Dimeric organization of the yeast oligosaccharyl transferase complex

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The enzyme complex oligosaccharyl transferase (OT) catalyzes N-glycosylation in the lumen of the endoplasmic reticulum. The yeast OT complex is composed of nine subunits, all of which are transmembrane proteins. Several lines of evidence, including our previous split-ubiquitin studies, have suggested an oligomeric organization of the OT complex, but the exact oligomeric nature has been unclear. By FLAG epitope tagging the Ost4p subunit of the OT complex, we purified the OT enzyme complex by using the non-denaturing detergent digitonin and a one-step immunoaffinity technique. The digitonin-solubilized OT complex was catalytically active, and all nine subunits were present in the enzyme complex. The purified OT complex had an apparent mass of ~500 kDa, suggesting a dimeric configuration, which was confirmed by biochemical studies. EM showed homogenous individual particles and revealed a dimeric structure of the OT complexes that was consistent with our biochemical studies. A 3D structure of the dimeric OT complex at 25-Å resolution was reconstructed from EM images. We suggest that the dimeric structure of OT might be required for effective association with the translocon dimer and for its allosteric regulation during cotranslational glycosylation.

electron microscopy | membrane protein purification | protein N-glycosylation

After translocation across the endoplasmic reticulum (ER) membrane, newly synthesized polypeptides are N-glycosylated by the oligosaccharyl transferase (OT) enzyme complex in the lumen of the ER (for recent reviews on OT, see refs. 1 and 2). The catalytic activity of the OT enzyme complex involves transfer of an oligosaccharide chain from the donor substrate, dolichol-P-P-oligosaccharide, to certain -Asn-X-Ser/Thr- (N-X-S/T-) sites in the nascent polypeptide chains. The OT enzyme complex is a multisubunit protein complex. In yeast, OT consists of nine different subunits, all of which contain one or more predicted transmembrane segments. In yeast, five of these proteins, Swp1p, Wbp1p, Ost2p, Ost1p, and Stt3p, are encoded by essential genes; four others that are not essential, Ost5p, Ost4p, Ost5p, and Ost6p, are also part of the OT complex. All yeast OT subunits have been cloned and sequenced (3–8), and the structure of one of them, Ost4p, has been elucidated by solution NMR (9). Homologs of all of the yeast OT subunits have been identified in higher eukaryotic organisms (3–6).

The OT enzyme complex catalyzes one of the most crucial and abundant posttranslational protein modifications, leading to the biosynthesis of an important class of macromolecules in the cell (namely, N-glycoproteins), very little information about its mechanistic aspects is available. Studies in the last two decades have provided clues to the possible functions of the OT subunits in the N-glycosylation reaction, but only recently has it been established that one of the OT subunits, Stt3p, bears the active site of the OT reaction (10–12). Ost3p and Ost6p are believed to perform redundant functions in the OT reaction because the two proteins exhibit sequence similarity and strikingly similar hydropathy plots (13). It has been postulated that two isoforms of the OT complex may exist in the yeast ER membrane, which are marked by the presence of either Ost3p or Ost6p (14–16). Ost3p and Ost6p may determine the association of the two isoforms with the two translocon complexes: namely, the Sec61 and Shs1 complexes, respectively (15). Ost4p is proposed to be responsible for recruiting Ost3p or Ost6p into the OT complex (16). Other studies have indicated that the donor substrate may be recognized by Wbp1p (17–19). Recently, it has been reported that the luminal domain of Wbp1p may possess the divalent metal ion-binding site (20), which has been shown to be essential for the catalysis of the OT reaction (21). Because Ost1p has been found to cross-link with a number of proteins, irrespective of their glycosylation status (22–24), it is proposed that the luminal domain of Ost1p may be involved in funneling the newly synthesized polypeptides into the active site of the OT reaction (24). However, it is important to note that with the exception of Stt3p, the contributions of the other OT subunits in the N-glycosylation reaction are highly speculative and based on indirect evidence. A possible conformational arrangement of the yeast OT complex has been proposed based on studies using a chemical cross-linker and the split-ubiquitin two-hybrid technique (22, 25). Other researchers have proposed the presence of three subcomplexes within the OT complex (26). Steady-state kinetic experiments have suggested an allosteric regulatory mechanism for the binding of the donor substrate (27). However, to date, no structural studies of the OT complex have been reported that could allow us to test these mechanistic or conformational proposals. In this report, we describe a one-step immunoaffinity chromatographic purification procedure for the OT complex. We show that all known nine subunits are present in the purified OT complex. Our biochemical analyses suggest a dimeric structure of the digitonin-solubilized yeast OT complex. We further report the structural analysis of the yeast OT complex by EM and single-particle image reconstruction. The structural result is in agreement with our biochemical studies.

Results and Discussion

Immunoaffinity Purification of the OT Complex. The OT complex is composed of nine subunits, so it was important to confirm the presence of all subunits in the purified preparation. Because we have antibodies to all of the OT subunits except Ost4p, Ost3p, and Ost6p, we generated a yeast strain in which genes encoding Ost4p, Ost3p, and Ost6p were epitope-tagged. We chose to epitope tag the C terminus of these three subunits because it has been previously demonstrated that the C-terminal tagging of these subunits does not lead to any growth defect (22, 26, 28, 29). We tagged the chromosomal copies of these three subunits and did not express them on an overexpression plasmid, thereby

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Abbreviations: OT, oligosaccharyl transferase; HRP, horseradish peroxidase; HA, hemagglutinin; ER, endoplasmic reticulum.

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eliminating the possibility of artifactual complex formation. The genes encoding Ost4p, Ost3p, and Ost6p were 3FLAG-, triple hemagglutinin (3HA)-, and 13MYC-tagged, respectively. Because Swp1p, Ost6p, and Ost5p are known to be of similar mobility on SDS/PAGE gels (13), addition of 13MYC and 3HA at the C termini of Ost6p and Ost3p increased their molecular mass to ~60 and ~37 kDa, respectively, allowing their easy identification by SDS/PAGE.

Because all components of the OT complex are transmembrane proteins that are residents of the ER membrane, it was important to solubilize the microsomes in an appropriate detergent that did not denature any of the subunits. Digitonin has been the detergent of choice of many researchers for the purification of the active OT complex (4, 30, 31), although others have reported purification of catalytically active OT complexes by use of detergents such as Nikkol and Nonidet P-40 (13, 14, 32). Although a comparative study of OT activity in various detergents has not been reported yet, digitonin seems to be an ideal detergent for the purification of the OT complex because we found that the digitonin-solubilized microsomal extract retains high OT activity even after storage for several days at 4°C (data not shown).

The OT complex has been purified from various mammalian and yeast sources by using a combination of chromatographic techniques (3–8, 18, 27, 30, 31, 33, 34), and other studies have used the immunoaffinity technique (13, 27, 34, 35). The purification of the OT complex was first carried out from canine pancreatic microsomes as a complex of three polypeptides: ribophorin I, ribophorin II, and OST48 (mammalian homologs of Ost1p, Swp1p, and Wbp1p, respectively) (3, 33). A similar composition was then reported for the avian, human, and pig OT complexes (6–8, 31). In early studies, the purification of the yeast enzyme complex was carried out, which indicated that the complex might be composed of four to six subunits (18, 30, 34, 35). In a later study, all subunits were reported to be present in the purified OT complex based on their migration on SDS/PAGE gels (27). However, the presence of some of the OT subunits could not be confirmed in this study by Western blotting, presumably because of the unavailability of antibodies against Ost3p, Ost4p, and Ost5p. Recently, the purification of the isoforms of the mammalian OT complex was reported, but the presence of homologs of Ost4p and Ost5p was not tested (4), again presumably because of an unavailability of antibodies. The purification of the yeast OT complex that detected all subunits except Ost4p has also been reported (13, 35). Because it has been previously demonstrated that, after solubilization with digitonin, one can coprecipitate all of the other OT subunits by immunoprecipitating one OT subunit (26), we decided to employ an immunoaffinity technique for the purification of the OT complex.

Having solubilized the microsomes in digitonin buffer, we immunoprecipitated Ost4p by using an anti-FLAG affinity gel. Analysis of the precipitate by Western blotting revealed that all of the other OT subunits coprecipitated along with Ost4p (Fig. L4). We used a 3XFLAG peptide to elute the OT complex in its native form from the anti-FLAG beads. The eluant was analyzed under nondenaturing conditions by using blue native gels. Blue native gels are believed to provide reliable estimates of the molecular mass of protein complexes (36). After silver staining of the blue native gel, a protein complex with an molecular mass of ~500 kDa was observed (Fig. 1B). Western blotting of the blue native gel using antibodies against Stt3p and Ost1p gave a single band corresponding to the OT complex. We further separated the components of the OT complex by SDS/PAGE followed by silver staining as seen in Fig. 1D. We also analyzed the sample by Western blot, which was probed with antibodies specific to all nine subunits of the OT complex (Fig. 1E). On comparison of the silver-stained gel and Western blots (Fig. 1D and E), we found that all of the subunits of the OT complex were present in the purified sample, and bands corresponding to each subunit were easily visible on silver-stained gels. No other extra band was visible on SDS/PAGE gel, suggesting that the one-step immunoaffinity procedure yields pure OT complex.

Fig. 1. Biochemical characterization of the immunopurified yeast OT complex. (A) All OT subunits coprecipitate along with Ost4p when microsomes are solubilized in digitonin buffer. The OT complex was immobilized on anti-FLAG beads as described in Materials and Methods. After washing with wash buffer, the anti-FLAG beads (20-μl settled bed) were resuspended in SDS/PAGE sample buffer (200 μl) containing 10% 2-mercaptoethanol and incubated at 55°C for 20 min. The supernatant was collected by centrifugation. Twenty microlitters of the sample was applied per lane on SDS/PAGE gels. After electrophoretic transfer, the blots were probed with polyclonal anti-HA or anti-Myc or monoclonal anti-FLAG antibodies to identify the Ost3-HAp, Ost6-Mycp, Ost1p, Ost2p, Ost5p, Stt3p, Wbp1p, or Swp1p to identify the respective proteins. The blots were also probed with polyclonal anti-HA or anti-Myc or monoclonal anti-FLAG antibodies to identify the Ost3-HAp, Ost6-Mycp, and Ost4-FLAGp subunits. (B) Analysis of purified OT on a blue native gel. After eluting the OT complex from anti-FLAG beads by using 3XFLAG peptide, 20 μl of the eluant was mixed with blue native gel sample buffer and applied to the gel, which was stained with silver reagent. (C) Western blot after blue native gel of purified OT using the indicated antibodies. Five microlitters of eluant was applied per lane on blue native gel for electroblotting. (D) Silver-stained SDS/PAGE of purified OT. The eluant (20 μl) was applied in one lane for SDS/PAGE. The gel was stained with silver reagent. The star indicates two bands that were identified as proteolytic C-terminal fragments of Wbp1p (data not shown). (E) Western blotting after SDS/PAGE of purified OT using polyclonal antibodies against Stt3p, Myc epitope, Ost1p, Ost6p, HA epitope, Swp1p, Ost2p, and Ost5p. A monoclonal M2 antibody against FLAG epitope was used to stain Ost4p. Two microlitters of eluant was applied per lane on SDS/PAGE gel for electroblotting. Molecular mass markers are indicated. For immunoblotting, primary and HRP-coupled secondary antibodies were used at a dilution of 1:3,000. The HRP-conjugated secondary antibodies were visualized with the LumiGLO kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD).
On blue native gel, besides the predominant 500-kDa band, we also observed a minor band at 570 kDa (Fig. 1B). Interestingly, both bands stained with antibodies specific to OT subunits (Fig. 1C). A similar finding has been reported by other researchers (16, 37). Because the 570-kDa band stains with antibodies to OT subunits, the presence of all OT subunits when resolved by SDS/PAGE in the second dimension (data not shown), it is clear that the minor band also represents the OT complex. However, the exact reason for the altered mobility is unclear. Because three OT subunits (Ost1p, Wbp1p, and Stt3p) are known to be glycoproteins (38), one possibility is that the 570-kDa band corresponds to the OT complex possessing glycosylated forms of the three OT subunits. Another possibility is that the 570-kDa band contains more copies of one or more of the OT subunits. The possibility that the two bands (500 and 570 kDa) correspond to the two OT isoforms (Ost3p and Ost6p isoforms) was ruled out by the finding that both bands react with antibodies specific to Ost3p and Ost6p (16). Because we immunoprecipitated the OT complex by targeting the Ost4p subunit, we believe that the purified OT complex is a total pool containing both of the OT isoforms (Ost3 and Ost6 isoforms) that are known to exist in the yeast ER membrane.

A typical yield of ~100 μg of pure OT was obtained from 10 mg of total microsomal proteins. The purified OT complex was found to be catalytically active by using the standard OT activity assay (data not shown). We obtained a 500-fold purification by using the one-step immunoprecipitation technique (data not shown).

Biochemical Studies Indicate That the Purified OT Complex Is a Dimer.

The observed mobility of 500 kDa of the purified OT complex on blue native gels is suggestive of a dimeric arrangement because the cumulative sum of the molecular masses of all of the nine subunits is ~250 kDa, a finding that has been previously reported by another group (16). Another independent study employing the genetic split-ubiquitin technique also indicated a higher order arrangement of the OT complex (25). It is important to note that the split-ubiquitin technique is a measure of in vivo interactions, suggesting that, under physiological conditions, OT may form higher order structures. If the OT complex indeed has a dimeric configuration, two copies of each OT subunit would coexist in one OT complex. Furthermore, the two copies should coimmunoprecipitate with each other when microsomes are solubilized with digitonin. To test the dimerization of the OT complex, we generated haploid OT strains in which two copies of an OT subunit exist; one plasmid-borne copy is hemagglutinin (HA) epitope-tagged, and the other chromosomal copy is Myc epitope-tagged. Four strains, each of which has two copies of Ost1p, Ost2p, Ost4p, or Stt3p, were generated. The microsomes of each of the four strains were solubilized in 1.5% digitonin, and the HA-tagged OT subunit was immunoprecipitated from the clarified supernatant by using the monoclonal anti-HA antibody. We analyzed the precipitate by using SDS/PAGE and Western blotting. The blot was probed with polyclonal anti-HA antibody, we found that the Myc-tagged OT subunit coimmunoprecipitated with its HA-tagged counterpart (Fig. 2B). This coexistence was found to be true for the four subunits (Ost1p, Ost2p, Ost4p, and Stt3p) that were tested, confirming that at least two copies of each of these subunits exist in one OT complex and suggesting that the digitonin-solubilized OT complex may be at least a dimer or possibly a higher oligomer. In view of these results and the apparent mass of 500 kDa based on blue native gels, we conclude that OT is a dimeric complex. Similar coimmunoprecipitation experiments carried out previously by others failed to yield these results, presumably because they used diploid strains for their experiments (27).

EM and 2D Image Classification Show a Dimeric Arrangement of the Purified OT Complex.

The purified OT complex appeared to be homogeneous after uranyl acetate staining with well separated individual particles of similar size, as shown in Fig. 3A. As revealed by blue native gel (see Fig. 1B), two species of OT complex were present in our preparation, one corresponding to the major band at 500 kDa and the other corresponding to the minor band at 570 kDa, which presumably is a glycosylated form of OT. At this low-resolution stage of structural characterization, we ignored the possible structural differences between the two species. This practice seems to be reasonable, because the masses of the two species are similar, and all nine subunits are present in both species. The estimated diameter size of the roughly spherical particles was ~120 Å, significantly larger than one would expect for a 250-kDa particle if OT were a monomer, even taking into consideration a layer of detergent molecules. Approximately 3,000 particle images were initially selected from the raw images and classified through multivariant statistical analysis. Some of the classes are shown in Fig. 3B. The OT complexes embedded in vitreous ice were also visualized by cryo-EM as well defined particles (Fig. 3C). Classification of these ice-embedded particle images resulted in class averages (Fig. 3D) that were very similar to those of negatively stained particles when these two types of class averages were aligned with each other. The major features correspond well between the two types of class averages (Fig. 3B and D). The good concordance of the structural features obtained in stain and in vitreous ice indicates that the OT structure was well preserved by both methods. However, the OT particles in ice were only visible when large underfocus values (3–4 μm) were used for imaging. The large defocus value resulted in blurred class averages with fewer structural details in the ice-embedded samples (Fig. 3D) as compared with the negatively-stained particles that were visible at smaller defocus values (1–2 μm) (Fig. 3B). We note that, unlike the much larger
particles such as ribosomes or viruses that cannot be fully embedded in heavy metal stain, the OT complexes could be fully stained because of their relatively small size, resulting in well-preserved structural features.

A near-mirror symmetry was found in a number of class averages, as indicated by dashed red lines across the middle of two images in Fig. 3B, suggestive of an in-plane twofold axis of these particles as positioned on the supporting carbon film. One such image is shown in Fig. 3E1 at a larger scale, and its mirror-symmetrized image is shown in Fig. 3E2. One class average displayed a near-twofold symmetry (Fig. 3E3), indicating a close to vertical orientation of a dimeric object on the carbon film. The twofold symmetrized version of the image is shown in Fig. 3E4. Based on the overall size of the OT particles and the presence of the apparent mirror symmetry and the near-twofold symmetry in some of the class averages, we conclude that the OT complex has a dimeric configuration, which is in agreement with the biochemical studies discussed above.

**Structural Description of the 3D EM Map of the OT Complex.** A 3D map of the OT complex was obtained from 4,900 negatively stained particles by 3D image reconstruction. The structure was estimated to have a resolution of 25 Å. Fig. 4 shows six surface-rendered views of the 3D reconstruction. Because the vast majority of oligomeric membrane proteins have their symmetry axes perpendicular to the membrane, the twofold axis of the OT dimer is highly likely to be arranged in the same way. The orientation of the structure with respect to the lipid bilayer has yet to be determined. We tentatively orient the larger end of the structure along the symmetry axis toward the ER lumen and the smaller end toward the cytosol, based on the fact that all nine OT subunits have their larger hydrophilic segments on the luminal side of the ER membrane. The two dashed white lines in Fig. 4A–C represent the potential bilayer site. OT was estimated to be ~120 Å in height. Excluding the 40 Å embedded on the lipid bilayer, there would be ~40 Å in either side of the membrane. This structural feature is consistent with the knowledge that the active site of the OT complex is located ~30–40 Å away from the membrane in the lumen (39).

The physiological role of the dimeric nature of the OT complex is currently unclear. However, we note that the actively translocating translocon was recently determined to form a dimer on ribosomes (40). Because OT is known to physically associate with and to function in collaboration with the translocon (41), we speculate that a dimeric configuration of the OT complex may be required for effective association with the translocon dimer and efficient N-glycosylation of newly synthesized polypeptides. Furthermore, a dimeric OT might facilitate the proposed allosteric regulation mechanism of the yeast complex (27).

**Materials and Methods**

Standard yeast media were used. T4 DNA ligase and shrimp alkaline phosphatase were obtained from Roche Diagnostics. Restriction enzymes and primers were obtained from Invitrogen. Anti-HA mAb HA.II was obtained from Covance (Richmond, CA). Anti-Myc and anti-HA polyclonal antisera were obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-labeled anti-rabbit IgG raised in goats was obtained from Chemicon International (Temecula, CA). Protein G-agarose beads were purchased from Invitrogen. Triple Master DNA polymerase was obtained from Eppendorf–Brinkman, and Pfu polymerase was obtained from Stratagene. Nitrocellulose membrane was obtained from Schleicher & Schuell. Anti-FLAG antibody, and 3XFLAG peptide were obtained from Genemed Synthesis (San Francisco) after coupling the peptide to keyhole limpet hemocyanin. Protein estimation was carried out using the standard bicinchoninic acid method (Pierce, Rockford, IL).
out by using the BCA protein estimation kit from Pierce. The lithium acetate protocol was used for all yeast transformations (42). Blue native gel electrophoresis was performed as described in ref. 43 by using a modification of a protocol described in ref. 44. Dolichol-P-oligosaccharide was prepared from microsomes of wild-type cells by using protocols outlined in refs. 45 and 46 and quantitated by the phenol-sulfuric acid assay. Silver staining of blue native gels and SDS/PAGE were carried out as described in ref. 47. The blue native gel was soaked in electro-transfer buffer for 10 min and electroblotted onto a nitrocellulose membrane at room temperature at 50 volts for 45 min.

**Strain Preparation.** The tagging of the chromosomal copies of Ost6p and Ost4p was carried out by using a one-step PCR-mediated protocol (48). The PCR products for tagging of OST6 were obtained as described by Longtine et al. (48) by using the pFA6a-13Myc-TRP1 plasmid. RGY330 (MATa ura3–52 leu2–Δ1 lys2–801 ade2–101 trpl–Δ1 his3–Δ200 OST3::HA3-UAS3) was the recipient strain for the OST6 PCR product. The colonies were selected on Trp plates, and the integration was confirmed by colony PCR. The expression of epitope-tagged protein was confirmed by SDS/PAGE and Western blot analysis. The resultant strain, LY500 (MATa ura3–52 leu2–Δ1 lys2–801 ade2–101 trpl–Δ1 his3–Δ200 OST3::HA3-UAS4 OST6::MYC9-TRP OST4::FLAG-G418), was used for purification of the OT complex.

**Purification of the OT Complex.** Yeast microsomes from the strain LY500 (MATa ura3–52 leu2–Δ1 lys2–801 ade2–101 trpl–Δ1 his3–Δ200 OST3::HA3-UAS3 OST6::MYC9-TRP OST4::FLAG-G418) were prepared as described earlier except that DTT was completely omitted from the buffer (0.2% digitonin, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4), 3.5 mM MgCl2, and 1 mM MnCl2). After incubation for 20 min on ice, the mixture was centrifuged for 20 min at 120,000 × g, and the clarified supernatant was diluted 100-fold to reduce the concentration of digitonin to 0.2% while keeping the concentration of all other components constant. The clarified supernatant was clarified by centrifugation for 20 min at 120,000 × g.

**Immunoprecipitation Experiments.** Conditions for immunoprecipitation were the same as those for OT purification, except that the supernatant was not diluted. The supernatant fraction was used for immunoprecipitation with monoclonal HA antibody. The immune complex was immobilized by using protein G-agarose beads. After incubation for 2 h with rocking at ambient temperature, the beads were recovered by centrifugation and washed five times with wash buffer containing 0.2% digitonin and once with TBS. Beads containing the immunoprecipitated proteins were incubated for 30 min at 60°C in SDS/PAGE sample buffer and centrifuged. The supernatant was analyzed by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane blots were probed with appropriate primary antibodies followed by secondary antibodies coupled to HRP.

**OT Activity Assay.** The OT activity assay was performed as described in ref. 52, except that a different peptide substrate, [H]Ac-Asn-Bpa-Thr-amide, was used instead of [H]Bz-Arg-Lys(BzN2)-Thr-NH2. Rather than spheroplasts, either 0.5 mg of microsomal proteins (positive control) or 5 μg of pure OT sample was used. When pure OT sample was assayed, 5 pmol of dolichol-P-oligosaccharide was added, and the concentration of digitonin was reduced to 0.002%.

**EM.** For negative staining, a 6-μl drop of sample containing the purified OT complexes was applied to a glow-discharged 300-mesh copper grid covered with a thin layer of carbon film. After removing the excess solution by blotting with filter paper, the sample grid was deeply stained by using three 6-μl drops of 2% uranyl acetate solution. Excess stain was removed by blotting, and grids were quickly dried by argon flow after final blotting. Images were recorded under low-dose conditions (10 e/Å2) in a JEOL JEM 1200EX electron microscope operating at 120 kV with a magnification of ×50,000 and defocus of 1.2–1.5 μm. The regions where the OT particles were fully embedded in stains, judged by the absence of a dark-stain accumulation ring around each particle, were selected with a charge-coupled device camera before recording the images. For samples embedded in vitreous ice, OT samples were applied to a quantifoil grid and rapidly frozen with liquid ethane in a Vitrobot. Frozen-hydrated samples were imaged in a JEOL 2010F electron microscope operating at 200 kV at a magnification of ×50,000 and a defocus value of ≈3 μm. All images were recorded on Kodak SO-163 negative films, and the films were developed for 12 min with full-strength Kodak D-19 developer at 20°C. Micrographs were inspected with an optical diffraction apparatus, and only micrographs without drift and astigmatism were further processed. The micrographs were digitized with a Nikon Supercool scanner 8000ED at a step size of 12.7 μm, corresponding to a pixel size of 2.54 Å at the sample level.

**Image Analysis and 3D Reconstruction.** We used SPIDER (53), EMAN (54), and MACiV (55) for processing images from negative-stained and vitrified OT specimens. The parameters of the contrast transfer function (CTF) for each micrograph were calculated, and these parameters were used for the CTF correction in SPIDER. Raw particle images were centered by auto-correlation in SPIDER, and multivariant statistical analysis and classification were performed on the centered particles. Selected well-centered class averages were used as references to realign all of the raw particle images in SPIDER. The aligned particle images were reclassified, and new class averages were calculated.
Multireference alignment and classification were further carried out in IMAGIC-V several times by using class averages from previous iterations as references until stable class averages were generated. A starting model was calculated in EMAN from selected final class averages based on the cross-common line technique. Selection of class averages was based on the structural features and their corresponding variance maps. The model was refined in a reprojection matching procedure with a total of 4,900 negatively stained particles to produce a refined map. The resolution of the resultant map (25 Å) was estimated by Fourier shell correlation of two maps calculated separately from two halves of the data set. The map was low-pass filtered to the estimated resolution and rendered into surface views with PYMOL.

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