The Motor Protein Prestin Is a Bullet-shaped Molecule with Inner Cavities

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Prestin is a transmembrane motor protein localized at the outer hair cells (OHCs) of the mammalian inner ear. Voltage-dependent conformational changes in prestin generate changes in the length of OHCs. A loss of prestin function is reported to induce severe auditory deficiencies, suggesting prestin-dependent changes of OHC length may be at least a part of cochlear amplification. Here we expressed the recombinant FLAG-fused prestin proteins in Sf9 cells and purified to particles of a uniform size in EM. The square-shaped top view of purified prestin, the binding of multiple anti-FLAG antibodies to each prestin particle, the native-PAGE analysis, and the much larger molecular weight obtained from size exclusion chromatography than the estimation for the monomer all support that prestin is a tetramer (Zheng, J., Du, G. G., Anderson, C. T., Keller, J. P., Orem, A., Dallos, P., and Cheatham, M. (2006) J. Biol. Chem. 281, 19916–19924). From negatively stained prestin particles, the three-dimensional structure was reconstructed at 2 nm resolution assuming 4-fold symmetry. Prestin is shown to be a bullet-shaped particle with a large cytoplasmic domain. The surface representation demonstrates indentations on the molecule, and the slice images indicate the inner cavities of sparse densities. The dimensions, 77 × 77 × 115 Å, are consistent with the previously reported sizes of motor proteins on the surface of OHCs.

The mammalian ear has specialized function in collecting sound signals and transmitting them to the nerve. Several amplifying systems are developed for sound collection. The pinna and ear canal funnel have suitable shapes for effective sound collection and condensation. The bones of the middle ear (malleus, incus, and stapes) convert the sound energy in the eardrum to the pressure waves in the fluid of the cochlea with 20–40-fold amplification.

In the inner ear, a positive feedback loop generates synchronous force by sensing the vibrations within the organ of Corti, which amplifies gained sound signals more than 100 times, although the mechanisms are not clearly understood (1, 2). One of the hypotheses is that voltage-dependent changes in the length of the outer hair cells (OHCs) generate the lateral membrane motility and amplify the sound (3–5). These changes are proposed to be caused by prestin (SLC26A5) (6, 7), which is a membrane integral protein and is categorized to the solute linked carrier (SLC) 26 family of anion transporters. It has been also reported that prestin responds to the membrane potential by harboring cytoplasmic anions (Cl⁻ or HCO₃⁻), as extrinsic voltage sensors, and changes its structure (8). Because prestin is densely embedded in the plasma membrane of the OHCs, the total length of the cell also changes as much as 5% as follows: decreases by depolarization and increases by hyperpolarization (9, 10). A voltage-dependent structural change was also observed in heterologously expressed cells, which strongly supports that prestin is a motor protein (6).

The prestin gene (81.4 kDa, 744 amino acids) was first identified using subtractive cloning between the motile OHC cDNA library and the nonmotile inner hair cell library (the other type of hair cell in the organ of Corti) (6). Hydropathic analysis of the sequence and antibody recognition experiments on each side of the membrane suggested the prestin has 10 (11) or 12 (8, 12) transmembrane segments with relatively large N and C termini in the cytoplasm (7, 12). Zheng et al. (13) concluded prestin as a tetramer by chemical cross-linking experiment and perfluorooctanoate electrophoresis, using an affinity-purified specimen. They also revealed that the tetramer is constituted of the dimer unit, and the component subunits are connected by disulfide bonds. An oligomeric structure of prestin was also supported by FRET analysis (11, 14).

Mutation in the prestin gene was reported to cause autosomal, recessive, nonsyndromic deafness (DFNB14 (15)).

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‡ The abbreviations used are: 3D, three-dimensional; AFM, atomic force microscopy; DDM, n-dodecyl β-D-maltoside; FSC, Fourier shell correlation; GNG, growing neural gas network; MRA, multireference alignment; NN, neural network; OHCs, outer hair cells; Rs, Stokes radius; SA, simulated annealing; SEC, size exclusion chromatography; TBS, Tris-buffered saline.
Homozygous mutant mice have a loss in OHC electromotility in vitro and a hundredfold loss of cochlear sensitivity in vivo, without disrupting mechano electrical transduction in OHCs. In heterozygotes, the electromotility was halved, and a significant elevation in cochlear thresholds was observed (16). Further study demonstrated that the prestin knock-out mice lose frequency selectivity in the compound action potential tuning curve, supporting the hypothesis of contributing prestin and OHC electromotility in the cochlear amplification (17). In addition, salicylate disrupts voltage-dependent length changes of the OHCs in vitro, through the competitive inhibition of anion incorporation to the prestin (8). Therefore, the disruption of prestin is considered as a cause of hearing loss and subjective tinnitus by administration of large doses of aspirin (18).

Prestin locates on the lateral membrane of the OHC in high densities (19, 20) and in much lower densities on the rest of the membrane, including the basal membrane (21). It has been observed as highly accumulated membrane integral proteins (2,500 particles/μm²) about 11 nm in diameter by using the freeze-fracture technique (22) and atomic force microscopy (AFM) (23). Recent AFM observation of Chinese hamster ovary cells expressing recombinant prestin showed similar particles of diameters between 8 and 12 nm on the cell surface (24), suggesting that reported particles on the surface of OHCs are the motor protein prestin.

The change in OHC length occurs in microseconds (as fast as 20 kHz) (7), the fastest movement among known motor proteins. Structural information of prestin is necessary for understanding the generation of the force and its regulation mechanisms. To address this issue, we purified recombinant prestin protein from baculovirus-infected Sf9 cells, observed the negatively stained molecules using EM, and reconstructed the 3D structure by single particle analysis (25–27).

**EXPERIMENTAL PROCEDURES**

**Molecular Biology and Infection of Sf9 Cells**—Rat prestin cDNA with an hemagglutinin tag at its N terminus (a gift from Dr. Fakler) was used in this study (7). The entire insert (Spel–HindIII fragment) was subcloned to pBluescript SK(−) vector. The original hemagglutinin tag was removed by replacing the 5′ end of the NotI (vector)–NruI (insert) with a PCR product starting with GCCG GCC GCC ATG. The FLAG tag was inserted at the C-terminal end of prestin by replacing the 3′ end (AccI insert–Xhol (vector)) with a PCR product that has a FLAG tag immediately after the prestin coding region in-frame and an Xhol site. The sequence was confirmed by DNA sequencing. The prestin cDNA tagged with the FLAG sequence was further subcloned to the pFastBac donor plasmid (Invitrogen) and used to transform the DH10Bac Escherichia coli strain to obtain the Bacmid DNA containing FLAG-tagged prestin. The Sf9 cells were infected with the recombinant Bacmid. After 96 h of infection, the supernatant, including the virus particles, was collected and applied to the next culture. By repeating this step five times, a high titer virus stock was obtained. For protein isolation, cells were harvested after 96 h of infection using Teflon cell scrapers, collected by centrifugation, immediately frozen, and stored at −80°C until use (28).

Frozen cells were homogenized in 10 volumes (v/w) of TBS (20 mM Tris-HCl, pH 7.4, at 4°C, 150 mM NaCl) with a Teflon homogenizer. Homogenates were first centrifuged for 15 min at 10,000 × g to remove debris, and the supernatant was further centrifuged at 100,000 × g for 60 min to obtain membrane fraction. All the procedures were performed on ice or at 4°C.

**Protein Preparation**—The membrane fraction was homogenized with a Teflon homogenizer in 4 ml of TBS (pH 7.4 at 4°C) containing 50 mM n-dodecyl β-D-maltoside (DDM) (Sigma), protease inhibitor mixture tablets (EDTA-free; Roche Diagnostics), and 0.02% sodium azide. After centrifuging for 10 min at 15,000 × g, the supernatant containing the solubilized FLAG-tagged prestin was loaded onto a column containing 1 ml of anti-FLAG affinity gel (Sigma) pre-equilibrated with the same buffer. The column was then washed with 20 ml of wash buffer (TBS containing 5 mM DDM, 300 mM MgCl₂, 0.02% sodium azide), and the bound protein was eluted with elution buffer containing 100 μg/ml FLAG peptide (Sigma). The elution was analyzed by silver staining and by Western blotting, and the mixture of two peak fractions (400 μl) was concentrated to 50 μl with a Microcon centrifuge filter unit YM-50 (Millipore). It was further purified by Superdex 200 (3.2/30) size exclusion chromatography (SEC) in a SMART system (GE Healthcare) with a TBS containing 5 mM DDM, 300 mM MgCl₂, and 0.02% sodium azide. The elution of protein was monitored by absorbance at 280 nm and collected 20-μl fractions with a flow rate of 40 μl/min. Protein concentrations were determined using the BCA method (29).

**SDS and Native Gel Electrophoresis**—The standard method of Laemmli (30) was applied for the SDS-PAGE. Samples were mixed with a sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.04% dithiothreitol, and 0.01% bromphenol blue and then incubated at 95°C for 3 min. Proteins were separated in a 2–15% gradient acrylamide gel with an electrophoresis buffer containing SDS and visualized by silver staining. For Western blotting, proteins electrophoresed in the gel were transferred to a polyvinylidene difluoride membrane and detected with an anti-FLAG antibody (Sigma) and a secondary antibody for chemiluminescent staining. Chemiluminescent images were obtained using a LAS-3000 mini image analyzer (Fujifilm). In the native gel electrophoresis (31), protein samples were combined with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, and 0.01% bromphenol blue), and electrophoresed for 5 h at 60 V in a 7.5% acrylamide gel containing 0.5 mM DDM or in a 2–15% gradient acrylamide gel that did not contain DDM. The running buffer (25 mM Tris-HCl, pH 8.4, and 192 mM glycine) was supplemented with 0.1 mM DDM for protein stabilization. The separated proteins were compared with molecular standards as follows: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa).

**Measurement of Molecular Weight and Stokes Radius by SEC**—To calculate the molecular weight of the FLAG-tagged prestin, high molecular weight standard proteins (GE Healthcare) were used for column calibration (32, 33). The distribution coefficient, K_d, was calculated from the equation K_d = (V_e - V_0) / (V_t - V_0), where V_e is the elution volume of each reference...
protein or prestin protein. Column void volume ($V_0$) was measured with blue dextran 2000, and $V_r$ represents total bed volume. All the standards and the prestin were solubilized in the same buffer for prestin purification. Prestin elution was repeated three times. The prestin data is presented by average ± S.D.

The same data set was applied to determine the Stokes radius ($R_g$) of FLAG-tagged prestin using a calibration curve, which was constructed by plotting $R_g$ of reference proteins versus $(-\log K_v)^{1/2}$ according to the relationship $(-\log K_v)^{1/2} = \alpha (\beta + R_g)$ (32). Table 1 lists the molecular weight, $R_g$, and obtained elution volume of each standard protein.

**Transmission Electron Microscopy**—The solubilized prestin of ~50 μg/ml was adsorbed by thin carbon films rendered hydrophilic by glow-discharge in low air pressure and supported by copper mesh grids. Samples were washed with 5 drops of double-distilled water, negatively stained two times with 2% uranyl acetate solution for 30 s, blotted, and dried in air. Micrographs of negatively stained particles were recorded in a JEOL 100CX transmission electron microscope at ×55,000 magnification with 100-kV acceleration voltages. Images were recorded on SO-163 films (Eastman Kodak Co.), developed with a D19 developer (Kodak), and digitized with a Scitex Leaf-scan 45 scanner (Leaf Systems Inc) at a pixel size of 1.82 Å at the specimen level.

**Automated Particle Selection and 3D Reconstruction**—Primary selection of prestin projections was performed automatically using the auto-accumulation method with simulated annealing (SA) (34). Three hundred particles were extracted into 140 × 140 pixel subframes and used to train the auto pickup system of a three-layer pyramidial-type neural network (NN) (35–37). Using the trained NN, a total of 14,346 particles was automatically selected from digitized EM films. Particles touching neighboring particles were discarded manually. In total, 13,149 images were analyzed after subtracting the uneven background. The selected particles were aligned rotationally and translationally by multireference alignment (MRA) (25, 26). To avoid introducing bias in reconstruction, reference-free alignment procedures are preferred for the first reference images (26). In this study, the references at the first cycle were calculated automatically only by the NN program (36), which includes neither subjective procedures by human beings nor any symmetry imposition. The aligned images were sub-grouped into 300 classes by the modified growing neural gas network (GNG) method (38). The class averages were used as new references, and this cycle was repeated 22 times.

The orientational Euler angles of the class averages were determined by the echo-correlated 3D reconstruction method using SA (39), assuming a 4-fold symmetry. These were used to calculate a 3D structure by the simultaneous iterative reconstruction technique method (40). The reprojections from the initial volume were employed as references for MRA. Each image in the library was aligned and clustered, providing improved class averages.

The 3D map was further refined until convergence by the projection matching method (41) together with intermittent MRA-GNG-averaging cycles. Particle images that correlated poorly with the 3D projections were rejected automatically using the cross-correlation function. Final reconstruction included 98.1% of all the selected images. The Fourier shell correlation (FSC) function was used to assess the resolution of the final 3D map at the threshold of 0.5 (42).

**Formation of the Prestin-Antibody Complex**—Purified prestin and anti-FLAG monoclonal antibody (Sigma) were mixed for 30 min at 4 °C, and excessive antibodies were removed by SEC. Fab fragment of anti-FLAG antibody was generated by papain digestion using a commercial kit (Pierce). The Fab fragments were conjugated with colloidal gold particles (BB International), and the conjugate was separated from nonreacted Fab molecules by 10–30% glycerol gradient centrifugation. The Fab-gold conjugates were mixed with purified prestin, left on ice for 30 min, and negatively stained. In obtaining averages of prestin-antibody complex, 353 prestin-antibody complexes were manually picked up from 32 EM films, averaged, and classified into 15 groups by modified GNG method using preexistent prestin two-dimensional averages without antibody as templates.

**RESULTS**

**Expression and Purification of the Prestin Protein**—Natural expression of prestin is limited to the outer hair cells of the inner ear and is too scarce for purification. To address this, we constructed a baculovirus containing FLAG-tagged prestin cDNA and infected Sf9 cells. We first expressed and purified either N- or C-terminally tagged prestin. As C-terminally tagged prestin expressed at higher efficiency, we have chosen this construct for further study (data not shown).

Expression of C-terminally FLAG-tagged prestin was detected in Sf9 cells using an anti-FLAG antibody. More than 90% of the cells were positive in immunological detection with the anti-FLAG antibody, and about 10% of the cells expressed the fused protein at a much higher level (Fig. 1). Recombinant prestin was strongly localized at the plasma membrane, suggesting that infected cells expressed the FLAG-tagged prestin well, and that the product was correctly integrated into the plasma membrane (Fig. 1). Prestin proteins were concentrated from solubilized membrane fractions using FLAG affinity chromatography. Most of the remaining contaminants, especially those of smaller size, were eliminated by further purification using SEC (Fig. 2A). Three absorbance peaks were observed in SEC analysis as follows: a large asymmetrical peak at 0.87 ml elution, a medium sized peak at 1.17 ml elution, and a small rise at 1.46 ml elution, besides a peak of FLAG peptides at 2.0 ml of elution (Fig. 2B).

Aliquots from each SEC fraction were separated in SDS-PAGE and detected by silver staining (Fig. 2C) and Western blotting using an anti-FLAG antibody (Fig. 2D). In both detections, predominant bands were observed at the size estimated from the amino acid composition of prestin (81.4 kDa), suggesting that the prestin protein is correctly expressed on the Sf9 membrane (Fig. 2, C and D). The band intensity is strongest at the elution of 1.16–1.24 ml, which corresponds to the second peak in SEC (Fig. 2B). The 81.4-kDa band is also observed in the fractions at 0.84 and 0.92 ml, together with the higher molecular weight bands, corresponding to the first asymmetrical high peak (Fig. 2, C and D).
In the fractions of the first peak, variously sized amorphous aggregations were observed using EM (Fig. 2B, inset), which appeared just after the void volume. Despite attempts at solubilization using various detergents and various mechanical methods (such as ultrasonication), formation of the large complexes could not be avoided. Indeed, these large complexes are tightly bound and could not be dissociated even by boiling in the reducing conditions for SDS-PAGE, as shown in the early fractions (Fig. 2, C, and D). In the fractions of the third peak at 1.46 ml of elution, FLAG-fused protein was very low by Western blotting. For further analysis of prestin molecule, we used an SEC fraction with a 1.17-ml peak (a small bump indicated by an arrow in Fig. 2B).

**Estimation of the Molecular Weight and Diameter of Purified Prestin**—SEC was used to estimate the molecular weight and diameters of prestin in aqueous solution (32, 33). We calculated the distribution coefficient ($K_{av}$) of purified prestin and estimated the molecular weight using the calibration curve obtained from standard proteins (Table 1). The $K_{av}$ of prestin was calculated to be 0.215 ± 0.003 (S.D. $n = 3$), which corresponds to 275.6 ± 4.8 kDa (Fig. 3A). The estimation, 3.4 times larger than that of the monomer (81.4 kDa), suggests the formation of a trimer or a tetramer.

The values of $(−\log K_{av})^{1/2}$ of the reference proteins were further plotted against the Stokes radii ($R_s$), and the size of
prestin was estimated from the calibration curve (Fig. 3B). The $R_s$ of prestin was determined to be 56.0 ± 0.3 Å, larger than that of aldolase (48.1 Å, 158 kDa) and catalase (52.2 Å, 232 kDa). The diameter of the prestin molecule can be roughly estimated as 112 Å, although this includes bound detergents, lipids, and hydrated water molecules and is affected by the molecular shape.

The size of prestin was further examined by the native gel electrophoresis. Purified prestin was separated in the native gel that did not contain SDS (instead, milder DDM was added at 0.5 mM in the gel for prestin stabilization). Although the mobility of proteins in the native gel is somewhat affected by their charge and shape as well as their molecular mass, the detection of prestin between 232- and 440-kDa standards supports 326 kDa of the tetramer (Fig. 3C, left). The same samples were then separated in a native gel that did not contain stabilizing DDM (Fig. 3C, right). The ladder of various multimer forms was observed, which shows partial dissociation from the tetramer under the denaturing condition.

In this study, we also tried the velocity sedimentation analysis to determine the molecular mass of prestin more precisely. However, we have not obtained the convincing result yet due to dissociation to multiple forms from tetramers and low yield of protein (data not shown). Application of this technique to the membrane proteins are limited so far, and further technical improvement seems necessary for wider application to various kinds of membrane proteins.

**Electron Microscopy and 3D Reconstruction**—Purified prestin was blotted onto a glow-discharged carbon grid, negatively stained by uranyl acetate, and observed by electron microscopy at ×55,000 magnification. Differently shaped particles of uniform size were observed, as demonstrated in Fig. 4. The divergence in shape represents the different projections of the molecule.

To perform the statistical analysis, we selected 13,149 well separated particles from 14,346 picked images by NN (using a combination of automated pickup programs, the auto-accumulation method (34) and utilized as training data for the three-layer NN auto-picking system (35, 36)). These images were then analyzed by the reference-free 3D reconstruction method; they were initially aligned both rotationally and translationally with respect to references, which had been automatically created as connection weights in the NN pickup program.
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(36). The aligned images were then classified into 300 groups using the modified GNG network method (38). The class averages were used as new references, and this cycle was repeated until convergence.

Orientational Euler angles of the class averages were automatically determined by the echo-correlated 3D reconstruction method using SA (39), and a 3D structure was reconstructed by the simultaneous iterative reconstruction technique method (40). Although a previous report suggested the prestin is a tetramer (13) and our biochemical studies (the SEC analysis and native gel electrophoresis) also support the large multimer structure of prestin, probably a tetramer, a possibility of trimer still remained. To confirm the stoichiometry and molecular symmetry of prestin, we determined the Euler angle and reconstructed the 3D reconstruction assuming 2-fold symmetry (C2), 3-fold symmetry (C3), or 4-fold symmetry (C4), independently from the same data set of two-dimensional averages (which were not imposed symmetry). Reprojections from the initial volume were employed as references for MRA. Each image in the library was aligned and clustered, providing improved class averages. The 3D map was further refined by projection matching (41). The averaging cycles (reconstruction-reprojection-MRA-GNG) were repeated six times for all reconstructions together with intermittent MRA-GNG-averaging cycles. Within the final cycle of each reconstruction, cross-correlation functions were calculated between reprojections from the 3D structure and the corresponding experimental two-dimensional averages. The scores of C2, C3, and C4 symmetries imposed reconstructions are 0.8467 ± 0.0113, 0.8437 ± 0.0100, 0.891 ± 0.0122, respectively (mean ± S.D. of higher correlated hundred image pairs), suggesting C4 symmetry is plausible. Although D2 is a common motif in tetramer assembly, this structure does not seem probable in the case of membrane-integral protein prestin. Immunological detection of all N and C termini in the cytoplasm (7, 8, 12) and the bullet-shaped side view of the particle (Fig. 5A, images 5 and 6) do not support the D2 symmetry.

Variously shaped raw images of prestin are presented (Fig. 5A, top line) with their corresponding class averages from MRA classification cycle number 21 (2nd line; imposed no symmetry), improved class averages from cycle number 32 (3rd line; reprojections from the 3D structure were used for MRA reference), and projection images from the 3D reconstruction (4th line). Despite the large divergence in the shapes of raw images, their size, shape, and inner structure fit very well with the corresponding averages, as well as with the projections from the final 3D structure. This indicates good consistency between the 3D reconstruction and the original particle images. Dispersed stain depositions in the molecule indicate patchy distribution of various levels of hydrophobicity.

The images in Fig. 5A are sequentially arranged in the order of putative top (Fig. 5A, images 1 and 2), oblique side views (Fig. 5A, images 3 and 4), and side views (Fig. 5A, images 5 and 6). Reprojections of homooligomeric protein structures often reflect their stoichiometries (28, 37, 43). In this study, we frequently observed square shapes in the raw images, and in the corresponding averages although symmetry was not imposed (Fig. 5A, left two columns in top and 2nd lines), which also supports the tetramer structure of prestin. A plot of the Euler angles of 282 adopted class averages shows that prestin is essentially randomly oriented on the carbon surface (Fig. 5B). According to the FSC function (42), the resolution of our final density map is 2 nm at a correlation coefficient >0.5.

The 3D structure (Fig. 6, contoured at 311 kDa; 95.5% of the calculated mass of the prestin tetramer) appears to be bullet-shaped with dimensions of 115 Å in height and 77 Å in side length at the widest position. A diagonal measurement is 96 Å. This size corresponds well to the dimensions obtained by SEC, which roughly estimated the diameter of hydrated prestin at 112 Å (Fig. 3B). The variance in width observed for the side views (Fig. 5A, images 5 and 6) corresponds to the variance between the side length and the diagonal length of the square (Fig. 6A, images 3 and 4). The latter images are rarely observed (Fig. 5B), because the flat side seems to fit the flat carbon support.

Sections parallel to the membrane plane are presented through the molecule at 5.5-Å intervals (Fig. 6B). The slices are square shaped in the upper part of the molecule (extracellular and transmembrane domains) and slightly twisted through the
top to the bottom of the particle. At the lower part of the cytoplasmic domain, the slice images are circular or octagonal (Fig. 6B, panels 15–19). Because the handedness of the images was not determined in this study, we show one of the alternatives. Although a detailed picture of inside the molecule is difficult to obtain using negative staining (25), protein density seems high at the outermost region of the prestin molecule and low inside the molecule (Fig. 6B). Further decreased density is observed in the center of slices 10–16. Indeed, weak stain permeations into the molecule are frequently observed within both raw images and averaged images (Fig. 5A), which indicates the likelihood of internal cavities. Although the density of molecular surface is basically high through the molecule, there are protein-sparse areas just beneath the plasma membrane (Fig. 6B, panels 11 and 12), which may be a possible entrance of ions into the molecule.

**Antibody Binding and Membrane Topology**—To address the molecular orientation across the membrane, we mixed prestin particles with anti-FLAG antibodies and, after negative staining, observed the prestin-anti-FLAG antibody complex by electron microscopy. Unbound antibodies were removed from the specimen by SEC. Preferable binding of antibodies to the wider end of prestin suggests the FLAG-fused cytoplasmic C terminus (7, 12) is located at the wider end (Fig. 7A). Because of its tetramer structure, prestin molecules bearing multiple anti-FLAG antibodies are frequently observed (Fig. 7A, columns 7 and 8). Similar to the observation in A, the gold conjugate binds to the wider end of prestin. Prestin particles bearing multiple Fab-gold conjugates are also observed (column 4). The scale bars represent 100 Å.
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negatively stained. The conjugates also bound to the wider end of prestin, and we frequently observed the binding of two Fab-gold particles with a single prestin particle (Fig. 7B). Prestin particles carrying three or four gold particles could not be easily observed, probably due to steric hindrance of large gold particles and small sized prestin particles.

The location of antibody binding to prestin was further analyzed by class averages of prestin-antibody complexes (Fig. 7A, columns 9 and 10). Despite the flexibility between prestin and antibodies at the binding epitopes, and between Fab and Fc fragments of antibody, the density of antibodies was obviously detected at the wider end of prestin. This agrees well with our EM observation of the complexes in raw images.

The putative position of the transmembrane domain (30 Å thickness, indicated in Fig. 6A, images 3 and 4) was determined so that the volumes of the extracellular, transmembrane, and cytoplasmic domains were close to 15, 33, and 52%, respectively, as calculated from the amino acid composition (Fig. 8A).

**DISCUSSION**

The observations of the OHC lateral membrane using the freeze-fracture technique (22) and AFM (23) demonstrated the accumulation of highly dense membrane proteins, assumed to be the motor protein prestin. This was supported by AFM observation of similar sized particles on the surface of Chinese hamster ovary cells in which prestin was heterologously expressed (24). The diameter of the particles was reported 8–15 nm, with the highest population at 11 nm (22–24). The dimensions presented in this study (77 Å in side length at the widest cytoplasmic region and 96 Å in diagonal length) agree with the previous observations; therefore, we conclude that the prestin molecules presented in this study correspond to individual particles on the OHC lateral membrane. Minor differences in size among the observations may be caused by dryness deformation of specimens in negative staining EM (25), by the thickness of additional metal layer in the freeze-fracture replica (44), or by a possible in vivo association with other related proteins, such as the glucose transporter GLUT-5 (45).

There may also be a difference in functional states of prestin among these observations. Prestin is thought to change its size and shape by liganded anionic ions and in a voltage-dependent manner. The functional state of prestin presented in this study cannot be easily determined, because it was purified in an environment containing Cl⁻ and analyzed without applying membrane potential. Creating a novel system for reproducing conformational changes of prestin in vitro (e.g. by removing Cl⁻ completely), in combination with EM observation, is necessary for explaining the mechanism of the rapid movement of prestin.

The functional stoichiometry of prestin has been speculated to be a multimer, and a tetramer is the most likely (11, 13, 14). Indeed, the size of the highly dense particles observed on the OHC surface is much larger than the size estimated for the monomer (22, 23). Even a spherical protein of 8 nm in diameter has an estimation of 226 kDa (if the commonly accepted density of 1.4 g/cm³ is applied), which is far larger than the 81.4 kDa of the monomer. All our results, the binding of multiple anti-FLAG antibodies to a single prestin particle, the much larger molecular weight for purified prestin by SEC than estimated for the monomer, the native-PAGE analysis, and the square-shaped top raw images and averages, support the tetramer formation. The projections from our 3D structure (imposed 4-fold symmetry) reflect well the corresponding raw images and the class averages without applying symmetry. Furthermore, the large mass of aggregated prestin observed in early elution of SEC suggests the possibility of forming higher order architecture by associating with neighboring prestin molecules. This may reflect the nature of prestin that densely accumulates at the lateral membrane of the OHCs.

Although prestin belongs to a family of anion transporters, recent studies suggest that prestin does not transport anions across the plasma membrane (8). Instead, prestin changes its structure by voltage-dependent translocation of anions within the molecule. In the depolarized state, anions locate near the cytoplasmic side; in the hyperpolarized state, the bound Cl⁻ moves to the external face. In this study, we postulate a low
density space inside the prestin molecule and a high density wall at the outermost region of the extracellular and transmembrane domains (Fig. 6B, panels 1–11), supporting the report that neither uptake nor exclusion of anions occurs at the extracellular domain.

In this study we present the 3D structure of prestin at 2 nm resolution using negatively stained EM and single particle analysis. To provide objective results, a large number of projections (13,149 particle) was used for the analysis that enabled obtaining averaged images even of rarely observed angles. Greater detail of the molecule, however, could not be determined through this study, probably due to uneven staining and to dryness deformation of the molecule. Further studies, such as cryo-EM-based single particle analysis, will be necessary to obtain the more precise details.

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