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Control of the Unidirectional Topological Orientation of a Cross-Linked Complex Composed of the Bacterial Photosynthetic Reaction Center and Horse Heart Cytochrome *c* Reconstituted into Proteoliposomes[1]

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Control of the unidirectional topological orientation was achieved for a cross-linked complex composed of the bacterial photosynthetic reaction center and horse heart cytochrome *c* (RC/cyt *c*) reconstituted into proteoliposomes. Using the method of Ueno *et al.* [Ueno *et al.* (1995) *Mater. Sci. Eng.* C3, 1-6], we prepared RC/cyt *c* by conjugating cyt *c* to the H-subunit of RC of *Rhodobacter sphaeroides* R-26 using a bifunctional cross-linking reagent, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), as previously reported. The freeze-thaw method was used to incorporate RC/cyt *c* into liposomes that contained dipalmitoyl-L-α-phosphatidylcholine and dipalmitoyl-L-α-phosphatidylglycerol (1:9). The topological orientation of RC/cyt *c* in the proteoliposomes was determined using three methods: (i) release of the cyt *c* moiety from the proteoliposomes by cleaving the disulfide bond in the linker residue, (ii) electron transfer from free cyt *c* outside the proteoliposomes to the RC moiety, and (iii) photo-induced membrane potential of RC- and RC/cyt *c*-reconstituted proteoliposomes. The results indicated that about 90% of the RC/cyt *c* in proteoliposomes was oriented with the H-subunit exposed on the outside of the liposomes, whereas only about 60% of the RC in proteoliposomes had this orientation. Thus, we successfully controlled the unidirectional topological orientation of the RC moiety in liposomes using the RC/cyt *c* complex.

Key words: cytochrome *c*, liposome, molecular orientation, photosynthetic reaction center, proteoliposome.

Photosynthetic reaction centers (RCs) in the purple non-sulfur photosynthetic bacterium, *Rhodobacter sphaeroides*, play a central role in the conversion of light energy into chemical energy. Charge separation in a bacteriochlorophyll dimer [$(Bchl)_2$] releases an electron at a high energy level (*i.e.*, low redox potential). The electron is transferred to bacteriopheophytin, an ubiquinone 10 (UQ-10) in the primary quinone (Q_A) site, and then to another UQ-10 molecule in the secondary quinone site (Q_B) (*1*). The UQ-10 in the Q_B site accepts an electron from UQ-10 in the Q_A site twice, binds two protons to become ubiquinol, and then is released into the quinone pool in the intracytoplasmic membrane (*2, 3*). Ubiquinol is oxidized by the cytochrome b/c_1 complex on the cytoplasmic side (*4*). Electrons are transferred back to the periplasmic side, and then finally to cytochrome c_2 in the periplasm, thereby generating a proton potential across the membrane. The oxidized $(Bchl)_2$ can be re-reduced by cytochrome c_2 to complete the redox cycle. This light-induced cyclic electron transfer in the intracytoplasmic membrane of photosynthetic bacteria is possible because the RCs have the same topological orientation (*5*).

The function of RC is of interest in applications for molecular electronic devices because RC is a stable protein causing photocurrent or photovoltage between electrodes (*6-8*). RC is also a promising protein for constructing biosensors for herbicides because many herbicides can bind to the Q_B site (*9*). Before these applications can be realized, methods must be developed for constructing the molecular assembly of RC, particularly for controlling the molecular orientation. We previously developed various methods for

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Abbreviations: RC, photosynthetic reaction center; cyt *c*, horse heart cytochrome *c*; DTT, dithiothreitol; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; LDAO, lauryldimethylamine *N*-oxide; β-OG, β-octyl-D-glucopyranoside; DMPC, L-α-phosphatidylcholine dipalmitoyl; DPPG, L-α-phosphatidylglycerol dipalmitoyl.

controlling this molecular orientation involving biological affinity (*e.g.*, avidin-biotin interaction), the Langmuir-Blodgett film technique, reconstitution of RC into liposomes, and chemical cross-linking of different proteins (*10, 11*). Continuing in this research area, we previously prepared a cross-linked complex between *R. sphaeroides* RC and horse heart cytochrome *c* (RC/cyt *c*) (*12*) that showed unique properties during electron transfer. After cross-linking between RC and cyt *c*, RC/cyt *c* was purified by gel filtration, hydroxylapatite column chromatography and affinity chromatography on a cyt *c*-immobilized column as described in our previous report (*12*). The cyt *c* moiety was cross-linked to only the H-subunit of RC in the purified RC/cyt *c* with the molar ratio of 1.5 cyt *c*:RC. The ratio was estimated from the calibration curve between the molar ratio (cyt *c*:RC) *versus* the ratio of absorbance (A_{800}:A_{415}) determined with the absorbance spectra of a mixture containing RC and cyt *c*. Because of the steric hindrance (*i.e.*, cyt *c* was linked to the H-subunit of RC), the complex did not show intramolecular electron transfer between the two moieties although the RC moiety and the cyt *c* moiety retained full redox activity (*12*). Recently, we reconstituted RC/cyt *c* into proteoliposomes and found that more than 90% of the complex had the same topological orientation, compared with only 60% when RC was reconstituted without cyt *c*. Encouraged by this finding, we characterized the topological orientation of RC/cyt *c* in proteoliposomes in line with the development of molecular assembly technology, as reviewed in the literature (*10, 11*).

MATERIALS AND METHODS

Materials—The cross-linking reagent, *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), was purchased from Pierce Chemical (Rockford, Illinois, USA). Using the method of Clayton and Wang (*13*), RCs were isolated and purified from *R. sphaeroides* R-26 using a detergent, lauryldimethylamine *N*-oxide (LDAO). Horse heart cyt *c* (type III), L-α-phosphatidylglycerol, dipalmitoyl (DPPG), L-α-phosphatidylcholine, dipalmitoyl (DPPC), and dithiothreitol (DTT) were purchased from Sigma Chemical (St. Louis, Missouri, USA). The concentration of RC was estimated using an extinction coefficient, ε_{802}, of 288 $mM^{-1}\cdot cm^{-1}$ (*14*), and the concentration of cyt *c* with ε_{550} = 27.6 $mM^{-1}\cdot cm^{-1}$ (*15*). The detergent, *n*-octyl β-D-glucopyranoside (β-OG), for the reconstitution of RCs into liposomes was purchased from Dojindo Laboratories (Kumamoto, Japan). A dye that is sensitive to a membrane potential, negatively charged diBA-C_4-(3) [bis(1,3-dibutylbarbituric acid-(5))trimethineoxonol], was purchased from Molecular Probes (Eugene, Oregon, USA).

Preparation of RC/cyt c—Preparation of RC/cyt *c* was carried out as described previously by Ueno *et al.* (*12*). Cytochrome *c* was conjugated to the H-subunit of RC using a cross-linking reagent, SPDP, as described by Carlsson *et al.* (*16*). Unconjugated cyt *c* was removed by gel filtration on a Sepharose CL-6B gel, and unconjugated RC by hydroxylapatite column chromatography. RC/cyt *c* was adsorbed to a cyt *c*-immobilized Sepharose 4B gel, and then eluted with 10 mM Tris-HCl buffer (pH 8.0) containing 30 mM β-OG and 0.2 M NaCl. In this affinity chromatography, RC/cyt *c* in which cyt *c* was attached to either the M- or L-subunit was removed, as reported by Rosen *et al.* (*17*). The molar ratio of RC moiety to cyt *c* moiety in the final preparation of RC/cyt *c* was 1:1.5 (*12*).

Reconstitution of RC/cyt c and RC into Liposomes—Liposomes were made as described by Mimms *et al.* (*18*). A mixture of 18 μmol of DPPG and 2 μmol of DPPC (molar ratio = 9:1) was dissolved in 0.5 ml of a chloroform solution in a glass tube, and then dried to a thin film on the inner wall of the tube using a stream of argon gas. The film was dispersed in 0.5 ml of 10 mM Tris-HCl buffer (pH 8.0) and then sonicated for 1 min at 28 kHz using a bath-type sonicator (VS-100 III, Iuchi). The dispersion became clear with this procedure. Before the reconstitution of the complex and RCs into liposomes, UQ-10 was also reconstituted to the Q_B site of RC as described by Rosen *et al.* (*17*). Either RC/cyt *c* or RC was added to a liposome solution at a lipid/RC molar ratio of 1,000. The detergent was then removed by dialyzing the mixture against 10 mM Tris-HCl buffer (pH 8.0) at 5°C for 2 days. The complex was reconstituted into the liposomes by the freeze/thaw method (*18*). After dialysis, the proteoliposomes were frozen by liquid nitrogen and then stored at −84°C until use. Before use, the proteoliposomes were thawed at room temperature and then collected by ultra-centrifugation for 1 h at 100,000 × *g*. The pellet was dissolved in 0.5 ml of buffer comprising 20 mM HEPES (pH 8.0), 50 mM KCl, and 0.5 mM $MgCl_2$, and then sonicated for 1 min at 0°C in a sealed tube at 28 kHz using the sonicator.

Cleavage of Disulfide Bonds by DTT—When the cyt *c* moiety was exposed to the outer surface of the RC/cyt *c*-proteoliposomes, we cleaved the disulfide bonds in the cross-linker between the RC moiety and the cyt *c* moiety (Fig. 1A) by adding 25 mM dithiothreitol (DTT), because DTT does not penetrate liposomal membranes. The released cyt *c* was separated from the proteoliposomes by loading these DTT-treated proteoliposomes on a 10 ml linear sucrose gradient comprising 10-50% (w/v) sucrose, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.5 mM $MgCl_2$. The loaded gradient was then ultra-centrifuged in a Beckman SW-40 rotor at 100,000 × *g* for 1 h at 4°C. After a sample had been collected from the bottom of the centrifugation tube, the absorbance of each fraction was measured to estimate the percentage of cyt *c* released from the liposomes.

Flash-Induced Oxidation of Cytochrome c—Flash-induced oxidation of cyt *c* in the presence of either RC or RC/cyt *c* was measured at 550 nm as previously described (*12*) with a xenon flash light of 25 μs duration. Stray light was minimized by using a filter for the near-infrared region (SC-70, Fuji Photo Film, Tokyo) and one for the visible region (C.S. 4-76, Corning, USA). The reaction mixture comprised 2 μM RC, 200 μM reduced cyt *c*, 20 mM HEPES buffer (pH 8.0), 50 mM KCl, and 0.5 mM $MgCl_2$. The data were collected for the first flash.

Light-Induced Proton Potential Across the Lipid Bilayer—The light-induced proton potential across the lipid bilayer in the RC/cyt *c*-proteoliposomes or that in the RC-proteoliposomes was measured using a potential-dependent fluorescence dye as described by Brauner *et al.* (*19*). A 2-ml sample comprising RC/cyt *c* proteoliposomes, 20 mM HEPES (pH 8.0), 50 mM KCl, 0.5 mM $MgCl_2$, and 200 μM reduced cytochrome *c* was kept at 20°C in a fluorescence cuvette made of quartz. Negatively charged diBA-C_4-(3) [bis(1,3-dibutylbarbituric acid-(5))trimeth-

ineoxonol] from a 1 mM ethanol stock solution was added to this sample solution until a final concentration of 0.02 μM was reached. The fluorescence emission at 524 nm with excitation at 497 nm was measured with a Hitachi F-4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The sample in the cuvette was illuminated with strong actinic light (108,000 lux) from a halogen lamp passed through a light guide made of glass-fiber bundles (Luminar Ace LA-50, Hayashi Watch-Works, Tokyo, Japan) and a cut-off filter (SC-70, Fuji Photo Film). The membrane potential was calibrated using the valinomycin-induced potassium diffusion potential. The mixture for the calibration standard comprised 2 μM RCs in proteoliposomes, 20 mM HEPES (pH 8.0), 0.5 mM $MgCl_2$, and various concentrations of KCl (50–1,125 mM). The potassium in the outer space of the vesicles was removed by centrifugation and resuspension with 20 mM HEPES buffer, pH 8.0, and 0.5 mM $MgCl_2$. The potassium diffusion potential was generated by adding 1 μM valinomycin to this potassium mixture.

RESULTS AND DISCUSSION

We examined the topological orientation of RC/cyt *c* using three methods. We performed all experiments to determine the topological orientation of RC/cyt *c* (1)–(3) twice or three times to check the reproducibility. The results were essentially the same.

Topological Orientation of RC/cyt c (1)—We examined the topological orientation of the RC/cyt *c* complex in proteoliposomes by using DTT to cleave the disulfide bonds in the linkage between the RC moiety and the cyt *c* moiety (Fig. 1A). The cyt *c* moiety exposed on the outer surface of the proteoliposomes was released, and then separated from the proteoliposomes by sucrose density gradient ultracentrifugation. In the same layer of the gradient [10–20% (w/v) sucrose] as native cyt *c*, 350 pmol cyt *c* was recovered (Fig. 1B), whereas in the lower layer, 278 pmol of RC in the proteoliposomes was recovered. The molar ratio of released cyt *c* and RC after the separation was 1.3. Using a molar ratio of 1.5 for cyt *c* moiety to RC moiety in RC/cyt *c* previously reported by Ueno *et al.* (*12*) as a reference, we calculated the direct recovery of cyt *c* from RC/cyt *c*-proteoliposomes to be 85%. As a control, we determined the recovery of cyt *c* with a sucrose gradient using a mixture of the RC-proteoliposomes and cyt *c*. The recovery yield of cyt *c* in this control was $92 \pm 2\%$ ($n = 3$). A small amount of cyt *c* might be adsorbed to the surface of RC-liposomes because of the affinity between RC and cyt *c*. When the loss due to adsorption of cyt *c* to proteoliposomes is taken into account, the actual recovery of released cyt *c* from RC/cyt *c*-proteoliposomes should be as high as 93%. This result indicated that 93% of RC/cyt *c* was oriented with the cyt *c* moiety exposed on the outer surface of the proteoliposomes (*i.e.*, H-out orientation).

Topological Orientation of RC/cyt c (2)—We also examined the topological orientation by measuring the efficiency of electron transfer from reduced cyt *c* (ferrocytochrome *c*) to flash-oxidized RC. It was reported that both RC in the presence of a detergent and RC reconstituted into liposomes showed similar kinetic properties as to the electron transfer from reduced cyt *c* (*20*). We measured the flash-induced oxidation of cyt *c* in the presence of free RCs (Fig. 2A), RC-proteoliposomes (Fig. 2B), and RC/cyt *c*-proteoliposomes (Fig. 2C). Using the amplitude of the flash-induced oxidation of cyt *c* in the presence of free RCs (Fig. 2A) as a reference (*i.e.*, 100%), the amplitudes of that in the presence of RC-proteoliposomes (Fig. 2B) and RC/cyt *c*-proteoliposomes (Fig. 2C) are 41 and 9%, respectively. Flash-oxidized RC can accept an electron from cyt *c* on the periplasmic surface of either the M- or L-subunit (P-side) in the native intracytoplasmic membrane (*17*). Flash-induced oxidation of cyt *c* occurred at 550 nm with RC in the presence of the detergent (Fig. 2D). The RC moiety with its P-side exposed on the outer surface (*i.e.*, H-in orientation) of proteoliposomes can accept an electron from reduced cyt *c* (Fig. 2E), whereas the RC moiety in the opposite orientation (*i.e.*, H-out orientation) can not accept an electron (Fig. 2F). We calculated the percentage of the RC moiety that has this H-in orientation (Fig. 2E) by dividing the amplitude of the flash-induced oxidation of cyt *c* with RC-proteoliposomes by that for free RC; the result

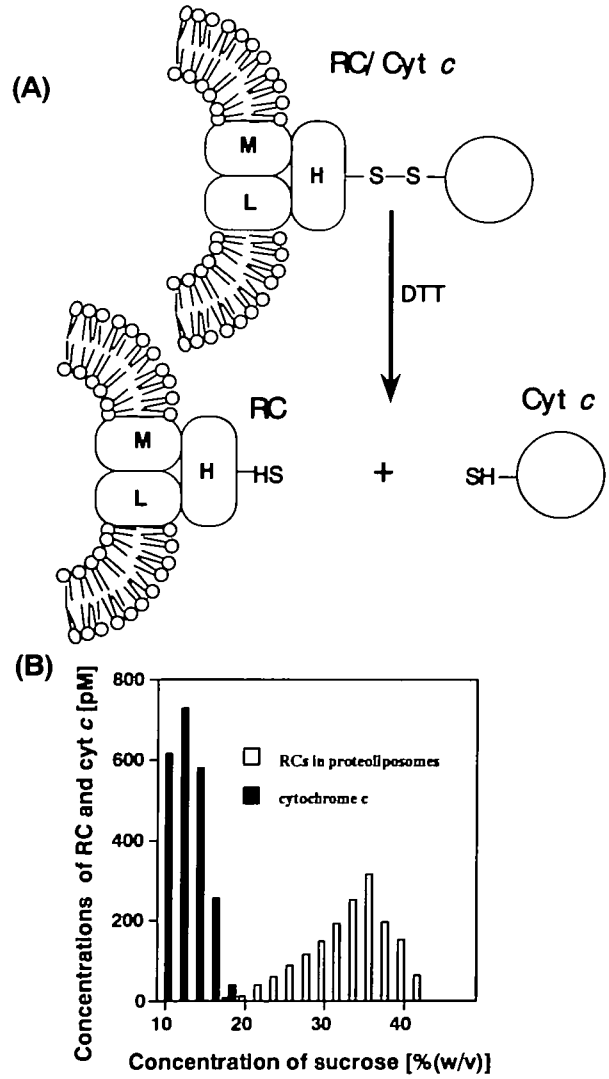

Fig. 1. Schematic representation of the cleavage of the disulfide bond between the RC moiety and the cyt *c* moiety in RC/cyt *c* (A), and the results of sucrose density gradient ultra-centrifugation to separate cyt *c* and RC-proteoliposomes after the cleavage (B).

was 9% for the RC/cyt *c*-proteoliposomes and 41% for the RC-proteoliposomes. This means that 91% of RC/cyt *c* was oriented with the cyt *c* moiety exposed on the outer surface of RC/cyt *c*-proteoliposomes (H-out orientation), whereas 59% of RC was oriented with the H-subunit exposed on the outer surface of RC-proteoliposomes (H-out orientation). This value agrees well with the 93% obtained on cleavage of the disulfide bond described in the preceding paragraph.

Topological Orientation of RC/cyt c (3)—For the final means of examining the topological orientation, we determined the continuous light-induced proton potential by measuring the fluorescence of diBa-C_4-(3). Control liposome without RC showed no light-induced fluorescence change (Fig. 3A); the RC-proteoliposomes showed strong fluorescence (Fig. 3B) due to the inside negative potential (−54 mV); and the RC/cyt *c*-proteoliposomes showed fluorescence in the opposite direction (Fig. 3C) due to the inside positive potential (20 mV). A calibration curve for these measurements (Fig. 3D) was obtained by generating a potassium diffusion potential by adding valinomycin to the mixture for measurement. We can explain these fluorescence results as follows. RC was reconstituted in two topological orientations: either the H-in orientation (Fig. 2E) or the H-out orientation (Fig. 2F). In the RC-proteoliposomes, 41% of the RCs were in the former orientation and therefore could accept electrons from reduced cyt *c* outside the proteoliposomes and under illumination could pump protons outward. Conversely, 59% of the RCs were in the H-out orientation and therefore pumped protons inward, but could not be supplied with a sufficient number of electrons from cyt *c* because reduced cyt *c* was not present in the interior on the proteoliposomes (Fig. 2F). Consequently, this orientation resulted in a weak ability to pump protons inward. Therefore, a strong net proton efflux induced by strong continuous light was observed in the RC-proteoliposomes (Fig. 3B). In the RC/cyt *c*-proteoliposomes, only 9% of the RCs were in the H-in orientation, and thus exhibited strong ability to pump protons inward (Fig. 2E). Conversely, 91% of the RC moiety was in the H-out

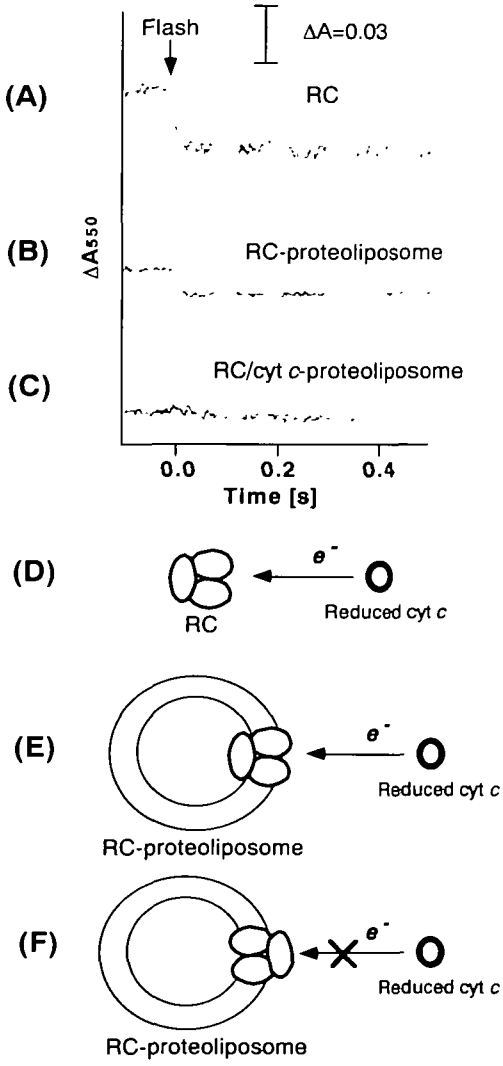

Fig. 2. **Flash-induced oxidation of cyt *c* in the presence of RC (A), RC-proteoliposomes (B), and RC/cyt *c*-proteoliposomes (C). Schematic representation of the electron transfer from reduced cyt c to RC (D), that to RC-proteoliposomes with the P-side exposed to the outside (E), and that to RC-proteoliposomes with the H-unit exposed to the outside (F).** The flash-induced decrease in absorbance at 550 nm was measured. The concentrations of RCs and reduced cyt *c* were adjusted to 2 and 200 μM, respectively. Each sample volume was 2 ml.

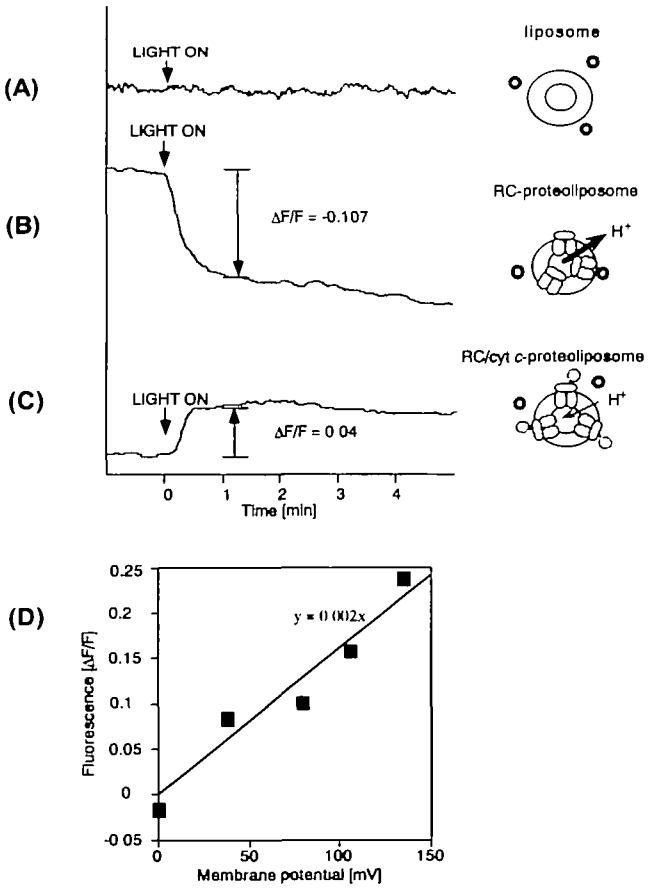

Fig. 3. **Continuous light-induced potential across a lipid bilayer measured as fluorescence for liposomes (A), RC-liposomes (B), and RC/cyt *c*-liposomes (C). Calibration standard curve for the measurements (D).** The insets to the right show schematic representation of the proton efflux in (B) and influx in (C). Thick circles represent reduced cyt *c* shown in Fig. 2.

orientation and pumped protons inward, but could not be supplied with a sufficient number of electrons from cyt *c* (Fig. 2F). Therefore, the RC/cyt *c*-proteoliposomes showed a small interior-positive membrane potential due to this weak net proton influx.

Mechanism of Topological Orientation—When only RCs were incorporated into liposomes, the orientation was relatively random (*i.e.*, 41% were in the H-in orientation). On the other hand, as confirmed by all three methods, the topological orientation of the RC moiety in the RC/cyt *c*-proteoliposomes was relatively unidirectional (*i.e.*, 91% of RC/cyt *c* was in the H-out orientation). These results demonstrate the effectiveness of using the cyt *c*-cross-linking method to control the orientation of RCs that is essential for the use of RCs in biosensors and in biomimetic materials.

One possible mechanism for the better orientation is interaction between the bulky hydrophilic positively charged cyt *c* moiety and the surface of negatively charged lipids. The result of this interaction is the incorporation of RC-cyt *c* with a specific orientation into liposomes. To determine why the unidirectional topological orientation was significantly higher for RC/cyt *c* (91-93%) than for RC (59%) in proteoliposomes, we looked at the three-dimensional structure of RC and that of cyt *c* (Fig. 4) elucidated by X-ray analysis (*21, 22*). The topological orientation of a reconstituted membrane protein in proteoliposomes may depend on two factors: (a) the distribution of electrostatic charges (dipole moment) and (b) the polarity of the hydrophilicity and hydrophobicity on the surface of the protein molecules. Most of the surface of an H-subunit, and only parts of the periplasmic surface of the L- and M-subunits of RC are hydrophilic, whereas the entire lateral surface of the L- and M-subunits is extremely hydrophobic (Fig. 4). Furthermore, Tiede *et al.* (*23*) reported that an RC molecule has an electrostatic dipole moment from the cytoplasmic side to the periplasmic side, thereby making the H-subunit side positively charged (*23*). Horse heart cyt

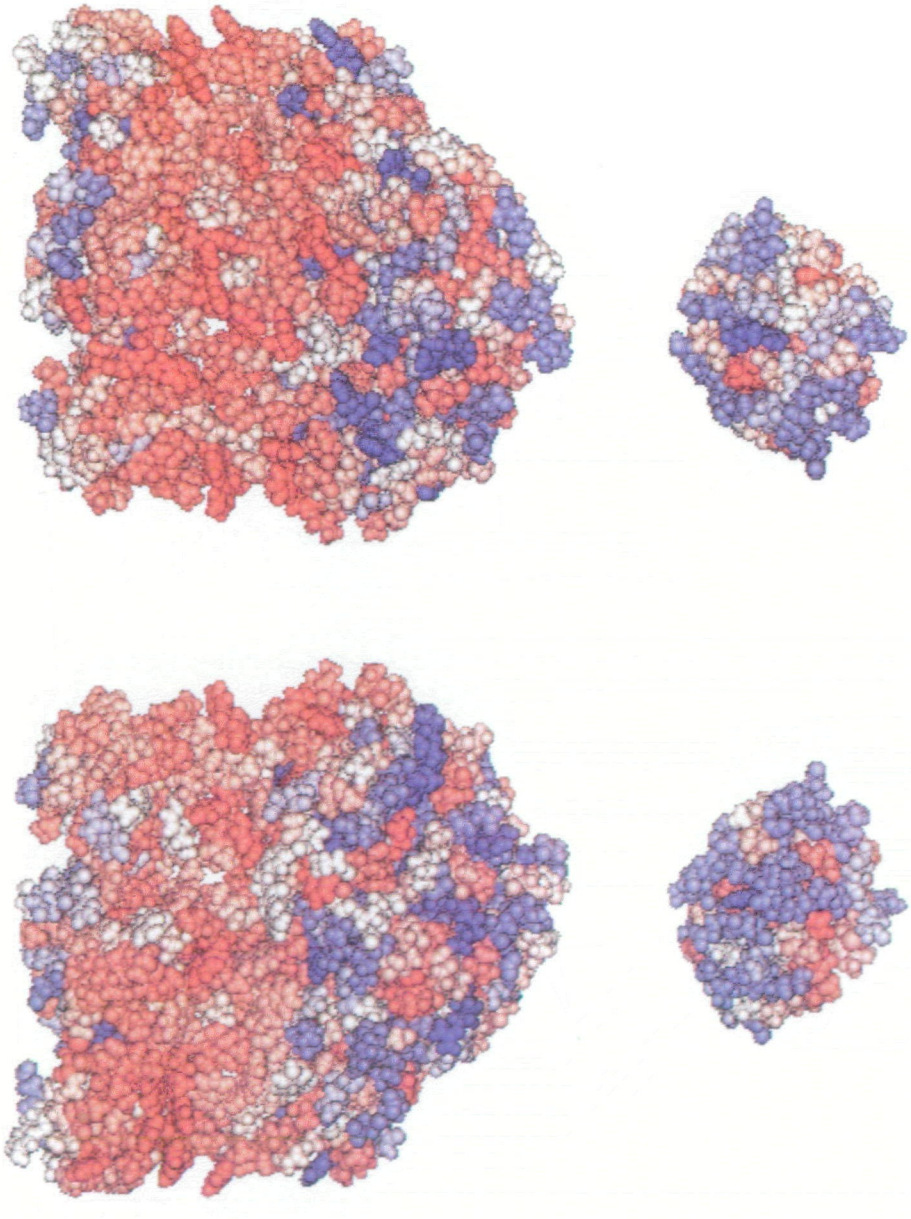

Fig. 4. Three-dimensional structures of *Rhodobacter sphaeroides* RC (upper figures) and horse heart cyt *c* (lower figures) using a space-filling model. The left figures are opposite views those in the right figures. Red and blue represent the hydrophobic and hydrophilic surfaces, respectively.

c is a positively charged hydrophilic protein at neutral pH. Cross-linking with cyt *c* on the H-subunit increased the polarity in the distribution of hydrophilicity and hydrophobicity, and increased the electrostatic dipole moment. Consequently, RC/cyt *c* was oriented much better than RCs in the proteoliposomes, although we were unable to determine which of the two factors (dipole moment or polarity) had the dominant role. The dipole moment of a protein can interact with the charges of lipids. Recently, we reported that the topological orientation of RCs in RC-proteoliposomes can be controlled by using various lipids with different charges (*24*).

The creation of artificial energy-conversion systems involving membrane proteins such as RCs requires the control of the orientation of RCs. This method can be applied to other membrane proteins (*e.g.*, cytochrome bc_1 complex, cytochrome *c* oxidase, *etc.*) in liposomes.

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