The Oxa1 protein is a ubiquitous constituent of the inner membrane of mitochondria. Oxa1 was identified in yeast as a crucial component of the protein export machinery known as the OXA translocase, which facilitates the integration of proteins from the mitochondrial matrix into the inner membrane. We have identified the Neurospora crassa Oxa1 protein which shows a sequence identity of 22% to the yeast homologue. Despite the low level of identity, the function of the homologues is conserved as the N. crassa gene fully complemented a yeast null mutant. Genetic analysis revealed that Oxa1 is essential for viability in N. crassa. Cells propagated under conditions that severely reduce Oxa1 levels grew extremely slowly and were deficient in subunits of complex I and complex IV. Isolation of the Oxa1 complex from N. crassa mitochondria revealed a 170–180-kDa complex that contained exclusively Oxa1. Since the Oxa1 monomer has a molecular weight of 43,000, our data suggest that the OXA translocase consists of a homooligomeric most likely containing four Oxa1 subunits.

The biogenesis of mitochondria requires delivery of several hundred gene products to their specific locations within the organelle. A small number of mostly hydrophobic proteins is encoded on the mitochondrial genome, whereas the great majority of mitochondrial proteins is encoded in the nucleus and synthesized in the cytosol. A series of protein translocation machineries is required for the import and sorting of newly synthesized mitochondrial proteins to their individual destinations. Three of these translocases facilitate protein import from the cytosol into mitochondria: the TOM complex (translocase of the outer membrane) in the outer membrane and two inner membrane TIM complexes (translocases of the inner membrane) which have differential substrate specificity (1–3).

A fourth translocase mediates the insertion of its substrates from the mitochondrial matrix into the inner membrane. Since the direction of translocation is opposite that of the other three translocases, the process is referred to as mitochondrial protein export. The export machinery is referred to as the OXA translocase since the only component identified thus far is the Oxa1 protein. Oxa1 was originally shown to be required for the formation of cytochrome c oxidase in Saccharomyces cerevisiae (4, 5). In yeast, the OXA translocase has now been shown to facilitate the insertion of several nuclear-encoded, matrix-targeted proteins and most, if not all, mitochondrially synthesized membrane proteins into the inner membrane (6–9).

The OXA translocase has been well conserved through evolution and homologues of Oxa1 are present in bacteria, chloroplasts, and mitochondria of various organisms (4, 10–12). The best characterized family member is the Oxa1 protein of S. cerevisiae. A knockout mutant of the S. cerevisiae OXA1 gene is viable, but mutants are unable to respire and grow only on fermentable carbon sources (4). Schizosaccharomyces pombe contains two genes encoding distinct Oxa1 proteins. Both are able to complement S. cerevisiae Oxa1 deficiency. Inactivation of both oxa1 genes in S. pombe is lethal to this petite negative yeast (13). The Escherichia coli homologue, YidC, is an essential component of the bacterial protein export machinery (11, 14).

To date, work on Oxa1 has concentrated on its genetics, function, and identification of substrates. The yeast Oxa1 contains five transmembrane domains with the N terminus of the protein residing in the intermembrane space and the C terminus in the matrix (15). Sequence comparisons suggest that this topology is highly conserved among Oxa1 family members, but little else is known about the structure of the OXA translocase. The E. coli YidC protein was reported to be at least partially associated with the Sec translocase (14). However, there is no homologue of the Sec machinery in mitochondria of yeast (16) or other organisms with completely sequenced genomes. It is unknown whether Oxa1 is associated with other components.

In this report we describe the characteristics of an N. crassa oxa1 mutant and show that a homooligomeric Oxa1 complex can be purified from mitochondria following lysis with nonionic detergents.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Growth and handling of N. crassa were as described (17). The N. crassa strains used in this study are listed in Table I. S. cerevisiae strains were isogenic to the wild type strain W303a and were cultivated at 30 °C on YP medium supplemented with 2% glucose, or 2% galactose, and 0.5% KOH-buffered lactate (18). In the Δoxa1 yeast strain the complete OXA1 reading frame was replaced by the HIS3 gene (8). The plasmid TPI-oxa1ΔHIS3 containing the coding sequence for N. crassa Oxa1, was transformed into this mutant resulting in strain Δoxa1 (oxa1ΔHIS3).

Cosmids and Plasmids—The amino acid sequence of the S. cerevisiae Oxa1 protein (4) was used to identify a N. crassa homologue in a BLAST search of the University of Oklahoma N. crassa EST (expression sequence tag) data bases. PCR primers were designed from the sequence of the EST and used to synthesize a N. crassa oxa1-specific probe. A

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cosmid library (19) was screened and several positive clones were identified. Cosmid 31-2 was chosen for further work. Plasmid XAB1–5 was constructed by cloning a 2.1-kb PCR-generated fragment containing the oxa1 gene and a 1.6-kb fragment encoding a blomycesin resistance gene from pAB520 (20) into the EcoRI and BamHI sites, respectively, of pBR325. This plasmid was used to transform strain HV of N. crassa to generate a duplication to serve as a RIP (repeat induced point mutation) substrate for oxa1. RIP is a natural phenomenon that occurs during the sexual phase of the N. crassa life cycle. Duplications present in the genome are detected and rendered non-functional by the generation of GC to AT transitions in both copies of the sequence (21).

Plasmid pSSOX-4 carries an oxa1-containing 5.5-kb BamHI-EcoRI fragment, derived from cosmid 31-2, cloned into pBSSKII. The plasmid was transformed into E. coli strain CJ236 for the generation of single-stranded DNA for site-directed mutagenesis. This single-stranded DNA was utilized to create plasmids pBR5-3 and pJ306-2 which contain mutations that generate stop codons at the positions corresponding to amino acid residues 322 and 247 of the preprotein coding sequence, respectively. A hygromycin resistance gene from pCSN44 (22) was inserted into each plasmid to give pH295-3-8 and pH306-2-12.

Plasmid pK5B-10 is a pBSSKII derivative containing the oxa1 coding sequence plus six histidine codons. The histidine tag was generated by PCR using a 3’ primer that contained the coding region of the C-terminus of oxa1 with six His codons inserted before the stop codon. The 5’ primer corresponded to a oxa1-specific sequence upstream of an AvrII site situated 237 base pairs upstream of the stop codon. The PCR fragment was polished with T4 DNA polymerase, cut with AvrII, and ligated into a blomycesin resistant containing derivative of pSSOX-4 that had been cut with KpnI, polished with T4 DNA polymerase, and then cut with AvrII. This released the C-terminal coding region of the oxa1 gene as well as the 3’-flanking sequence. Thus, the PCR product containing the histidine tag replaced the normal C terminus and flanking region of the oxa1 gene. Plasmid TPI-oxa1NC was constructed for transformation of yeast D. oxaxa1 strain and contained an intron-less version of N. crassa oxa1 in yeast under control of the constitutive trisophosphate isomerase promoter.

Sheltered RIP of the oxa1 Gene—The oxa1 gene was inactivated by the process of RIP (21). Because we suspected that null mutants of oxa1 would be inviable in N. crassa, we utilized sheltered RIP to obtain heterokaryotic strains in which one nucleus contained no functional oxa1 alleles. Sheltered RIP requires knowledge of the chromosome that carries the target gene. RFLP mapping studies (23–25), using cosmid 31-2 as a probe, showed that the N. crassa oxa1 gene was located on chromosome V. The principles, strains, and procedures of sheltered RIP for chromosome V have been described (26) and are analogous to those used previously to inactivate the tom20 gene on linkage group IV (27).

Briefly, the sheltered RIP Host V strain was transformed with plasmid pXAB1-5 to generate a duplication of oxa1 to create the RIP substrate. Transformants were selected on blomycesin containing medium, taken through one round of purification on blomycesin containing plates, and examined by Southern analysis for evidence of ectopic integration of a single copy of oxa1. Strain OX80-1 was chosen to act as the male parent in a cross to the Mate V strain. The duplication of oxa1 in OX80-1 stimulated RIP of both copies of the duplication during the sexual cycle. The desired ascospores produced from this cross were disomic for linkage group V and were selected by growth on minimal medium. Upon vegetative growth, the disomic ascospores break down into heterokaryons containing two different nuclei. The potential lethality caused by the inability of the nucleus containing the oxa1RIP gene to make functional Oxa1 is sheltered, or complemented, by a nucleus containing an wild-type oxa1 gene. The nuclei may also contain the introduced ectopic copy of oxa1 depending on its segregation through meiosis. To screen for heterokaryons containing both wild type and RIPed oxa1 sequences, genomic DNA was prepared from each isolate and used in PCR reactions with oxa1-specific primers. The PCR products were sequenced directly and two strains, OX80-7-1 and OX80-15-1, gave mixed sequence with evidence of the GC to AT transitions characteristic of RIP. Strain OX80-15-1 was chosen for further analysis. Cloning and sequence analysis of oxa1 sequences from this strain revealed that the RIP nucleus contained both the endogenous and ectopic alleles of oxa1. Both contained multiple RIP mutations including an identical mutation which generates stop codons at position 137 of the 462-amino acid coding sequences. We consider these to be effectively null alleles since the proteins encoded would be terminated before the first predicted membrane spanning domain.

Antibody Production—Antisera were raised against a fusion protein comprising glutathione S-transferase and the C terminus of N. crassa Oxa1. For expression of this protein in E. coli, the sequence encoding amino acid residues 345 to 462 of the Oxa1 preprotein was amplified by PCR using the primers HH13 (5’-GGGAGATCTAGTTTTGCTCCCGGTCGGCTCAGGTCC- 3’) and HH14 (5’-GGGGGGATCCATCTTATTCTCATATCCC- 3’). The PCR product was digested with BamHI/BglII and subcloned into the BamHI site of the E. coli expression vector pETGEX (28). The antisera used against yeast Oxa1 and Cox2 were described previously (15). Antiserum against purified N. crassa complex I and a sample of purified complex I, used as a control for the specificity of the antiserum, were generously provided by Ulrich Schulte and Hans Weiss, Düsseldorf, Germany.

Purification of the Oxa1 Protein—To facilitate isolation of Oxa1, we developed strain K5-15-23-1 (Table I). The strain was obtained by chromosomal insertion of a modified oxa1 gene, encoding a C-terminal hexahistidinyl-tagged version of the protein, into the oxa1RIP nucleotid of heterokaryon OX80-15-1. The resulting strain is a lysine-lysine-requiring homokaryon that expresses exclusively a His-tagged version of Oxa1 at wild type levels. Mitochondria from strain K5-15-23-1 were dissolved in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM imidazole, 2 mM phenylmethylsulfonyl fluoride, plant protease inhibitor mixture (Sigma), and either 1% dodecyl maltoside or 1% Triton X-100) at a concentration of 1 mg of mitochondrial protein per ml of buffer. After gentle mixing for 30 min at 4 °C, the sample was clarified by centrifugation at 27,000 × g for 20 min at 2 °C. The cleared lysate was diluted with 4 volumes of lysis buffer, without the protease inhibitor mixture, and mixed gently for 60 min with Ni-NTA-agarose beads (Qiagen, Hilden, Germany). The slurry was poured into a column, washed with lysis buffer containing 25 mM imidazole, and eluted with lysis buffer containing 250 mM imidazole. The protein was further purified by binding the Ni-NTA column eluate to SP-Sepharose (Amersham Biosciences AB, Uppsala, Sweden) in binding buffer (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.1% dodecyl maltoside or 0.1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride). The column was washed with binding buffer containing 150 mM NaCl and eluted with binding buffer containing either 350 or 500 mM NaCl. Size exclusion chromatography was performed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM
Identification of the N. crassa oxal1 Gene—An N. crassa EST for oxal1 was identified in a BLAST search using the sequence of the S. cerevisiae Oxal1 protein as the query. Primers designed from the EST sequence were used to generate PCR products to probe a cosmid library of N. crassa (Sc), S. cerevisiae (Sc), and H. sapiens (Hs). The number of amino acid residues in the precursor protein are indicated on the right. Solid bars under the sequence indicate predicted membrane spanning domains. Shading indicates residues that are identical in two or three of the proteins. Arrows above residues 247 and 352 of the N. crassa sequence indicate the positions converted to stop codons by site-directed mutagenesis. The first residue following the confirmed matrix processing peptidase cleavage site of the N. crassa protein (see Fig. 7) is indicated by an asterisk at position 70. B, prediction of coiled coil structure in the three Oxal1 proteins using the parameters of Lupas et al. (44) with a window size of 21 amino acid residues. The prediction was obtained using the EMBnet web site (www.ch.embnet.org/software/COILS_form.html).

EDTA, 0.1% detergent, and 1 mm phenylmethylsulfonyl fluoride using either a Superose 6 (10/30) column (Amersham Biosciences AB) in a Pharmacia Äktan explorer chromatography unit or Superose 6 (16/50) and Superose 12 (10/30) columns arranged in series in a Pharmacia LCC500 chromatography unit. Identical sizes were obtained for the Oxal1 complex, analyzed relative to molecular weight calibration standards, under both separation conditions. Oxa1 complex, analyzed relative to molecular weight calibration standards, under both separation conditions.

Other Techniques—Western blots (29), Southern blots (30), transformation of yeast cells (31), transformation of N. crassa cells (32, 33), and solid phase Edman degradation (34) were according to published procedures. Site-directed mutagenesis was done with the Mutagen system. The chemically cleaved products, solid phase Edman degradation (34), were according to published procedures. Site-directed mutagenesis was done with the Mutagen system.

RESULTS

Identification of the N. crassa oxal1 Gene—An N. crassa EST for oxal1 was identified in a BLAST search using the sequence of the S. cerevisiae Oxal1 protein as the query. Primers designed from the EST sequence were used to generate PCR products to probe a cosmid library of N. crassa (Sc). The oxal1 gene sequence was determined from a single cosmid and has been deposited in the NCBI data base (accession number AF435863). A BLAST search of the N. crassa genome sequence revealed no oxal1 paralogues. The amino acid sequence of the N. crassa Oxal1 protein is shown in Fig. 1A, aligned with its Homo sapiens and S. cerevisiae orthologues. The sequence of the mature N. crassa protein is 22% identical to both the predicted mature yeast and human homologues. Despite this low level of identity, the overall structure of the proteins is very similar. All three Oxal1 proteins are comprised of a well conserved core region of about 200 amino acid residues that contains five transmembrane domains with highly conserved lengths and spacing. This core region is flanked by N- and C-terminal extensions which contain little sequence identity. However, a high probability for coiled coil structure formation is predicted in the C-terminal matrix domain of all homologues (Fig. 1B).

Oxal1 Is Essential for Viability in N. crassa—Previously described mutants of the S. cerevisiae oxal1 gene were found to exhibit a petite phenotype (4). An Oxal1 null mutant in N. crassa was created by the procedure of sheltered RIP to give a heterokaryon in which one nucleus contains a functional version of oxal1, while the other does not (Fig. 2). Sequence analysis demonstrated that the oxal1 alleles present in the RIPed nucleus were effectively null alleles (see “Experimental Procedures”). Macromonia (conidia), the asexual spores formed by N. crassa for propagation, typically contain between one and five nuclei. When the sheltered heterokaryon produces conidia, the two nuclear types should be distributed randomly giving rise to three types of conidia: nucleus 1 homokaryons, nucleus 2 homokaryons, and heterokaryotic conidia containing both nuclei. To determine whether conidia containing only nucleus 1 were viable, conidia formed from the sheltered heterokaryon were streaked onto media containing lysine, leucine, and inositol to obtain single colonies. As shown in Table II, analysis of these colonies revealed no lysine-, leucine-requiring homokaryons, whereas both inositol-requiring homokaryons and heterokaryotic colonies were identified. In contrast, lysine and leucine auxotrophs could be isolated following transformation of the sheltered heterokaryon with plasmid pK5B-10, encoding the hexahistidinyl-tagged version of Oxal1. Taken together, these data indicate that Oxal1 is essential for viability in Neurospora. This agrees with the observation that S. pombe strains lacking both of their Oxal1 isoforms were also inviable (13).

To demonstrate directly that Oxal1 is required for growth, the sheltered RIP strain was propagated in the presence of cycloheximide to increase the ratio of nuclei containing the non-functional oxal1 allele relative to nuclei containing the wild type allele (Fig. 2). This caused an almost complete growth arrest of the culture, whereas the cycloheximide-resistant control strain grew only slightly slower than cells in inhibitor free medium (Fig. 3A). Western blotting revealed that growth in increasing concentrations of cycloheximide resulted in reduced Oxal1 levels in the sheltered heterokaryon whereas no detectable changes occurred in control strains grown under similar conditions (Fig. 3B). The level of Oxal1 in the sheltered hetero-
TABLE II
Inability to isolate homokaryons carrying the oxa1<sup> RIP </sup> allele

| Strain      | Colonies examined | Inositol requiring homokaryons | Lysine and leucine requiring homokaryons | Heterokaryons |
|-------------|-------------------|--------------------------------|-----------------------------------------|---------------|
| OX80–7–1    | 94                | 26                             | 0                                       | 68            |
| OX80–15–1   | 182               | 56                             | 0                                       | 126           |

FIG. 3. Oxa1 function is necessary for growth of N. crassa. A, the OX80–15–1 sheltered heterokaryon (oxa1<sup> RIP </sup>) and control strain (HV) were grown either with or without lysine, leucine, and cycloheximide (CHI, 50 µg/ml). The presence of lysine and leucine frees the oxa1<sup> RIP </sup> nucleus from its dependence on its heterokaryotic partner nucleus for the synthesis of these compounds. The presence of cycloheximide forces the oxa1<sup> RIP </sup> nucleus to predominate in the heterokaryon as it supplies resistance to the inhibitor. After the indicated growth periods, the mycelia were harvested and weighed. B, deficiency of Oxa1 protein in the oxa1<sup> RIP </sup> sheltered heterokaryon (OX80–15–1). Mitochondria were isolated from the control strains or the oxa1<sup> RIP </sup> sheltered heterokaryon following growth with or without cycloheximide. All cultures also contained lysine and leucine with the exception of the sheltered heterokaryon grown without cycloheximide. Mitochondrial proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunodecoration with the indicated antibodies. mtHsp70, mitochondrial Hsp70.

FIG. 4. N. crassa oxa1 complements a yeast oxa1 null mutant. A, yeast wild type (wt), and oxa1 null mutant without, (Δoxa1), or with a plasmid for expression of the N. crassa Oxa1 protein (Δoxa1 (oxa1<sup>H9004</sup>)), strains were grown to log phase on YP medium containing 2% glucose. Serial 10-fold dilutions of the cultures were spotted on YP plates containing 2% glucose (left panel) or 2% glycerol (right panel). The plates were incubated at 30°C for 2 days (Glucose) or 4 days (Glycerol). B, mitochondrial extracts (50 µg of protein) of the yeast strains indicated were subjected to SDS-PAGE, blotted to nitrocellulose membrane, and decorated with antisera against N. crassa Oxa1 (Oxa1<sup>Nc</sup>), yeast Oxa1 (Oxa1<sup>H9004</sup>), Cox2, and the outer membrane protein Tom70 as a control. For comparison, a control blot using a mitochondrial extract from wild type N. crassa cells decorated with the N. crassa Oxa1 antiserum is shown on the right.
protein is required for the biogenesis of complex I. Since a number of complex I subunits are mitochondrially encoded in *N. crassa*, it seems likely that the membrane insertion of at least some of them requires the OXA translocase.

The C-terminal Matrix Domain of Oxa1 Is Not Required for Its Function in *N. crassa*—Oxa1 contains five transmembrane domains and a large C-terminal tail that is localized in the mitochondrial matrix (15). As discussed above, computer predictions strongly suggested the existence of a coiled coil structure in the C-terminal matrix domain (Fig. 1B). To determine whether this domain plays a critical role in Oxa1 function, we constructed a mutant version of Oxa1 that lacks the last 110 residues of the protein where the predicted coiled coil domain resides (Fig. 6A). This variant was transformed into the sheltered heterokaryon and selection for strains in which the truncated variant had rescued the *oxa1RIP* nucleus (Fig. 2) was imposed. Mitochondria isolated from the rescued homokaryotic strains contained no detectable Oxa1 (Fig. 6B) since our antibody was raised against the C-terminal 117 residues of the protein which are missing in the truncated form. No full-length Oxa1 is present because the rescued strains are homokaryons containing only the *oxa1RIP* nucleus (Fig. 2). Thus, the fact that the cells are viable provides evidence that the truncated form of the protein is present and functional in these rescued strains. Growth rates of the isolates containing the variant were indistinguishable from control strains (Fig. 6C). In contrast, an Oxa1 variant containing only the N-terminal 246 residues of the 462 residue preprotein, did not rescue the *oxa1RIP* nucleus. Taken together, these data demonstrate that the predicted coiled coil domain found in the C terminus of the *N. crassa* Oxa1 protein, is not essential for Oxa1 function.

Purification of Oxa1 from *N. crassa* Mitochondria—The yeast Oxa1 protein was reported to be part of a high molecular weight complex of unknown composition (8). To study the complex further we wished to isolate it from *N. crassa* mitochondria. To enhance the purification procedure, we constructed strain K5-15-23-1, which expresses exclusively a hexahistidinyl-tagged version of Oxa1. Following lysis of purified mitochondria with either dodecyl maltoside or Triton X-100, a strongly enriched Oxa1 fraction was eluted from Ni-NTA columns (Fig. 7A). When the protein pattern of this fraction was compared with a control obtained from mitochondria of an untagged wild type strain, the Oxa1 band was the only additional visible band (not shown). This indicates that no other protein was co-isolated after this first purification step. Ion exchange chromatography of the Ni-NTA eluate on SP-Sepharose allowed purification of *N. crassa* Oxa1 to near homogeneity and no components co-isolating with the protein were detectable (Fig. 7A). About 0.5 to 2.0 mg of Oxa1 could be isolated per gram of mitochondrial protein. Thus, Oxa1 makes up at least 0.05 to 0.2% of total mitochondrial protein content.

To verify that the purified protein was Oxa1, the band from a Ni-NTA fraction on a Coomassie-stained SDS gel was excised and the N-terminal 17 amino acid residues were sequenced by Edman degradation. The resulting sequence was identical to the *N. crassa* Oxa1 sequence starting at position 70 of the mature protein (36).

*Oxa1 Forms a Homooligomeric Complex*—To analyze the size of the Oxa1 complex in *N. crassa*, we employed size exclusion chromatography after lysis of purified mitochondria with the mild detergent dodecyl maltoside. Upon fractionation on a Superose 6 FPLC column, both the wild-type (Fig. 8A, open circles) and hexahistidinyl-tagged version (not shown) of Oxa1 were found exclusively in the size range of 200–300 kDa. Purified Oxa1 complex, isolated from mitochondria dissolved in dodecyl maltoside, fractionated in a sharp peak of about the same size (Fig. 8A, filled circles). Since the purified complex contains only Oxa1, it appears that the protein forms a homooligomeric complex containing several Oxa1 subunits.

Solubilization of membrane proteins with mild detergents,
The consensus for the mitochondrial processing peptidase (MPP) was identified. The maturation site is indicated by an arrow.

The amino acid residue at position 3 of the mature protein (upper row) was determined by Edman degradation. The resulting sequence is shown in the lower row.

The N terminus of the purified protein. The isolated protein was cut out from dodecyl maltoside extracts and was subjected to SDS-PAGE, gel filtration chromatography on SP-Sepharose, and gel stained with Coomassie Blue. The positions of molecular weight markers that were run in parallel are indicated. B, mitochondria were lysed in 1% Triton X-100 at a protein concentration of 0.6 mg/ml and the solution was clarified by centrifugation. Either 1 ml of extract, or 1 ml of the Oxa1 containing fractions obtained following purification by Ni-NTA and SP-Sepharose chromatography, were subjected to gel filtration chromatography on Superose 6 and 12 columns joined in series. Fractions of 1.2 ml were collected and the amounts of Oxa1 were determined as described in A.

**DISCUSSION**

We have identified the Oxa1 protein of *N. crassa*. The protein shows limited sequence identity to other Oxa1 homologues but the number and spacing of transmembrane segments is highly conserved between the *N. crassa* protein and Oxa1 from other organisms. The function of these proteins is also conserved, since both the *N. crassa* (this study) and human (10) homologues can rescue the respiratory defect of *S. cerevisiae oxa1* null mutants. We have shown that the *N. crassa* homologue restored normal levels of yeast Cox2, thus proving that it fulfills the function of the yeast Oxa1 with respect to insertion of proteins into the mitochondrial inner membrane. In yeast, defects in Oxa1 abolish the function of the cytochrome oxidase complex and lead to strongly reduced levels of complex III and F$_1$F$_0$-ATPase. Consistent with these observations, depletion of Oxa1 in *N. crassa* resulted in strongly reduced levels of cytochrome oxidase subunits. In addition, accumulation of two subunits of complex I was found to be dependent on Oxa1. In contrast to most eukaryotes, *S. cerevisiae* does not have a complex I-type NADH oxidoreductase. Thus, our observations provide evidence for a dependence of complex I subunits on Oxa1 for their assembly. Taken together, these data show that the Oxa1 protein is critical for the biogenesis of at least four out of the five respiratory chain complexes and likely explain why the protein is essential in obligate aerobic organisms.

Analysis of the primary sequence of Oxa1 proteins revealed a region in the C-terminal matrix domain which is strongly predicted to form coiled coil structures, even though the amino acid sequence in the region is not significantly conserved. Coiled coil motifs are versatile structures which mediate protein-protein interactions and are involved in a variety of biological functions including molecular recognition (38, 39). We consider it unlikely that these domains mediate the association of monomers in the Oxa1 complex since the complex was found to be stable in the high salt concentrations used during the purification procedure. This suggests that the association of the subunits is mediated mainly by hydrophobic interactions between the transmembrane segments. Attractive models for the role of a protein-protein interaction domain in the Oxa1 molecule would be to provide a site of interaction with its substrate proteins, with mitochondrial ribosomes, or with components that collaborate with the OXA translocase. However, since the deleted form lacking this domain is able to rescue the oxa1 mutant and to restore the growth rate to that of control cells, it is apparent that the function of the domain is not essential.

Given the conservation of the predicted coiled coil among Oxa1 homologues, this finding was somewhat surprising. It is conceivable that another protein provides a redundant function that fulfills the role of the C-terminal domain in the deletion.
strains. In yeast, the Mba1 protein has been shown to overlap in function and substrate specificity with Oxa1. Mba1 can also cooperate with Oxa1 since it was required for the activity of a temperature-sensitive oxa1 allele, even at the permissive temperature. Mba1 was suggested to play a role as a receptor that recuits substrates and facilitates their membrane insertion by the OXA complex (40). Thus, the effects of deletion of the coiled coil domain of Oxa1 might be masked if a protein of similar function to Mba1 exists in *N. crassa*.

Size exclusion chromatography of mitochondria lysed in Triton X-100 revealed an Oxa1 complex of 170–180 kDa. In contrast, solubilization with the mild detergent dodecyl maltoside resulted in a complex with a significantly larger apparent molecular weight. Since we did not detect any proteins co-isolating with purified Oxa1 using either detergent, we regard it as unlikely that the different lysis conditions result in different protein composition of the complexes. Thus, the shift in apparent molecular weight is likely due to the size of the associated detergent micelles. Bound detergent can contribute significantly to the size of solubilized membrane complexes. This effect was shown to be much smaller for strong detergents like Triton X-100 than for milder ones like dodecyl maltoside, where size increases of 30 to 50% were observed (37). This degree of variability in apparent molecular weight is consistent with our findings for the size of the complex following solubilization in dodecyl maltoside (200,000–300,000) compared with Triton X-100 (170,000). The sizes of the yeast Oxa1 complex determined using mitochondrial polypeptides into the membrane, thereby reducing the risk of unproductive aggregation of proteins after their synthesis in the matrix.

Future work will involve the reconstitution of the purified Oxa1 complex into liposomal membranes. The rather simple architecture of the complex makes functional reconstitution of the OXA translocation machinery highly promising and should allow further experimental dissection of the mechanisms of protein export in mitochondria.

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