAAACTTCAATACGCAA   839
  29  P  A  H  I  R  H  L  H  D  I  R  P  P  A  S  N  F  N  T  Q    48

 840 GAATCGGCCCCCATACCGGAGTCTCCAGCAAACTCACCAACTCGACCACAGATGGCACCT   899
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 900 AAACCCAATTTGAAAAAAAAAAATCGTAGTTTAATGTATTCTATTATTGGGGTTTCCATA   959
  69  K  P  N  L  K  K  K  N  R  S  L  M  Y  S  I  I  G  V  S  I    88

 960 GTAGGTTTATATTTTTGGTTTAAAAGTAACTCCAGGAAACAAAAACTACCTCTTTCGGCG  1019
  89  V  G  L  Y  F  W  F  K  S  N  S  R  K  Q  K  L  P  L  S  A   108

1020 CAAAAAGTCTGGAAGGAAGCCATATGGCAAGAAAGTGATAAAATGGATTTTAATTACAAA  1079
 109  Q  K  V  W  K  E  A  I  W  Q  E  S  D  K  M  D  F  N  Y  K   128

1080 GAAGCGTTAAGGCGGTATATTGAGGCGTTGGATGAATGCGATCGCTCTCATGTCGATTTA  1139
 129  E  A  L  R  R  Y  I  E  A  L  D  E  C  D  R  S  H  V  D  L   148

1140 TTGTCAGATGATTATACCAGAATAGAGCTGAAAATTGCTGAAATGTATGAAAAGCTCAAT  1199
 149  L  S  D  D  Y  T  R  I  E  L  K  I  A  E  M  Y  E  K  L  N   168

1200 ATGCTTGAAGAAGCCCAAAATTTGTACCAAGAATTATTAAGTCGGTTTTTCGAAGCGCTG  1259
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1260 AATGTTCCTGGCAAAGTTGATGAGAGTGAAAGAGGCGAGGTTTTAAGAAAAGACTTGAGA  1319
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1440 TTTGAAAACAGAAAAAAGAAGCTCTCGATGGTAAAAGACATCAATAGAGACCCTAATGAT  1499
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 289  L  D  L  E  K  N  S  S  A  W  E  P  F  K  E  E  F  F  T  A   308

1620 AGAGATTTATATACAGCTTATTGTCTGTCATCAAAAGACATAGCTGCAGCTCTAAGTTGC 1679
 309  R  D  L  Y  T  A  Y  C  L  S  S  K  D  I  A  A  A  L  S  C   328

1680 AAGATAACTAGTGTGGAATGGATGGTTATGGCAGACATGCCACCAGGACAGATATTGCTA 1739
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1740 TCACAGGCAAATTTGGGGTCATTGTTCTATCTTCAAGCAGAAAAGCTAGAAGCTGACTTA 1799
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1800 AATCAATTAGAGCAAAAGAAAAGTAAAGAGTCCAACCAAGAGTTAGATATGGGAACATAC 1859
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1860 ATAAAAGCCGTTAGATTCGTACGCAAAAATCGTGACTTATGTCTGGAAAGAGCACAAAAA 1919
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1920 TGTTACGACAGCGTTATTGCGTTTGCCAAAAGAAACAGAAAAATTAGGTTTCATGTGAAG 1979
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1980 GATCAACTGGATCCTTCAATTGCACAGTCAATTGCTCTATCTACCTATGGAATGGGGGTT 2039
 429  D  Q  L  D  P  S  I  A  Q  S  I  A  L  S  T  Y  G  M  G  V   448

2040 TTAAGCCTTCATGAAGGTGTTTTGGCTAAAGCTGAAAAACTATTCAAAGATTCGATCACT 2099
 449  L  S  L  H  E  G  V  L  A  K  A  E  K  L  F  K  D  S  I  T   468

2100 ATGGCCAAGGAGACTGAATTTAATGAACTCCTTGCAGAAGCTGAAAAGGAACTAGAAAAG 2159
 469  M  A  K  E  T  E  F  N  E  L  L  A  E  A  E  K  E  L  E  K   488

2160 ACGACAGTCTTGAAAGCGGCCAAAAAAGAGGGTTTAAACTAATACCACAATAAAGGAAAT 2219
 489  T  T  V  L  K  A  A  K  K  E  G  L  N  *                    501

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2460 CATAACTTGCCAAACTCTAATGACGTTGTCGGTGTA                          2495
```
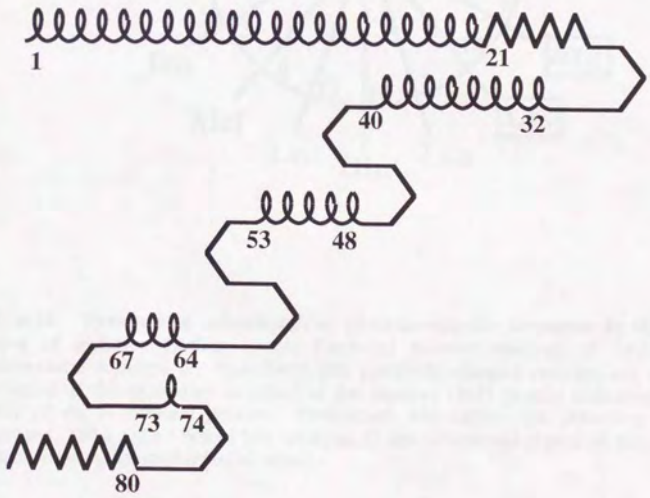

Fig. 6-9. Sequence analysis of *OSD3* gene. 2.5 kb region within 3.8 kb XbaI fragment was sequenced (see Fig. 6-7). Sequence of the predicted protein for the open reading frame was also indicated below the corresponding nucleotide sequence.

A

1 11
MetLeuLeuGlnGlyMetArg LeuSer GlnArg LeuHisLysArg HisLeuPheAlaSer
21
Lys IleLeuThr TrpThrThrAsnProAsaHisIle -- --

B



C

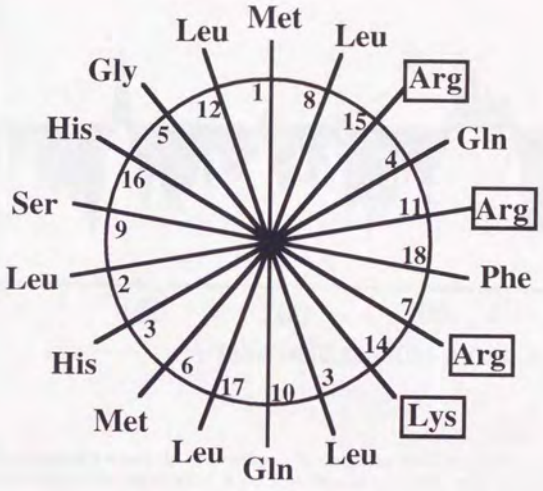


Fig. 6-10. Presence of mitochondrial presequence-like sequence in the N-terminal region of putative Osd3p. (*A*) Predicted primary structure of Osd3p is shown. Hydroxylated residues are underlined and positively-charged residues are outlined. (*B*) Prediction of the secondary structure of the putative Osd3 protein indicating an α-helical nature of the N-terminal portion. Predication was carried out according to Chou and Fassmann, 1974. (*C*) Wheel plot analysis of the N-terminal region of the putative Osd3 protein showing its amphiphilical nature.

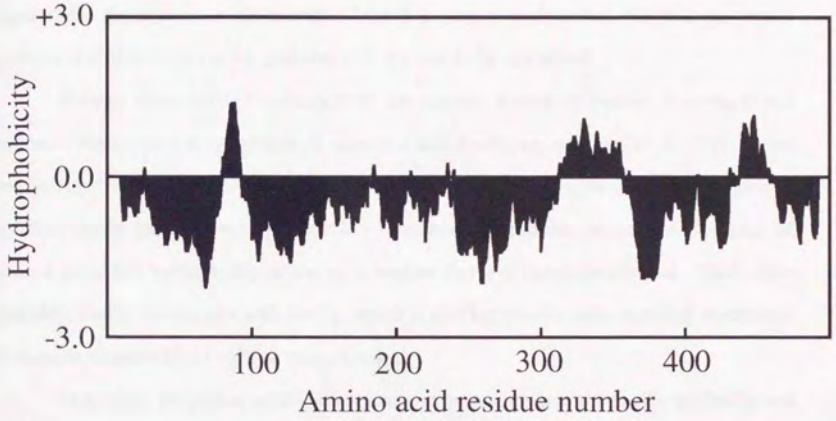


Fig. 6-11. Hydropathy analysis of Osd3p. Hydropathy profile of the putative Osd3p was predicted according to the method of Kyte and Doolittle, 1982, with a scanning window of 19 amino acid residues.

6. 7 Discussion for chapter 6

In this chapter, we described the two genes of *Saccharomyces cerevisiae* that are essential for the degradation of unassembled Cox II. Before this study, a matrix ATP-dependent protease was the only known factor involved in mitochondrial protein degradation. In addition to the *OSD1* gene, study in this chapter revealed that also other two genes are different from the matrix protease. Thus, at least three factors different from the matrix protease are required for degradation of unassembled Cox II in yeast mitochondria. Whether the matrix protease is also involved in this pathway still remains to be elucidated.

Though intracellular localization of the putative Osd2p is unclear, for N-terminal region of it possesses no properties of mitochondrial presequences (Hartl et al. 1989; Roise and Schatz 1988), existence of typical presequence-like structure in the N-terminal region of putative Osd3p implies strongly that it is a mitochondrial protein. Moreover, existence of several predicted hydrophobic region in it implies that it is membrane-bound. Thus, most probably, Osd3p co-operates with Osd1p, which is also localized to mitochondrial membrane, to degrade unassembled Cox II in mitochondria.

At present, no protein with known function homologous to the Osd2p or Osd3p was found, nor any characteristic sequence in these proteins implying their function These proteins may function by interacting physically with Osd1p or with each other. Alternatively, some of them may regulate an activity of some other by chemically modifying it. Osd1p is shown to have ATP-binding cassette. Though no biochemical evidence exists, existence of those sequences suggests that Osd1p can bind or hydrolyze ATP. It is possible that either or both of Osd2p and Osd3p function by regulating ATP-binding or hydrolyzing activity of Osd1p. Biochemical approaches including purification and reconstitution of the three *OSD* gene products in *vitro* is important for further analysis of their functions in a molecular level.

Though all of the three *osd* mutants, when with *COX IV*$^{+}$ genetic background, show temperature sensitivity on a glycerol medium, severity of growth suppression is different among them. As described in chapter 4, *osd2-1* and *osd3-2* mutants showed milder phenotypes than *osd1-1* mutants. The results described in this chapter showing that the *OSD2* or *OSD3* gene disruption also caused the milder temperature sensitivity compared to that of the *OSD1* disruptant eliminated the possibility that the difference is due to some leakiness of the *osd2-1*

and *osd3-2* mutant alleles. However, severity of the growth suppression seems not to be correlated to that of the degradation defect of unassembled Cox II. In fact, pulse-chase experiments described in chapter 4 suggested that severity of the degradation defect is apparently similar among the different *osd* mutants. Thus, the *OSD1* may be additionally required for some biological process, distinct from the degradation pathway of the unassembled Cox II, for which the *OSD2* and *OSD3* are not required, though a possibility that it is due to a subtle difference of the efficiency of Cox II degradation that could not be detected in our experiments.

Chapter 7 Discussion

In this work, I showed that multiple, at least three, nuclear genes (the *OSD* genes) are required for the degradation of unassembled Cox II in yeast mitochondria. Furthermore, it also suggested that degradation pathway of unassembled Cox III is at least partly distinct from that for Cox II, implying the existence of multiple protein degradation pathways with distinct substrate specificities, at least for unassembled mitochondrial translation products. The three *OSD* genes involved in the degradation of unassembled Cox II were molecular cloned and sequence analysis of the *OSD1* gene revealed the existence of ATP-binding cassette and Zn-binding motif (HEXXH motif) in its predicted gene product, which seems to explain well the results of pulse-chase experiments *in vivo*.

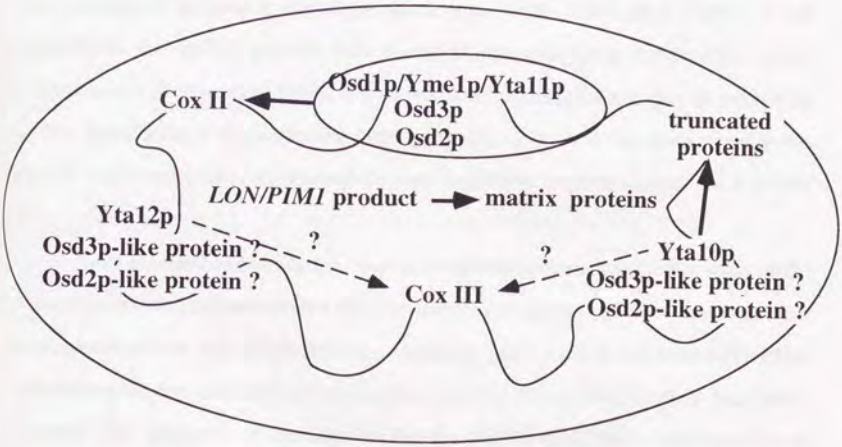


Fig.8-1. Schematic overview of the mitochondrial protein degradation system in *Saccharomyces serevisiae*. Arrows indicate the identified or predicted factor-substrate relatioships.with arrow-heads pointing from factors to substrates. Possible but not proven relationships are shown by dashed lines and predicted but not identified factors are indicated by question marks. Precise localizations of Osd2p and Yta12p are not yet known.

In this section, I attempt to overview the mitochondrial protein degradation system in yeast taking account of the results obtained in this study and those from other groups. I also tried to make some predictions for constructing a model of its molecular integrity. A model is depicted in the diagram of Fig. 8-1. Mitochondrial signal peptidases were excluded from it since involvement of these proteases in metabolic protein degradation has not been reported.

First, ATP-dependent soluble protease bearing homology with bacterial La protease is responsible for the degradation of matrix proteins. This type of protease has been purified from the mitochondrial matrix fraction of rat liver (Desautels and Goldberg 1982a), bovine adrenal cortex (Watabe and Kimura 1985a; Watabe and Kimura 1985b) and yeast (Kutejová et al. 1993). Putative human gene for it has been also reported (Wang et al. 1993). Cloning and disruption of the gene for yeast homolog of this protease, the *LON/PIM1*, revealed that degradation of at least one of matrix proteins is suppressed in the disruptant and lack of it causes respiratory-deficient ρ^- phenotype (Dyck et al. 1994; Suzuki et al., 1994). It was reported that the purified protease from bovine adrenal cortex can degrade some matrix proteins *in vitro* (Watabe et al. 1993). It is unclear whether this is the sole enzyme responsible for the degradation of mitochondrial matrix proteins. Also it is uncertain whether this enzyme is also responsible for some of the inner membrane proteins such as Cox II or Cox III.

Next, in regard to degradation of inner membrane proteins, the present study on the degradation pathway for unassembled Cox II revealed two important features. First is possible existence of multiple degradation pathways. Selective suppression of unassembled Cox II in *osd* mutants implied that degradation pathways for Cox II and Cox III are at least partly different. The present work also showed that the *OSD1/YME1/YTA11*, which possess an ATP-binding cassette and a Zn-binding motif, is involved in the degradation of unassembled Cox II. Recently, Schnal et al. reported cloning of two other genes, the *YTA10* and *YTA12*, from *Saccharomyces cerevisiae*, which are highly homologous to the *OSD1/YME1* and to bacterial FtsH (Schnall et al. 1994). Furthermore, very recently, they reported that ATP-dependent degradation of puromycin-containing truncated mitochondrial translation products is significantly suppressed in the mitochondria of *YTA10*-disruptant (Pajic et al. 1994), suggesting that this gene is involved in elimination of truncated polypeptides translated in

mitochondria. However, whether it is involved in the degradation of some complete but unassembled mitochondrial translation products is still unclear. Though it is also still unclear whether the *YTA12* are involved in the degradation of some of mitochondrial proteins, its sequence similarity to the *OSD1/YME1/YTA11* and the *YTA10* suggests strongly that it is so. Thus, probably at least three degradation pathways exist for inner membrane proteins, each for unassembled Cox II, unassembled Cox III, and truncated proteins. Furthermore, involvement of a member of this subfamily of putative ATPases seems to be a common feature of the degradation of inner membrane proteins. I consider it highly possible that the *YTA10*, *YTA12* or both of them are involved in the degradation of unassembled Cox III.

Other important implication for the degradation of inner membrane proteins obtained in the present work is that the *OSD1* is not sufficient for the degradation of unassembled Cox II. At least two other gene, the *OSD2* and *OSD3*, are also required for this pathway. Though functions of the *OSD2* and *OSD3* gene products remains to be elucidated, the results of this study indicated that they are not required for the degradation of Cox III. Whether they are required for truncated proteins remains to be elucidated. Similarity of the *YTA10* and *YTA12* to the *OSD1/YME1/YTA11* suggests a possibility that a factor or factors similar to Osd2p or Osd3p are involved in the biological pathways, most likely proteolytic pathway, in which the *YTA10* or *YTA12* are involved. In this regard, it will be interesting to examine whether genes homologous to the *OSD2* or *OSD3* exist in yeast.

Unassembled mitochondrial translation products other than Cox II and Cox III can be also degraded rapidly. In fact, mutants in which apo-cytochrome *b* or Cox I are shown or suggested to be unstabilized have been reported (Krummeck and Rödel 1990; Schoppink et al. 1989). It is also important to examine whether the *OSD1/YME1/YTA11*, *OSD2*, *OSD3*, *YTA10* or *YTA12* are involved in the degradation of those proteins.

Protein degradation pathways for intermembrane space or outer membrane may also exist, but there is no reports indicating it at present.

Recent studies, including this work, of mitochondrial degradation pathways, especially for inner membrane proteins, have revealed its unexpectedly intricate nature, though not so as can be seen in the ubiquitin-dependent pathway, at least for the present. The physiological significance of such complexity such as the existence of multiple pathways for the degradation

of inner membrane proteins remains to be elucidated. In future work, genetic approaches as described in this work will be helpful to further identify factors involved in the mitochondrial degradation system for different physiological substrate proteins *in vivo*. On the other hand, biochemical approaches including purification of those factors and reconstitution *in vitro* will be crucial for elucidating the molecular functions of those factors and their relationships to each other.

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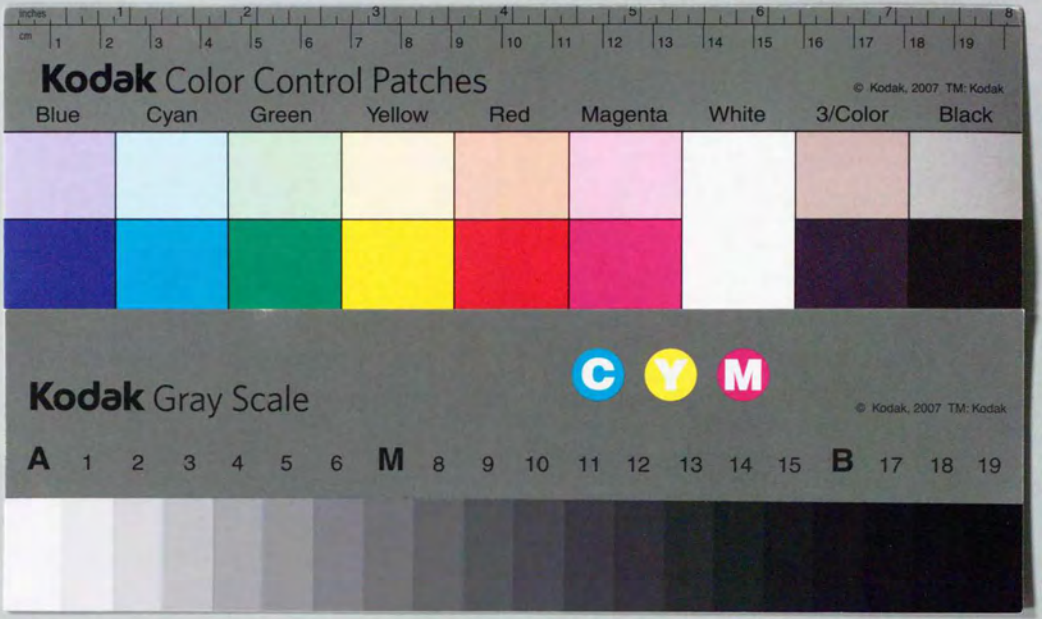
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