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Acyl-CoA oxidase is imported as a heteropentameric, cofactor-containing complex into peroxisomes of *Yarrowia lipolytica*

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Five isoforms of acyl-CoA oxidase (Aox), designated Aox1p to Aox5p, constitute a 443-kD heteropentameric complex containing one polypeptide chain of each isoform within the peroxisomal matrix of the yeast *Yarrowia lipolytica*. Assembly of the Aox complex occurs in the cytosol and precedes its import into peroxisomes. Peroxisomal targeting of the Aox complex is abolished in a mutant lacking the peroxin Pex5p, a component of the matrix protein targeting machinery. Import of the Aox complex into peroxisomes does not involve the cytosolic chaperone Pex20p, which mediates the oligomerization and import of peroxisomal thiolase. Aox2p and Aox3p play a pivotal role in the formation of the Aox complex in the cytosol and can substitute for one another in promoting assembly of the complex. In vitro, these subunits retard disassembly of the Aox complex and increase the efficiency of its reassembly. Neither Aox2p nor Aox3p is required for acquisition of the cofactor FAD by other components of the complex. We provide evidence that the Aox2p- and Aox3p-assisted assembly of the Aox complex in the cytosol is mandatory for its import into peroxisomes and that no component of the complex can penetrate the peroxisomal matrix as a monomer.

Introduction

One view of protein translocation across membranes is that only unfolded polypeptides are competent for translocation and that folding, cofactor attachment and oligomerization of proteins into active structures occur only after proteins have been completely translocated (McNew and Goodman, 1996; Schatz and Dobberstein, 1996). However, recent evidence has shown that not all proteins are translocated and assembled in this way (Teter and Klionsky, 1999). Pathways of protein transport through the membrane that are known to accommodate folded, cofac-

tor-containing and oligomerized polypeptides have been described, including the cytoplasm-to-vacuole targeting (Cvt)* of aminopeptidase I (API) in the yeast *Saccharomyces cerevisiae* (Klionsky and Ohsumi, 1999), the ΔpH-dependent translocation of proteins across the plant thylakoid membrane and the related twin-arginine translocation (Tat) of proteins across the bacterial plasma membrane (Dalbey and Robinson, 1999), and the peroxisomal import of some matrix proteins (Subramani, 1998; Titorenko and Rachubinski, 2001). For peroxisomes, import of folded and oligomeric proteins is not an exclusive pathway, as alcohol oxidases in two yeast species, *Candida boidinii* (Stewart et al., 2001) and *Pichia pastoris* (Waterham et al., 1997), are imported into peroxisomes as monomers, and their octamerization occurs only in the organelle matrix.
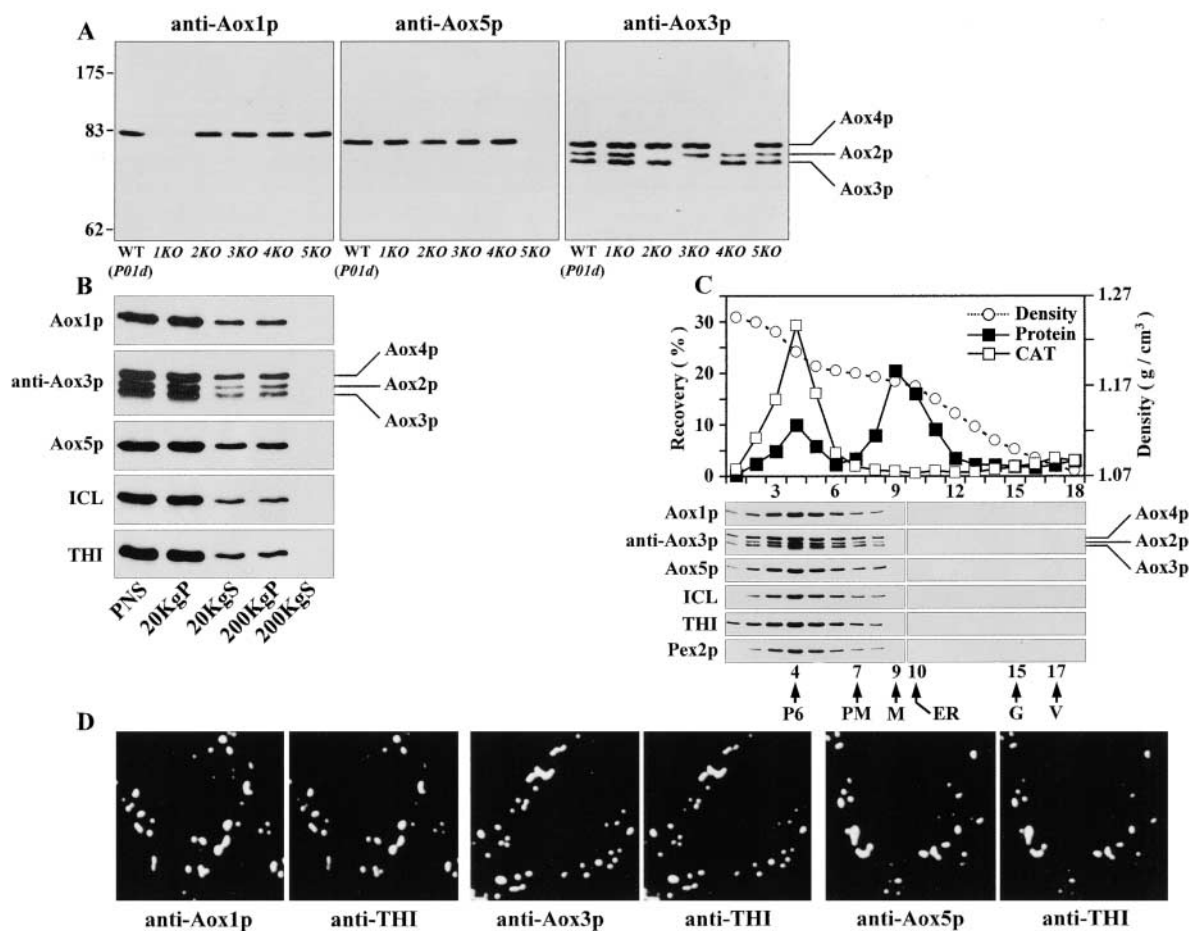
Although the import of folded and oligomerized proteins into peroxisomes has been reported for a variety of organisms from yeast to humans (Glover et al., 1994; McNew and Goodman, 1994; Walton et al., 1995; Elgersma et al., 1996; Häusler et al., 1996; Leiper et al., 1996; Lee et al., 1997; Titorenko et al., 1998; Stewart et al., 2001), many mechanistic aspects of this import pathway remain to be elucidated. Oligomerization of the matrix enzyme thiolase (THI) of the yeast *Yarrowia lipolytica* in the cytosol is known to require the chaperone Pex20p

Figure 1. **The five Aox isoforms are peroxisomal.** (A) Immunoblot analysis of lysates of whole cells of the wild-type strain *P01d* (WT) and of the mutant strains *aox1KO* (*1KO*), *aox2KO* (*2KO*), *aox3KO* (*3KO*), *aox4KO* (*4KO*) and *aox5KO* (*5KO*) probed with anti-Aox1p, -Aox3p, and -Aox5p antibodies. Strains were grown in YPBO for 9 h. Proteins were resolved by SDS-PAGE in 5–7.5% acrylamide gradient gels and visualized by immunoblotting. The positions of Aox2p, -3p, and -4p are indicated. (B) Equal portions (0.1% of total volume) of the indicated subcellular fractions from YPBO-grown wild-type cells were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to the indicated proteins. (C) The 20KgP fraction of the wild-type strain *P01d* grown in YPBO for 9 h was fractionated by isopycnic centrifugation on a discontinuous sucrose gradient. Sucrose density (g/cm^3) of fractions and percent recoveries of loaded protein and of catalase (CAT) activity are presented. Equal volumes of gradient fractions were analyzed by immunoblotting with anti-Aox1p, -Aox3p, and -Aox5p antibodies and with antibodies to peroxisomal matrix (ICL, THI, and MLS) and membrane (Pex2p) proteins. Numbers and arrows indicate peak fractions for peroxisomes (P), plasma membrane (PM), mitochondria (M), endoplasmic reticulum (ER), Golgi region (G), and vacuoles (V). (D) Double-labeling, indirect immunofluorescence microscopy of YPBO-grown wild-type (*P01d*) cells using rabbit anti-Aox1p, -Aox3p, or -Aox5p and guinea pig anti-THI primary antibodies. Primary antibodies were detected with rhodamine-conjugated goat anti–rabbit IgG and fluorescein-conjugated donkey anti–guinea pig IgG secondary antibodies.

(Titorenko et al., 1998), yet it remains unknown whether the acquisition and maintenance of the oligomeric conformation by other proteins imported into the peroxisome are assisted by a specialized set of cytosolic chaperones or whether they can occur spontaneously in the cytosol. Furthermore, it is unknown if any peroxisomal protein can preassemble with its cofactor in the cytosol and if cofactor attachment to the protein is a prerequisite for its transport through the peroxisomal membrane, as has been demonstrated recently for the translocation of some proteins across the bacterial plasma membrane by the Tat pathway (Santini et al., 1998; Rodrigue et al., 1999). Another intriguing question is whether any component of a heterooligomeric protein complex imported into peroxisomes may be required for assembly of the complex in the cytosol, for acquisition of cofactor or for

membrane targeting of the complex, while other components are not required for any of these functions. Such a division of labor amongst different components has recently been reported for the heterodimeric hydrogenase 2 enzyme translocated across the bacterial plasma membrane by the Tat pathway (Rodrigue et al., 1999).

Here, we report that acyl-CoA oxidase (Aox) of *Y. lipolytica* is imported into peroxisomes as a heteropentameric, cofactor-containing complex that is preassembled in the cytosol. We show that two subunits of the complex, Aox2p and Aox3p, play a pivotal role in its formation in the cytosol. We also provide evidence that none of the components of the Aox complex is import-competent in the monomeric form and that Aox2p- and Aox3p-assisted assembly of the complex in the cytosol is a requirement for its import into peroxisomes.

Results

All five isoforms of Aox are peroxisomal

Recently, five genes, designated *AOX1* to *AOX5*, encoding five isoforms of Aox in the yeast *Y. lipolytica* were identified (Wang et al., 1999a). Antibodies raised against His_6-Aox1p and His_6-Aox5p fusion proteins specifically recognized isoforms Aox1p and -5p, respectively (Fig. 1 A, left and middle). Antibodies raised against His_6-Aox3p recognized isoforms Aox4p, -2p, and -3p (Fig. 1 A, right; top, middle, and bottom bands, respectively), as judged from immunoblot analysis of cell lysates of the wild-type strain *P01d* and mutant strains carrying single *AOX* knock-out mutations.

Subcellular fractionation of wild-type cells grown in oleic acid–containing (YPBO) medium showed that, similar to the peroxisomal marker proteins isocitrate lyase (ICL) and THI, 80% or more of all five Aox isoforms associated with the 20KgP, while up to 20% localized to the 200KgP (Fig. 1

B). The 20KgP fraction is enriched for mature peroxisomes, whereas the 200KgP fraction is enriched for high-speed pelletable peroxisomes (Titorenko et al., 1998, 2000). Fractionation of the 20KgP by isopycnic centrifugation on a discontinuous sucrose density gradient revealed that all five isoforms of Aox localized to fractions containing mature peroxisomes (Fig. 1 C). The peroxisomal location of all five Aox isoforms was confirmed by double-labeling, indirect immunofluorescence microscopy of oleic acid-grown wild-type cells using anti-Aox1p, -3p, or -5p antibodies and antibodies to THI. The fluorescence patterns generated by these antibodies were superimposable and showed the punctate pattern characteristic of peroxisomes (Fig. 1 D). Therefore, similar to Aox proteins identified previously in humans, mammals, plants, and various yeast species (van den Bosch et al., 1992; Mannaerts and van Veldhoven, 1996), all five isoforms of Aox are peroxisomal in *Y. lipolytica*. Impor-
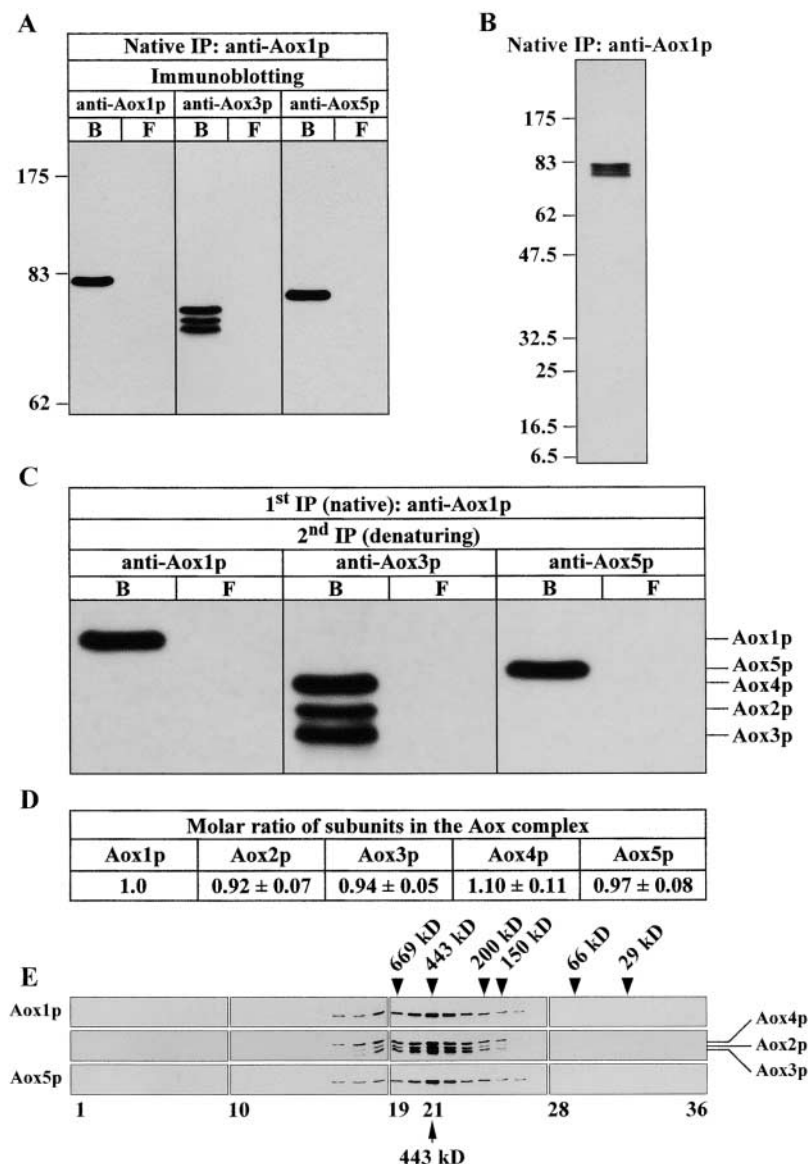
Figure 2. **In the peroxisomal matrix, the five Aox isoforms constitute a 443-kD heteropentameric complex.** (A) Peroxisomes purified from the 20KgP fraction of YPBO-grown wild-type (*P01d*) cells were lysed by addition of LC buffer and subjected to centrifugation to yield a supernatant enriched for matrix proteins. Matrix proteins were subjected to immunoaffinity chromatography under native conditions using anti-Aox1p antibodies covalently coupled to protein A-Sepharose. Proteins bound to the column (B) and eluted with 100 mM glycine (pH 2.8) and unbound proteins recovered in the flow-through (F) were divided into equal aliquots that were analyzed by immunoblotting with anti-Aox1p, -Aox3p, or -Aox5p antibodies. (B) Spheroplasts of the wild-type strain *P01d* grown in YPBO for 9 h were pulse-labeled with L-[^{35}S]methionine for 2 min, chased with unlabeled L-methionine for 30 min, and subjected to subcellular fractionation to isolate peroxisomes from the 20KgP fraction. Peroxisomal matrix proteins were subjected to immunoprecipitation under native conditions as described in the legend to A. Immunoprecipitated proteins were resolved by SDS-PAGE in 7.5–20% acrylamide gradient gels and visualized by fluorography. (C) Pulse-labeled and chased peroxisomal matrix proteins were subjected to a first immunoprecipitation under native conditions, as described in the legend to B. Immunoprecipitated proteins that eluted with 100 mM glycine (pH 2.8) were divided into three equal aliquots that were subjected to a second immunoprecipitation under denaturing conditions with anti-Aox1p, -Aox3p, or -Aox5p antibodies linked to protein A-Sepharose. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to fluorography. (D) To quantify the stoichiometry of the Aox complex, the densities of the signals for Aox1p to Aox5p (C) were divided by 15, 13, 16, 19, and 17, the number of methionine residues in Aox1p, -2p, -3p, -4p, and –5p, respectively. (E) Matrix proteins recovered from peroxisomes of the wild-type (*P01d*) strain were fractionated by centrifugation on a glycerol gradient. Equal volumes of each fraction were analyzed by immunoblotting with anti-Aox1p, -Aox3p, and -Aox5p antibodies. The fractions are numbered. Arrow indicates peak fraction 21 for the Aox complex, which cofractionated with a 443-kD molecular weight standard. Arrowheads indicate peak fractions for molecular weight standards.

tantly, no Aox isoform contains a conserved variant of a COOH-terminal peroxisomal targeting signal (PTS)1 or a NH$_2$-terminal PTS2 (Wang et al., 1999b).

The five Aox isoforms are components of a heteropentameric complex in the peroxisomal matrix

Immunoaffinity chromatography of matrix proteins from peroxisomes of the wild-type strain showed that all five isoforms of Aox coimmunoprecipitated under native conditions with anti-Aox1p (Fig. 2 A), -Aox3p, or -Aox5p (unpublished data) antibodies. Therefore, the five isoforms of Aox form a complex in the peroxisomal matrix. The different Aox isoforms are present in equimolar amounts in the complex (Fig. 2 C), as judged by quantitation of their stoi-

chiometry in L-[^{35}S]methionine-labeled Aox complex immunoprecipitated from the peroxisomal matrix (Fig. 2 D; the number of methionine residues in Aox1p, -2p, -3p, -4p, and -5p are 15, 13, 16, 19, and 17, respectively). No other peroxisomal matrix protein coimmunoprecipitated with the components of the Aox complex under native conditions (Fig. 2 B). The molecular weight of the Aox complex recovered from the peroxisomal matrix was ~443 kD (Fig. 2 E), based on cofractionation with a molecular weight standard. From these observations and a consideration of the molecular weights of each isoform of Aox (~80 kD), we conclude that Aox is present in the peroxisomal matrix of wild-type cells as a 443-kD heteropentameric complex composed of one polypeptide chain of each of Aox1p to Aox5p. Our data

Figure 3. **Assembly of the heteropentameric Aox complex occurs in the cytosol and precedes its import into the peroxisome.** (A) Spheroplasts of the wild-type strain *P01d* grown in YPBO for 9 h were pulse-labeled with *L*-[^{35}S]methionine for 0.5, 1 or 2 min, treated with ice-cold 10 mM NaN$_3$ to terminate incorporation of radiolabeled methionine into cell protein, and lysed. Lysed spheroplasts were immediately subjected to centrifugation at 200,000 *g* to yield 200KgS (cytosolic, *C*) and 200KgP (organellar, *P*) subcellular fractions. Both fractions were incubated with, or without, the noncleavable cross-linker, DSS. Samples were then subjected to immunoprecipitation under denaturing conditions with anti-Aox1p antibodies. (B) Fluorograms in A were quantitated by densitometry. To quantitate the percentages of Aox1p monomers detected in samples treated with DSS at the indicated times of pulse-labeling, the levels of Aox1p detected in untreated samples at the same times of labeling were set to 100%. To quantitate the percentages of Aox1p-containing oligomers at the indicated times of pulse-labeling, the maximal level of oligomer, which was detected in the DSS-treated sample by 2 min of labeling, was set to 100%. (C) Spheroplasts of the wild-type strain as in A were pulse-labeled with *L*-[^{35}S]methionine for 2 min and subjected to subcellular fractionation to yield a 200KgS (cytosolic) fraction. Cytosolic proteins were subjected to immunoprecipitation under native conditions as described in the legend to Fig. 2 A. (D) Pulse-labeled but unchased cytosolic proteins were subjected to a first immunoprecipitation under native conditions, as described in the legend to Fig. 2 A. Immunoprecipitated proteins that eluted with 100 mM glycine (pH 2.8) were divided into three equal aliquots that were subjected to a second immunoprecipitation under denaturing conditions with anti-Aox1p, -Aox3p or -Aox5p antibodies linked to protein A-Sepharose. (E) The stoichiometry of the cytosolic Aox complex was quantitated as described in the legend to Fig. 2 D. (F) Pulse-labeled but unchased cytosolic proteins of the wild-type (*P01d*) strain were fractionated by centrifugation on a glycerol gradient. Equal volumes of each fraction were subjected to immunoprecipitation under denaturing conditions with anti-Aox1p, -Aox3p, or -Aox5p antibodies. Immunoprecipitated proteins in (A), (C), (D) and (F) were resolved by SDS-PAGE and visualized by fluorography.
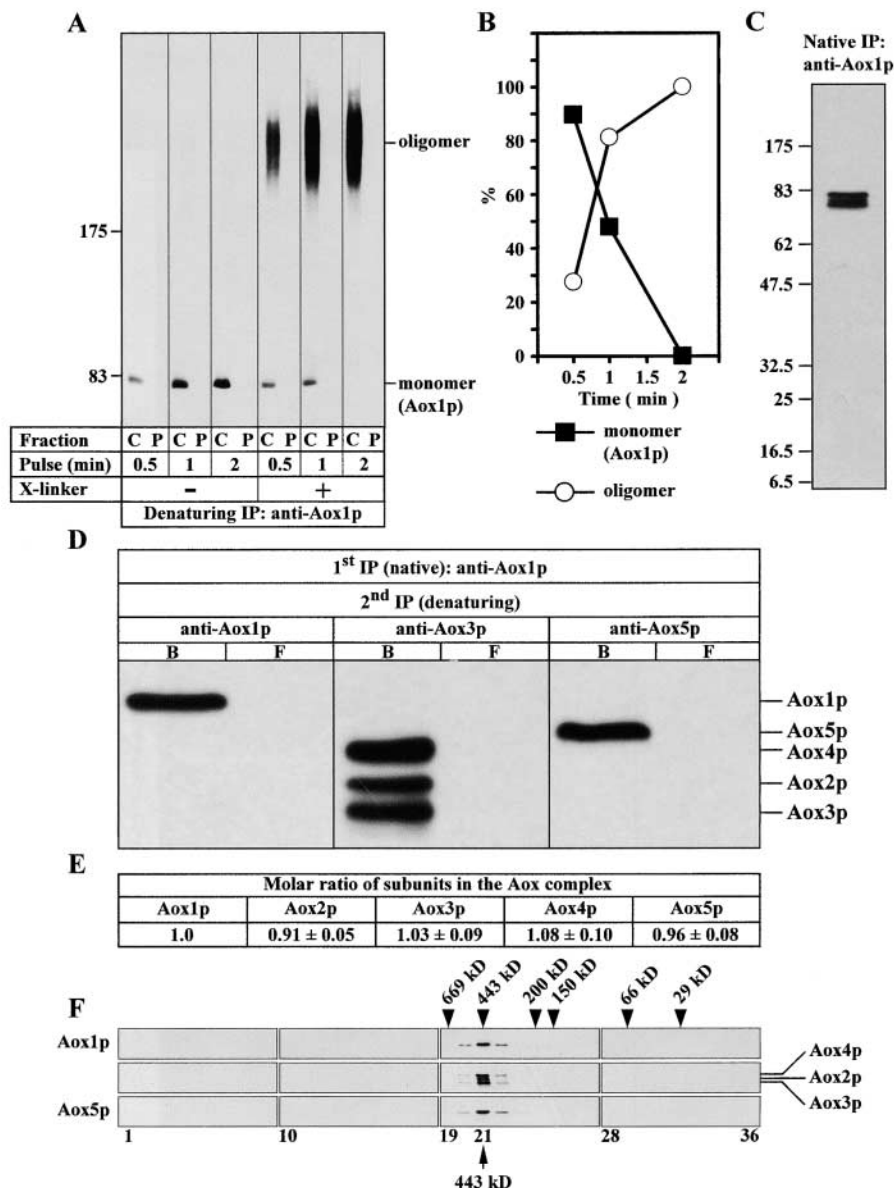
also show that this heteropentameric complex is the only Aox complex in the peroxisomal matrix and that no Aox isoform is present in the peroxisomal matrix as a monomer. Indeed, all five Aox isoforms from the peroxisomal matrix peaked in concentration in fraction 21 of a linear glycerol gradient (Fig. 2 E), cofractionating with the 443-kD molecular weight standard. Furthermore, no Aox isoform was recovered in the flow-through when native immunoprecipitation of peroxisomal matrix proteins was performed with anti-Aox1p (Fig. 2 A), -Aox3p, or -Aox5p (unpublished data) antibodies.

## The heteropentameric Aox complex assembles in the cytosol

We evaluated the ability of all five Aox isoforms to undergo oligomerization in the cytosol before their import into peroxisomes. The 200KgS (cytosolic) and 200KgP (organellar) fractions of wild-type cells pulse-labeled with L-[^{35}S]methionine for 0.5, 1 or 2 min were incubated with, or without, the noncleavable, amine-reactive, homobifunctional cross-linker, disuccinimidyl suberate (DSS). Samples were then subjected to immunoprecipitation under denaturing conditions with antibodies specific to the different

Aox isoforms. In samples not exposed to cross-linker, the levels of radiolabeled Aox1p (Fig. 3 A, X-linker −), -2p, -3p, -4p, and -5p (unpublished data) in the cytosol gradually increased with increasing time of pulse-labeling, reflecting the ongoing synthesis of Aox monomers in the cytosol. In contrast, in samples treated with cross-linker, the levels of radiolabeled Aox1p (Fig. 3 A, X-linker +, and B), -2p, -3p, -4p, and -5p (unpublished data) monomers in the cytosol decreased with increasing time of pulse-labeling, whereas the levels of cytosolic oligomers containing Aox1p (Fig. 3 A, X-linker +, and B), -2p, -3p, -4p, and -5p (unpublished data) increased proportionally. The oligomerization of Aox monomers in the cytosol occurs with a half-time of ∼1 min (Fig. 3 B), and, by 2 min of pulse-labeling, Aox1p (Fig. 3 A, X-linker +, and B), -2p, -3p, -4p, and -5p (unpublished data) can be detected only in oligomers. It should be stressed that the observed changes in the levels of cytosolic Aox monomers and oligomers were not due to peroxisome leakage during subcellular fractionation, as neither monomeric nor oligomeric forms of Aox1p (Fig. 3 A, X-linker +, and B), -2p, -3p, -4p, or -5p (unpublished data) were detected in the 200KgP (organellar) fraction, even after 2 min of pulse-labeling. Importantly, the integ-
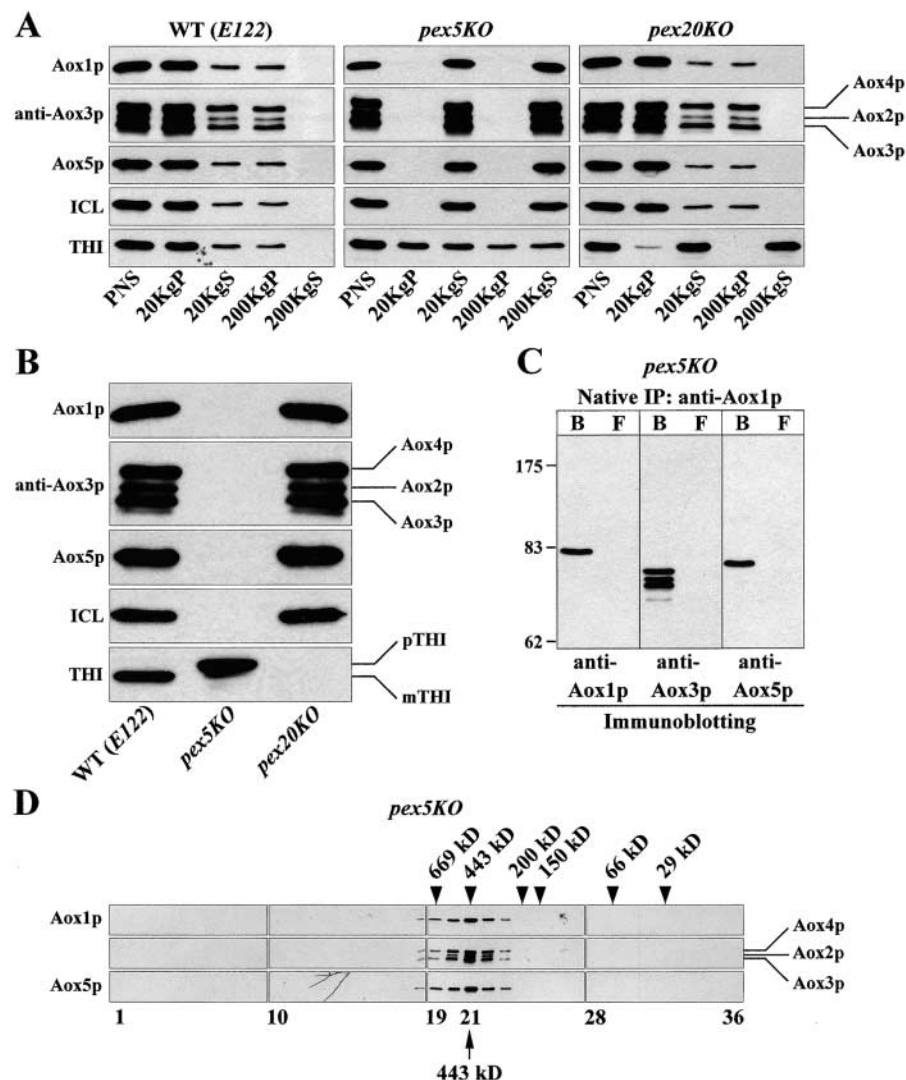
Figure 4. **Peroxisomal targeting, but not assembly in the cytosol, of the Aox complex is abolished by the *pex5KO* mutation.** (A) Equal portions (0.1% of the total volume) of the indicated subcellular fractions isolated from YPBO-grown wild-type (*E122*), *pex5KO*, and *pex20KO* cells were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies to the indicated peroxisomal matrix proteins. (B) Equal amounts (10 μg) of protein from peroxisomes purified from YPBO-grown wild-type (*E122*), *pex5KO*, and *pex20KO* cells were analyzed by immunoblotting with antibodies to the proteins indicated. The positions of the 47-kD precursor form (pTHI) and of the 45-kD mature form (mTHI) of THI are indicated. (C) The 200KgS (cytosol) fraction of YPBO-grown *pex5KO* cells was subjected to immunoaffinity chromatography under native conditions with anti-Aox1p antibodies linked to protein A-Sepharose. Samples were then processed and analyzed by immunoblotting with the indicated antibodies as described in the legend to Fig. 2 A. (D) The 200KgS (cytosol) fraction of YPBO-grown *pex5KO* cells was subjected to centrifugation on a glycerol gradient. The gradient was fractionated, and equal volumes of fractions were analyzed by immunoblotting with anti-Aox1p, -3p, or -5p antibodies.

Figure 5. **Effects of single and multiple *AOX* knock-out mutations on the subcellular localization of Aox isoforms.** Equal portions (0.1% of the total volume) of the indicated subcellular fractions from YPBO-grown wild-type (*P01d*) and *aox* mutant cells were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.
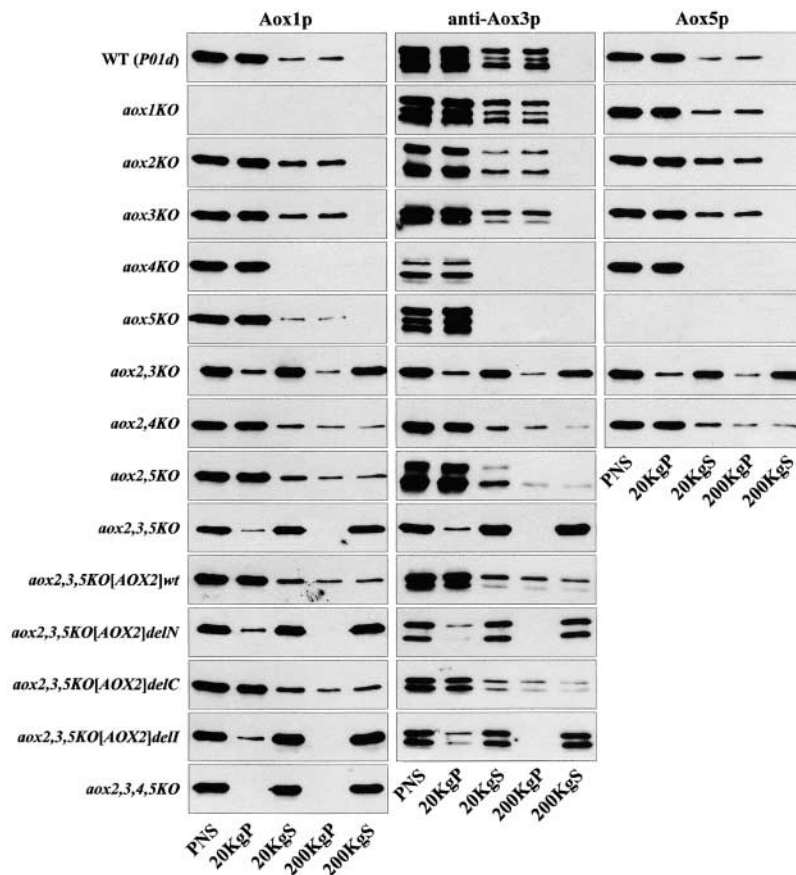
rity of peroxisomes was preserved under these experimental conditions, as shown by the results of pulse-chase analysis of the peroxisomal import of preassembled cytosolic Aox complexes (see Fig. 7 B). After a 2-min pulse of wild-type cells with L-[^{35}S]methionine, all labeled isoforms of Aox were found exclusively in the 443-kD heteropentameric cytosolic complex (Fig. 3 F) composed of one polypeptide chain of each of the five isoforms (Fig. 3, D and E). No other cytosolic protein coimmunoprecipitated under native conditions with the components of the Aox complex (Fig. 3 C). Similar to the Aox complex from the peroxisomal matrix, the 443-kD heteropentameric Aox complex is the only form of Aox complex found in the cytosol (Fig. 3 F). By 2 min of pulse-labeling, no Aox isoform was detected in the cytosol as a monomer (Fig. 3, A, D, and F). Together, these data indicate that assembly of the 443-kD heteropentameric Aox complex from the five newly synthesized Aox monomers occurs in the cytosol and precedes import of the complex into the peroxisome.

Peroxisomal targeting of the Aox complex is abolished in a pex5KO mutant strain

We have previously identified the *Y. lipolytica* peroxin Pex5p as a component of the peroxisomal targeting machinery for most matrix proteins (Szilard et al., 1995). We have also shown that the cytosolic chaperone Pex20p is required for oligomerization of THI in the cytosol and for its targeting to the peroxisome (Titorenko et al., 1998). We assessed the effects of mutations in the *PEX5* and *PEX20*

genes on the assembly of the Aox complex in the cytosol and its targeting to the peroxisome. In *pex5KO* cells, all five Aox isoforms were found exclusively in the 200KgS (cytosol) fraction (Fig. 4 A) and were accessible to externally added protease (unpublished data). Similarly, the *pex5KO* mutation abolished peroxisomal targeting of ICL (Fig. 4 A) (Szilard et al., 1995), which is targeted to the matrix by a COOH-terminal PTS1 (Barth and Scheuber, 1993). No Aox isoform and none of ICL associated with peroxisomes purified from YPBO-grown *pex5KO* cells (Fig. 4 B). All five Aox isoforms accumulated in the cytosol of *pex5KO* cells and were found exclusively as components of the 443-kD heteropentameric Aox complex (Fig. 4, C and D). Therefore, the *pex5KO* mutation completely abolishes peroxisomal targeting of the Aox complex but does not affect its assembly in the cytosol.

In *pex20KO* cells, as in wild-type cells, no Aox isoform was found in the cytosol (Fig. 4 A). Moreover, all five isoforms were peroxisomal in *pex20KO* cells (Fig. 4 B) and were found in the matrix only as components of the 443-kD heteropentameric Aox complex (unpublished data). In contrast, the *pex20KO* mutation completely abolished both the assembly in the cytosol (Titorenko et al., 1998) and the peroxisomal targeting (Fig. 4, A and B) (Titorenko et al., 1998) of the oligomeric peroxisomal matrix protein, THI. Therefore, unlike the oligomerization and peroxisomal import of THI, neither the assembly of the Aox complex in the cytosol nor its targeting to the peroxisome is mediated by the cytosolic chaperone, Pex20p.
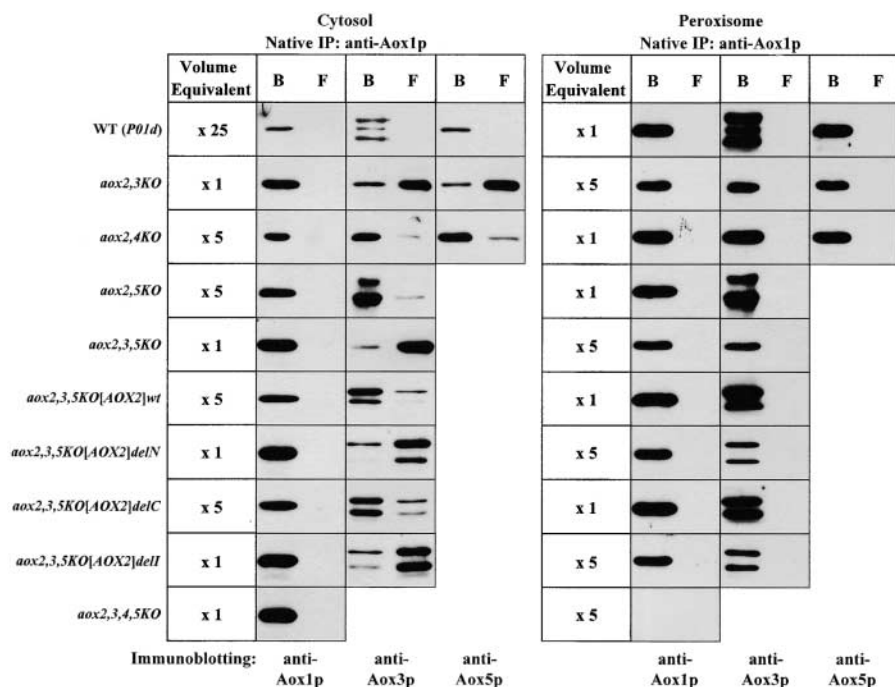
Figure 6. **Effects of multiple *AOX* knock-out mutations on the oligomeric state of Aox complex in the cytosol and in the peroxisomal matrix.** The 200KgS (cytosol) fraction (left) and matrix proteins from purified peroxisomes (right) of YPBO-grown wild-type (*P01d*) and *aox* mutant cells were subjected to immunoaffinity chromatography under native conditions using anti-Aox1p antibodies coupled to protein A-Sepharose. Samples were subsequently processed and analyzed by immunoblotting with the indicated antibodies as described in the legend to Fig. 2 A. Volume equivalents of the 200KgS fraction and protein equivalents of the peroxisomal matrix proteins are indicated. One volume equivalent is equal to 200 µl of 200KgS. One protein equivalent is equal to 10 µg of matrix proteins.

Lack of both Aox2p and Aox3p affects assembly of the remaining Aox isoforms into a cytosolic complex and their targeting to the peroxisome

We evaluated a possible role for the different Aox isoforms in assembling the Aox complex in the cytosol and in importing the complex into the peroxisome. No mutation knocking out a single *AOX* gene affected the subcellular localization of the isoforms encoded by the remaining *AOX* genes (Fig. 5). Similar to the heteropentameric Aox complex in wild-type cells, the heterotetrameric complexes of Aox were found exclusively in the matrix of peroxisomes in all five single *AOX* knock-out mutants (unpublished data). In contrast, Aox1p, -4p, and -5p were for the most part not targeted to peroxisomes and remained cytosolic in the double *aox2KO aox3KO* mutant (Fig. 5). Aox1p, -4p, and -5p accumulated primarily as monomers in the cytosol of *aox2KO aox3KO* cells, as the major fraction of the cytosolic pool of any of them could not be coimmunoprecipitated under native conditions by either anti-Aox1p (Fig. 6, left) or anti-Aox3p (unpublished data) antibodies. Neither of the two other double *AOX* knock-out mutants tested, *aox2KO aox4KO* and *aox2KO aox5KO*, nor the *aox2KO aox3KO aox5KO* [*AOX2*]*wt* strain carrying a chromosomal copy of a full-length *AOX2* gene (i.e., genotypically *aox3KO aox5KO*) was as severely affected either in assembly of the Aox complex in the cytosol (Fig. 6, left) or in the peroxisomal targeting of the remaining intact Aox isoforms (Fig. 5) as was the double *aox2KO aox3KO* mutant. Therefore, lack of both Aox2p and Aox3p causes the most severe defects in assembly of the remaining Aox monomers into a cytosolic complex and in their targeting to the peroxisome. Accordingly, these two isoforms of Aox play a key role in Aox complex formation in the cytosol and can substitute for one another in promoting assembly of the complex.

At least two portions of Aox2p, namely its NH$_2$-terminal 15 amino acids and its segment between amino acids 165 and 175, but not its COOH-terminal 15 amino acids, play a role in assembly of the Aox complex in the cytosol and in the peroxisomal targeting of the other Aox isoforms. In contrast to the full-length *AOX2* gene and to its [*AOX2*]*delC*-truncated copy, neither the [*AOX2*]*delN* nor the [*AOX2*]*delI*-truncated copy of the *AOX2* gene was able to restore either the assembly of Aox monomers into complex (Fig. 6, left) or their targeting to the peroxisome (Fig. 5) when chromosomally integrated into the *aox2KO aox3KO aox5KO* mutant. Interestingly, hydropathy analysis predicted that the Aox2p segment between amino acids 165 and 175 contains a membrane-associated helix, which may act in targeting or tethering Aox2p to the peroxisomal membrane.

Assembly of the Aox complex in the cytosol is required for its import into peroxisomes

Quantitation of the immunoblots presented in Figs. 5 and 6 showed that, in any of the multiple *AOX* knock-out mutants tested, the efficiency of peroxisomal import of different Aox monomers is directly proportional to the efficiency of their assembly into a cytosolic complex (Fig. 7 A). Moreover, all isoforms recovered in the peroxisomal matrix of any of the *AOX* mutants tested associate into heterooligomeric complexes, and none of them is present in monomeric form. Indeed, not only were all peroxisomal Aox isoforms coimmunoprecipitated under native conditions by either anti-Aox1p (Fig. 6, right) or anti-Aox3p (unpublished data) antibodies, but also no isoform was recovered in the flow-through. Together, these data indicate that only oligomeric Aox complexes are competent for targeting (binding) to the peroxisome and are able to penetrate into the peroxisomal matrix. In contrast, no component of the Aox complex can be imported into the peroxisome as a monomer. Therefore, the Aox2p- and Aox3p-assisted assembly of Aox complex in the cytosol is required for and drives import of the complex into the peroxisomal matrix.
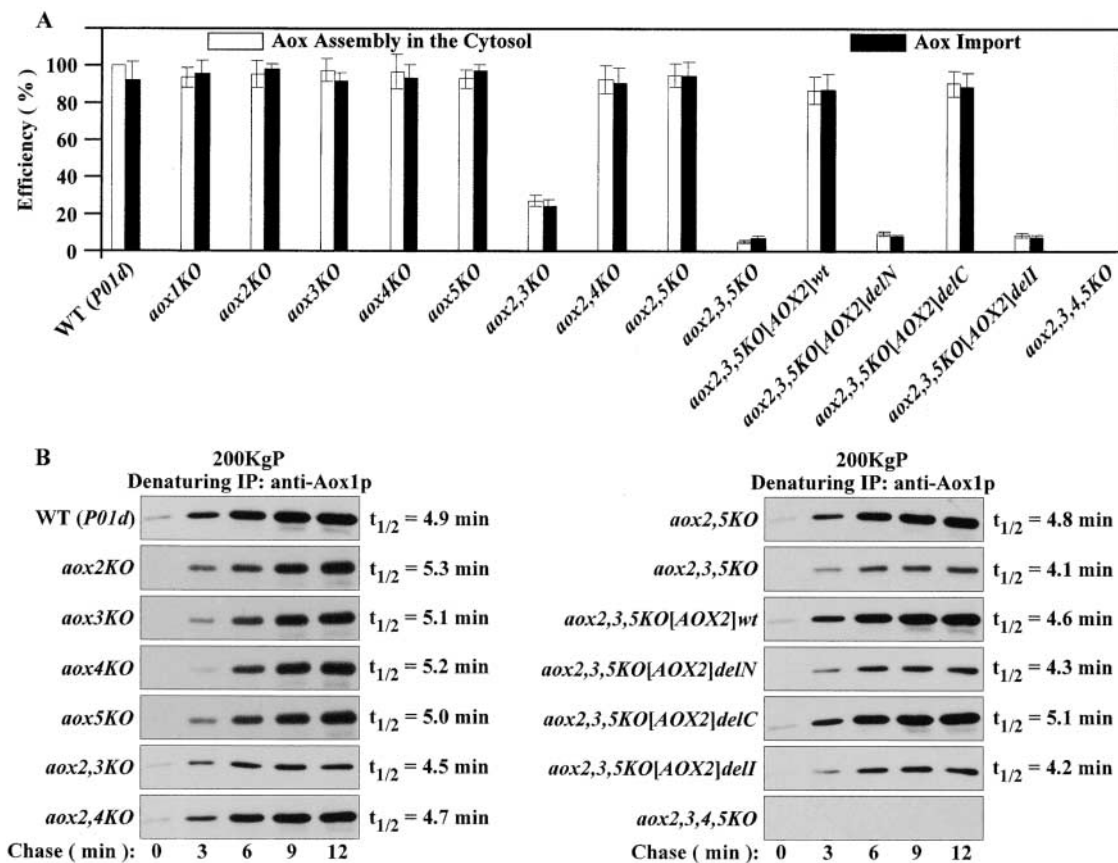
Figure 7. **Effects of multiple *AOX* knock-out mutations on the efficiency and rate of Aox complex assembly in the cytosol and import into peroxisomes.** (A) The efficiencies of Aox complex assembly in the cytosol were quantitated by densitometric analysis of the immunoblots presented in Fig. 6 (left) and are expressed as a percentage of the different Aox isoforms coimmunoprecipitated under native conditions by either anti-Aox1p or anti-Aox3p (unpublished data) antibodies relative to their total recoveries (protein bound to the immunoaffinity column plus unbound protein in the flow-through). The efficiencies of peroxisomal import of the Aox complex were quantitated by densitometric analysis of the immunoblots presented in Fig. 5 and are expressed as a percentage of the different Aox isoforms recovered in the 20KgP plus 200KgP fractions relative to their recoveries in the postnuclear supernatant fraction. The values reported are the means ± SD of two independent experiments for the five isoforms of Aox. (B) Spheroplasts of cells of the wild-type strain (*P01d*) and of *aox* mutant strains grown in YPBO for 9 h were pulse-labeled for 2 min with L-[^{35}S]methionine and chased with unlabeled L-methionine. Samples were taken at the indicated times after chase. Spheroplasts were subjected to subcellular fractionation to yield a 200KgP fraction. Aox1p was immunoprecipitated under denaturing conditions from the 200KgP. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Fluorograms were quantitated by densitometry. The maximal level of Aox1p in the 200KgP fraction of a particular strain was set to 100%. Half-times for the import of Aox1p into the 200KgP were calculated.

Remarkably, while the efficiencies (steady-state levels) of peroxisomal targeting/import of Aox complexes composed of different Aox isoforms vary significantly and are directly proportional to the efficiencies (steady-state levels) of their assembly in the cytosol, the rates of peroxisomal targeting/import of preassembled cytosolic complexes appear to be independent of the subunit number and composition of the complexes. The half-times for the targeting of different Aox complexes from the cytosol to high-speed pelletable peroxisomes localized to the 200KgP (Titorenko et al., 1998, 2000), quantitated for pulse-labeled and chased Aox1p, were similar for the wild-type strain and for any of the multiple *aox* mutants tested (Fig. 7 B). These data also demonstrate that, in contrast to their key role in Aox complex assembly in the cytosol, neither Aox2p nor Aox3p is directly required for the peroxisomal targeting/import of the Aox complex once it is assembled in the cytosol.

One possible explanation for our data on the half-times for peroxisomal import of preassembled Aox complexes with different numbers and compositions of subunits is that although a heteropentamer is always translocated across the peroxisomal membrane (inside the peroxisomal matrix of single, double, and triple *aox* mutants), redundant Aox subunits used to compensate for missing Aox subunits dissociate more readily from the complex. However, this possibility was ruled out in experiments involving the cross-linking and subsequent denaturing immunoprecipitation of pulse-labeled, newly synthesized cytosolic Aox complexes and radiolabeled Aox complexes newly chased from the cytosol to the peroxisomal matrix. In fact, the oligomeric states of the Aox heteropentamer, heterotetramer, heterotrimer, or heterodimer assembled in the cytosol of a particular wild-type or *aox* mutant strain were the same as those found in the peroxisomal matrix soon after translocation of the complex across the peroxisomal membrane (Fig. 8).
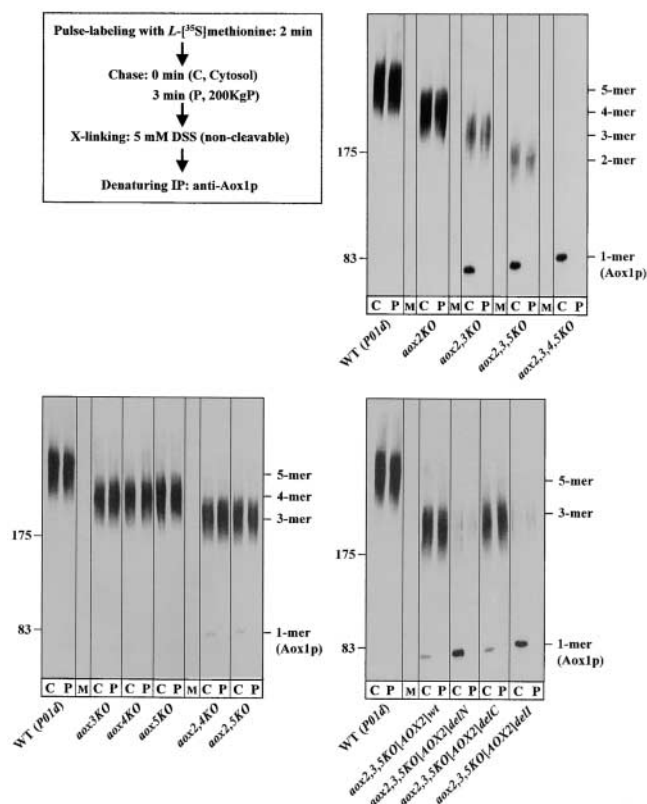
Figure 8. **Aox complexes of different subunit number and composition are imported into the peroxisomal matrix in the oligomeric state in which they were preassembled in the cytosol.** Spheroplasts of wild-type and *aox* mutant strains grown in YPBO for 9 h were pulse-labeled with *L*-[^{35}S]methionine for 2 min. One aliquot of each sample was not subjected to a chase period and was immediately used for subcellular fractionation to yield a 200KgS (cytosolic, *C*) fraction. The other aliquot was chased with unlabeled *L*-methionine for 3 min, and was subsequently used to isolate a 200KgP (organellar, *P*) fraction as described in the legend to Fig. 3 A. Both the *C* and *P* fractions were incubated with the noncleavable cross-linker, DSS. Samples were then subjected to immunoprecipitation under denaturing conditions with anti-Aox1p antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by fluorography. *M*, protein molecular weight markers.

Aox2p and Aox3p are not required for the acquisition of the cofactor FAD by other components of the Aox complex

Peroxisomal and cytosolic Aox complexes from the wild-type strain and mutant strains carrying various combinations of knock-out mutations in different *AOX* genes were purified as described in Materials and methods. UV-visible absorption spectroscopy of a cofactor extracted from the purified complexes demonstrated that, like peroxisomal Aox previously identified in humans, other mammals, plants, and various yeast species (van den Bosch et al., 1992), Aox complexes isolated from the peroxisomes of wild-type *Y. lipolytica* cells and the cytosol of *pex5KO* mutant cells contain FAD as a cofactor (Fig. 9 A). Quantitation of the FAD attached to the wild-type peroxisomal or the *pex5KO* cytosolic Aox complex showed that five FAD molecules are associated with each of these heteropentameric complexes (Fig. 9 F). These data suggest that each subunit of an Aox complex assembled in the cytosol is associated with one

molecule of FAD. Importantly, cytosolic Aox1p monomers purified from *aox2KO aox3KO aox4KO aox5KO* and *aox2KO aox3KO aox5KO* cells (Fig. 9, B and F), as well as Aox3p, -4p, and -5p monomers isolated from the cytosol of various *aox* mutants (Fig. 9, C, D, and E, respectively), are each attached to one FAD molecule (Fig. 9 F). Therefore, attachment of FAD to any one of the five different Aox monomers occurs in the cytosol and precedes assembly of these monomers into heterooligomeric, peroxisome import–competent complexes in the cytosol of wild-type and *aox* mutant cells. The above data also demonstrate that, in contrast to their essential role in the assembly of Aox complex in the cytosol, neither Aox2p nor Aox3p is required for the attachment of FAD to the other components of the Aox complex.

Aox2p and Aox3p retard GuHCl-induced disassembly of the Aox complex

The quaternary structure of the Aox complex was dramatically affected at 500 mM GuHCl, as complexes isolated from the cytosol of wild-type and *aox* mutant cells were almost completely disassembled into their monomeric subunits at this concentration of GuHCl (Fig. 10, A and B). We therefore examined the GuHCl-induced disassembly of purified cytosolic Aox complexes composed of various combinations of Aox monomers at concentrations of GuHCl $\leq$500 mM. The heterotrimeric Aox complex lacking both Aox2p and Aox3p disassembled into monomers at significantly lower concentrations of GuHCl ($C_{50\%}$ = 46 mM) than the heteropentameric cytosolic Aox complex purified from wild-type cells ($C_{50\%}$ = 171 mM) (Fig. 10, B and C). In contrast, no significant differences in the efficiencies of in vitro disassembly were observed for cytosolic Aox complexes isolated from wild-type cells (Fig. 10, B and C) and from cells of any of five single *AOX* knock-out mutants (unpublished data), cells of the other two double *AOX* knock-out mutants tested, *aox2KO aox4KO* and *aox2KO aox5KO* (Fig. 10, B and C), and cells of the *aox2KO aox3KO aox5KO[AOX2]wt* mutant carrying a chromosomal copy of a full-length *AOX2* gene (Fig. 10, B and C). Considering that, as in any chemical reaction, assembly of the Aox complex is a reversible process, these data strongly suggest that Aox2p and Aox3p specifically decrease the rate of the reverse reaction and, therefore, retard disassembly of the complex. The above data also demonstrate that Aox2p and Aox3p can substitute for one another in retarding Aox complex disassembly in vitro. In contrast to the products of the full-length *AOX2* gene and its [*AOX2*]*delC*-truncated copy, the products of both the [*AOX2*]*delN* and [*AOX2*]*delI*-truncated copies of the *AOX2* gene were unable to retard disassembly of the Aox complex in vitro (Fig. 10 C) when expressed in the *aox2KO aox3KO aox5KO* mutant. Therefore, at least two portions of Aox2p, namely its NH$_2$-terminal 15 amino acids and the segment between amino acids 165 and 175, but not its COOH-terminal 15 amino acids, play a role in retarding Aox complex disassembly.

Reassembly of the Aox complex lacking both Aox2p and Aox3p is retarded

Cytosolic Aox complex purified from wild-type cells was disassembled into monomers by treatment with 500 mM GuHCl. Following dilution in buffer D to yield a concentration of GuHCl of 20 mM, these monomers efficiently reassembled into
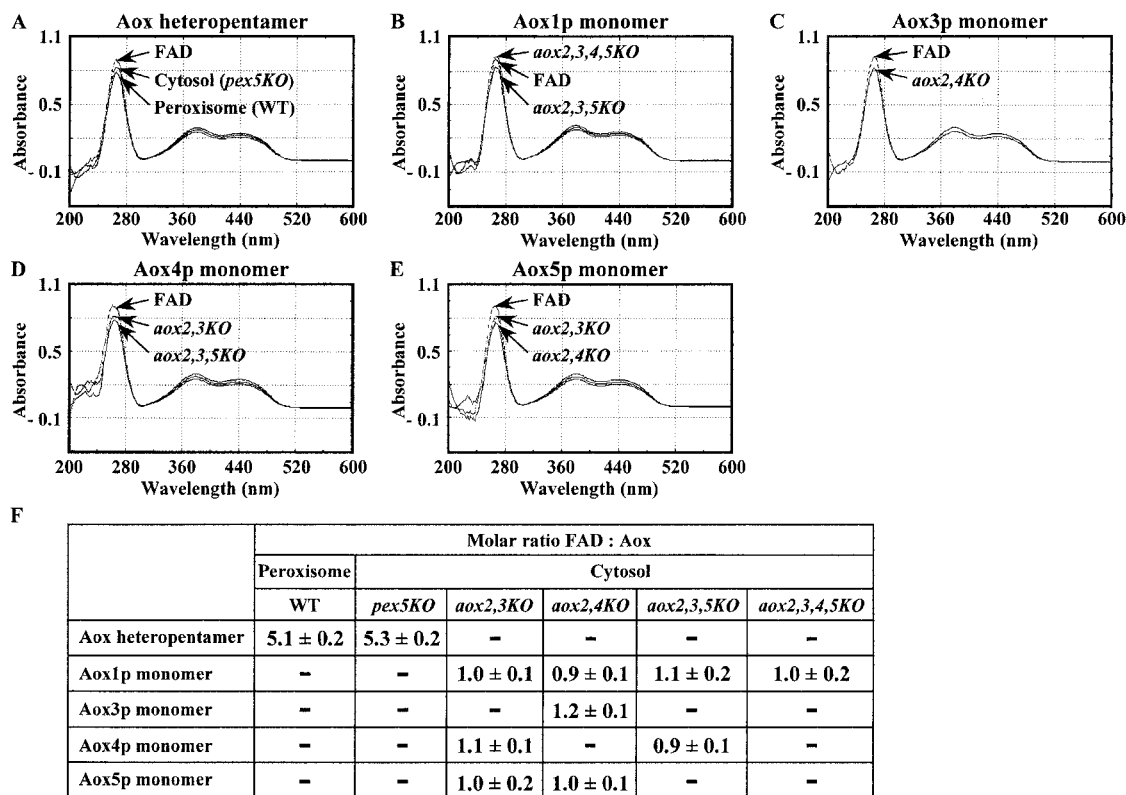
Figure 9.   **Aox2p and Aox3p are not required for attachment of the cofactor FAD to the remaining components of the Aox complex.** (A) Absorption spectrum of the cofactor extracted from Aox complex purified from peroxisomes of wild-type cells or the cytosol of *pex5KO* mutant cells. For comparison, the absorption spectrum of commercial FAD is presented. (B–E) Absorption spectrum of the cofactor extracted from Aox1p (B), -3p (C), -4p (D), or -5p (E) monomers purified from the cytosol of the indicated *aox* mutants. (F) Molar ratios "FAD:Aox" determined for peroxisomal and cytosolic Aox heteropentamers purified from wild-type and *pex5KO* mutant cells, and for cytosolic Aox monomers purified from *aox* mutant cells.

the heteropentameric Aox complex (Fig. 11). Under the same conditions, the heterotrimeric Aox complex lacking both Aox2p and Aox3p reassembled much less efficiently (Fig. 11). In contrast, cytosolic Aox complexes purified from any of the single *AOX* knock-out mutants (unpublished data), from the double *AOX* knock-out mutants *aox2KO aox4KO* and *aox2KO aox5KO* (Fig. 11), and from the *aox2KO aox3KO aox5KO[AOX2]wt* mutant carrying a full-length *AOX2* gene (Fig. 11) reassembled with approximately the same efficiency as Aox complex purified from the cytosol of wild-type cells. However, in contrast to the products of the full-length *AOX2* gene and to its *[AOX2]delC*-truncated copy, the products of the *[AOX2]delN* and *[AOX2]delI*-truncated copies of the *AOX2* gene were unable to promote the in vitro reassembly of the Aox complex when expressed in the *aox2KO aox3KO aox5KO* mutant (Fig. 11). Together, these data support the above suggestion that, in the reversible process of Aox complex formation, Aox2p and Aox3p specifically retard disassembly of the complex and therefore increase its steady-state concentration.

Discussion

Assembly of Aox complex in the cytosol is mandatory for its import into peroxisomes

Here we report that the attachment of the cofactor FAD to the five isoforms of Aox, followed by their assembly into a

heteropentameric complex, occur in the cytosol and precede import of the Aox complex into the peroxisome. We also demonstrate that oligomeric Aox complexes composed of various combinations of different isoforms are equally competent for peroxisomal targeting/import. In contrast, no component of the Aox complex can be imported into the peroxisome as a monomer. Therefore, similar to the peroxisomal targeting/import of another matrix protein, THI (Titorenko et al., 1998), formation of Aox complex in the cytosol is a prerequisite for its targeting to, and import into, the peroxisome. Assembly of a heterooligomeric complex is also required for the plasma membrane targeting and translocation of bacterial hydrogenase 2 that is exported by the Tat pathway (Rodrigue et al., 1999), which is known to accommodate folded, cofactor-containing, and oligomeric polypeptides (Dalbey and Robinson, 1999; Teter and Klionsky, 1999). Thus, while protein unfolding is mandatory for the membrane translocation of many proteins (McNew and Goodman, 1996; Schatz and Dobberstein, 1996), folding, cofactor attachment and oligomerization of some peroxisomal and bacterial proteins appear to be required for their transport across membranes.

Assembly of Aox complex in the cytosol

Our kinetic data show that at the end of a short pulse with radiolabeled methionine, all labeled isoforms of Aox assembled in the cytosol into a heteropentameric Aox complex, and no iso-
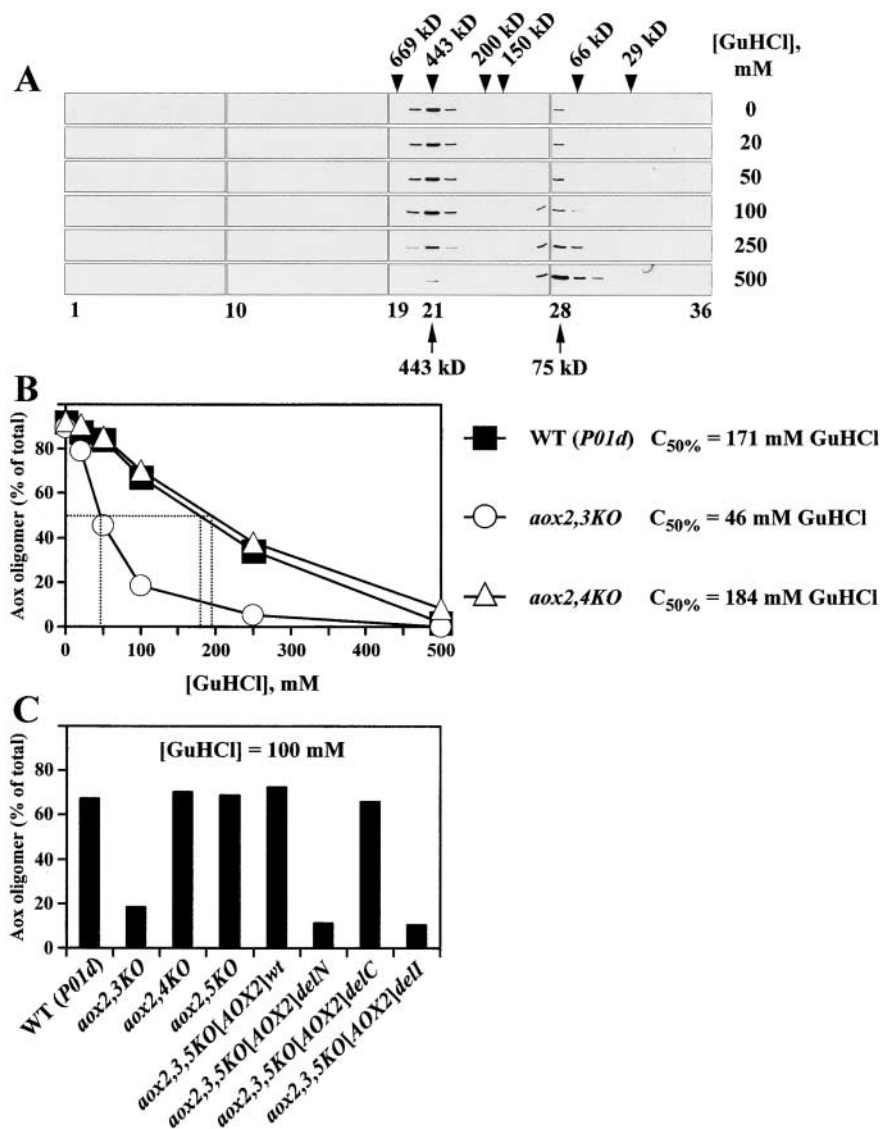
Figure 10. **Effects of multiple *AOX* knock-out mutations on the disassembly of Aox complexes in vitro.** (A) The in vitro disassembly of Aox complexes purified from the cytosol of wild-type (*P01d*) cells was performed at the indicated concentrations of GuHCl as described in Materials and methods. Samples were subjected to centrifugation on glycerol gradients in buffer D containing GuHCl at the concentrations used for disassembly. Proteins recovered from equal volumes of gradient fractions were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Aox1p antibodies. Arrows indicate peak fractions 21 and 28 for the 443-kD heteropentameric Aox complex and the 75-kD monomer of Aox1p, respectively. (B and C) The percentage of heterooligomeric Aox complex recovered at the indicated concentrations of GuHCl was quantitated by densitometry of the immunoblots presented in A. Data for Aox complexes purified from the cytosol of wild-type and *aox* mutant cells are presented. $C_{50\%}$, the transition midpoint of the GuHCl-induced disassembly of a particular Aox complex.

form was found in the cytosol as a monomer. Therefore, similar to two other peroxisomal matrix proteins whose assembly in the cytosol precedes peroxisomal targeting/import, THI and ICL (Titorenko et al., 1998), formation of Aox complex in the cytosol is very rapid. API, which is transported to yeast vacuoles by the Cvt pathway, another nonclassical protein transport pathway, is also rapidly assembled in the cytosol with a rate comparable to that for the Aox complex (Kim et al., 1997). We speculate that rapid assembly of oligomeric complexes in the cytosol could provide an advantage to nonclassical mechanisms of protein transport and has been evolved to ensure rapid binding of oligomeric proteins to their target membrane.

We have also demonstrated that, similar to the assembly of some other peroxisomal (Titorenko et al., 1998), vacuolar (Klionsky and Ohsumi, 1999), and bacterial (Rodrigue et al., 1999) oligomeric proteins, assembly of Aox complex in the cytosol does not require the attachment of any Aox monomer to the target organelle membrane. In fact, in the *pex5KO* mutant deficient in Aox binding to the peroxisomal membrane, the efficiency of self-assembly of Aox complex in the cytosol was not compromised, and none of the Aox monomers was associated with the peroxisomal membrane.

We have identified FAD as a cofactor of *Y. lipolytica* Aox and shown that each subunit of the Aox complex is associated with one molecule of FAD. We have also shown that neither Aox2p nor Aox3p is required for acquisition of FAD by the other components of the complex. This is in contrast to dimeric, nickel-containing hydrogenase 2 of *Escherichia coli*, which is exported by the Tat pathway. The small subunit of this protein complex is required for acquisition of nickel by the large subunit (Rodrigue et al., 1999).

Analysis of the assembly of the Aox complex revealed that two subunits of the complex, Aox2p and Aox3p, play a pivotal role and can substitute for one another in establishing the quaternary structure of the Aox complex. In vitro, Aox2p and Aox3p retard disassembly of the Aox complex and thereby increase the efficiency of its reassembly from individual subunits.

Two distinct pathways for the peroxisomal import of oligomeric proteins

At least two distinct pathways for the peroxisomal import of oligomeric proteins exist in *Y. lipolytica*. One pathway is mediated by the cytosolic chaperone Pex20p, which is required for the oligomerization of THI in the cytosol and its target-

Figure 11. **Effects of multiple *AOX* knock-out mutations on reassembly of Aox complexes in vitro.** (A) In vitro reassembly of Aox complexes disassembled by treatment with 500 mM GuHCl was performed as described in Materials and methods. Following dilution with buffer D to yield a concentration of GuHCl of 20 mM and incubation for 1 h at room temperature, samples were subjected to centrifugation on glycerol gradients in buffer D containing 20 mM GuHCl. Proteins recovered from equal volumes of gradient fractions were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Aox1p antibodies. Data for complexes purified from the cytosol of wild-type (*P01d*) and *aox* mutant cells are presented. Arrows indicate peak fractions 21, 23, and 28 for the 443-kD heteropentameric Aox complex, the 250-kD heterotrimeric Aox complexes and the 75-kD monomer of Aox1p, respectively. (B) The immunoblots presented in A were quantitated by densitometry. The percent reassembly of the indicated heterooligomeric Aox complexes is presented.
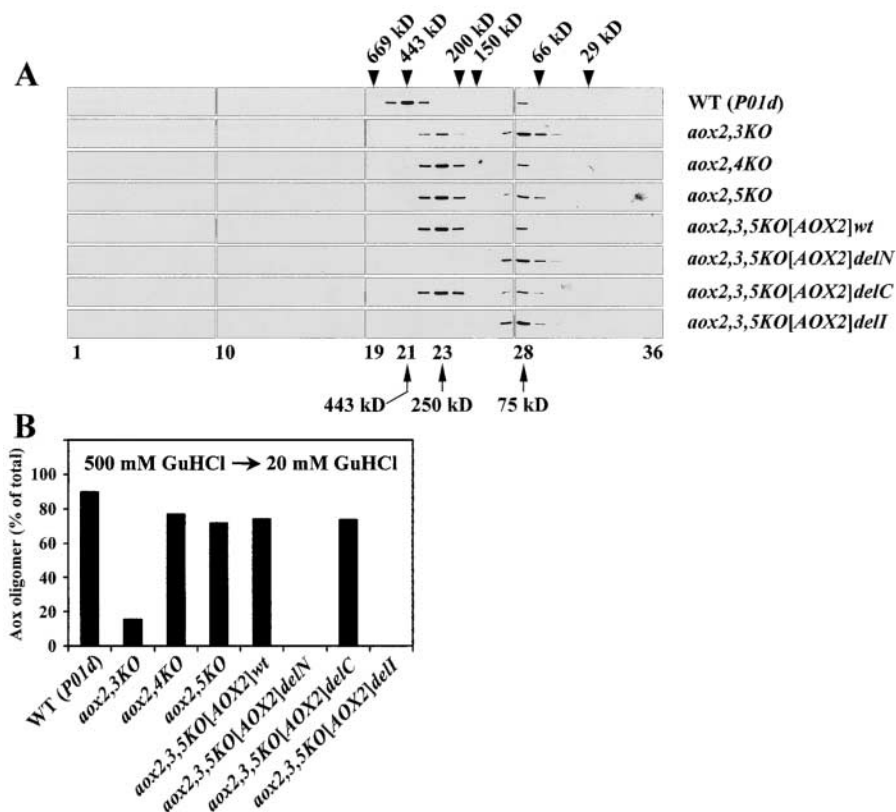
ing to the peroxisome (Titorenko et al., 1998). This pathway does not involve the Pex5p-dependent targeting machinery that acts as the major pathway for protein penetration into the peroxisomal matrix (Szilard et al., 1995). A second oligomeric peroxisomal matrix protein, Aox, is imported into peroxisomes via a Pex20p-independent pathway. Assembly of the Aox complex in the cytosol requires two components, Aox2p and Aox3p, and results in the acquisition of an oligomeric, import-competent conformation. We also demonstrate that the peroxisomal targeting of the Aox complex, but not its assembly in the cytosol, is abolished in the *pex5KO* mutant. Therefore, the peroxin Pex5p, a component of the major targeting pathway for peroxisomal matrix proteins in *Y. lipolytica*, may directly or indirectly (see below) be involved in the import of the Aox complex into the peroxisome.

A mechanism for the translocation of oligomeric Aox complex across the peroxisomal membrane

At present, the best understood mechanism for the translocation of folded, oligomeric proteins is the Cvt pathway for API translocation operating in *S. cerevisiae* (Klionsky and Ohsumi, 1999). The API homododecameric complex, which is preassembled in the cytosol, is sequestered in subvacuolar (nonvacuolar) vesicles that subsequently fuse with the vacuole (Klionsky and Ohsumi, 1999). In *cvt* mutants deficient in the vacuolar targeting of API, oligomers of this protein accumulate either as membrane-associated/protease-accessible or vesicle-sequestered/protease-resistant forms (Teter and Klionsky, 1999). In contrast, in the *Y. lipolytica pex5KO* mutant, preassembled Aox complex accumulates in

the cytosol as a protease-sensitive form and does not associate with a membrane. Therefore, targeting of Aox to the peroxisomal membrane is unlikely to involve the envelopment of preassembled cytosolic Aox complexes by a subperoxisomal (nonperoxisomal) vesicle as an intermediate step.

Two mechanisms for the membrane translocation of oligomeric proteins have been proposed, a poretype mechanism and an endocytosis-like mechanism that is not pore mediated (McNew and Goodman, 1994; Teter and Klionsky, 1999). Morphological studies have not revealed any structure on the peroxisomal surface reminiscent of a nuclear pore (see McNew and Goodman [1994] and references therein). Therefore, it has been suggested that the binding of oligomeric proteins to the peroxisomal membrane could lead to the assembly of a specific set of membrane proteins into dynamic pores that would rapidly dissociate into their component proteins after the completion of protein translocation (McNew and Goodman, 1994). Similar to the pores mediating the translocation of folded proteins by the ΔpH-dependent thylakoid membrane machinery (Teter and Theg, 1998), putative peroxisomal pores might be gated, as the peroxisomal membrane is impermeable to protons, NADH, and NADPH (see Dansen et al. [2000] and references therein). An alternative model for the translocation of oligomeric proteins across peroxisomal membranes proposes that they are internalized in vesicular structures generated by invagination of the peroxisomal membrane in response to the binding of an oligomeric protein (McNew and Goodman, 1994).

Both the pore and endocytic models for the translocation of oligomeric protein complexes predict the peroxisomal

membrane to be a very dynamic structure, undergoing rapid conformational changes. Two alternative scenarios for the translocation of the Aox complex across the peroxisomal membrane can therefore be envisaged. First, the Aox complex could target to the peroxisome via Pex5p-dependent machinery, use a distinct docking site(s) on the peroxisomal membrane and be translocated through a distinct pore. Second, the Aox complex could penetrate into the peroxisomal matrix by an endocytosis-like mechanism that involves invagination of the peroxisomal membrane as an essential step. In the *pex5KO* mutant, the association of the bulk of matrix proteins with the peroxisomal membrane (Szilard et al., 1995) could lead to dramatic changes in its conformation, and thereby abolish the normal functioning of a specific docking factor(s) required for targeting of the Aox complex to the membrane. The resulting impairment of Aox complex binding to the peroxisomal membrane could either prevent its translocation through the pore served by the Pex5p-dependent machinery (first scenario) or could abolish the invagination of the peroxisomal membrane normally resulting from the binding of the Aox complex to the membrane (second scenario).

In conclusion, the results reported herein provide evidence that the Aox2p- and Aox3p-assisted assembly of Aox complex is a mandatory step in the peroxisomal import of this heteropentameric, FAD-containing complex. We have also demonstrated the existence of at least two pathways for the translocation of oligomeric proteins, Aox and THI, across the peroxisomal membrane. The mechanisms by which these two pathways function and the identity of the peroxisomal membrane–associated components serving these translocation pathways are currently being investigated.

Materials and methods

Y. lipolytica strains

The *Y. lipolytica* wild-type strains *E122* (Szilard et al., 1995) and *P01d* (Wang et al., 1999a), the mutant strains *pex5KO* (Szilard et al., 1995) and *pex20KO* (Titorenko et al., 1998), and the single and multiple *AOX* gene knock-out strains (Wang et al., 1999a) have been described. The plasmids pINA1067 and pHSS6 (Neuvéglise et al., 1998) linearized by digestion with NotI were ligated to create the plasmid JMP1. JMP1 was cleaved with HindIII and EcoRI, and a HindIII-ClaI-MluI-HpaI-BamHI-KpnI-EcoRI polylinker was introduced to make the plasmid JMP3. To construct *aox2KO aox3KO aox5KO* strains carrying chromosomal copies of the genes [*AOX2*]wt, [*AOX2*]delN, [*AOX2*]delC, and [*AOX2*]delI, DNA fragments encoding these *AOX2* constructs flanked by HindIII and EcoRI sites were amplified by PCR. The amplified products were placed under control of the *AOX2* promoter by a three-way ligation reaction involving the ClaI-EcoRI fragment from JMP3 and the ClaI-HindIII fragment of the *AOX2* promoter from SMT25 (Wang et al., 1999a). The resultant plasmids were digested with NotI to liberate expression cassettes for the wild-type and truncated copies of the *AOX2* gene carrying the *URA3* gene. These linear constructs were used to transform the *aox2KO aox3KO aox5KO* mutant strain, and Ura$^+$ transformants were selected as described (Wang et al., 1999a). The [*AOX2*]wt gene encodes full-length Aox2p, whereas the [*AOX2*]delN, [*AOX2*]delC and [*AOX2*]delI genes code for truncated forms of Aox2p lacking the NH$_2$-terminal 15 amino acids, the COOH-terminal 15 amino acids or amino acids 165–175, respectively. Media, growth conditions, and genetic techniques for *Y. lipolytica* have been described (Szilard et al., 1995).

Antibodies

Rabbit polyclonal antibodies to the Aox1p, -3p, and -5p isoforms were produced against hexahistidinyl (His$_6$)-tagged versions of the full-length

proteins expressed in *E. coli* (Wang et al., 1999a). Antibodies to isocitrate lyase (ICL), Kar2p, malate synthase (MLS), Pex2p, Pex16p, THI, and Sec14p have been described (Titorenko et al., 1998).

Subcellular fractionation and peroxisome isolation

Subcellular fractionation of *Y. lipolytica* cells grown in oleic acid–containing YPBO medium and isolation of highly purified peroxisomes from a 20,000 *g* pellet fraction (20KgP) by isopycnic centrifugation on a discontinuous sucrose density gradient (Titorenko et al., 1998) have been described.

Glycerol gradients

For gradient analysis of peroxisomal matrix proteins, purified peroxisomes were lysed by incubation on ice for 20 min in LC buffer (20 mM Hepes-KOH, pH 8.0, 50 mM NaCl, and a protease inhibitor cocktail [Szilard et al., 1995]), and lysates were subsequently clarified by centrifugation at 200,000 *g* for 30 min at 4°C in a Beckman Coulter TLA120.2 rotor. For gradient analysis of cytosolic proteins, the 200KgS (cytosol) fraction in homogenization buffer (Titorenko et al., 2000) was dialyzed in a Tube-O-Dialyzer (7.5-kD cutoff) (Chemicon) against LC buffer. The cytosolic proteins were then concentrated by centrifugation through a Biomax-30 filter (Millipore) for 30 min at 4°C. 800-μl aliquots of peroxisomal matrix or cytosolic proteins in LC buffer were loaded onto 35.2-ml, linear 5–35% (wt/vol) glycerol gradients in LC buffer. Samples were subjected to centrifugation at 120,000 *g* for 18 h at 4°C in a Beckman SW28 rotor. 36 fractions of 1 ml each were collected from the top. Identical gradients were calibrated with molecular weight standards (MW-GF-1000 kit; Sigma-Aldrich).

Purification of cytosolic Aox complexes and Aox monomers

50–160 ml of the 200KgS fraction recovered from wild-type and mutant strains was concentrated to a volume of 10–20 ml (12–25 mg protein/ml) by centrifugation through a Biomax-30 filter and dialyzed against LC buffer, as described above. Dialysate was subjected to centrifugation on a linear 5–35% (wt/vol) glycerol gradient in LC buffer, and gradients were fractionated as described above. Fractions containing Aox complexes or Aox monomers were pooled and concentrated to a final volume of 5 ml (5–16 mg protein/ml) by centrifugation through a Biomax-30 filter. LC buffer was exchanged for NIP buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% [vol/vol] Triton X-100, and a protease inhibitor cocktail [Szilard et al., 1995]) by dialysis. Samples were subjected to immunoaffinity chromatography with anti-Aox1p, -Aox3p, or -Aox5p antibodies covalently coupled to protein A-Sepharose (Sigma-Aldrich) as described (Szilard et al., 1995), except that bound proteins were successively washed five times in NIP buffer, three times in NIP buffer without Triton X-100, once in 50 mM Tris-HCl, pH 7.5, containing 20 mM MgCl$_2$, and once in 50 mM Tris-HCl, pH 7.5, containing 100 mM MgCl$_2$ and were eluted with 50 mM Tris-HCl, pH 7.5, containing 500 mM MgCl$_2$.

In vitro disassembly and reassembly of purified cytosolic Aox complexes

Aliquots of purified cytosolic Aox complex were dialyzed against buffer D (25 mM Tris-HCl, pH 7.5, 0.2 M KCl, 3 mM DTT), concentrated by centrifugation through a Biomax-30 filter, and clarified by centrifugation at 200,000 *g* for 30 min at 4°C in a TLA120.2 rotor. To analyze the disassembly of Aox complexes in vitro, equal aliquots of complex in buffer D were adjusted to a concentration of GuHCl from 0 to 500 mM and incubated for 1 h at room temperature. Samples were then subjected to centrifugation on linear 5–35% (wt/vol) glycerol gradients in buffer D containing GuHCl at the same concentrations used for disassembly of the Aox complexes. To monitor reassembly of Aox complexes in vitro, equal aliquots of complex in buffer D were diluted with buffer D containing 6 M GuHCl to yield a concentration of GuHCl of 500 mM. After incubation for 1 h at room temperature, disassembled Aox complexes were diluted with ice-cold buffer D to yield a concentration of GuHCl of 20 mM. Following an incubation for 1 h at 15°C, samples were subjected to centrifugation on linear 5–35% (wt/vol) glycerol gradients in buffer D containing 20 mM GuHCl. 36 fractions of 1 ml each were collected from each gradient and analyzed by immunoblotting with anti-Aox1p antibodies.

Other methods

Lysates of whole cells were prepared as described (Eitzen et al., 1997). SDS-PAGE and immunoblotting (Titorenko et al., 1998), double-labeling indirect immunofluorescence microscopy (Szilard et al., 1995), pulse-chase analysis and crosslinking (Titorenko et al., 1998), and immunoprecipitation under native (Szilard et al., 1995) or denaturing (Titorenko et al., 2000) conditions were performed according to established procedures.

Activities of marker enzymes for peroxisomes, mitochondria, ER, Golgi region, vacuoles, and plasma membrane were determined as described (Titorenko et al., 1998). UV-visible absorption spectroscopy was performed with a Beckman DU640 spectrophotometer operating at 23°C.

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