A giant liposome for single-molecule observation of conformational changes in membrane proteins

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Abstract

We present an experimental system that allows visualization of conformational changes in membrane proteins at the single-molecule level. The target membrane protein is reconstituted in a giant liposome for independent control of the aqueous environments on the two sides of the membrane. For direct observation of conformational changes, an extra-liposomal site(s) of the target protein is bound to a glass surface, and a probe that is easily visible under a microscope, such as a micron-sized plastic bead, is attached to another site on the intra-liposomal side. A conformational change, or an angular motion in the tiny protein molecule, would manifest as a visible motion of the probe. The attachment of the protein on the glass surface also immobilizes the liposome, greatly facilitating its manipulation such as the probe injection. As a model system, we reconstituted ATP synthase (F0F1) in liposomes tens of μm in size, attached the protein specifically to a glass surface, and demonstrated its ATP-driven rotation in the membrane through the motion of a submicron bead.

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1. Introduction

Single-molecule techniques reveal dynamics of membrane proteins that are not directly accessible with traditional biochemical measurements [1]. Single-channel recordings have amply demonstrated the significance and power of studying individual behaviors, allowing detailed kinetic analysis of the opening and closing of ion channels [2]. Indeed, the channel current was the first proof that proteins work as single. Arrangement and architecture of individual membrane proteins can now be imaged with atomic force microscopy [3,4], and their movement in membranes by single-particle tracking [5,6]. Manipulation of a single membrane protein in situ, such as unfolding and refolding under force, is also feasible [7,8]. Here we focus on conformational changes in a membrane protein that underlie its function. Observation of individual molecules in real time reveals details of the kinetics of conformational changes, as demonstrated by fluorescence resonance energy transfer (FRET) which is sensitive to a small change in the distance between two chromophores [9–12]. At the single-molecule level, however, FRET signals are rather noisy, particularly in membrane environments where lipids (and other components) could give rise to a background fluorescence (and also scatter light). Also, photobleaching and possible blinking of the fluorophore limit the observation time. Our preference is a probe that is “huge” compared to the size of a protein molecule, such as an actin filament [13,14] or a micron-sized bead [15], that allows long-time imaging at high spatial and temporal precisions. Movies of the motion of a huge probe can often be interpreted without analysis, enabling seeing-is-understanding type experiments. In addition, a huge probe by itself magnifies angular changes in a small protein molecule, and thus is particularly suited for the detection of a conformational change which necessarily accompanies an angular motion of one part against another. As examples, details of rotations in a flagellar motor [16] or F1-ATPase [13,17] have been observed clearly. Mechanical properties such as a force (or torque) generated by a protein machine can also be assessed by a huge probe that moves against viscous drag [18–20] or that works against an external force set by optical or magnetic tweezers [21–24]. A critical disadvantage with huge probes is that, if a conformational change (reorientational motion) is to be observed, a part of the protein must be fixed on a solid substrate in addition to the attachment of the probe on another part. To fulfill the last requirement, fixation on a substrate, for a protein molecule in a membrane, we chose to work with a giant liposome. Supported membranes also allow immobilization of protein molecules on a surface [25,26], but aqueous environments on both sides of the membrane cannot be controlled independently. Planar membrane...
systems have two independent compartments [27], but fixing a membrane protein in space is not easy. A liposome attached to a surface through the protein molecules of interest (Fig. 1A) suits both purposes, fixing the protein and controlling the media on two sides of the membrane independently. A giant liposome tens or hundreds of microns will allow injection of micron-sized beads (or other huge probes) that will be attached to the protein and move freely in the internal space of the liposome.

We selected F0F1-ATP synthase as a model membrane protein in this study. The ATP synthase consists of two rotary motors, the membrane-embedded F0 motor driven by the flow of hydrogen ions (protons or H+) across the membrane and the water-soluble F1 motor driven by hydrolysis of ATP [28–31]. Subunit compositions of the two motors are \( \alpha_3\beta_3\gamma_2\varepsilon_1\) for F1 and \( \alpha_2\beta_{10.15}\) for F0 in bacteria. When F0F1 functions, the subunits \( \gamma_3\epsilon_{10.15}\) rotate as the rotor, against the stator subunits \( \alpha_3\beta_3\sigma_{\alpha\beta}\) (darker subunits in Fig. 1B). That is, the rotors of the F0 and F1 motors are fused against each other, and the stators of both motors are also joined. Yet the genuine directions of rotation in the two motors are opposite to each other: when F0 wins, as in most in vivo conditions, F1 is forced to rotate in reverse and synthesizes ATP from ADP and inorganic phosphate (Pi). When hydrolysis of ATP by F1 is favored, protons are pumped back by F0. Both ATP-driven [32–34] and proton-driven [35–37] rotations of F0F1 have been observed.

We have reconstituted F0F1 of thermophilic origin in a membrane and produced giant liposomes by de-hydration and re-hydration (Fig. 1C) [38]. The liposomes were immobilized on a glass surface by covalent attachment of nickel nitrilotriacetic acid (Ni-NTA), through histidine-tags genetically introduced in the stator part of F0F1. Finally, we injected streptavidin-coated beads into a liposome to let the beads attach to the biotinylated rotor part of F0F1. In the presence of ATP, we observed that the beads rotated continuously.

2. Materials and methods

All experiments were performed at room temperature unless indicated otherwise.

2.1. Chemicals

Alexa Fluor 488 C5 maleimide (Alexa 488-maleimide), 5- (and 6)-carboxyfluorescein (mixed isomers) and N-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine-lipid) were purchased from Molecular Probes (Eugene, OR). NHS-PEG-MAL-3400 was from Nektar (San Carlos, CA), \( \alpha\)-phosphatidylcholine from soybean, type II-S from Sigma-Aldrich (Saint Louis, MO), N-(5-amino-1-carboxypentyl)iminodiacetic acid (AB-NTA), n-decyl-[\( \beta\)-o]-maltoside and N-6-(biotinylamino)hexanoyl-N-[2-(N-maleimidomethyl)ethyl]piperazinone, hydrochloride (biotin-maleimide) from Dojindo Laboratories (Kumamoto, Japan), tris[2-carboxyethyl]phosphine hydrochloride (TCEP) solution (0.5 M) from Pierce Biotechnology (Rockford, IL), and 3-mercaptopropyltrimethoxysilane (SH-silane) from GE Toshiba silicone (Tokyo, Japan).

Buffers used in this study are: buffer G (0.1 M K-Pi, pH 7.5, 1 mM MgSO4, 1% (v/v) glycerol); buffer MD (10 mM K-Pi, pH 7.5, 100 mM KCl, 5 mM n-decyl-[\( \beta\)-o]-maltoside); buffer R (10 mM HEPES-KOH, pH 7.5, 2 mM MgSO4, 50 mM KCl, 1 mM ATP); buffer RT (10 mM HEPES-KOH, pH 7.5, 2 mM MgSO4, 50 mM KCl, 0.05% (v/v) Triton X-100).

2.2. Preparation of F0F1

An expression vector, pTR-ISBS2-CNCR3 for a mutant F0F1 (c-Ser2Cys, \( \alpha\)-Cys193Ser, \( \alpha\)-Trp463Phe, \( \beta\)-10 histidines at N terminus) was used to express the recombinant thermophilic Bacillus PS3 F0F1 in Escherichia coli cells. The plasmid vector was constructed from pTR19-ASDS-CNCR3 [34], by additionally introducing uncl gene that codes a molecular chaperon for c-ring assembly (to be published elsewhere). F0F1 was expressed in F0F1-deficient E. coli strain DK8 [39] and purified as described [40]. Briefly, the E. coli cells were cultured in 2×YT medium containing 100 µg/ml of ampicillin, and inverted membranes were prepared by French Press. F0F1 were solubilized from the membranes with 2% (v/v) TritonX-100 and 0.5% (w/v) sodium deoxycholate and purified on a Ni-NTA column (Qiagen, Hilden, Germany). Eluted F0F1 was concentrated by the addition of ammonium sulfate. The concentration of F0F1 was estimated from the extinction coefficient of 249,000 (M⁻¹ cm⁻¹) predicted from the amino acid sequence [41] and converted to mg/ml assuming a molecular weight of 532 kDa.

2.3. Modification of F0F1

Biotinylation of F0F1, of which the sole cysteine residues were the introduced Ser2Cys on the ten c subunits and an intrinsic a-Cys27, was performed as follows. 200 µl of purified F0F1 at 24 mg/ml was reduced with 1 mM TCEP in 50 mM Tris-SO4, pH 8.0, 5 mM n-decyl-[\( \beta\)-o]-maltoside. After 1 h, excess TCEP was removed on an NAP-5 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer MD. To 700 µl of the eluent, 5 µl of 10 mM biotin-maleimide dissolved in DMSO was added. After 1 h, 10 mM DTT was added to quench the reaction. After another 1 h, the biotinylated F0F1 was frozen in liquid nitrogen and stored at −80 °C until use.

Biotinylation was confirmed by Western blotting. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at
16% (w/v) acrylamide, the gel was blotted onto a PVDF-membrane (Bio-Rad Laboratories, Hercules, CA) in Tris buffer (25 mM Tris, 192 mM glycine, 20% (w/v) methanol) with 0.1% (w/v) SDS, and stained with streptavidin followed by biotinylated horse radish peroxidase (Vectastain ABC kit, Vector laboratories, Burlingame, CA) and its fluorescent substrate (ImmunoStaining HRP-1000, Konica Minolta Medical & Graphic, Tokyo, Japan).

Labeling F2F1, with the fluorescent dye Alexa 488 was performed as the biotinylation above with several differences. Reduction was by 5 mM TCEP instead of 1 mM. To 700 μl eluate from NAP5, 18 μl of 2 mM Alexa 488-maleimide in DMSO was added. After quenching the reaction with DTT, unreacted dye and DTT were removed with a centrifugal filter device (Ultrafree-0.5 Biomax-30, Millipore, Billerica, MA) as follows: 500 μl of the quenched sample was loaded on a centrifugal device pre-rinsed with buffer MD and centrifuged at 9500 × g for 7 min at 4 °C. 400 μl of buffer MD was added to the concentrated sample (≈100 μl) and centrifuged again. After another round, the labeled F2F1 was recovered in 200 μl of buffer MD, frozen in liquid nitrogen, and stored at −80 °C until use.

Labeling was confirmed on the 16% (w/v) gel illuminated with ultraviolet light. The dye/protein molar ratio in the final purified sample was estimated from the Alexa 488 and protein absorbances. For Alexa 488, its peak absorbance of 77,100 M−1 cm−1 (supplied by the manufacturer) at 493 nm in buffer MD was assumed to remain the same after labeling, although labeling shifted the peak wavelength to 498 nm. To estimate the protein concentration from A280 nm, the contribution of Alexa 488 absorption at 280 nm was subtracted by assuming its A280 nm/A493 nm was equal to the A280 nm/A493 nm of 0.157 measured for free dye in buffer MD.

2.4. Preparation of lipid and proteolipid suspensions

To prepare giant liposomes by dehydration and rehydration, we first prepared suspensions of lipid and of the mixture of lipid and F2F1, as starting materials. The suspensions were presumably in the form of multilamellar liposomes, although we did not confirm their nature.

Pure lipid suspension was prepared as described [42]. The soybean lipid was washed with acetone [43] and dissolved in chloroform at 20 mg/ml. 250 μl of chloroform was added and mixed, and the lipid was dried under vacuum with a centrifugal device pre-rinsed with buffer MD and centrifuged at 800 × g for 7 min at 4 °C. 400 μl of buffer MD was added to the concentrated sample (≈100 μl) and centrifuged again. After another round, the labeled F2F1 was recovered in 200 μl of buffer MD, frozen in liquid nitrogen, and stored at −80 °C until use.

These were dissolved in 0.5 ml of 10 mM Tricine-NaOH, pH 8.0, 5 mM Na2SO4, 0.157 measured for free dye in buffer MD.

2.5. Preparation of giant liposomes

Giant liposomes were prepared by de-hydration and re-hydration [38,45]. Two slightly different procedures were used for preparation and observation. One was for the confirmation of successful incorporation of F2F1 in the liposomal membrane. For this purpose, 5 μl each of the lipid and proteolipid suspensions above were mixed. The mixture was put on a coverslip (24 × 60 mm2, NEO, Matsunami Glass Industry, Osaka, Japan) and de-hydrated under vacuum. After ≈10 min 200 μl of buffer R was added to the de-hydrated film. After ≈30 min the sample was observed under a microscope.

The other procedure was for the immobilization of giant liposomes on a Ni-NiTA modified glass surface. On the Ni-NTA modified coverslip (24 × 32 mm2) described below, we placed a silicon rubber sheet (1 mm thick) with a central rectangular hole ~16 × 22 mm2 to make an observation chamber. 20 μl each of the lipid and proteolipid suspensions were mixed. When rhodamine-lipid was to be included, 10 μl each of pure lipid and rhodamine-lipid containing suspensions described above were mixed with 20 μl of the proteolipid suspension including Alexa 488-labeled F2F1; with these ratios, the fluorescence intensities of rhodamine and Alexa 488 became comparable. The 40 μl mixture was divided into four 10-μl spots on a coverslip and de-hydrated under vacuum. After 20 min 50 μl of buffer R was added to each de-hydrated film. After 5 min, all four drops were poured on the top of 0.5 ml of buffer R overfilling the open observation chamber. After ~3 h when most of the liposomes had settled on the bottom, observation under a microscope was started.

2.6. Modification of coverslips with Ni-NiTA

Glass surface was modified with Ni-NiTA as follows. First, 24 × 32 mm2 coverslips (Matsunami Glass Industry) were coated with SH-silane [46]. The silanized surfaces were reduced by incubation with 0.1 M DTT for 1 h and washed with ultra pure water. Then the coverslips were immersed in 20 mM NHS-PEG-MAL-3400, 200 mM AB-NTA and 300 mM NaOH in 50 mM MOPS-NaOH, pH 7.0 (final pH was 8.6–8.8) for >4 h and washed with ultra pure water. Finally, they were incubated in 10 mM NiSO4 for >6 h, washed with ultra pure water and stored at room temperature under air until use.

2.7. Preparation of streptavidin-coated beads

Streptavidin-coated magnetic beads with a nominal diameter of 0.711 μm (3015-2105, Seradyn, Indianapolis, IN) were washed [47] as follows. 10 μl of the bead suspension was mixed with 2 ml ultra-pure water and centrifuged at 800 × g for 9 min at 4 °C in a swing rotor. Supernatant was recovered and centrifuged at 8000 × g for 10 min at 4 °C. The pellet was dissolved in 400 μl of ultra-pure water and centrifuged at 8000 × g for 5 min at 4 °C. The final centrifugal washing was repeated four more times. The pellet in the last wash was dissolved in 10 μl of ultra-pure water and stored on ice. Beads thus prepared were used within two days.

2.8. Observation of rotation of F2F1 in giant liposomes

After giant liposomes containing biotinylated F2F1, settled and attached to the Ni-NTA modified bottom of the observation chamber, streptavidin-coated beads were injected into a lipidome under a microscope [42]. Glass pipettes were drawn of borosilicate glass capillaries of outer and inner diameters 1.0 mm and 0.78 mm (GC100T-10, Harvard Apparatus, Holliston, MA) with a micropipette puller (P-2000, Sutter Instrument, Novato, CA). A pipette was filled
with the mixture of 5 μl of the washed beads and 5 μl of buffer R, and set on a home-made capillary holder mounted on a micromanipulator (TransferMan NK2, Eppendorf, Hamburg, Germany). The pipette tip was inserted into a liposome with the help of electric pulses at 30 V and duration 2 ms generated by a D-A converter (PCI-3335, Interface, Hiroshima, Japan) in a personal computer and amplified with an NF 4015 amplifier (NF, Kanagawa, Japan). An Ag/AgCl electrode was placed in the external solution to serve as the counter electrode. Injection of the beads was controlled pneumatically by a microinjector (CellTram vario, Eppendorf) and took 60–150 s to fill a liposome with tens of beads. After the injection the pipette was withdrawn from the giant liposome. In long observations (1.5–2 h), evaporation from the open observation chamber decreased the solution volume from the initial ∼700 μl to ∼500 μl, implying an increase in solute concentrations by ∼50%.

2.10. Imaging system

We used a conventional microscope (IX71, Olympus, Tokyo, Japan). Both differential-interference-contrast (DIC) and fluorescence images were observed through an UPlan FI objective (40×, numerical aperture 0.75, Olympus). The fluorescence cassette used for Alexa 488 and carboxyfluorescein observation was U-MWB2 (excitation 460–490 nm, emission >510 nm, Olympus), and, for rhodamine-lipid, U-MWCG2 (excitation 510–550 nm, emission >590 nm). Images were captured with an EM-CCD camera (MC081SDP-ROBO, Texas Instruments, Dallas, TX). The analogue output of the camera was connected to an LCD monitor (LMD-1410, Sony, Tokyo, Japan) for a visual check. The digital output was saved to a personal computer through a CL61 (BitFlow, Woburn, MA) frame grabber and a CLT-301L (Vivid Engineering, Shrewsbury, MA) camera link translator. The image-capture software was CiView (BitFlow) for still pictures and VideoSavant 4.0 (IO Industries, Ontario, Canada) for movies (30 Hz).

2.11. Data analysis

Rotation time courses were analyzed by eye by watching the video sequence and assigning a rotary angle to the bead image in each frame with a resolution of 30°. When the rotation was slow, the assignment was made in every other frame or every 3 frames. To convert the instantaneous angles into cumulative revolutions, we restricted the angular difference between successive frames to be between −150° and +180°. For a fast-moving bead that occasionally rotates more than +180° per frame, this procedure will report a negative rotary step instead of a positive +180° step. Because we could not be 100% sure that the step was positive, we left the negative values untouched (although negative steps with a size exceeding 90° were virtually absent in slowly rotating beads).

3. Results

3.1. Modification of FOF1

To attach a streptavidin-coated bead(s) as a probe of rotation, c subunits in the rotor part of FOF1 were biotinylated by mixing reduced FOF1 with biotin-maleimide. SDS-PAGE (Fig. 2A) did not show detectable differences between biotinylated and untreated FOF1 (δ and b subunits are resolved in Fig. 2E). Western blot stained for biotin (Fig. 2B) showed no bands for the untreated FOF1, whereas several bands with molecular weights corresponding to the c subunit and its oligomers appeared in the lane of biotinylated FOF1. The bands labeled c, c2, and c10 were also detected in anti-c immunoblot (data not shown). Although the FOF1 we used had another cysteine residue at the a subunit (a-Cys27), the corresponding band was not seen, suggesting that the a subunit is less reactive to biotin-maleimide.

We used FOF1 labeled with Alexa 488-maleimide to confirm incorporation in liposomal membranes (see below). The results were similar to the biotinylation above: no change in the subunit composition (Fig. 2C), and apparently exclusive labeling of the c subunit (Fig. 2D). Lane 2 of Fig. 2D is the Alexa 488-labeled FOF1 before

![Fig. 2](image-url). Confirmation of modification of FOF1 with biotin or a fluorescent probe (Alexa 488). Purified FOF1 was labeled with biotin-maleimide (A and B) or Alexa 488-maleimide (C and D) and subjected to SDS-PAGE at 16% (w/v) acrylamide (A, C and D) followed by Western blotting (B). In (E), 14% gel was used to resolve δ and b bands. Lanes M, Mb, 1, 2, 3 and 4 are respectively molecular markers, biotinylated molecular markers, biotinylated FOF1, Alexa 488-labeled FOF1, before free-dye removal by filtration, purified Alexa 488-labeled FOF1 and untreated FOF1, (A), (C) and (E) are gels stained with CBB. In (B), biotinylated proteins were detected after blotting on PVDF membrane. (D) is the gel without staining imaged under ultra violet illumination to detect Alexa 488-fluorophore. The amount of FOF1 applied was 20 μg in all lanes. Molecular weights of molecular markers in kDa are shown on the left of each gel and subunit identification for FOF1 on the right.
filtration for the removal of free dye. The bottom band, which disappeared after soaking the gel in the SDS-running buffer for 1 h, shows the free dye, of which the intensity is consistent with the labeling stoichiometry below. We used filtrated sample (lane 3) in all experiments described below.

The labeling yield for Alexa 488 was estimated from the absorption spectra. The peak wavelength of the Alexa 488 absorption shifted from 493 nm to 498 nm upon binding to F$_{2}$F$_{1}$, but we assumed that the extinction coefficient did not change appreciably and estimated the dye concentration accordingly. The dye/protein molar ratio, [Alexa 488]/[F$_{2}$F$_{1}$], was 2.47 in the final purified sample, while that of the reaction mixture was 4.43. Thus the reaction yield was ∼50%, consistent with lane 2 of Fig. 2D.

### 3.2. Reconstitution of F$_{2}$F$_{1}$ into giant liposomes

Several methods have been reported for the reconstitution of membrane proteins into giant liposomes: de-hydration and re-hydration [38,45], peptide-induced fusion [48], and electro-formation [49]. We selected the de-hydration and re-hydration procedure because it works at physiological ionic strengths. To confirm successful reconstitution, we prepared liposomes with the Alexa 488-labeled F$_{2}$F$_{1}$ and observed them under a microscope. A typical liposome is shown in Fig. 3A. Thin, circular contour of the ∼75 μm liposome seen in the DIC image (arrow) matches the fluorescent ring in the bottom image, indicating incorporation of the Alexa 488-labeled F$_{2}$F$_{1}$ in the membrane.

Most of liposomes were attached to a large aggregate of lipids (the dark object, indicated by an asterisk, in Fig. 3A) as previously reported [38,42]. The lipid appendage contained the Alexa 488-labeled F$_{2}$F$_{1}$, as seen in the fluorescence image. Immediately after preparation, we often observed that a liposome with an appendage grew in size, indicating that the aggregate is the seed of the liposomal membrane. We also observed, though not many, appendage-free liposomes which were presumably pinched off an aggregate.

Liposomes prepared with unlabeled F$_{2}$F$_{1}$ were indistinguishable from the labeled ones in the DIC image (Fig. 3B). The lipid aggregate in unlabeled liposomes also fluoresced, due to impurities in the soybean lipid, but the intensity was obviously weaker than the labeled liposomes. The liposomal membrane is hardly visible in Fig. 3B.

### 3.3. Immobilization of a giant liposome on a glass surface via the embedded F$_{2}$F$_{1}$

Attaching the proteoliposome to a glass surface via the incorporated membrane protein to be studied serves three purposes: (i) the immobilized protein stays in the field of view and allows long-time observation, (ii) conformational changes of the protein on the surface can be monitored as an angular movement of a probe attached to the protein, and (iii) immobilization of the liposome greatly facilitates its manipulation (injection of the probes, ligands, etc.). The F$_{2}$F$_{1}$ we used carried a histidine-tag at the N-terminus of each of the three β subunits (Fig. 1B), which would specifically bind to a Ni-NTA modified glass surface. To test if this specific interaction leads to the desired immobilization, we prepared giant liposomes that contained the Alexa 488-labeled F$_{2}$F$_{1}$ and a small amount of fluorescent lipid (rhodamine-lipid) and transferred the proteoliposomes into an open chamber whose bottom glass surface was modified with Ni-NTA. After ∼3 h when most liposomes have settled on the bottom, we observed their morphology by DIC and the locations of the Alexa 488-F$_{2}$F$_{1}$ and the rhodamine-lipid separately in the fluorescence images at respective wavelengths. The outermost contours of the liposomes on the chamber bottom were only slightly above the glass surface (dotted line in Fig. 4C, left), and most were oval (Fig. 4A) compared to the circular appearance as in Fig. 3A or 4B (see below). A similar but somewhat smaller and more irregular contour was observed when the focus was shifted to the position of the glass surface. These observations indicate strong adhesion of the liposomal membrane to the Ni-NTA surface over a wide area, as diagramed in Fig. 4C.

Comparison of the Alexa 488 and rhodamine fluorescence images show that the histidine-tagged F$_{2}$F$_{1}$ was concentrated on the bottom surface and its concentration was low in other parts of the membrane including the outermost edge (also compare with Fig. 4B). A similar phenomenon has previously been reported where diffusible protein molecules in one liposome were concentrated at the interface with another liposome whose surface presented dense high-affinity sites for the protein [50]. A closer look at the surface images in Fig. 4A reveals that the distribution of F$_{2}$F$_{1}$ on the glass surface is heterogeneous, consisting of zones with a relatively high-density periphery. These are not membrane wrinkles or overlapping membranes, because we never saw such heterogeneity in the lipid image (except for the very bright lipid aggregates). The likely cause is that the adhesion of the membrane to the glass surface proceeded in several phases, beginning with a small area(s) of contact. The F$_{2}$F$_{1}$ molecules in the initial contact area should be immobile, while the molecules in the rest of the membrane can freely diffuse and bind to the glass surface at the edge of the initial contact area. This way the contact area would slowly expand, accumulating F$_{2}$F$_{1}$ densely at the periphery. If, for some reason (e.g., by flow), the expansion of the contact area became fast, then the density of F$_{2}$F$_{1}$ in the fast growing contact zone would be low. Or, another part of the free membrane might touch the surface, starting a similar process.

The extensive attachment of the liposomal membrane to the glass surface was due to the specific interaction between the histidine-tags of F$_{2}$F$_{1}$ and the Ni-NTA modified glass surface. In the presence of imidazole that competes with histidine, the outermost edges of the giant liposomes were high above the glass surface (Fig. 4C, right) and were always circular (Fig. 4B, DIC). The bottom contact was not clear and there was no sign of F$_{2}$F$_{1}$ depletion from the free membrane area.

![Fig. 3. Reconstitution of F$_{2}$F$_{1}$ into giant liposomes. Giant liposomes into which Alexa 488-labeled F$_{2}$F$_{1}$ (A) or unlabeled F$_{2}$F$_{1}$ (B) was reconstituted were observed with transmitted light (DIC) or fluorescence illumination. Fluorescence images were spatially averaged using a Gaussian smoothing filter. Arrows indicate liposomal membranes (circular), whereas asterisks indicate lipid aggregates which fluoresced strongly due to the Alexa 488-labeled F$_{2}$F$_{1}$ (A) or less strongly due to fluorescent impurities in the soybean lipid (B). The liposomal membrane is invisible in (B). Scale bar, 20 μm.](image-url)
In the absence of imidazole, the FOF1 molecules in the adhesion area must be immobilized on the glass surface through the three histidine-tags that bind to Ni-NTA.

3.4. Binding of streptavidin-coated beads to biotinylated F0F1 in membrane

To attach a submicron bead to the rotor of F0F1 to observe its rotation (a conformational change), we reconstituted the biotinylated F0F1, which would bind streptavidin on the c subunits (Fig. 2B), into giant liposomes. After the liposomes settled on the Ni-NTA modified glass surface, streptavidin-coated beads were injected into giant liposomes selected for an oval shape which is a sign of good adhesion (large contact area with glass surface). In ~10 min, many beads settled on the bottom of the liposome (black dots in Fig. 5A). In contrast, few beads were seen on the bottom if the FoF1 had not been biotinylated (Fig. 5B); the white to gray unsharp dots are out-of-focus images of beads that were floating above the surface. With the biotinylated F0F1, most beads on the bottom surface were tightly bound to the FoF1 immobilized on the glass surface. The beads did not move: they are clearly visible in the image averaged over 10 s (bottom of Fig. 5A). With unbiotinylated F0F1, on the other hand, most of the (out of focus) bead images disappeared after averaging (bottom of Fig. 5B), showing they were mobile (undergoing Brownian motion).

Fig. 4. Specific binding of the proteoliposomes to the Ni-NTA modified glass surface through the histidine-tags on F0F1. (A) A giant liposome containing Alexa 488-labeled F0F1 and rhodamine-lipid is seen in the DIC and fluorescence images (at Alexa 488 and rhodamine wavelengths). The focus of the images on the left was at the outermost edge of the liposome (dotted line in C), whereas the focus was at the glass surface on the right. Scale bar, 20 μm. (B) The same set of images for a liposome in the presence of 0.2 M imidazole. Arrows indicate liposomal membranes, and asterisks lipid aggregates. All fluorescence images were spatially averaged using a Gaussian smoothing filter. (C) Schematic cross sections for the case of adhesion (left) and no adhesion (right). Adhesion occurs in the absence of imidazole (A), resulting in flattening of the liposome and condensation of F0F1 on the bottom surface. Dotted lines indicate positions of outermost edges of liposomes.

Fig. 5. Specific binding of streptavidin-coated beads to biotinylated F0F1 in giant liposomes. DIC images focused at the glass surface obtained 10 min after injection of streptavidin-coated beads (black particles in the image) into a giant liposome with biotinylated (A) or untreated (B) F0F1. Arrows indicate membranes and asterisks lipid aggregates. Images at bottom have been accumulated for 300 frames (10 s), whereas those at top are snapshots (1 frame = 33 ms): moving beads disappear in the accumulated images. Scale bar, 20 μm.
Thus, the biotinylated F$_{0}$F$_{1}$ sitting in the membrane and anchored to the glass surface can still bind a bead tightly through the specific streptavidin–biotin linkage.

3.5. ATP-driven rotation of F$_{0}$F$_{1}$ in the liposomal membrane

Observation of F$_{0}$F$_{1}$ rotation requires that a bead is bound by only one F$_{0}$F$_{1}$ molecule; if two molecules immobilized on the surface bind a bead simultaneously, rotation would be hindered. To reduce the probability of multiple binding, we diluted the biotinylated F$_{0}$F$_{1}$ with untreated F$_{0}$F$_{1}$ in the reconstitution. Several minutes after the injection of streptavidin-coated beads into an oval-shaped giant liposome immobilized on the Ni-NTA modified glass surface, we began to observe some beads settled on the surface to start rotating counterclockwise (1 mM ATP was present in the solution). A typical example is shown in Fig. 6A. Only a few among tens of beads settled on the bottom rotated. Some started to rotate as soon as they came down to the bottom, and others started at variable times. Most rotating beads stopped, and some resumed rotation again, during an observation for ~2 h; MgADP inhibition [51,52] is the likely cause of these behaviors. Detachment of rotating beads was also observed.

Time courses of rotation of beads that made more than 10 revolutions are shown in Fig. 6B. The rotary speed varied significantly. The primary reason is the heterogeneity in both size and shape of the beads that we used in this research, which resulted in a wide distribution of the viscous drag against the beads; aggregates of beads also rotated, and these were slow. Some beads lapsed into short pauses frequently, probably due to steric hindrances because the pauses usually occurred at the same angle(s). The apparent backsteps seen only in the fastest beads (Fig. 6B) are presumably an artifact of the data analysis: when a bead rotates more than 180° in one video frame (33 ms), the event is recorded as a negative rotary artifact of the data analysis: when a bead rotates more than 180° in one video frame (33 ms), the event is recorded as a negative rotary artifact.

3.6. F$_{0}$F$_{1}$ must be in the lipid membrane to rotate

It could be argued that, at the bottom contact between a giant liposome and the Ni-NTA glass surface, lipid membrane might be distorted or non existent and the F$_{0}$F$_{1}$ molecules in this region are not embedded in the lipid environment. Although the images of fluorescent lipid (Fig. 4A) suggest this is not the case, we sought additional warranty that the rotating F$_{0}$F$_{1}$ was indeed embedded in the lipid membrane, by asking whether F$_{0}$F$_{1}$ can rotate in the absence of lipids.

That F$_{0}$F$_{1}$ can rotate in detergent micelles has been documented [32,34]. Here we made a similar system and exchanged the solution alternately with or without detergent (Triton X-100) to see whether bare F$_{0}$F$_{1}$ can rotate. As shown in Fig. 7, ~70 beads per ~100,000 μm$^2$ rotated in the first solution with the detergent. Exchanging to detergent-free solution abolished rotation except for a couple of beads. Further solution changes gave essentially similar results. F$_{0}$F$_{1}$ in a detergent-free solution, even though some residual detergent may well remain, cannot rotate. A detergent micelle, or lipid membrane, is necessary for its function.

3.7. Confinement of a fluorescent dye in the immobilized proteoliposome

To test if the giant liposomes immobilized on a surface through the embedded F$_{0}$F$_{1}$ are capable of retaining solute, we injected a water-
soluble fluorescent dye carboxyfluorescein and monitored its fluorescence intensity under a microscope. Under continuous excitation, the fluorescence intensity decreased exponentially with a time constant of $75 \pm 6$ s (mean $\pm$ s.d.) (lines without symbols in Fig. 8), due mostly to photobleaching, whereas intermittent excitation at symbols lengthened the time constant to $170 \pm 61$ s. The giant liposome system we have developed here retains the dye for hundreds of seconds.

4. Discussion

We have shown that the giant-liposome system we have developed fulfills the requirements for direct observation of conformational changes in single membrane proteins: (i) the target membrane protein ($F_{0}F_{1}$) was embedded in the lipid membrane that separates two aqueous compartments, (ii) part of the protein molecules, while sitting in the membrane, could be specifically immobilized on the glass surface, and (iii) thanks to the immobilization, ATP-induced conformational changes (rotation) of the protein was visualized through the motion of a submicron-sized probe. An additional advantage in our method is the ease in repeating many experiments. Once we prepare a sample, many independent giant liposomes are available in one observation chamber. If, for example, one liposome is broken during the manipulation of bead-injection, or if a liposome fails to produce a rotating bead, we can test a next liposome in the same chamber without losing time. With a planar membrane system, in contrast, breakage or failure of a membrane would be the end of an experiment and a new membrane must be prepared afresh.

A probe that is sufficiently large for direct optical imaging allows straightforward interpretation of what is going on under a microscope. There is little doubt, just by looking into a microscope to unambiguously or sound interpretation, the molecule under observation, a rotary molecular motor. An essential point here is that, for an membrane system, in contrast, breakage or failure of a membrane will be broken during the manipulation of bead-injection, or if a liposome fails to produce a rotating bead, we can test a next liposome in the same chamber without losing time. With a planar membrane system, in contrast, breakage or failure of a membrane would be the end of an experiment and a new membrane must be prepared afresh.

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References

[8] K. Misler, K.E. Gittes, J. Howard, The force exerted by a single kinesin molecule driven by proton motive force across the liposomal membrane is not trivial. Most other probes are much less compared to a huge probe such as the bead we used. Atomic force microscopy [54,55] is more damaging and disturbing, but it provides detailed structural information rather than just motion, and all molecules in the field of view can be studied simultaneously. Diffraacted X-ray tracking may be regarded a kind of optical imaging with a huge probe, with possibly higher precision in angular movements. All methods are complementary to each other, and what we have shown in this work is one adaptation of the huge-probe method to membrane proteins.

A natural application of the current system is to observe rotation of $F_{0}F_{1}$ driven by proton flow. We have been trying to show this, so far without the final success. One difficulty is that the rotation, leading to ATP synthesis, requires a lot of energy in terms of the proton motive force. A typical requirement is a transmembrane voltage of $\sim 70$ mV in addition to a pH difference of $\sim 3.3$ units [57]. Producing and maintaining this much across the liposomal membrane is not trivial. Most other molecular machines in membranes, such as channels, receptors, transporters and pumps, do not need such an enormous voltage/concentration difference for function. We hope that the method we have developed will be of use in the elucidation of structure–function relationships in various membrane machines.


