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TECHNOLOGY FOR MOLECULAR ASSEMBLY OF ELECTRON TRANSFER PROTEINS

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We have developed various techniques to construct a molecular assembly system with controlled molecular orientation of electron transfer proteins, especially for bacterial photosynthetic reaction centers. The molecular orientation of an immobilized reaction center was controlled using specific biological affinity ligands, Langmuir-Blodgett film techniques, and reconstitution into liposomes. We also constructed biological photocells, herbicide sensors and bioreactors for decomposition of toxic compounds and also for production of hydrogen.

INTRODUCTION

Historical overview and recent trend in biomolecular engineering of electron transfer proteins

From the engineering point of view, downsizing of electrical devices has almost arrived at the stage of molecular device or atomic device in the last decade. The words "molecular machine", "bioelectronics" and "biocomputer" became common about 10 years ago because many researchers in the field of chemistry or physics focused their interest on bio-mimetic molecular assembly systems such as Langmuir-Blodgett films, lipid bilayer vesicles (liposomes), self assembled monolayers, polymer micelles, etc. The research field of nanotechnology directed by probe microscopy (AFM, STM etc.) also expanded simultaneously in a last decade. Sometimes nanotechnology and micromanufacturing-technology (photolysography etc.) have been fused to successfully construct very small devices on atomic scale.

From the scientific point of view, the last 15 years has been a revolutionary era in structural analysis of functionally important membrane proteins in bioenergetics including photosynthesis, respiration and transport. The three-dimensional structure of many proteins has been elucidated as follows. Bacterial photosynthetic reaction centers (RCs) (Deisenhofer, Epp, Miki, Huber & Michel, 1985, Feher, Allen, Okamura & Rees, 1989), bacterial light-harvesting pigment protein complexes (LHs) (McDermott, Prince, Freer, Hawthornthwaite-Lawless, Papiz, Cogdell & Isaacs, 1995; Koepke, Hu, Muenke, Schulten & Michel, 1996), plant light-harvesting complexes (Kuhlbrandt, Wang & Fujiyoshi, 1994), cyanobacterial photosystem I (PS I) (KrauB, Hinrichs, Witt, Fromme, Pritzkow, Dauter, Betzel, Wilson, Witt & Saenger, 1993), plant photosytem II (PS II) (Boekema, Hankamer, Bld, Kruip, Nield, Boonstra, Brber & Rogner, 1995; Rhee, Morris, Zheleva, Hanskamer, Kuhlbrandt & Barber, 1997), bacteriorhodopsin (BR) (Kimura, Vassylyev, Miyazawa, Kidera, Matsushima, Mitsuoka, Murata, Hirai & Fujiyoshi, 1997; Pebay-Peyroula, Rummel, Rosenbusch & Landau, 1997), potassium channel (Doyle, Cabral, Pfuetzner, Kkuo, Gulbis, Cohen, Chait & MacKinnon, 1998), F1 ATPase (Abrahams, Lesli, Lutter & Walker, 1994), cytochorme *c* oxidases (Tsukihara, Aoyama, Yamashita, Tomizaki, Yamaguchi, Shinzawa-Itoh, Nakashima, Yaono & Yoshikawa, 1995; Iwata, Ostermeier, Ludwig, Michel, 1995) and cytochrome *b/c*1 complexes (Kim, Xia, Yu, Xia, Kachurin, Zhang, Yu & Deisenhofer, 1998; Zhang, Huang, Shulmeister, Chi, Kim, Hung, Crofts, Berry & Kim, 1998; Iwata, Lee, Okada, Lee, Iwata, Rusmussen, Link, Ramaswamy & Jap, 1998) were analyzed in detail. The molecular mechanism of protein functions (enzyme catalysis, membrane transport, energy transfer, electron transfer, etc.) can be now discussed not in terms of biochemistry but in terms of molecular physics.

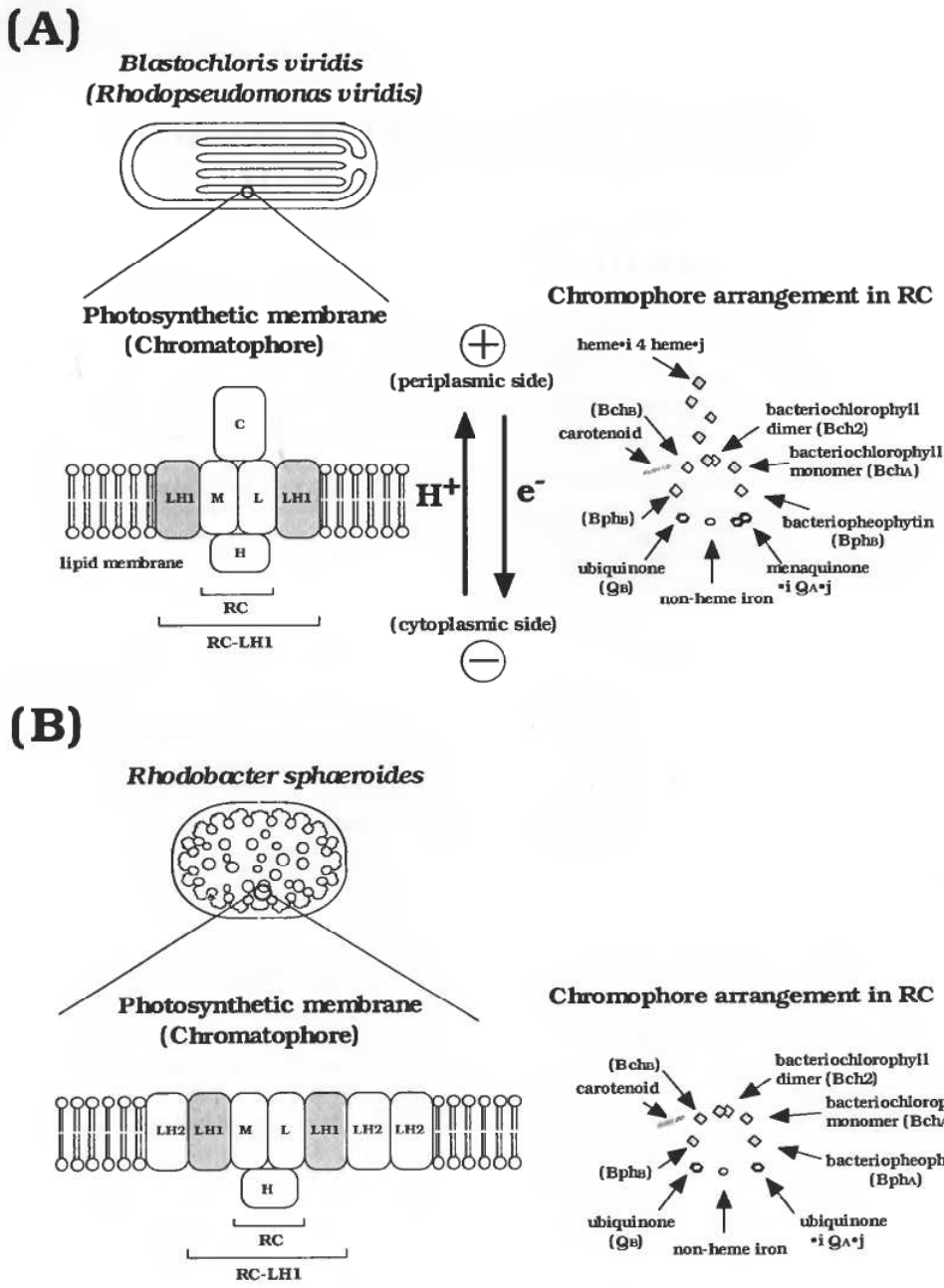


Fig. 1 Schematic representation of cell, chromatophore membrane and RC of Blc. viridis (A) and those of Rba. sphaeroides (B). H-, M-, L- Subunit and bound cytochrome (C-subunit) is contained in Blc. viridis RC that was surrounded by light-harvesting complex 1 (LH1) as shown in panel (A). Direction of light-induced electron transfer and that of proton uptake across the membrane is shown by thick arrows. Arrangement of cofactors in RC is shown in the right side of panel (A). H-, M- And L-subunit are contained in Rba. sphaeroides RC that was surrounded by LH1 and LH2 as shown in panel (B).

Three-dimensional structural information obtained by structural biology has enabled the creation of new frameworks for bio-molecular engineering in terms of physics. For example, three dimensional arrangements and mutual distance of cofactors (bacteriochlorophylls, bacteriopheophytins, quinones, carotenoids, etc.) were precisely determined in RC (Deisenhofer *et al.*, 1985, Feher

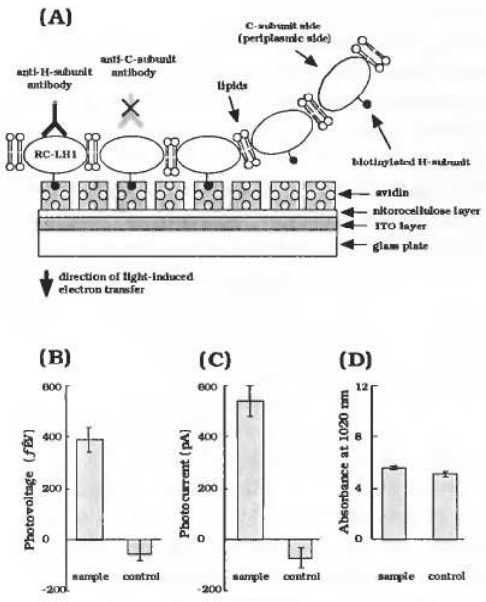


Fig. 2 Schematic representation of oriented immobilization with Blc. viridis chromatophore membranes on the solid surface (ITO electrode) by avidin-biotin interaction (A), photovoltage from the chromatophore-immobilized device (B), photocurrent of it (C), and absorbance of it (D).

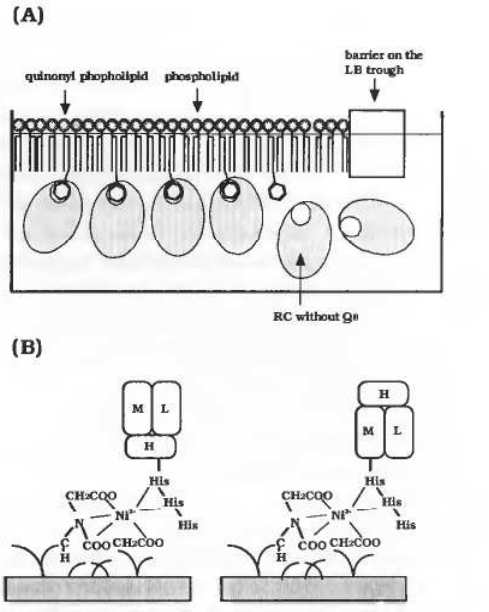


Fig. 3 Schematic representation of LB film consisting of Blc. viridis RC and quinonyl lipids (A) and oriented immobilization of histidine-tagged Rba. sphaeroides RC (B). Histidine tag was attached to H-subunit (left side) or M-subunit (right side) in panel (B).

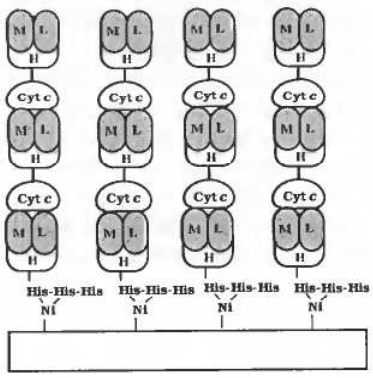
et al., 1989). Therefore the rate of inter-cofactor electron transfer could be discussed in detail in terms of quantum mechanics according to the Marcus theory (Marcus & Sutin, 1985; Moser, Keske, Warncke, Farid & Dutton, 1992). The knowledge from theoretical calculations answered the question why efficient photoelectrical energy conversion was realized in RC by charge separation in bacteriochlorophyll dimer (Bch2) and subsequent electron transfer from Bch2 to quinones through bacteriopheophytin (Bph). This theoretical knowledge could also contribute to the construction of artificial photosynthesis systems composed of synthetic materials (Wasielski, Niemczyk, Svec & Pewitt, 1985; Robertson, Farid, Moser, Urbauer, Mulholland, Pidikiti, Lear, Wand, Degrado & Dutton, 1994).

The intermolecular arrangement of proteins, phospholipids and quinones in the thylakoid membrane can be now discussed in much more detail than before. For example, the interaction between RC, cytochrome *b/c*1 complex, cytochrome *c*2 etc. in the pathway of bacterial photosynthetic electron transfer can be discussed with the structural models. In the natural biological membranes, protein molecules and lipid molecules are well oriented to ensure efficient electron and proton transfer. However it is difficult to artificially control orientation of each molecule again after isolation from the membrane. There is no universal methodology to control the molecular orientation of various proteins *in vitro*. If we hope to construct functional molecular assembly systems like artificial photosynthetic membranes composed of proteins, lipids, and other biological molecules, we need to develop techniques to control the molecular orientation of those molecules.

Difference between artificial systems and molecular assembly systems in organisms

If we compare the man-made machine (car, TV, air plane etc.) with biological molecular assembly systems (cells, organelles, thylakoid membranes in photosynthetic organisms, etc.), there are several essential differences. The first and second clear differences are size and principle of their construction, respectively. Man-made machines are in cm – m scale and their components are artificially assembled in the factory. However biological molecular assembly systems are in nm – μm scale and their components (proteins, lipids and other molecules) are self-assembled achieving a local minimum of Gibbs free energy (G) in aqueous solution. The third difference is the energy level of their function. Usually thermal fluctuation and disturbance of the component is negligible in man-made

machines. However biological molecular assembly systems work in the lower range of energy that is similar to the energy level of thermal fluctuation (Brownian movement etc.) of biological molecules. Therefore the function of the system is sometimes disturbed by thermal fluctuation of the components. The fourth difference is the information. All the information necessary for construction of the system is given as a two-dimensional blueprint or a picture and therefore can be clearly visible in case of man-made machine. However, the information is given as a one-dimensional DNA sequence and difficult to understand at a glance in the biological molecular assembly system. A fifth difference is the principle that drives the system. Rotary motions in the man-made machines are mostly driven by electromagnetic motors, or by combustion engines (gasoline engine, diesel engine, etc.). However, rotary motion in the F1/FoATPase is driven by the proton current across the lipid bilayer membrane (Noji, Yasuda, Yoshida & Kinoshita, 1997). Rotary motion of bacterial flagella is driven by proton current or by a current of other ion (Atsumi, McCarter & Imae, 1992). Another example is information processing. Memory, calculation and other information processing works are mostly carried out electrically or magnetically in man-made machines (computer, TV, telephone, etc.). However information processing (transcription of DNA, translation of mRNA, receptor-mediated signal transduction of hormones, cytokines, neurotransmitters, etc.) is mostly carried out chemically in the biological molecular assembly system (Bray, 1995). Application of bacteriorhodopsin, a protein-pigment complex with photo-driven proton pumping activity for information processing devices were also reported Miyasaka *et al.* (1992), Birge (1992).



Generally speaking, any small machines can save energy if they work as well as large ones. The characteristic properties of biological molecular assembly systems as described in the previous paragraph are the basis of a highly efficient energy conversion without futile energy loss (photosynthesis, respiration, transport, muscle contraction, etc.) in organisms. Therefore we believe that it is necessary to establish a universal technology for the construction of a molecular assembly systems which function as natural biological membranes, if we want to realize intelligent bio-molecular devices such as biocomputer, bio-memory device, intelligent biosensors, and so on. The mutual distance between technology for devices and the science of bioenergetics is decreasing, little by little. With that perspective, 10 years ago we started our research to establish technology for controlling molecular orientation of membrane proteins and have pursued the technology for bio-molecular assembly. We chose the RC as a model protein in development of the technology as described in our previous review articles (Miyake & Hara, 1997a, b). We summarize the result of our efforts and perspective of this technology to applications in near future.

Bacterial photosynthetic reaction centers

Figure 1a and 1b show the schematic representation of photosynthetic reaction center from purple non-sulfur photosynthetic bacterium *Blastochloris viridis* (formerly called as *Rhodopeudomonas viridis*) and that from *Rhodobacter spaeroides*.

Blc. viridis, a bacteriochlorophyll *b*-containing bacterium has highly stacked lamellar intracytoplasmic membranes in those many photosynthetic reaction center-light-harvesting complexes 1 (RC-LH1s) are densely packed like two-dimensional crystal (Giesbrecht & Drews, 1966). Intracytoplasmic membranes separated from mechanically disrupted cells (chromatophores) of this bacterium are mostly sheets or broken vesicles, and lack the light-harvesting complex 2 (LH2).

The photosynthetic reaction center of *Blc. viridis* consists of four protein subunits and 14 cofactors. Two bacteriochlorophylls form a special pair (also called as bacteriochlorophyll dimer; Bch2) and an additional two bacteriochlorophyll molecules exist as monomers (also called as accessory bacteriochlorophylls: Bch). Two bacteriopheophytins, a menaquinone as a primary quinone (QA), a ubiquinone as a secondary quinone (QB), a carotenoid and a non-heme iron. All these cofactors are non-cavalently bound to M- and L-subunit. Each of these two subunits is extremely hydrophobic and

forms 5 trans-membrane helices. The H-subunit has only one trans-membrane helix and a less hydrophobic subunit without any cofactors. The C-type bound cytochrome containing 4 hemes is strongly bound to the periplasmic side of M- and L-subunit.

When the RC is excited by light energy, charge separation occurrs in Bch2. The electron formed by charge separation is transferred from Bch2 to Bph and then to QA and QB. Another electron is supplied by the bound cytochrome to prevent the back reaction of electron transfer in RC. Hemes in the bound cytochrome can be re-reduced by another water-soluble cytochrome *C*2 in the periplasmic space. If QB is reduced twice, it binds to protons to become a quinol (QB H2) and then is exchanged with the ubiquinone in the quinone pool of the membrane. The spatial arrangement of Bch2, BchA, BphA, BchB, BphB, QA and QB in the M- and L-subunit is almost symmetrical. BchA, BphA and QA are shown in the right side whereas BchB, BphB and QB are in the left side in Figure 1a. The light-induced electron transfer occurs through the one pathway from Bch2 to QA through BphA (never from Bch2 to QB through BphB) although the both pathways have similar (pseudo-symmetrical) arrangement of cofactors.

The direction of electron transfer is from the periplasmic side to the cytoplasmic side, while the direction of proton uptake accompanied with quinol formation is conversely from cytoplasmic side to periplasmic side.

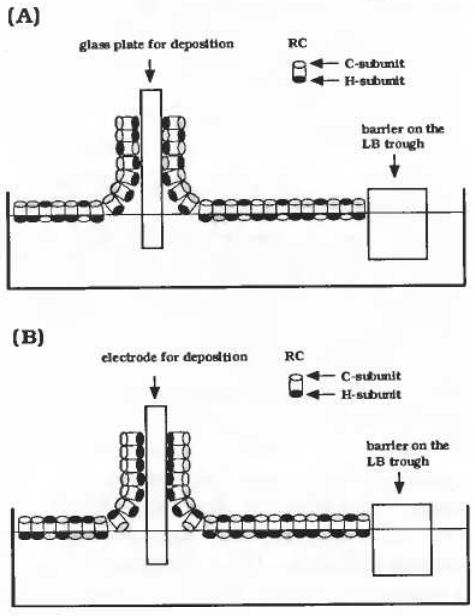


Fig. 5 Schematic representation of LB film composed of Blc. viridis RC deposited on the glass plate (A) and that on the electrode with the controlling molecular orientation of RC (B).

Rba. spaeroides, a bacteriochlorophyll *a*–containing bacteria has vesicular intracytoplasmic membranes in which RC-LH1s are surrounded by many LH2s like islands. The intracytoplasmic membrane isolated from mechanically disrupted cells (chromatophores) and those from enzymatically disrupted cells (spheroplast-derived vesicles) are well-sealed vesicles (Matsuura & Nishimura, 1997).

The RC of *Rba. sphaeroides* consists of three subunits; H-, M- and L-subunit but lacks the bound cytochrome. The primary quinone is a ubiquinone, while other structural and functional features are similar to those of *Blc. viridis* RC. Light-oxidized Bch2 is directly re-reduced by cytochrome *C*2 in the periplasmic space in *Rba. sphaeroides*. *Blc. viridis* RC and *Rba. sphaeroides* RC are relatively stable, structurally analysed membrane proteins, and retain their function (light-induced electron transfer) in the form of dried or frozen samples (Kleinfeld, Okamura & Feher, 1984). These characteristic properties are advantageous for our research to establish the general methodology for controlling their molecular orientation. We describe the each technique to control the molecular orientation of RC with different principles in the next section.

TECHNIQUES FOR CONTROLLING THE MOLECULAR ORIENTATION OF RCS

Oriented immobilization of RC using biological affinity ligands

Biological affinity ligands are a highly specific molecular recognition system and have been widely used in affinity chromatography and biochemical assays such as enzyme-linked immunosorbent assay (ELISA), avidin-biotin detection method, DNA-RNA hybrydization (Nothern blotting), DNA-DNA hybrydization (Southern blotting), etc. These affinity probes and other natural sets of protein/affinity ligands are also useful also for oriented immobilization of membrane proteins like RC.

We showed oriented immobilization of a *Blc. viridis* chromatophore membrane using avidin-biotin interaction by selective biotinylation of the protein subunit and avidin adsorbed on the solid surface as shown in Fig. 2a (Hara, Majima, Miyake, Ajiki, Sugino, Toyotama & Kawamura, 1990). Orientation of immobilized membranes was

evaluated. Amplitude of the photo-voltage or that of photo-current is greatly improved by oriented immobilization of the membranes as shown in Fig. 2b and 2c, respectively while the amount of immobilized membranes was almost identical for the sample and control as shown in Fig. 2d. Orientation of the immobilized membrane was also evaluated by ELISA with both anti-H-subunit and anti-C-subunnit (bound cytochrome) antibody. It proved that 80% of the membrane was oriented by that immobilization. Multilayers of immobilized chromatophore membranes could also be prepared by repeated immobilization (Hara, Majima, Ajiki, Sugino, Toyotama, Ueno, Asada & Miyake, 1996).

Another example is based on RC-quinone affinity combined with Langmuir-Blodgett film (LB film) technique. A monolayer of phospholipid containing the quinonyl lipid was formed on the subphase. QB-Depleted *Blc. viridis* RC was gently added to the subphase in the LB-trough and then bound to the monolayer film of lipids containing quinonyl lipids. Binding of RC to quinonyl residues was proven when the ability of light-induced spectral change of QB-depleted RC was recovered. That recovery reflected the electron transfer from Bch2 to QB (Hirata & Miyake, 1994).

Expression of histidine-tagged protein is a widely used technique based on the genetic engineering for purification of proteins, especially membrane proteins. It is also useful for oriented immobilization of proteins on the solid surface. A histidine tag was connected to either the H-subunit (Nakamura, Hasegawa, Yasuda & Miyake, in press a) or the M-subunit (Goldsmith & Boxer, 1996) of *Rba. sphaeroides* RC. These RC were then immobilized on the solid surface (sensor tip for biosensors) with chelation of Ni^{2+} ion as shown in Fig. 3b.

We prepared a genetically engineered fusion protein from *Rba. spaeroides* RC and horse herart cytochrome *c*. Cytochrome *c* was connected to H-subunit of RC, the heme pocket was facing the outside in this genetically engineered complex. Cytochrome *c* has a special affinity for the periplasmic side of RC (M- and L-subunit) as a couplet of electron donor and acceptor (Tiede, Vashishta & Gunner, 1993). Therefore it is possible to construct a multilayer assembly of this complex when it is combined with immobilized histidine-tagged RC as shown in Fig. 4 (Nakamura, Kaneko, Hasegawa, Yang, Hara, Shirai & Miyake, 1998). The RC-cytochrome *c* complex over the second layer attached to another complex by specific affinity between RC and cytochrome *c*.

We also prepared another type of RC-cytochrome *c* complex by the chemical coupling method as described in later paragraph (Ueno, Hirata, Hara, Arai, Sato, Miyake & Fujii, 1995; Ueno, Hara, Kamo, Fujii & Miyake, 1998). The heme pocket of cytochrome *c* in that complex was facing the H-subunit of RC in that complex and could not bind to another RC. Therefore a covalent RC-cytochrome *c* complex could not be used for multilayer assembly. Instead, it is very useful for controlling the topological orientation in reconstituted liposomes as described later.

Langmuir-Blodgett films of RC

The Langmuir-Blodgett film (LB film) technique is a special technique used to prepare a monolayer film of amphiphilic molecules on the air/water interface and then deposit it onto a solid support. Amphiphilic molecules (surfactants, lipids, etc.) spread on the surface of aqueous solution (subphase) are compressed horizontally by an barrier on the subphase surface and are then deposited onto solid support (glass plate etc.). The relationship between surface pressure on the subphase (π) and area of the monolayer on the subphase (A) is shown as π-A curve. The vertical deposition method as shown in Fig. 5a, or otherwise a horizontal lifting method is used to deposite the monolayer to solid support. Amphiphilic molecules can be uniaxially oriented by compression before deposition. LB Films of *Blc. viridis* RC were prepared by us and other groups (Alegria & Dutton 1991a; Alegria & Dutton 1991b). If the polarity of molecules with respect to hydrophobicity and hydrophilicity is very clear, the molecules can be highly oriented on the subphase because the hydrophilic part is dipped into the aqueous phase while exposing their hydrophobic part to the air. Unfortunately a 3-dimensional structure of RC showed that the polarity of the *Blc. viridis* RC was not so clear (Deisenhofer *et al.* 1985; Feher *et al.* 1989). Bound-cytochrome and a major part of H-subunit with relatively hydrophilic surface protrude in opposite directions to each other from the transmembrane region consisting of M- and L-subunit. Therefore the *Blc. viridis* RC has a random up/down orientation as shown in Fig. 5a. (Alegria & Dutton, 1991a, b) prepared the LB film in which *Blc. viridis* RC s are uniaxially highly oriented and successfully addressed the spatial arrangement of four different hemes in the bound cytochrome (Alegria & Dutton 1991a, Alegria & Dutton 1991b). However, the up/down orientation was not controlled in the LB film.

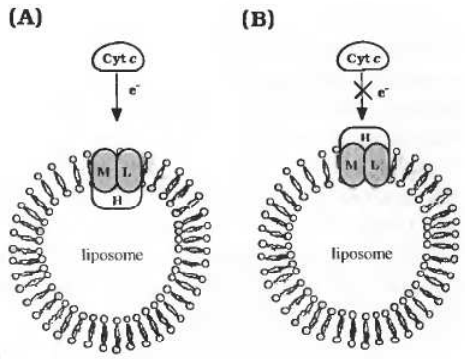


Fig. 6 Schematic representation of electron transfer reaction from horse heart cytochrome c to Rba. sphaeroides RC reconstitute into liposomes. Periplasmic surface of RC is exposed (A) or not exposed (B) to outside.

We could partly control the up/down orientation by different deposition methods (Yasuda, Sugino, Toyotama, Hirata, Hara & Miyake, 1994). *Blc. viridis* RCs in a Z-type LB film deposited on the hydrophilic plate have a different molecular orientation from those in a X-type LB film deposited on the hydrophobic plate. Cytoplasmic side (bound cytochrome) and periplasmic side (H-subunit) are facing the surface of the plate in the former and the latter case, respectively. We evaluated the molecular orientation of *Blc. viridis* RC by both the measurement of photocurrent response and by the ELISA method.

We focused our attention not only on the polarity between hydrophilicity and hydrophobicity on the surface of *Blc. viridis* RC but also on the electrostatic charge distribution. When we look at charge distribution of *Blc. viridis* RC, M- and L-subuunit are less charged because they are embedded in the lipid bilayer of photosynthetic membranes. H-subunit and bound-cytochrome have charged surfaces because they protrude into the cytoplasm and periplasm, respectively. Bound cytochrome is positively charged and the H-subunit is negatively charged. Therefore there is a electrostatic dipole along the long axis of the *Blc. viridis* RC.

Recently we developed a novel method to control the molecular orientation of *Blc. viridis* RC in the LB film. When a small voltage was applied between the electrode as a plate for deposition and the subphase solution, molecular orientation of RC was well controlled as shown in Fig. 5b (Yasuda, Hara, Miyake & Toyotama, 1997). Molecular orientation of RC in the depositied LB film was evaluated by the measurement of light-induced

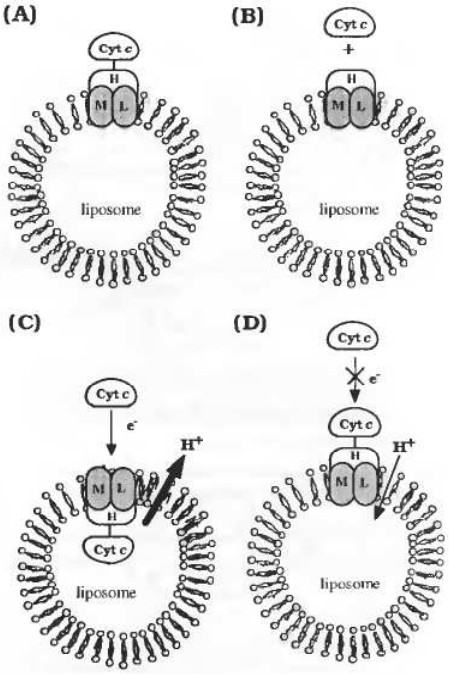


Fig. 7 Schematic representation of RC-cyt c complexes reconstituted into liposomes. Periplasmic surface of Rba. sphaeroides RC moiety is exposed outside (C) or not exposed (A), (B), (D). Panel (B) shows the cleavage between RC and cyt c. Large amount of proton efflux and small amount of proton influx are shown by a thick arrow and an thin one in (C) and (D), respectively.

displacement current. We believe that the RC was rotated or re-oriented while deposited on the surface of the electrode. The addition of a charged polymer (poly-L-lysine) as a counter ion suppressed the effect of bias voltage because the polymer could be adsorbed to the RC by electrostatic interaction and then hampered the rotation of RC (Yasuda, Toyotama, Hara & Miyake, 1998). The result supported the explanation as described above.

Reconstitution of RC into liposomes

In natural biological membranes, proteins are topologically completely oriented because the translated polypeptide is transported or secreted across the membrane and folded into a proper 3-dimensional structure with the help of molecular machinery such as Sec proteins. However if we solubilize the membrane proteins and reconstitute them into liposomes, topological orientation of proteins is uncertain. Sometimes they can be

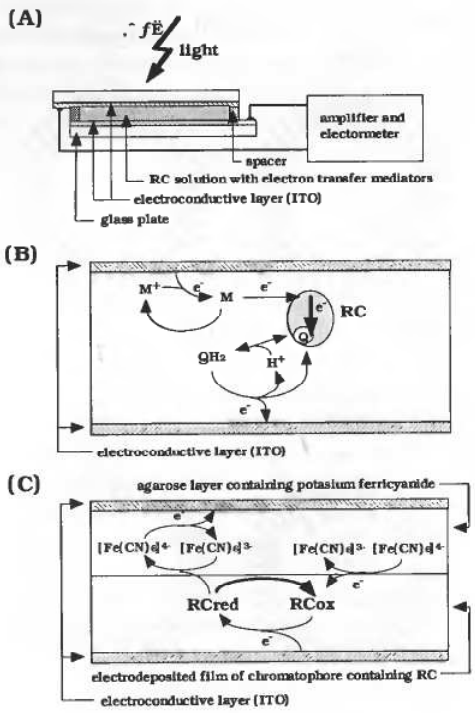


Fig. 8 Schematic representation of the simple photocell (A), reaction mechanism in it (B) and reaction mechanism in the advanced photocell with more complex structure (C).

randomly oriented while some other proteins can be oriented automatically. We investigated how the topological orientation of protein is determined when it is reconstituted into liposomes. Reconstitution experiments were carried out with the *Rba. sphaeroides* RC and the covalently linked complex between RC and horse heart cytochrome *c* (RC-cyt *c*) (Hara M., Ueno T., Fujii T., Yang Q., Asada Y. & Miyake, 1997b; Ueno *et al.*, 1998).

When the *Rba. sphaeroides* RC was reconstituted into liposomes composed of phosphatidylcholine by detergent dialysis method combined with freezing and thawing, topological orientation of RC depend on the charges of lipids. More than half (60-73%) of RCs exposed their periplasmic side to outside of the liposomes consisted of neutral lipids or negatively charged lipids (Hara *et al.*, 1997b). However only 30-33% of RCs were in the same orientation as positively charged lipids. That topological orientation was estimated by the ability of light-induced electron transfer from cytochrome *c* added in the suspension of liposomes. A photooxidized RC exposing the periplasimc surface could be reduced by cytochrome *c* as shown in Fig. 6a. However, a RC exposing the cytoplasmic surface could not be re-reduced as in Fig. 6b. The *Rba. sphaeroides* RC has a slight polarity in distribution of hydrophobic surface and hydrophilic surface: the H-subunit has a large hydrophilic surface in the cytoplasmic side whereas the L- and M-subunits have a limited hydrophilic surface only on the periplasmic side (Feher *et al.*, 1989; Ueno *et al.*, 1998).

When we attached horse heart cytochrome *c* , another hydrophilic protein to H-subunit using a bifunctional chemical coupling reagent in the preparation of RC-cyt *c*, the polarity in distribution of hydrophobic/hydrophilic surface in RC-cyt *c* was clearer than that in native RC. The RC-cyt *c* was reconstituted into liposomes by the same procedure as RC. It was topologically more oriented in reconstituted liposomes than RC (Ueno *et al.*, 1998).

We estimated the topological orientation of RC-cyt *c* reconstituted in the proteoliposomes using three different experiments. They were

(i) release of the cytochrome *c* moiety from the proteoliposomes by cleaving the disulfide bond in the linker residue,

(ii) electron transfer from free cytochrome *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* was oriented with H-subunit exposed on the outside of the proteoliposomes.

From all of the results as described above, we conclude that topological orientation of the membrane protein such as RC depend on both charges of lipids and the polarity in distribution of hydrophobic/hydrophilic surface of proteins.

Liposomes can be used not only to control the topological orientation of RC but also in the environment where the chlorophylls exist and work as light-harvesting pigments for the RC. We reported the reconstructed light-harvesting system of liposomes in which both *Rba. sphaeroides* RC and chlorophylls were reconstituted (Goc, Hara, Tateishi & Miyake, 1996).

Comparison with other methods and strategies

We reviewed three different strategies for the controlling the molecular orientation of membrane proteins such as RC: using biological affinity ligand, LB film, and reconstitution into liposomes. Each strategy depends on a different principle and works in a different phase. The first one works in aqueous solution (liquid phase). The second one

works in the air/water interface (gas/liquid interface). The third one works in the lipid/water interface (semi-liquid/liquid interface). Not all the methods to control the molecular orientation of proteins can be classified into these three categories. There are also other strategies. For example, crystallization of proteins or physical adsorption of proteins onto the solid surface works in the solid/liquid interface depending on the hydrophobic interaction and electrostatic interaction between the molecules. Other kinds of forces such as gravitational force (g) and magnetic force (gauss) may affect the molecular orientation of proteins although we do not discuss these in detail in this section.

APPLICATION OF ELECTRON TRANSFER PROTEINS FOR ELECTRICAL DEVICES

Biotechnology and biomimetics

When we applied the scientific knowledge regarding biological electron transfer systems such as the photosynthetic electron transfer chain or the respiratory chain, we had two strategies to construct an electrical device: biotechnology and biomimetics. The approach with biotechnology is to construct bio-electrical devices such as biosensors or bioreactors with biological components: proteins, lipids, biological membrane fragments and cells. The approach with biomimetics involves construction of an the electrical device using synthetic materials such as organic and inorganic compounds although the principle of efficient electron transfer system in bioenergetics is used for the design of the device. For example, photosystem II in the chloroplast has water-splitting activity and can evolve O_2 under illumination. An oxygen-evolving photo-bioreactor with immobilized chloroplasts is the biotechnological approach and that with titanium oxide is the biomimetical approach. Both approaches can be combined in application, if necessary. We generally took the biotechnological approach to construct bio-electrical devices as described below.

Photocells

A solution of *Blc. viridis* RC or chromatophore suspension was sandwiched by two transparent electrodes to construct simple photocell as shown in Fig. 8a (Tamura, Sato, Ajiki, Sugino, Hara & Miyake, 1991). The photocurrent from the anode to the cathode was measured using that device, Fig. 8b shows the possible mechanism of photocurrent generation. When light-induced electron transfer occurs in RC, a photo-oxidized RC was reduced by

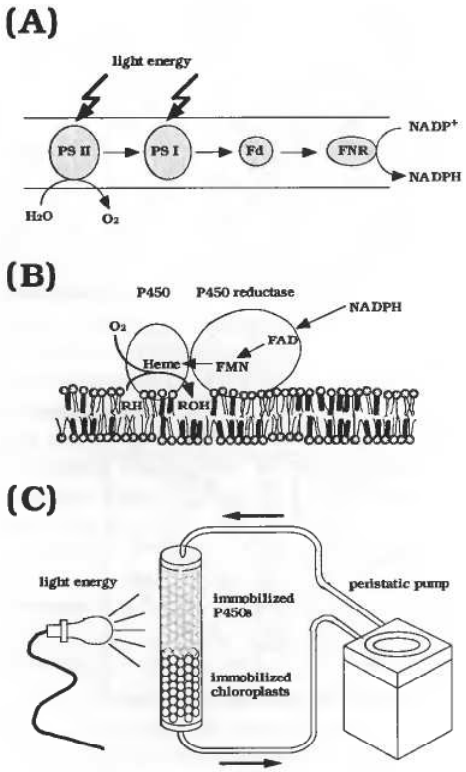


Fig. 9 Schematic representation of electron transfer in chloroplast (A), that in microsomal membranes including P450, and a photo-bioreactor with immobilized chloroplasts and immobilized P450s.

electron from electrode through the electron transfer mediator. When two different electrodes have different work functions (the energy required for an electron to escape a solid surface), a net photocurrent results from anode to cathode as the difference between the electron transfer from anode to solution and that from cathode. It is better to use different electrodes to construct the photocell, or otherwise to apply the small bioas voltage between anode and cathode to get a larger current (Tamura, Miyake, Yasuda, Hara & Sato, 1992). The addition of electron transfer mediators enhanced the amplitude of photocurrent although photovoltage was rather small (Tamura *et al.*, 1992). When we sandwiched a dried film of chromatophore between the anode and the cathode, instead of an aqueous chromatophore suspension, we got a higher amplitude of transient photovoltage although it was difficult to get higher amplitude of constant photocurrent because of the low conductivity in the dried film. Therefore it is difficult to get an efficient photocurrent and photovoltage simultaneously.

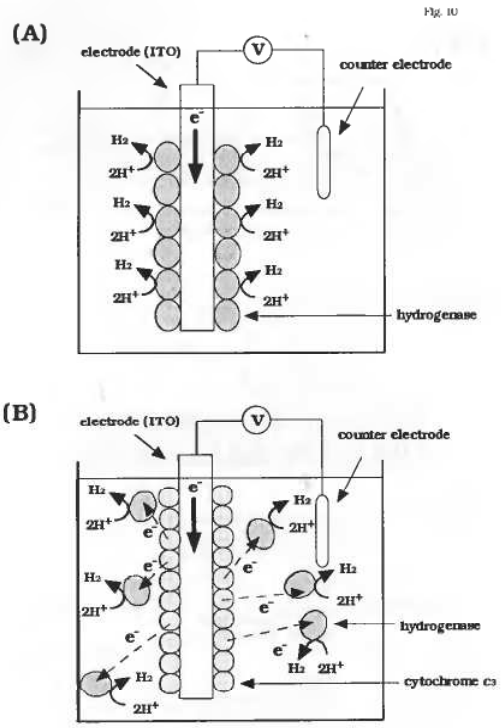


Fig. 10 Schematic representation of hydrogen evolving bioreactor with the hydrogenase LB film (A) and the cytochrom c3 LB film with hydrogenase in solution (B).

We invented an efficient photocell consisting of an electrodeposited film of *Blc. viridis* chromatophore membranes on the cathode side and an thin agarose layer containing potassium ferrocyanide on the anode side as a electron transfer carrier (Ajiki, Toyotama, Hara & Miyake, 1997). This photocell showed a higher amplitute of photocurrent although the current derived not only from light energy but also from the chemical energy by consuming the ferrocyanide as an electron donor.

Herbicide sensors

Photocurrent generation in the photocells as in Fig. 8a and Fig 8b depends on the species of electron transfer mediators contained in the solution between anode and cathode. If the solution contains a triazine compound, which is a well known herbicide and can bind to the QB site of RC as a inhibitor of electron transfer (Tamura, Sato, Hara, Asada & Miyake, 1994), the photocurrent is suppressed or reduced. Therefore, the photocell can be used as a herbicide sensor. Chloroplasts isolated from spinach can also be used for this purpose (Tamura *et al.*, 1994).

We are trying to construct another herbicide sensor based on a different principle, surface plasmon resonance (SPR) (Nakamura, Shimada, Hasegawa, Shirai & Miyake, 1999). For this purpose, RC would be immobilized on the sensor tip of SPR equipment as shown in Fig. 3b with the correct molecular orientation.

3.4 Bioreactors

Spinach chloroplasts catalyse the Hill reaction to reduce $NADP^+$ to NADPH using light energy as shown in Fig. 9a (Hara, Ohkawa, Narato, Shirai, Asada, Karube & Miyake, 1997a). Various enzymes involved in oxidation or reduction need NADPH as a substrate. Therefore immobilized chloroplasts can supply NADPH to drive the enzymatic reaction of those enzymes by recycling NADPH from $NADP^+$. Microsome-type P450s are the enzymes which need NADPH for their enzymatic reaction in the presence of P450 reductase as shown in Fig. 9b. We constructed a photobioreactor consisting of immobilized spinach chloroplasts and immobilized P450s as shown in Fig. 9c. We used a genetically engineered fusion enzyme comprised of rat P4501A1 and yeast P450 reductase expressed in yeast microsomal membranes. The reactor converted substrate (7-ethoxycoumarin) to product (7-hydroxycoumarin) under illumination quite efficiently. Bioreactors with immobilized P450s have been reported before (King, Azari & Wiseman, 1998; Yawetz, Perry, Freeman & Katchalski-Katzir, 1984), they used an NADPH-recycling system containing coupling enzymes which consume their own substrate. Our system contained immobilized chloroplasts instead of those enzyme-coupled NADPH-recycling systems and therefore could recycle NADPH without consuming the substrate. P450s have wide variety of molecular species with diversed substrate specificity (Guengerich, 1993). Many of them can decompose the hydrophobic toxic substances. Therefore, various P450s can be used in the bioreactors although we showed an example only with rat P4501A1.

Another example of our bioreactor with electron transfer proteins is a hydrogen evolution system with immobilized hydrogenase, a molecular hydrogen-evolving enzyme as shown in Fig 10a. We prepared the LB film of hydrogenase isolated from the photosynthetic bacterium *Thiocapsa roseopersicina* on the electrode (Noda, Zorin, Nakamura, Miyake, Gogotov, Asada, Akutsu & Miyake, 1998). When a small bias voltage was applied between the electrode and counter electrode, hydrogen evolved from the hydrogenase-immobilzed electrode. Hydrogen did not evolve from the naked

electrode without hydrogenase as a control experiment. Therefore, the hydrogen evolution derived not from simple electrolysis of water by electrode but from the enzymatic reaction with the assistance of a small bias voltage to change the energy gap on the electrode surface.

We also constructed another hydrogen evolving reactor as shown in Fig. 10b. Cytochrome *c*3, a water-soluble protein containing four hemes was immobilized on the ITO by LB techniques (Nakamura *et al.*, in press a).

This protein is thought to be an electron donor to hydrogenase in the cells of bacteria *Desulfovibrio sp*. Cytochrome *c*3-immobilized electrode can reduce hydrogenase in the solution and therefore hydrogen was evolved. A small bias voltage was also applied in that case.

TECHNOLOGY FOR BIO-MOLECULAR ASSEMBLY IN NEAR FUTURE

Self assembly and hierarchical structure

The self assembling-properties of biological molecules (proteins, nucleic acids, lipids etc.) allows formation of complex systems like a molecular machines such as multi-subunit protein complexes. The protein complexes are assembled as organelles. They are also assembled to be cells. The principle in construction of living organisms has hierarchical properties: components are assembled to comprise large parts, and parts are assembled to make a whole system. We can explain the concept of hierarchical structure with an example of striated muscle. An actin filament (F-actin) is formed by polymerization of actin monomers (G-actin) according to the condensation-polymerization mechanism as in case of crystallization (Oosawa & Asakura, 1975). Many actin filaments are regularly assembled to form a bundle called a thin filament. Many molecules of myosin, another protein, are also regularly assembled to form a thick filament. Numerous thin filaments and thick filaments are arranged regularly as a hexagonal lattice forming a myofibril. Sarcoplasmic reticulum containing calcium ATPase, and other muscle proteins (troponin, tropomyosin, etc.) were also attached to the myofibril. Numerous regularly arranged many bundles of myofibrils are contained in a single muscle fiber. A huge number muscle fibers are contained in a muscle. Therefore the muscle is composed of hierarchical structure: there are many different levels of components from a single protein (actin, myosin, etc) to the whole muscle. This hierarchical structure enables the quick and efficient muscle contraction without loss of energy supplied as ATP.

Presently, it is somehow possible to construct the artificial molecular assembly system (LB films, liposomes, complex formation of different proteins, etc) composed of membrane proteins and lipids as we described in the previous sections. However, it is still very difficult to construct molecular assembly systems which have a highly ordered hierarchical structure like cells or organelles of living organisms. We can find hierarchical structure not only at the molecular level but also at the cellular or tissue level in animals, plants and other organisms. For example, blood vessels in the human body are highly branched, dendrite structures (or fractal structures) from large arteries in the vicinity of the heart to micro vessels in the peripheral tissues. That highly branched structure ranging in size from centimeter to several tens of micrometers in scale is inevitably necessary for quick circulation of blood from the heart to peripheral tissues. It is still difficult to artificially fabricate industrial materials (plastics, metals, etc.) in such forms.

Lakes also proposed an idea that structural hierarchy is very important and can play a large part in determining the bulk material properties (Lakes, 1993). We believe that the new technology for constructing the hierarchical structure in materials from molecular level (nm order in size) to visible level (mm – cm order in size) will be very important in the next decade.

Protein / metal interface

Computers and other man-made devices for information-processings (memory disk, videotape, TV, etc.) are always based on the principle that information is processed by electronic or magnetic properties of materials. However information processing in the human brain is carried out by neurons. The information-processing-process in the human brain is carried out by axonal conduction and synaptic transmission. Information is transferred and processed by electrical signals (conduction of active potential in neuronal membranes along the axon) and chemical signal (neurotransmitter release from the pre-synaptic membranes and their action to the receptors in the post-synaptic membranes). Interface between neurons and metal electrodes will be extensively developed in near future. The axonal conduction will be directly converted to current in the electronic circuit. The synaptic transmission is can be functionally controlled by a metal electrodes. These techniques will enable to construct biological/electronic hybrid computers. In other words, R&D for functioning protein/metal

interface is very important. This is also a very promising breakthrough in the molecular assembly technology for electron transfer proteins in near future.

CONCLUSION

Technology for molecular assembly systems of biological molecules, especially electron transfer proteins is necessary for the construction of bioelectrical devices such as photocells, biosensors, bioreactors. It has been partly developed in the last decade although many important problems still remain to be solved. New technology for the creation of hierarchical structure in material and that for the protein / metal interface will be very important in the coming decade.

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