A New Method for the Reconstitution of Membrane Proteins into Giant Unilamellar Vesicles

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ABSTRACT In this work, we have investigated a new and general method for the reconstitution of membrane proteins into giant unilamellar vesicles (GUVs). We have analyzed systematically the reconstitution of two radically different membrane proteins, the sarcoplasmic reticulum Ca^{2+}-ATPase and the H^+ pump bacteriorhodopsin. In a first step, our method involved a detergent-mediated reconstitution of solubilized membrane proteins into proteoliposomes of 0.1–0.2 μm in size. In a second step, these preformed proteoliposomes were partially dried under controlled humidity followed, in a third step, by electroswelling of the partially dried film to give GUVs. The physical characteristics of GUVs were analyzed in terms of morphology, size, and lamellarity using phase-contrast and differential interference contrast microscopy. The reconstitution process was further characterized by analyzing protein incorporation and biological activity. Both membrane proteins could be homogeneously incorporated into GUVs at lipid/protein ratios ranging from 5 to 40 (w/w). After reconstitution, both proteins retained their biological activity as demonstrated by H^+ or Ca^{2+} pumping driven by bacteriorhodopsin or Ca^{2+}-ATPase, respectively. This constitutes an efficient new method of reconstitution, leading to the production of large unilamellar membrane protein-containing vesicles of more than 20 μm in diameter, which should prove useful for functional and structural studies through the use of optical microscopy, optical tweezers, microelectrodes, or atomic force microscopy.

INTRODUCTION

Biological membranes are differentially permeable barriers separating two compartments. These membranes are formed by a mixture of lipid molecules organized as a bilayer in which are embedded amphiphilic proteins involved in a wide range of important biological processes such as transport phenomena, energy transduction, signaling, cell recognition, and motility (Alberts et al., 1994). However, the study at the molecular level of the roles of membrane proteins in these processes is often hampered by the complexity of the native membranes. Thus, as a first step toward a better understanding of complex biological membranes, it is often necessary to focus on more simple systems, consisting of membrane proteins reconstituted into artificial lipid membranes. In this context, incorporation of purified membrane proteins into proteoliposomes by detergent-mediated reconstitutions has allowed for the acquisition of a large amount of information about the functions and the structures of different membrane proteins (Rigaud et al., 1995, 2000). Despite the usefulness of these detergent-mediated reconstitutions, one main limitation is often related to the relative small size (0.1–0.2 μm) of the reconstituted proteoliposomes. Indeed, this small size impedes the analysis of structure/function relationships of membrane proteins through optical microscopy techniques such as fluorescence correlation spectroscopy, dynamic force spectroscopy, and single particle tracking or through proteoliposomes manipulation using pipettes, tweezers, electrodes, or atomic force microscopy (AFM).

Giant unilamellar vesicles (GUVs) with sizes ranging from 10 to 100 μm are potentially attractive models and are becoming objects of intense scrutiny in diverse areas focusing on biological functions such as adhesion, fusion and fission, or motility (Luisi and Walde, 2000). Several methods to produce giant vesicles have been developed and reported in the literature. One of the methods that has been applied successfully to produce large proteoliposomes amenable to patch-clamp recording and electrophysiological measurements is based on a dehydration-rehydration procedure described by Criado and Keller (1987). Such a method consists of the dehydration of biological membranes or preformed proteoliposomes in the presence of added exogenous lipids followed by a rehydration process in the desired buffer (Ajouz et al., 2000). As a variant of this method, the rehydration of thin dried films obtained after the evaporation of the solvent from lipid-protein complexes solubilized in organic solvents has been used to produce giant proteoliposomes (Darszon et al., 1980). The main advantage of this dehydration/rehydration method is that these vesicles can be prepared in a large range of buffer compositions and more specifically in

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buffers with high ionic strengths. However, this method produces a very heterogeneous population of proteoliposomes with a large proportion of multilamellar vesicles related to the uncontrolled rehydration process. This bottleneck has been overcome by the electroformation technique (Angelova et al., 1992), in which a lipid film is rehydrated in the presence of an alternating current (AC) electric field. Thus, GUVs can be produced with a more homogeneous distribution in size, centered around 20 μm in diameter. The use of this electroformation technique was first described by Manneville and co-workers to reconstitute an integral membrane protein, bacteriorhodopsin (BR) (Manneville et al., 2001). The GUVs were formed by rehydration under an AC electrical field of a dried film prepared from a solution of lipids and bacteriorhodopsin in diethyl ether. Unfortunately, such a reconstitution of membrane proteins by electroformation is very limited, due to the use of organic solvents that denature most amphiphilic membrane proteins. Another approach has been recently reported in which lipidic GUVs prepared by electroformation were fused with small preformed proteoliposomes containing bacteriorhodopsin. However, such a procedure limits the amount of protein that can be incorporated and leads to the insertion of foreign molecules, namely the fusogenic peptides that have been covalently attached to the preformed proteoliposomes to allow their fusion with GUVs (Kahya et al., 2001).

In this work, we present a new method, to our knowledge, for the reconstitution of transmembrane proteins into GUVs. This method involves three successive steps: 1), incorporation of the solubilized proteins into the membranes of submicrometer sized unilamellar vesicles by reconstitution upon detergent removal (Rigaud et al., 1995); 2), partial dehydration of these preformed proteoliposomes on conductive glass surfaces; and 3), controlled rehydration in the presence of an AC electric field to form giant vesicles (Angelova et al., 1992). We checked the usefulness of this new method by studying the reconstitution of two radically different transmembrane proteins. One is the Ca^{2+}-ATPase from the sarcoplasmic reticulum (SR), an ATP-driven Ca^{2+} pump of 110 kDa consisting of a large hydrophilic domain connected to a hydrophobic domain composed of 10 transmembrane α-helices (for review see Lee, 2002). The other protein is the bacteriorhodopsin from *Halobacterium salinarium*, a light-driven H^+ pump of 27 kDa that is a very hydrophobic membrane protein formed by seven transmembrane α-helices (for review see Oesterhelt and Tittor, 1989). The reconstituted giant vesicles produced by our new procedure were analyzed with respect to size and morphology and with respect to protein incorporation, protein distribution, and biological activity. In summary, our results allow us to define important parameters involved in the efficient and the functional reconstitution into GUVs of two prototypical membrane proteins, which should prove of general use for the reconstitution of other membrane proteins.

MATERIALS AND METHODS

Chemicals

Purified egg yolk L-α-phophatidylcholine (EYPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), stearolyloleoyl-phosphocholine (SOPC), egg yolk phophatidic acid (EYPA), 1,2-diacyl-*sn*-glycero-3-(phospho-L-serine) (DOPS), 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 2-(12-(7-nitro2-oxa-1,3-dialzol-4-yl)amino)dodecanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (NBD-C12-HPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Triton X-100 was from Roche Diagnostics (Mannheim, Germany), and octaethylene glycol mono-*n*-1-dodecyl ether ($C_{12}E_8$) was from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Polystyrene Bio-Beads SM-2 (25–50 mesh) were obtained from Bio-Rad (Hercule, CA). The fluorescent probes, fluorescein 5′-isothiocyanate (FITC), Fluo-5N impermeant form, 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (pyranine), and 9 amino-acridine (9-AA) were purchased from Molecular Probes (Eugene, OR). All other reagents were of analytical grade.

SR membranes were provided by Dr. P. Champeil (Commissariat à l'Énergie Atomique Saclay, France) and prepared from rabbit white skeletal muscles as described in Champeil et al. (1985). For reconstitution experiments, detergent-solubilized monomeric Ca^{2+}-ATPase was prepared by incubating SR vesicles in $C_{12}E_8$ at a detergent/protein ratio of 2 w/w in a buffer containing 20 mM Mops-Tris, pH 7.0, supplemented with 0.1 mM $CaCl_2$ to prevent the inactivation of the protein (Levy et al., 1992).

Purple membranes were purified from *H. salinarium* according to the procedure described in Oesterhelt and Stoeckenius (1974). For reconstitution experiments, detergent-solubilized monomeric bacteriorhodopsin was prepared by incubating overnight purple membrane in Triton X-100 at a detergent/protein ratio of 5 w/w in a buffer containing 20 mM Pipes, pH 6.8 (Rigaud et al., 1988).

Preparation of submicrons proteoliposomes

BR and Ca^{2+}-ATPase were reconstituted into proteoliposomes according to the general procedure developed by Rigaud and co-workers (Rigaud et al., 1988; Levy et al., 1992; Rigaud and Levy, 2003). Typically, pure liposomes at 4 mg lipid/ml were prepared by sonication in a buffer containing 2 mM Mops-Tris, pH 7.0, and completely solubilized into mixed micelles at detergent/lipid ratios of 2 (w/w) for Triton X-100 and $C_{12}E_8$. Then aliquots of solubilized proteins were added to the detergent-lipid mixture under stirring at the desired lipid/protein ratio. After 30 min incubation for micellar equilibration, the detergent was removed at room temperature by adding prewashed SM2 Bio-Beads (Rigaud et al., 1998). For BR reconstitution, Triton X-100 was removed by a slow removal procedure consisting in two successive additions of 10 mg Bio-Beads per milligram of Triton X-100 for 1 h each, followed by a third addition of 20 mg Bio-Beads per milligram of Triton X-100 to ensure full detergent removal. For Ca^{2+}-ATPase reconstitution, inhomogeneous protein incorporation (Levy et al., 1992) was avoided by fast detergent removal through the addition of 30 mg Bio-Beads per milligram of $C_{12}E_8$ at once, followed by an incubation of 4 h. After complete detergent removal, the reconstituted proteoliposomes were separated from the beads by pipetting the supernatant.

Preparation of GUVs

Giant unilamellar vesicles were generated by the electroformation technique (Angelova, 2000). Preformed proteoliposomes were diluted to 0.8 mg lipid/ml and droplets of $\sim$2 μl were carefully deposited on indium tin oxide (ITO) treated glass slides. The film was then partially dried overnight in a desiccator under saturated vapor pressure of a saturated NaCl solution. After partial dehydration, the two ITO slides were sealed with paste Sigillum wax (Vitrex, Copenhagen, Denmark) and separated with 1-mm Teflon spacers. About 1 ml buffer containing 0.1 M sucrose, 1 mM Mops-Tris, pH 7.0, and supplemented with either 1 mM $MgCl_2$ for Ca^{2+}-ATPase or 2 mM KCl for

BR was added, and the conductive glasses were connected to copper electrodes. For electroformation, an AC electric field provided by a pulse generator was applied for 4 h across the chamber and incremented every 5 min from 20 mV to 1.1 V at 12 Hz frequency. Then, the AC frequency was lowered to 4 Hz, and the voltage was raised to 2 V for 30 min to detach the giant vesicles from the glass slides.

Observation of GUVs

Giant vesicles were observed either by phase contrast or by differential interference contrast (DIC) with an inverted microscope (Axiovert 135, Carl Zeiss, Germany). Twenty microliters of the reconstituted GUVs were transferred into an observation chamber (22×22 mm^2 wide and 1 mm high) containing a buffer in which the sucrose buffer was replaced with an equiosmolar glucose buffer containing 5 mM Mops-Tris, pH 7.0, and supplemented either with 5 mM $MgCl_2$ and 0.5 mM $CaCl_2$ for Ca^{2+}-ATPase or with 5 mM KCl for BR. The density difference between the outside (glucose) and the inside (sucrose) of the vesicles allowed their sedimentation, and the refractive index gradient enhanced the image contrast. After 10 min, when all liposomes settled down to the bottom area, the whole area was scanned, and the size distribution of GUVs was scored.

Unilamellarity of the GUVs was checked by a fluorescence quenching assay based on the measurement of the distribution of fluorescent lipids between inner and outer monolayers (Gruber and Schindler, 1994). For such measurements, 0.5 mol % NBD-C12-HPC was included in the initial lipidic composition. After electroswelling, GUVs were transferred in a 2-ml cuvette, and the fluorescence was monitored in a Perkin Elmer LS 50B spectrofluorimeter (Foster City, CA) using 2-nm and 4-nm slits for excitation ($\lambda_{ex} = 465$ nm) and emission ($\lambda_{em} = 534$ nm), respectively. The contribution of the outer lipid layer was determined by measuring the fluorescence quenching induced by the addition of 20 μl solution containing 1 M sodium hydrosulfite in 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), pH 9.0. Then, addition of a solubilizing Triton X-100 concentration induced a further fluorescence quenching that allowed determining the contribution of inner lipid layers.

Labeling of proteins

Ca^{2+}-ATPase was labeled with fluorescein 5′-isothiocyanate by incubating SR vesicles at 2 mg protein/ml for 30 min at 25°C in darkness and in a buffer containing 0.3 M sucrose, 1 mM Mg^{2+}, 10 μM Ca^{2+}, 50 mM Tricine-Tris, pH 8.0, and a 10-fold excess of fluorescent label. The labeling reaction was stopped by centrifugation at $100{,}000 \times g$ for 15 min at 4°C. The pellet was suspended in 20 mM Mops-Tris, pH 7.0.

BR was labeled with FITC after a protocol adapted from Heberle and Dencher (1992). The fluorescent labeling was performed by incubating purple membranes in the presence of 1.9 μmol FITC per milligram of protein for 4 h at room temperature in 50 mM Tris-HCl, pH 8.5. The excess of nonbound fluorescein was removed by centrifugation at $60{,}000 \times g$ for 20 min. The labeled purple membranes were then washed twice in 50 mM Tris-HCl, pH 8.5, and left overnight at 4°C. The day after, membranes were washed three times, first in 0.2 M KCl and then in distilled water.

The concentration of bound fluorescein was measured spectrophotometrically in the presence of 1% sodium dodecyl sulfate, assuming a molar absorption coefficient of 8×10^4 M^{-1}cm^{-1} at 496 nm for the fluorescein bound to the protein (Mitchinson et al., 1982).

Assays for monitoring protein incorporation

Incorporation of FITC labeled proteins into GUVs was analyzed by confocal fluorescence microscopy using a Leica TCS SP2 Confocal Microscope (Leica Lazer Tecknik, Heidelberg, Germany) equipped with a Zeiss HCX-Apochromat 63×, 1.4-numerical-aperture oil immersion lens. An argon ion laser operating at 488 nm was used, close to the maximum of absorption of fluorescein. The epifluorescence was converted into a static beam by an *x-y* scanner device and focused onto a photomultiplier (PMT). The fluorescence emission, after passing a 505-nm high pass filter, was focused into a pinhole in front of the photomultiplier to eliminate all the out-of-focus light. The pinhole blocking aperture was at 230 μm for BR and at 320 μm for Ca^{2+}-ATPase. Commercial confocal software (Leica) was used for image analysis.

Assays for monitoring protein activities

The calcium-dependent hydrolytic ATPase activity of the Ca^{2+}-ATPase was measured at 25°C in an ATI Unicam UV2-100 spectrophotometer (Cambridge, Great Britain), using an enzyme-coupled method (Möller et al., 1979). With this method, 1 mol ADP produced leads to the oxidation of 1 mol α-nicotinamide adenine dinucleotide (NADH) and therefore to a decrease of the absorbance at 340 nm. Phosphoenol pyruvate was used to regenerate ATP. Typical experiments were performed in a continuously stirred 1-cm cuvette containing 40 μg Ca^{2+}-ATPase in 2 ml reaction buffer containing 50 mM Mops-Tris, pH 7.5, 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM phosphoenol pyruvate, 5 units/ml pyruvate kinase, 5 units/ml lactate dehydrogenase, and 1 mg/ml $C_{12}E_8$. The reaction was started by the addition of 1 mM ATP and stopped by 2.5 mM EGTA. The rate of ATP hydrolysis could be deduced from the difference of the two slopes obtained in the presence and in the absence of EGTA assuming a molar absorption coefficient ε_{340}(NADH) = 6220 M^{-1}cm^{-1}. Protein concentration was determined separately by absorbance measurements at 280 nm ($\varepsilon = 120{,}480$ M^{-1}cm^{-1}).

Ca^{2+} pumping activity was measured as changes in the fluorescence intensity of a low-affinity calcium indicator, Fluo-5N, entrapped inside vesicles. The Ca^{2+}-ATPase was reconstituted via electroformation in the sucrose buffer supplemented with 20 μM Fluo-5N. After electroswelling, GUVs were transferred into a microchamber containing the equiosmolar glucose buffer. Changes in internal calcium concentration upon addition of 1 mM ATP were followed in a confocal microscope by measuring the fluorescent intensity of the entrapped Fluo-5N ($\lambda_{ex} = 495$ nm and $\lambda_{em} = 515$ nm).

Light-induced transmembrane pH gradients by BR reconstituted into proteoliposomes were measured as changes in the fluorescence intensity of the Delta-pH-probe 9-AA (Cladera et al., 1996). Fluorescence was monitored with a Perkin Elmer LS 50B spectrofluorimeter using 400 and 460 nm for excitation and emission, respectively. Illumination was performed with a 250-W xenon lamp through a flexible glass fiber guide equipped with a low wavelength cutoff at 500 nm and a heat filter.

For BR pumping activity in GUVs, we measured the variations in the internal pH as changes in the fluorescence intensity of the membrane-impermeable pH sensitive probe pyranine entrapped into the GUVs lumen (Rigaud et al., 1988). Here, the GUVs containing BR were formed by electroswelling in the sucrose buffer supplemented with 10 μM pyranine. After electroswelling, GUVs were transferred into a microchamber containing the equiosmolar glucose buffer. Changes in internal pyranine fluorescence after 10 min illumination at 458 nm with a xenon lamp were monitored between 505 and 530 nm in a confocal microscope.

RESULTS

Reconstitution procedure

Our procedure for reconstituting membrane proteins into GUVs involves three successive steps. In the first step, solubilized proteins are reconstituted into the membranes of submicron proteoliposomes according to the detergent-mediated reconstitution protocol described by Rigaud and co-workers (Rigaud et al., 1995; Rigaud and Levy, 2003). This protocol, which consists of removing detergent from

lipid-protein-detergent micellar solutions, has been described as very efficient and relevant for the reconstitution of many membrane proteins, including the Ca^{2+}-ATPase (Levy et al., 1992) and BR (Rigaud et al., 1988). The use of polystyrene beads as a detergent-removing agent is a highly reproducible way to generate unilamellar proteoliposomes of ~0.1–0.2 μm in size and with homogenous protein incorporation in a large range of lipid/protein ratios. In addition, it allows for quasicomplete detergent removal, minimizing the possible interference of residual detergent molecules on the further formation of giant vesicles (for review see Rigaud et al., 1998). Finally, it is of note that, starting from a micellar lipid-protein-detergent mixture, this reconstitution procedure produces proteoliposomes with an asymmetric but preferential protein orientation in the membrane: ~80% of the Ca^{2+}-ATPase molecules are oriented with their large cytoplasmic domain outside, leading to efficient ATP-dependent Ca^{2+} accumulation (Levy et al., 1992), whereas ~75% of the BR molecules are oriented with their C-terminus outside, leading to an efficient light-induced H^+ accumulation inside the proteoliposomes (Rigaud et al., 1988).

In the second step, these preformed proteoliposomes are dehydrated on ITO treated glass slides. Preliminary experiments indicated that complete dehydration under high vacuum had deleterious effects on the biological activities of BR and Ca^{2+}-ATPase. Thus, it is essential to perform a partial dehydration of the proteoliposomes under controlled humidity, i.e., under saturated vapor pressure of a saturated salt solution.

The third step of the reconstitution process is a rehydration step in the presence of an AC electric field. Growing of large GUVs from the rehydrated lipid-protein film was promoted with an AC electric field with increments from 20 mV to 1.1 V at 12 Hz frequency. Once the GUVs were formed, the AC frequency was lowered to 4 Hz and the voltage raised to 2 V for 30 min. This helped the mature giant vesicles to adopt a spherical shape and to separate them from the glass slides. It is important to stress that the buffer composition used for the preparation of the preformed liposomes and for the rehydration step is crucial, considering that low ionic strengths impede the formation of giant vesicles by electroformation (see below).

Morphology and size

The effects of protein content, lipid composition, or buffer composition on the morphology and the size of reconstituted GUVs were monitored by phase contrast microscopy (Fig. 1 *a*). For Ca^{2+}-ATPase and BR reconstitution, GUV formation was dependent upon the lipid/protein ratio of the initial detergent-reconstituted proteoliposomes. Giant vesicles could be directly formed from native SR vesicles (lipid/Ca^{2+}-ATPase ratio of 0.8 w/w), but they exhibited tubular, ovoid, and pear-like shapes, probably related to the geometry

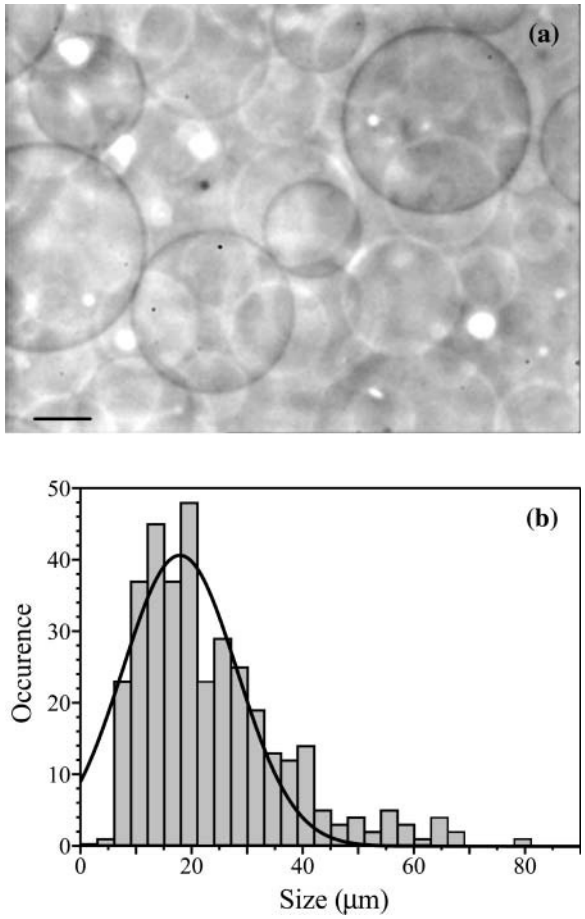

FIGURE 1 Size distribution of reconstituted GUVs. (*a*) Phase-contrast image of EYPC/EYPA (9:1) GUVs containing Ca^{2+}-ATPase at a lipid/protein ratio of 6.5 w/w. Scale bar = 10 μm. (*b*) Size distribution of the Ca^{2+}-ATPase reconstituted GUVs. Vesicles with a diameter lower than 5 μm were not counted.

of Ca^{2+}-ATPase. Starting from proteoliposomes reconstituted at lipid/Ca^{2+}-ATPase ratios of ~3.5 w/w (i.e., 490 mol/mol), spherical GUVs began to form, and the fraction of nonspherical vesicles decreased with increasing lipid/protein ratios. Preparations with only spherical GUVs could be obtained from proteoliposomes with lipid/Ca^{2+}-ATPase ratios ranging from 5 to 40 w/w (i.e., from 680 to 5500 mol/mol). As already observed for the electroformation of pure lipidic giant vesicles, the size distribution of the GUVs containing Ca^{2+}-ATPase was very broad with diameters varying from 5 to 100 μm, centered around 20 μm (Fig. 1 *b*). Similar results were obtained for the reconstitution of BR into GUVs. In particular, no GUVs could be formed starting from BR-proteoliposomes reconstituted at lipid/protein ratios smaller than 5 w/w (160 mol/mol), whereas spherical and large GUVs could be formed from proteoliposomes reconstituted at lipid/BR ratios ranging from 5 to 40 w/w (160–1350 mol/mol).

The general morphology and size of the reconstituted GUVs were independent of the nature of the lipids. Spherical GUVs with diameters varying from 5 to 100 μm could be formed from Ca^{2+}-ATPase or BR proteoliposomes reconstituted into neutral lipids (EYPC, DOPC, or SOPC) or with EYPC or DOPC mixtures containing charged lipids such as EYPA or DOPS, respectively. GUVs could also be produced from different phospholipid mixtures including DOPC/DOPE/DOPS (at 9:1:0, 8:1:1, and 7:2:1 ratios), brain lipids, or asolectin.

The composition of the buffers used for the preparation of the preformed liposomes and for the rehydration buffer is very important since the process of GUV formation by electroformation requires low ionic strengths (Angelova, 2000). Indeed, we have found that GUVs could not be formed with buffers containing more than 5 mM monovalent ions or 1 mM divalent ions. Since these low ionic strengths could be deleterious for biological activities, we have determined the minimal salt concentration needed to maintain the activity of the reconstituted proteins. For BR, we analyzed the amplitudes of the light-induced transmembrane pH gradients at different points in our reconstitution procedure by monitoring the changes in 9-AA fluorescence depending upon the buffer composition. When reconstitutions were performed in pure water, no activities could be detected. However, buffers containing 1 mM Mops-Tris pH 7.0 and 2 mM KCl were sufficient to observe a large light-induced 9-AA fluorescence quenching ($\Delta F/F = 30\%$) in the initial preformed proteoliposomes and after the dehydration/rehydration step (data not shown). For Ca^{2+}-ATPase, reconstitution and rehydration buffers containing 1 mM Mops-Tris pH 7.0 and 1 mM $MgCl_2$ were sufficient to retain the hydrolytic ATPase activity in preformed liposomes and after the dehydration/rehydration step (data not shown; see also Fig. 6 *a*).

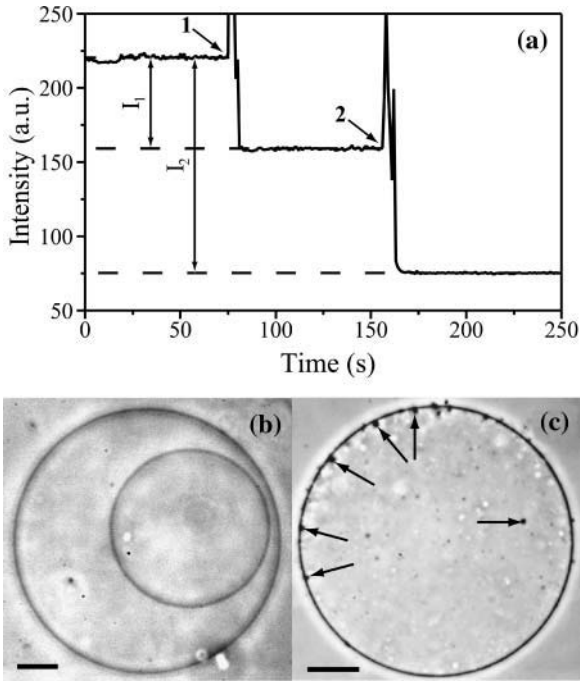

FIGURE 2 Unilamellarity of reconstituted GUVs using a fluorescent quenching assay. Giant vesicles were prepared with a lipid mixture containing EYPC/NBD-C12-HPC (99.5:0.5 mol/mol) at a lipid/Ca^{2+}-ATPase ratio of 6.5 w/w. (*a*) Unilamellarity of the GUVs was checked by measuring the distribution of fluorescent lipids between inner and outer monolayers as described in Materials and Methods. Addition of sodium hydrosulfite to reconstituted GUVs (*arrow 1*) induced a rapid quenching of the fluorescent lipids present in the outer lipid layer. Solubilization of the vesicles by an excess of Triton X-100 (*arrow 2*) induced a second decrease in fluorescence due to the quenching of the inner lipids. The I_1/I_2. ratio is an index of the unilamellarity of the vesicles and should be equal to 0.5 for a perfect unilamellarity. The value of 0.6 found for reconstituted GUVs could be accounted for by the presence of small vesicles (*b*) or other lipidic structures (*arrows* in *c*) that were sometimes visible encapsulated by the GUVs. Scale bars of phase-contrast images correspond to 5 μm.

Unilamellarity

The unilamellarity of the reconstituted GUVs was checked by analyzing the distribution of fluorescent lipids between the inner and outer lipid layers with a fluorescence quenching assay (Gruber and Schindler, 1994). For such measurements, 0.5 mol % NBD-C12-HPC was included in the initial lipidic composition. The addition to reconstituted GUVs of sodium hydrosulfite, an impermeant quenching agent (*arrow 1* in Fig. 2 *a*), decreased the fluorescence of NBD-C12-HPC lipids by reducing the dye located only in the outer leaflet of the bilayer. Then, a subsequent solubilization of the vesicles, done by adding an excess of detergent (*arrow 2*), allowed the NBD-C12-HPC lipids present in the inner leaflets to be exposed to the quencher. For the evaluation of the lamellarity of the vesicles, we assume that the first quenching step I_1 is proportional to the concentration of NBD-C12-HPC accessible in the outer leaflet of the bilayer, whereas I_2, the difference between the initial and final fluorescent values, is proportional to the total concentration of NBD-C12-HPC present in the sample. The I_1/I_2 ratio should be equal to 0.5 for a perfect unilamellar vesicle. For pure lipidic GUVs as well as for BR or Ca^{2+}-ATPase reconstituted GUVs, we obtained I_1/I_2 ratios between 0.38 and 0.42. Such ratios are consistent with a quasiequal distribution of dye on the outer and inner layer of unilamellar vesicles. The limited (10%) difference between outer and inner layer fluorescence intensities could be accounted for by the presence of small vesicles (Fig. 2 *b*) or other lipidic structures (*arrows* in Fig. 2 *c*) that sometimes appeared encapsulated inside the GUVs.

The unilamellarity of the reconstituted GUVs was confirmed by measuring the mechanical stretch properties of the vesicle with the micropipette aspiration technique (Kwok and Evans, 1981). The bending modulus κ of a membrane is proportional to the number of bilayers, and when a vesicle is aspirated with a suction pressure ΔP by a micropipette, part of the area stored in the membrane fluctuation is pulled inside the

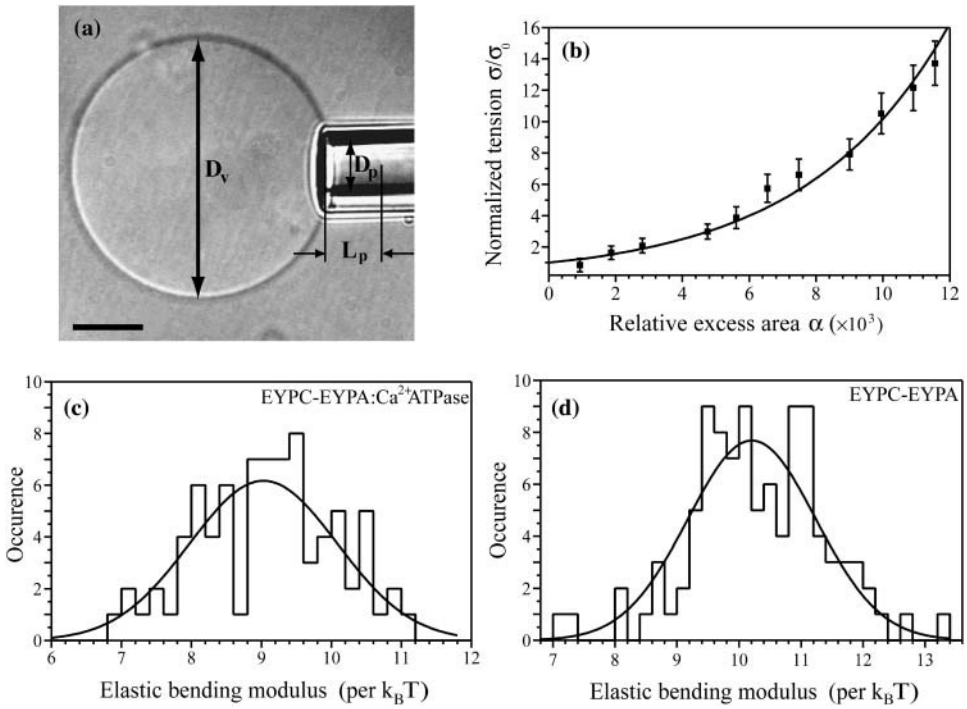

FIGURE 3 Unilamellarity of reconstituted GUVs using elastic bending measurement. Giant vesicles were prepared in EYPC/EYPA (9:1) at a lipid/Ca^{2+}-ATPase ratio of 6.5 w/w. (*a*) Microscopy image of a reconstituted GUV aspirated with a suction pressure by a micropipette and observed with a 63× differential interference contrast (DIC) oil immersion objective. D_P, pipette diameter; D_v, vesicle diameter; and L_p, projection length inside the micropipette. Scale bar = 5 μm. (*b*) Plot of membrane tension versus the area dilation of a reconstituted vesicle. The solid curve is fitted with an exponential function based on a bending rigidity modulus $\kappa = (9.3 \pm 1.1) \times k_BT$. Histograms of elastic bending moduli from micropipette aspiration experiments on 80 Ca^{2+}-ATPase reconstituted GUVs (*c*) and on 100 pure lipidic GUVs (*d*).

pipette with a length L_p as shown in Fig. 3 *a*. The relative excess area α and the membrane tension σ can be deduced from measurements of the projection length L_p, the pressure difference ΔP, the pipette diameter D_P, and the vesicle diameter D_v (Olbrich et al., 2000). We deduced the bending modulus from the dependence of the applied tension σ on the relative excess area $\alpha = (A_0 - A_p)/A_0$ by the equation (Evans and Rawicz, 1990):

$$\sigma = \sigma_0 \exp\left(\frac{8\pi\kappa\alpha}{k_BT}\right), \quad (1)$$

in which A_0 is the optically measured surface at the initial value σ_0 of the tension, A_p is the projected surface for consecutive tension values σ, and the thermal energy k_BT is 4×10^{-21} J. As depicted in Fig. 3 *b*, the tension of a GUV reconstituted at a lipid/Ca^{2+}-ATPase ratio of 6.5 w/w (900 mol/mol) increases exponentially with its excess area. These data (*solid squares*) can be fitted perfectly with Eq. 1 (*solid line*) using an elastic bending modulus κ of $(9.3 \pm 1.1) \times k_BT$. Fig. 3 *c* shows the results for 80 independent tests that give an average modulus of $9.0 \times k_BT$ with a standard deviation of $1.5 \times k_BT$. The order of magnitude of this modulus value is in good agreement with the value of $(10.2 \pm 1.5) \times k_BT$ found for pure lipid vesicles (Fig. 3 *d*). Even if the presence of the Ca^{2+}-ATPase induces a small decrease in the bending rigidity of the membrane, the vesicles are clearly unilamellar, as no higher modulus multiple of κ was measured. Moreover, this small decrease may be due to the renormalization of the bending rigidity in the presence of inclusions (endogenous lipids and proteins) (Leibler, 1986). Similar results have been obtained with BR, confirming the unilamellarity of reconstituted GUVs (data not shown).

Homogeneity of the reconstitution of transmembrane proteins into GUVs

An important issue of membrane protein reconstitution is the homogenous incorporation of proteins among the reconstituted vesicles and the homogeneity of the distribution of the proteins inside the membranes. We have checked these two points using confocal fluorescence microscopy. The measurements were performed at different lipid/protein ratios on 50 vesicles containing FITC-labeled Ca^{2+}-ATPase (Fig. 4 *a*) or FITC-labeled BR (Fig. 5 *a*). The laser intensity was kept constant for the different sets of experiments, ensuring that the fluorescence measurements were directly comparable.

Using Scion Image software (Scion, MD), the fluorescence intensity (in gray levels varying from 0 to 255 a.u.) was recorded at four different points of the membrane: equator, pole, and two latitudes (Fig. 4 *a*). These measurements gave similar results for each vesicle indicating that the proteins were incorporated homogeneously in the membrane (data not shown). The normalized fluorescence intensities, which correspond to the fluorescence intensities divided by 255, were then plotted for 50 vesicles and for different lipid/Ca^{2+}-ATPase ratios (see Fig. 4 *b*). The distribution was Gaussian for each ratio, and with a higher halfwidth for the lower protein concentrations, due to the lower signal/noise ratio of the fluorescence images. The center of the distribution was assigned to the average intensity of the

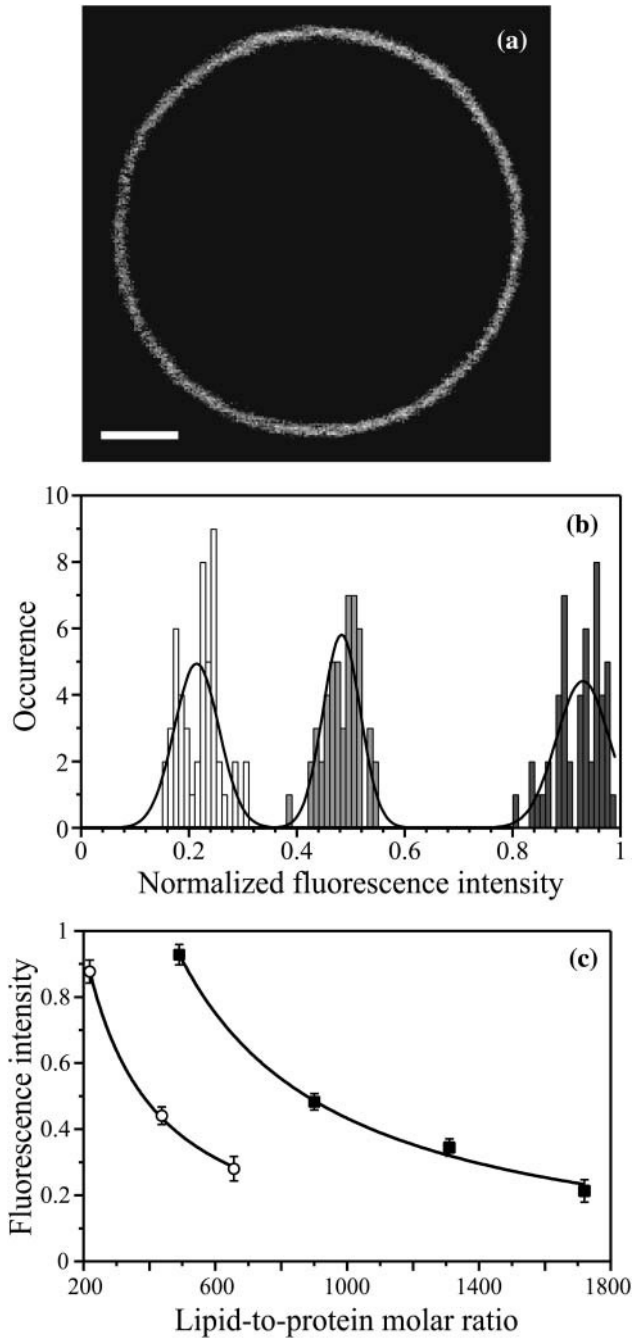

FIGURE 4 Homogeneity of Ca^{2+}-ATPase incorporation in GUVs. (*a*) Fluorescence intensity image (false color representation) of a GUV reconstituted at a SOPC/FITC labeled Ca^{2+}-ATPase ratio of 6.5 w/w. Scale bar = 5 μm. (*b*) Fluorescence distribution of 50 GUVs reconstituted at lipid/Ca^{2+}-ATPase ratios of 3.5 (*dark shading*), 6.5 (*light shading*), and 12.5 w/w (*open*). A normal distribution function has been superimposed for each ratio giving the mean and standard deviation. (*c*) The average of the normalized fluorescence intensity, obtained in *b*, is reported as a function of the lipid to Ca^{2+}-ATPase (■) or lipid/BR (○) ratios.

membrane. As shown in Fig. 4 *c*, the variation of the average fluorescence intensity of the GUV membrane is inversely proportional to the lipid/protein ratio and thus linearly related to the protein content in the initial preformed proteoliposomes. Similar results were obtained for the BR reconstitution (see Fig. 5 *a*), indicating homogeneous incorporation of this membrane protein into GUVs. Most GUVs show a continuous ring of fluorescence, which is homogenous overall, suggesting that proteins are homogeneously distributed in the reconstituted membranes. At this point, it is important to note that using the protocol of Manneville and co-workers (Manneville et al., 2001), i.e., electroformation from a film of lipid-BR in organic solvent, FITC-labeled BR was not homogeneously distributed on a vesicle as indicated by the presence of fluorescence patches, reflecting the presence of BR aggregates (Fig. 5 *b*).

Proteins retain biological activities in GUVs

The efficiency of the reconstitution of Ca^{2+}-ATPase into GUVs was analyzed by measuring the calcium-dependent hydrolytic activity and the calcium pumping activity of the reconstituted protein. Using an enzyme coupled assay, which couples ATP hydrolysis to the consumption of NADH (Fig. 6 *a*), we measured an ATPase activity of ~4 μmol ATP/min/mg protein in reconstituted GUVs, whatever the lipid/protein ratio. This value is only 30% lower than that measured in the native SR, indicating that the different steps of our new reconstitution procedure do not significantly affect protein function. In theory, the fraction of Ca^{2+}-ATPase accessible from the outside of the GUVs could have been determined by comparing the calcium-dependent ATPase activity with and without added detergent in the medium of measurement. Unfortunately, such a comparison could not be done since the integrity of the GUVs could not be maintained due to the mixing necessary for this cuvette assay. We have also analyzed the calcium pumping activity of Ca^{2+}-ATPase reconstituted into GUVs, using a fluorescent calcium indicator, Fluo-5N, encapsulated by GUVs. In the absence of ATP, the fluorescence intensity of the GUV's lumen remained at a basal level. ATP addition in the presence of Ca^{2+} resulted in a significant time-dependent increase in fluorescence, as shown in Fig. 6 *b*, demonstrating efficient ATP-dependent calcium pumping into the inner compartment of GUVs.

The efficiency of the reconstitution of BR into GUVs was analyzed by measuring the light-dependent proton pumping activity, using pyranine as a fluorescent pH-sensitive probe, encapsulated by GUVs. For each vesicle, pictures were recorded with the same settings (e.g., laser intensity, acquisition time, and photomultiplier's sensitivity) to discriminate the effects of photobleaching from the pH changes in the GUV's lumen induced by the H^+ pumping. Interestingly, the analysis of pyranine fluorescence of 15 independent GUVs revealed the existence of two popula-

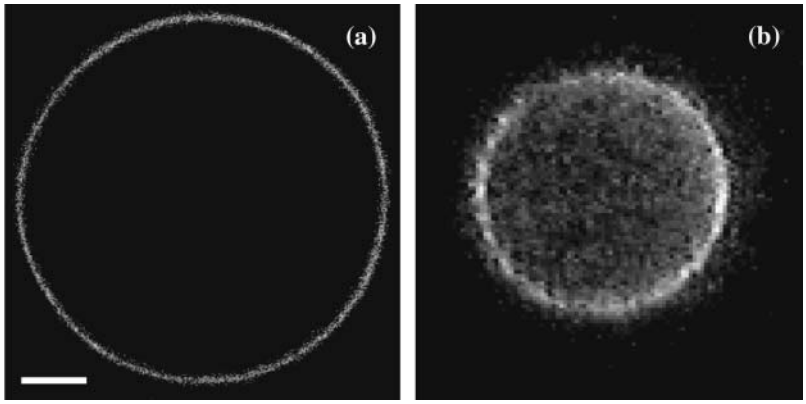

FIGURE 5 Homogeneity of BR incorporation in GUVs. (*a*) Confocal image of FITC-labeled BR reconstituted into SOPC vesicles. The fluorescence is homogenously distributed in the membrane of the vesicle. (*b*) Epifluorescence videomicroscopy frame (courtesy of Dr. J.-B. Manneville, Unité Mixte de Recherche 144 Centre National de la Recherche Scientifique/Institut Curie, Paris) of FITC-labeled BR reconstituted into SOPC vesicles according to the protocol of Manneville et al. (2001). The fluorescence of the membrane is not homogenous suggesting the presence of BR aggregates. Scale bars = 5 μm.

tions. Fig. 7 depicts the changes observed in two-thirds of the BR containing GUVs. Upon illumination (*shaded area* in Fig. 7), a large decrease in the pyranine fluorescence intensity is observed, reaching a steady state after about a 5–6-min illumination period. This is indicative of an internal acidification and thus of an inward proton pumping activity mediated by BR. When the illumination is stopped, the fluorescence of pyranine increases as a function of time due to the passive diffusion of the H^+ previously accumulated in the lumen of the vesicle. However, in one-third of the GUVs, we observed a light-dependent increase of the pyranine fluorescence intensity that reverses when the light is turned off (data not shown). All these data indicate that BR keeps its activity in the reconstituted GUVs. They also indicate that although most of the GUVs have a preferential BR orientation that leads to inward light-dependent H^+ pumping activity, a significant proportion of the GUVs have an opposite preferential orientation of BR.

DISCUSSION AND CONCLUSION

Giant unilamellar vesicles provide an excellent membrane model system, as they are suitable for optical microscopy and exhibit a cell-like curvature, with sizes ranging from 10 to 100 μm. In addition, manipulations of these large vesicles by optical tweezers or micropipettes provide a unique opportunity in membrane research to monitor a closed vesicle exposed to controlled mechanical, thermal, and/or chemical perturbations. For these reasons, GUVs have been instrumental in analyzing bending stiffness of lipid bilayers and free energies of interactions between membranes (Lipowsky, 1995; Parsegian and Rand, 1995), membrane deformation (Brochard-Wyart et al., 1976), lipid-membrane phase transitions (Chen et al., 1995), and the adhesion of vesicles on various supports (Helfrich, 1989; Evans, 1991). In addition, GUV membrane interactions with colloidal particles have made possible the observation of endocytotic phenomena (Dietrich et al., 1997), the effects of local delivery and interactions of biologically active substances (Bucher et al., 1998), and DNA-lipid interactions (Angelova et al., 1999). However, the harsh preparation procedures of GUVs have precluded so far their widespread use in membrane protein reconstitution. Indeed, in addition to preserving the activity of the protein, any method of membrane protein reconstitution should fulfill a number of important criteria, including an efficient and homogeneous insertion of protein into regular and unilamellar bilayer structures. In this work, we present a novel method fulfilling these main requirements and leading to the successful reconstitution of the Ca^{2+}-ATPase and the BR into GUVs.

The main criteria for a successful reconstitution experiment is the conservation of the protein activity. This was systematically monitored at each step of our reconstitution protocol. Concerning the first step of the protocol, which relies on the incorporation of solubilized membrane proteins into submicron liposomes, we used a detergent-mediated procedure that allows for very efficient reconstitution of Ca^{2+}-ATPase and BR (Rigaud and Levy, 2003). Interestingly, the reconstitution of any membrane protein of interest can be rapidly optimized using this procedure, by testing different detergents, different lipids, and analyzing the resulting biological activities. The following steps of our reconstitution protocol involve dehydration of the preformed proteoliposomes followed by rehydration in an electric field. These two steps can be deleterious for membrane proteins. We found that it was necessary to carry out the dehydration step of the preformed proteoliposomes at a low rate and also under a controlled humidity atmosphere to preserve proteoliposomes from complete dehydration. In addition, we took into account that the process of electroformation imposes an ionic strength below 5 mM for the initial buffer used to incorporate the protein into small proteoliposomes as well as for the buffer used during the rehydration. We show that these low ionic conditions do not significantly affect the biological activities of two radically different membrane proteins, the Ca^{2+}-ATPase and the bacteriorhodopsin. Noteworthily, reconstituted GUVs were produced that sustained a high level of activity as demonstrated by the light-induced internal pH changes due to the H^+ pumping activity of BR or the changes in internal Ca^{2+} concentration due to the ATP-dependent Ca^{2+} pumping activity of the Ca^{2+}-ATPase.

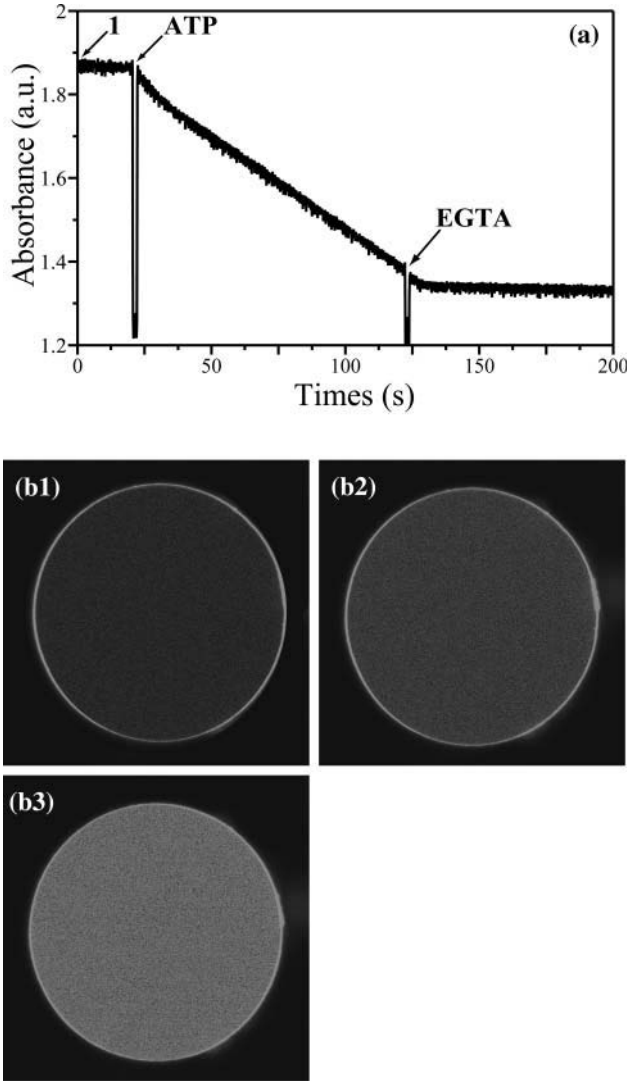

FIGURE 6 Activity of Ca^{2+}-ATPase into GUVs. Giant vesicles were prepared in EYPC/EYPA (9:1) at a lipid/Ca^{2+}-ATPase ratio of 6.5 w/w. (*a*) ATPase activity was performed at 25°C as described in Materials and Methods. The reconstituted GUVs were transferred to a cuvette and solubilized with $C_{12}E_8$ (*arrow 1*). The reaction was started by the addition of ATP and stopped with EGTA. (*b*) Calcium pumping activity. Confocal images of a Ca^{2+}-ATPase GUV (30 μm in diameter) reconstituted in EYPC/EYPA/NBD-C12-HPC s and encapsulating Fluo-5N. After ATP addition, the fluorescence intensity in the interior of the vesicles increases as a function of time. (*b1*) $t = 5$ min; (*b2*) $t = 15$ min; and (*b3*) $t = 25$ min.

We also characterized the unilamellarity of the vesicles, together with the quality of protein incorporation in terms of distribution in the reconstituted membranes. Besides preserving the biological activity of the reconstituted proteins, one of the advantages of our method is that it generates spherical unilamellar giant vesicles at a high yield whatever the lipid composition and in a large range of lipid/protein ratios. In particular, it is of note that giant unilamellar vesicles could be reconstituted at lipid/protein ratios as low as 5 w/w (i.e., 160 or 680 mol/mol) for BR and Ca^{2+}-ATPase, respectively. At this lipid/protein ratio of 5 w/w, one can estimate that a vesicle of 20 μm in diameter contains $\sim 3 \times 10^6$ or 6.5×10^5 molecules of BR and Ca^{2+}-ATPase, respectively. Also important, it is shown (Figs. 4 *a* and 5 *a*) that after reconstitution, fluorescently labeled proteins are smoothly distributed over the entire membrane proteins and do not form aggregates detectable with a microscope. In addition, the analysis of different reconstituted GUVs indicates that their composition is homogeneous in the vesicle suspension, ensuring reproducibility in the experiments. This shows definite improvement compared to previous reports on the reconstitution of bacteriorhodopsin. Using the protocol of Manneville and co-workers (Manneville et al., 2001), by which GUVs were produced by electroformation from a lipid-BR film in organic solvent, BR was found heterogeneously distributed in the vesicle preparation and, more drastically, formed aggregates in the membrane. On the other hand, using the protocol of Kahya et al. (2001) in which preformed proteoliposomes are fused with lipidic GUVs, despite a homogenous protein distribution, the amount of proteins that could be incorporated remained limited due to the presence of the fusogenic agents, which have been reported to induce some lysis at high lipid/protein ratios.

Another critical parameter for membrane protein reconstitution is related to the final orientation of the protein

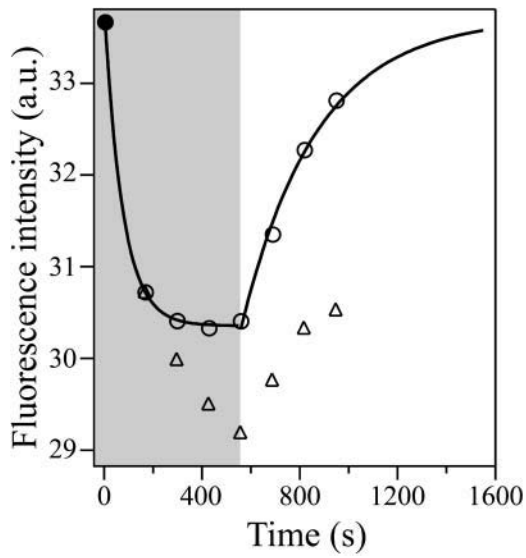

FIGURE 7 Proton pumping activity of BR into GUVs. Light-induced fluorescence responses of pyranine trapped inside giant vesicles. Giant vesicles containing pyranine were prepared in SOPC at a lipid/BR ratio of 7 (w/w) and analyzed by confocal microscopy. In the shaded part, BR is illuminated and pumps H^+. In the open part, the illumination is stopped, and the pH reequilibrates. △ correspond to the direct measured variation of fluorescence intensities. ○ correspond to the fluorescence intensities corrected for the photobleaching. ● correspond to the extrapolated initial fluorescence intensities since the vesicles were already illuminated in the confocal microscope during the installation of the sample and selection of the vesicle, leading to a pumping of protons before the fluorescence measurement.

within the membrane. Activity measurements performed on BR-reconstituted GUVs reveal the presence of two distinct populations of protein-reconstituted GUVs, which undergo opposite internal pH variations upon illumination (Fig. 7). About two-thirds of the GUVs have a preferential inside-out orientation of BR leading to an inwardly directed H^+ transport (Fig. 7), whereas about one-third have a preferential right-side out orientation leading to an outwardly directed H^+ transport. In this context, it has been shown that two orientations of the protein were also present in the initial preformed BR proteoliposomes, with the inside out orientation (75%) predominant. However, by using the pH sensitive fluorescence probe approach at acidic pHs (Seigneuret and Rigaud, 1985, 1988), it was demonstrated that these two orientations were not segregated in different subclasses of proteoliposomes but homogeneously distributed over the entire population of the initial preformed BR proteoliposomes (Rigaud et al., 1988). Thus, the reason for two GUVs populations with opposite preferential protein orientations must be related to the dehydration/rehydration steps. It is difficult to provide an explanation of how two such types of GUVs are formed in parallel, since little is known about the mechanisms of vesicle dehydration and rehydration. Concerning the Ca^{2+}-ATPase reconstitution, we have only detected ATP-dependent calcium pumping into the inner compartment of GUVs. However, as opposed to light driven proton pumps, only those Ca^{2+}-ATPases oriented right-side out respond to the substrate (ATP) added in the external medium, and, thus, only precise measurements of the Ca^{2+} pumping rates would permit the detection of GUVs with different Ca^{2+}-ATPase orientations.

In conclusion, our method of membrane protein reconstitution into giant vesicles fulfills a number of important criteria. This method is relatively simple and also gentle with respect to the protein activity conservation. In addition, it produces giant proteoliposomes of more than 20 μm in diameter, which are spherical and unilamellar, and in which membrane proteins are homogeneously inserted and distributed. Although our method has only been evaluated for the reconstitution of two membrane proteins, the sarcoplasmic reticulum Ca^{2+}-ATPase and BR, presumably it will apply to the reconstitution of other transmembrane proteins. Such giant vesicles are critical for studying the mechanisms of transport by membrane proteins as demonstrated here for BR and Ca^{2+}-ATPase. More specifically, it will be applicable to single-molecule optical microscopy studies of lateral and rotational mobility and, more generally, folding and association-dissociation equilibriums of individual protein molecules, lipid-protein, and protein-protein interactions. The GUVs' approach could provide an interesting complement to preparative techniques such as, for example, patch-clamp (Walz et al., 2002). High protein incorporation into the membranes of giant vesicles will be of value for high resolution imaging of the surface topography of membrane proteins by atomic force microscopy, keeping in mind that a limitation of this approach is related to the size of the samples to be imaged (Scheuring et al., 2001). Also, the ability to have a controlled concentration of transmembrane proteins in giant vesicles will be of use in the analysis of the adhesion of decorated membranes (Abdelghani-Jacquin et al., 2002), the propulsion of biomimetic bacteria (Giardini et al., 2002), or the effect of the protein activity on the fluctuations of the membrane (Manneville et al., 2001). In this context, using the pipette experiments presented in Fig. 3 *a*, we have shown that, in the presence of ATP, the activity of the Ca^{2+}-ATPase contributed to the amplification of membrane fluctuations, leading to a modulation of the slope of the log(tension) versus relative excess area (our unpublished data).

In the long term, we can also consider interesting biotechnological applications for this technique, for example, reconstituting transport proteins to control the composition inside the GUVs, which could then be used as chemical microreactors (Karlsson et al., 2001). Also, preparations of functionalized GUVs could be used for building up biosensors (Barth et al., 2003). Finally, considering the increasing number of membrane proteins that have been identified in the sequencing of the genomes of different organisms, the reconstitution of many different proteins can be tested in GUVs, and new applications in pharmaceutics or chemistry will emerge.

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