Evidence That the Yeast Desaturase Ole1p Exists as a Dimer in Vivo*

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Desaturase enzymes are composed of two classes, the structurally well characterized soluble class found predominantly in the plastids of higher plants and the more widely distributed but poorly structurally defined integral membrane class. Despite their distinct evolutionary origins, the two classes both require an iron cofactor and molecular oxygen for activity and are inhibited by azide and cyanide, suggesting strong mechanistic similarities. The fact that the soluble desaturase is active as a homodimer prompted us to test the hypothesis that an archetypal integral membrane desaturase from Saccharomyces cerevisiae, the Δ⁹-acyl-Co-A desaturase Ole1p, also exhibits a dimeric organization. Ole1p was chosen because it is one of the best characterized integral membrane desaturase and because it retains activity when fused with epitope tags. FLAG-Ole1p was detected by Western blotting of immunoprecipitates in which anti-Myc antibodies were used for capture from yeast extracts co-expressing Ole1p-Myc and Ole1p-FLAG. Interaction was confirmed by two independent bimolecular complementation assays (i.e. the split ubiquitin system and the split luciferase system). Co-expression of active and inactive Ole1p subunits resulted in an ~75% suppression of the accumulation of palmitoleic acid, demonstrating that the physiologically active form of Ole1p in vivo is the dimer in which both protomers must be functional.

Unsaturated fatty acids are found in the membranes of prokaryotes and eukaryotes. The balance between saturated and unsaturated fatty acids is a key variable in maintaining membrane fluidity. Postsynthetic desaturation of fatty acids is mediated by desaturase enzymes (1–5). Fatty acid desaturases are derived from two evolutionary lineages, the soluble acyl-ACP desaturases found primarily in the plastids of higher plants (e.g. the castor Δ⁸-18:0-acyl² carrier protein (ACP)³ desaturase (6) and the integral membrane desaturases typified by the yeast ole Δ⁹-desaturase (Ole1p) (7)). The reactions require iron cofactors, molecular oxygen, and reducing equivalents and are both inhibited by azide and cyanide but are insensitive to carbon monoxide. All desaturases investigated to date show remarkable stereo-selectivity in abstracting pro(R) hydrogens from adjacent carbons in the fatty acid substrate (8). As noted by Bloch (1), desaturase selectivity “would seem to approach the limits of the discriminatory power of enzymes” by a mechanism(s) that remain to be determined almost 40 years later.

Ole1p is a bifunctional 53-kDa protein (7), composed of two distinct domains: an N-terminal 42-kDa desaturase domain that has 36% homology to the rat stearoyl-CoA desaturase and a C-terminal 11-kDa domain that has strong homology to cytochromes b₅ (9).

Although integral membrane desaturases, like many membrane proteins, have proved difficult to overexpress and purify, sequence analysis has provided important information regarding the structure of the enzyme. For example, Martin and co-workers (7) employed hydropathy analysis to predict that the yeast desaturase consists of four transmembrane domains with the N and C termini facing the cytosolic side of the endoplasmic reticulum. Under their model, all three elements of a tripartite motif composed of 8 conserved histidine residues (10) would also be located on the cytoplasmic face. Experimental support for this model came from topology analysis of the closely related mouse stearoyl-CoA desaturase with the use of SC1D (stearoyl-CoA desaturase 1) fused with epitope tags at various positions along the its sequence, in which the N and C termini, along with the histidine-containing regions, are located on the cytosolic face of the endoplasmic reticulum, and the enzyme is anchored by four transmembrane helices connected by two short hydrophilic loops within the endoplasmic reticulum lumen (11). Similar analysis was performed on the distantly related (11% homology with Ole1) Bacillus subtilis Δ⁵-desaturase, and although the equivalent structural elements were found on the inner membrane face, six rather than four transmembrane-spanning domains were proposed (12).

Much of what we know about the structure and function of desaturase enzymes comes from studies on the soluble acyl-ACP desaturases, which are readily expressible in Escherichia coli in quantities sufficient for biochemical investigation (13). The crystal structure of the castor and soy acyl-ACP desaturases confirmed their dimeric structure with deep interdigitation between the monomers adjacent to four helix bundles in which the dimer active site is located (14, 15). A half-of-the-sites mechanism was hypothesized for desaturase function, in which...
the energy released upon substrate binding is transferred to the second subunit to facilitate product release (16).

To date, no information is available concerning the oligomeric state of the integral membrane class of enzymes. Here we present evidence from co-immunoprecipitation/immunoblot analysis, yeast two-hybrid analysis, and analysis of the co-expression wild type (WT) along with catalytically inactive Ole1p that is consistent with the hypothesis that Ole1p forms dimers in vivo.

**EXPERIMENTAL PROCEDURES**

**Fatty Acid Analysis**—Cultures were initiated from single yeast colonies. Yeast cells L8-14C (MATa ole1Δ::LEU2 leu2-3 leu2-112 trp1-1, ura3-52 his4) defective in Tergitol Nonidet P-40, 0.5 mM oleic acid). Cell pellets were collected by centrifugation and then washed with SD-Leu-Ura lacking dextrose. Cells were induced at an A600 of 1.0 at 30 °C for 16 h in SD-Leu-Ura supplemented with 2% galactose. After centrifugation, the cell pellets were washed with 1% Tergitol Nonidet P-40 and water sequentially and dried under N2. Fatty acid methyl ester was prepared with 1 ml of 1 N HCl-methanol at 80 °C 1 h. After the addition of 1 ml of 0.9% NaCl, fatty acids were extracted with 2 ml of hexane. The resulting fatty acid methyl esters were concentrated by evaporation under a stream of nitrogen. Samples were analyzed on a Hewlett-Packard 6890 gas chromatograph equipped with a 5973 mass-selective detector (gas chromatography/mass spectrometry) and a Supelco 60 m × 250-µm SP-2340 cyanocapillary column. The oven temperature was raised from 100 to 240 °C at a rate of 15 °C/min and held at 240 °C for 5 min. The helium flow rate was 1.1 ml/min.

**Yeast Protein Extraction and Co-immunoprecipitation Analysis**—Yeast cells were collected by centrifugation, washed once with water, and disintegrated in YEB buffer (50 mM HEPES, pH 7.4, 10 mM EDTA, 0.1% Triton X-100) by vortexing with glass beads. Following 15 min of centrifugation at 12,000 × g, the supernatant was supplemented with 0.5% protease inhibitor (Sigma) and incubated with antibody for 1 h. One-twentieth volume of protein A-Sepharose (Amersham Biosciences) suspension was added, and the mixture was incubated at 4 °C with gentle shaking overnight. Beads were washed twice with 10 volumes of Buffer A (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) and Buffer B (same as buffer A without NaCl and Triton X-100). Bound proteins were eluted with elution buffer (0.1 M glycine, pH 3.0). The eluted proteins were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by Western blot. The reactive bands on membranes were visualized after incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**Yeast Transformation and Bimolecular Complementation**—Yeast transformation was performed with the Yeastmaker Yeast Transformation System 2 (Clontech). Experiments employing the membrane protein split ubiquitin system were performed according to the protocols of the Frommer laboratory (17). The split luciferase system was employed to provide quantitative analysis of the Ole1p-Ole1p interaction in vivo (18). For luminescence detection, yeast cells were grown at 30 °C overnight in SD-Ura supplemented with 2% glucose. Cells were collected by 1 min centrifugation at 16,000 × g, and cell pellets were washed with SD-Ura (lacking dextrose). Cells were induced at an A600 of 0.4) in SD-Ura supplemented with 2% galactose at a temperature of 30 °C for 16 h. Cell cultures (90 µl) were transferred to a 96-well plate, and assays were initiated with the addition of 10 µl of a 60 µM concentration of a coelenterazine derivative (ViviRen live cell substrate, Promega). The assay plate was incubated for 5 min at room temperature in the darkness before luminescence measurement with the use of a TECAN Ultra 384 microplate reader.

**RESULTS**

**C-terminal FLAG- and Myc-tagged Ole1p Retains Activity**—To facilitate the detection and immunocapture of various Ole1 constructs, we explored the consequences of fusing Saccharomyces cerevisiae Ole1p with Myc and FLAG epitope tags (see Fig. 1A). Ole1p was fused with C-terminal tags encoding either FLAG or Myc. Ole1p-FLAG was placed under the control of the GAL10 promoter. A second construct was engineered in which the Ole1p-Myc, under the control of the GAL1 promoter, was added to the plasmid containing the GAL10-Ole1p-FLAG cassette, such that Myc- and FLAG-tagged Ole1p could be co-expressed.

To evaluate whether C-terminal Myc- or FLAG-tagged Ole1p retains activity, constructs containing either Ole1p-Myc or Ole1p-FLAG were expressed in the yeast ole1 deletion strain L8-14C cultured in medium supplemented with oleic acid (18:1Δ9) as a source of unsaturated fatty acid. Cells were washed to remove exogenous oleic acid, and protein expression was induced for 30 h. Levels of oleate increased from ~28% in...
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Co-immunoprecipitation Provides Immunological Support for Ole1p-Ole1p Association—Yeasts cells harboring empty vector or Ole1p-Myc alone or co-expressing FLAG- and Myc-tagged Ole1p fusions were subject to immunoprecipitation with anti-FLAG antibodies and protein A capture. The resulting proteins were separated by PAGE and immunoblotted with anti-Myc antibodies after transfer to polyvinylidene difluoride membrane. Fig. 2A shows an immunoreactive signal only upon co-expression both Ole1p-FLAG- and Ole1p-Myc-tagged proteins. Controls show that cells lacking Ole1p-FLAG show negative Western signal, indicating that the capture of anti-Myc-reacting polypeptide was dependent on the co-expression of anti-FLAG-tagged Ole1p. The fact that the signal was lost upon omission of anti-FLAG antibodies for immunoprecipitation further confirms that the anti-Myc-reactive polypeptide was recovered specifically by the anti-FLAG antibody. To validate this result, we performed the same experiment with anti-Myc antibodies for capture and anti-FLAG antibodies for immunodetection (Fig. 2B). Controls confirm that the positive signal is dependent on expression of both tagged forms of Ole1p and the presence of anti-Myc capture antibody. Together, these results provide compelling immunological evidence for Ole1p-FLAG-Ole1p-Myc association.

Bimolecular Association Analysis Confirms Ole1p-Ole1p Association in Vivo—As an independent approach to the immunological experiments described above, we employed split ubiquitin analysis to probe for potential Ole1p-Ole1p interactions in vivo. To achieve this, Ole1p was cloned into a bait vector such that it was expressed with a C-terminal fragment of ubiquitin (Cub) and the artificial transcription factor PLV (protein A-LexA-VP16) at its C terminus. For the split ubiquitin system to function, CubPLV and the Nub peptides must both be located on the cytosolic face of the membrane. In the absence of an empirically tested topology model, two test prey vectors were constructed, a Nub G-Ole1p fusion and an Ole1p-Nub G fusion, the former to test for tail-to-head interaction and the latter to test for tail-to-tail interaction (Fig. 3A) (the designation head or tail is used to refer to the location of the reporter at the N or C terminus, respectively, as fusions to the target protein). Yeast harboring constructs illustrated in A on nonselective minimal medium, SD-L-T, (left) and selective minimal SD-T-L-H-A medium (middle) and X-gal assay (right) in which LacZ activity with X-gal as substrate is indicated by dark coloration.

FIGURE 2. Ole1p-Myc and Ole1p-FLAG association revealed by co-immunoprecipitation analysis. A, yeast extracts from strain YPH499 expressing combinations of Ole1p-Myc and Ole1p-FLAG, as indicated, were subject to immunoprecipitation (IP) with anti-FLAG antibody or lacking the addition of antibody as negative control (NC). Samples were separated by SDS-PAGE, immunoblotted, and visualized with the use of anti-Myc antibodies. The bands corresponding to Myc- and FLAG-tagged Ole1p are indicated. B, same as A except that immunoprecipitation was performed with anti-Myc antibodies and immunoblotting was performed with anti-FLAG antibodies. Empty vector controls to ~38% in cells harboring Ole1p-FLAG. Oleate levels increased by a further 14% to ~52% when Ole1p-Myc was co-expressed along with Ole1p-FLAG, showing that Ole1p-Myc is also active. Because Ole1p is capable of desaturating both 16- and 18-carbon fatty acids, the accumulation of palmitoleic acid (16:1ω9) is the best indicator of Ole1p function because host cells lack palmitoleic acid, and there is therefore no background to subtract (see Fig. 1B). Ole1p-FLAG results in the accumulation of ~16% palmitoleic acid, and Ole1p-Myc accounts for ~20% accumulation of palmitoleic acid. These data confirm that Ole1p is active in the presence of C-terminal FLAG and Myc tags and that both constructs have similar in vivo activities.

FIGURE 3. Membrane split ubiquitin yeast two-hybrid analysis shows that Ole1p self-associates in vivo. A, schematic representation of the pairs of bait and prey plasmid constructs. The bait Ole1-Cub-artificial transcription factor PLV (protein A-LexA-VP16) construct is driven by the met25 promoter, and prey expression is driven by the ADH promoter. PC, positive control; NC1, NC2, and NC3, negative controls; Kan MX, kanamycin resistance module; Kat 1, Arabidopsis K+ channel protein; T-H, tail to head; T-T, tail to tail. B, challenge of yeast harboring the constructs illustrated in A on nonselective minimal medium, SD-L-T, (left) and selective minimal SD-T-L-H-A medium (middle) and X-gal assay (right) in which LacZ activity with X-gal as substrate is indicated by dark coloration.
membrane protein negative control, the Arabidopsis K+ channel protein, KAT1. As an additional negative control, the plant membrane bound desaturase Fad3 was tested for association with Ole1p, with no interaction being detected (see supplemental Fig. 1). The host strain employed for the experiments shown in Fig. 3 also contains a LexA: lacZ such that the yeast can be plated on X-gal-containing medium and on which β-galactosidase activity can be estimated qualitatively by the degree of blue coloration of the colony. In Fig. 3B (right), dark coloration corresponds to the ability of the colony to grow on -H-A plates (Fig. 3B, middle). The degree of dark coloration is stronger for tail-to-head than for the tail-to-tail orientation. The restoration of His and Ade prototrophy demonstrates in vivo Ole1p-Ole1 association; however, the detection of β-galactosidase activity was performed after SDS lysis of the yeast cells and can thus be considered an in vitro assay. To corroborate the split ubiquitin analysis and to quantitate the observed in vivo interactions, Ole1p was subjected to a second bimolecular fluorescence analysis with the use of the Renilla split luciferase (RLUC) system.

In general, the two complementary fragments of the split luciferase system can be fused to either N or C termini of the target protein. In practice, the functioning of combined luciferase fragments is dependent on their orientation with respect to the protein of interest due to specific steric and/or folding constraints (19). Thus, to optimize the luciferase activity, constructs were engineered to test four relative orientations: tail to tail, head to tail, head to head, and tail to head (Fig. 4A). The tail-head and tail-tail orientations showed no significant increase in luminescence (p < 0.001 and p < 0.02 levels, respectively) relative to the negative controls, whereas the head-to-tail and head-to-head orientations showed no significant increase in luminescence (p < 0.2 level). These data reflect the possibility that the two fragments of the RLUC are not sufficiently close to facilitate productive interaction or that their relative orientations are incompatible with productive interaction (19). An alternative interpretation is that the short FLAG tag sequence present on these two constructs results in a reduction of luciferase activity; however, this seems unlikely because the maximally active combination T-H contains a linker plus C-terminal luciferase fragment that represents a much larger C-terminal extension of Ole1p. Similar issues have been reported for homo-FRET (fluorescence resonance energy transfer) interactions (20). The RLUC data are consistent with the qualitative in vitro β-galactosidase activity results presented above.

Co-expression of Wild Type and Inactive Mutant Desaturase Provides Evidence for Subunit Poisoning of Heterodimers—Sequence alignment of integral membrane desaturases identified a series of 8 histidine residues that are strongly conserved between members from evolutionarily distant lineages, such as bacteria, fungi, and humans (10). These conserved histidine residues have been proposed as ligands to a diiron active site cluster identified by Mossbauer spectroscopy in the distantly related alkane ω-hydroxylase enzyme from Pseudomonas oleovorans (21). Functional relevance of the histidine cluster has been determined for rat and cyanobacterial desaturases and also in the alkane ω-hydroxylase (22). However, the relevance of these residues has not been described for Ole1p. We therefore chose several conserved histidines at positions 161, 162, 201, 202, and 338 along with several non-conserved histidines at positions 172 and 196 as targets for alanine-scanning mutagenesis (Fig. 5A). Each of the alanine mutants was introduced behind a GAL1 promoter as a C-terminal Myc-tagged fusion protein (Fig. 5B). The constructs were transformed into the ole1p mutant unsaturated fatty acid auxotroph yeast strain, which was challenged to grow on medium supplemented with or lacking unsaturated fatty acids (Fig. 5C). The ole1 deletion strain L814C expressing wild type ole1p or non-conserved substitutions (H172A and H196A) grew on medium lacking unsaturated fatty acid, whereas substitution of conserved histidine residues for alanines resulted in Ole1p variants that were unable to grow in the absence of unsaturated fatty acid-containing medium, demonstrating the essential nature of these five conserved histidine residues for Ole1p function.

In some enzymes, component protomers act independently, whereas in others, a single inactive subunit can result in an inactive oligomer, a phenomenon that has been referred to as subunit poisoning. The availability of inactive subunits in which conserved histidine residues are replaced by alanines provides a way of assessing whether the desaturase subunits act independently by coexpression of wild type and mutant desatu-
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From experiments described above, the tail-to-head orientation results in the highest functionality and is therefore used to test for desaturase subunit poisoning (Fig. 6A). We employed a plasmid pESC-Ura designed for the expression of two proteins simultaneously, one under the control of the GAL10 promoter and the other under the control of the GAL1 promoter. We therefore first evaluated the relative strengths of the GAL1 and GAL10 promoters, comparing the fluorescence generated by expression of an Ole1p-luciferase fusion under each promoter separately (Fig. 6B). Curves of fluorescence versus time showed no significant differences in luminosity between Ole1p-luciferase constructs under the control of the GAL1 and GAL10 promoters, indicating that both promoters are equivalent in strength.

Each of the mutants was expressed in a tail-to-head orientation along with wild type Ole1p, and the respective strains were subjected to luminescence assay (Fig. 6C). All WT-mutant pairs resulted in a similar magnitude of bimolecular fluorescence complementation, demonstrating that the mutants retain their ability to co-localize and associate with WT Ole1p. Minor differences in fluorescence may reflect minor differences in localization and/or stability of individual mutants.

To evaluate the activity of the co-expressed desaturase constructs, we assayed the accumulation of palmitoleic acid because it is absent from the host strain (Fig. 1B). Co-expression of non-conserved (i.e. active) histidine to alanine mutants (H172A and H196A) with WT Ole1p led to the accumulation of high levels of palmitoleic acid equivalent to those of the cells expressing two WT genes (Fig. 6D). In contrast, co-expression of loss-of-function histidine-to-alanine mutants with WT resulted in a significant (p < 0.001) decrease in accumulation of palmitoleic acid to an average of 24.9% of that of the WT control.

**DISCUSSION**

In contrast to the soluble desaturases, integral membrane desaturases are notoriously recalcitrant to overexpression in heterologous systems and with several notable exceptions (23, 24) have been very difficult to purify. Several crystal structures of soluble desaturases have been determined, which show that the enzyme exists as a dimer with a deeply interdigitated interface (14, 15, 25). However, the integral membrane desaturases...
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A dimeric organization can also play an important role for homodimers in that the state of component protomers can influence each others’ biochemical properties. For instance, the soluble desaturase dimer exhibits high affinity binding for one acyl-ACP substrate, but the affinity for binding a second substrate becomes much reduced, implying that a signal is transmitted from the substrate-bound protomer to the unoccupied protomer that reduces its substrate binding affinity (30). This may have implications for coordinating desaturase-substrate interactions. A half-of-the-sites mechanism was proposed for the soluble desaturase (16) in which the energy of desolvation released upon binding of the hydrophobic substrate to one protomer is transferred to the second protomer, providing a driving force to facilitate the energy-demanding release of the hydrophobic product into the aqueous phase. Binding of substrate to only one of the two protomers would facilitate an unbound substrate that would be available for substrate binding, facilitating the coordination of substrate binding with product release. This is an appealing scenario for the soluble desaturase and acyl-CoA desaturases, such as Ole1p, because the substrates and products exist in the aqueous phase. However, it is not clear if this mechanism would apply to desaturases in which the substrates are lipid-bound acyl chains for which binding of substrate is unlikely to be coordinated with product release.

An alternate imperative for the dimeric organization of desaturases could relate to their requirement for two-electron reduction of the active site iron center for catalysis. In both the soluble and membrane desaturases, electrons are supplied singly by interaction of the desaturase with ferredoxin and cytochrome b₅, respectively. In vivo redox titration of the soluble desaturase at ambient temperatures failed to reveal the presence of a meta (Fe²⁺-Fe³⁺) state of the iron center that is observed upon reduction at low temperatures (77 K), suggesting that the meta state is unstable at ambient temperatures (31). A dimeric structure of the desaturase would potentially allow for concerted reduction of the iron centers within each protomer to produce two meta state iron centers. These could undergo disproportionation to produce one reduced, Fe²⁺-Fe²⁺, and one oxidized, Fe³⁺-Fe³⁺, site.

At this time, it is unknown whether the formation of desaturase dimers is related to the general economies associated with the formation of homodimers (described above) or whether it relates to desaturase chemistry-specific imperatives imposed by the energetics of product release or requirement for two-electron reduction. It may be possible to test the desaturase-specific hypotheses empirically. This approach would be analogous to the subunit poisoning experiments presented herein; the activities of heterodimers composed of wild type protomers paired with protomers impaired in either substrate-or redox-partner binding could be evaluated. Inactivation of wild type activity upon pairing of wild type protomers with protomers disrupted in substrate or redox-partner binding would provide empirical support for these hypotheses.

The expression of Ole1p is highly regulated, responding to factors such as carbon source, metal ions, and the availability of oxygen (32). Multiple levels of regulation have been reported, including at the transcriptional, RNA stability, and transla-
tional levels. It remains to be determined whether the forma-

tion of dimers is a dynamic process that could potentially con-

tribute to this process.

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