Volume 324, number 3, 287–292 FEBS 12599 June 1993

Visualization of the morphology of purple membrane surfaces by monoclonal antibody techniques

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Received 16 April 1993; revised version received 28 April 1993

A monoclonal antibody technique for determining the surface morphology of the purple membrane (PM) has been developed. Two types of antibodies for distinguishing the extracellular surface and the cytoplasmic surface of PM were produced by immunizing the corresponding synthetic peptides to mice. The Langmuir–Blodgett (LB) method was employed to form a single-layer two-dimensional array of PM fragments on an electron microscope grid. The LB film was then treated with the antibodies which had been labeled with colloidal gold particles. Electron microscope observations visualized the morphology of the two surfaces of PM to be rough and smooth regions, respectively; thus the orientation of PM fragments was determined.

Bacteriorhodopsin; Purple membrane; Monoclonal antibody; Immuno-gold labeling; Electron microscopy

1. INTRODUCTION

Bacteriorhodopsin (BR), the only protein in the purple membrane (PM) of *Halobacterium halobium*, transports protons from the cytoplasm to the extracellular space under light excitation, generating a transmembrane electrochemical gradient [1]. The amino acid sequence of the protein has been established [2] and a three-dimensional model has been developed [3] which is consistent with this sequence and its diffraction data [4,5]. According to this model, the orientation of the protein in the membrane is such that the carboxyl terminus is on the cytoplasmic side with about 20 amino acid residues protruding into the cytoplasm while the amino terminus appears to exist outside the cell membrane.

Since PM can easily be isolated without any apparent impairment in function and is extremely stable against light, attempts have been made to devise bioelectronic systems such as optical computers and memories [6–9]. We have demonstrated that a liquid-junction photocell with ultra-thin LB films of PM has differential responsivity to light intensity, which is characteristic of vertebrate photoreceptors [10]. This BR photocell was shown to be capable of detecting and processing optical information in a manner similar to that found in visual functions [11].

Such artificial systems have been thought to exhibit photoelectric activity only when the incorporated BR molecules are nonrandomly oriented so that unidirectional currents can be produced upon illumination. It was further believed that the orientation of PMs is based on physical properties of the asymmetric membrane structure, such as a difference in the surface charge densities on both sides of the membrane [12] and the permanent dipole moment associated with the surface potential of PM [13]. These asymmetrical properties have been used as the basis of a number of procedures for controlling the orientation of PM, such as dispersion at the air/water interface, adsorption to charged membrane filters, and electric-field application [14]. The orientation of PM has thus been postulated on the basis of the direction of the photoelectric response in the device form and the actual degree of the oriented membranes remains unknown.

Few morphological aspects of the PM surfaces have, however, been characterized by direct observation. Fisher et al. showed that it is possible to distinguish between the two surfaces using electron microscopy with an air-dried, platinum–carbon-shadowed PM [15], suggesting that the C-terminal side was exposed to the cytoplasmic surface on the basis of a morphological comparison between the sidedness of PM fragments and freeze-fracture of whole cells. This technique, however, requires a great deal of skill and the experience and the determination will be affected to a considerable degree by the presence of 'cracked' and 'pitted' areas which vary with the drying procedure.

In the present paper, we report a method for detecting a specific side of PM fragments based on immuno-gold labeling that utilizes a monoclonal antibody labeled with colloidal-gold particles.

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2. MATERIALS AND METHODS

Purple membrane fragments were isolated from *Halobacterium halobium* strain S9 using the standard procedure [16]. Peptides comprising the C- and N-terminal sequences of BR were synthesized on an ABI 430A peptide synthesizer, coupled to Keyhole-limpet haemocyanin (KLH) and used to immunize mice. Inhibition experiments and immunoblot analyses are described in the figure legends.

PM monolayer (LB) films were prepared as described previously [17]. In short, a monolayer of PM fragments spread on a neutral aqueous subphase was transferred onto a solid substrate either by vertical dipping of the substrate across the interface or by the horizontal attachment thereof on the supernatant fragments ELISA in this experiment was carried out as follows. The PM film deposited on a glass plate through the dipping method was incubated with an anti-BR antibody and then with horseradish peroxidase (HRP) coupled goat anti-mouse IgG (Sigma). Antibodies labeled with gold particles were prepared by mixing colloidal gold solution (approx. 5 nm diameter, BioCell) with IgG in borax-HCl buffer at pH9 and adding BSA (bovine serum albumin) after 10 min, to yield a final concentration of 1%. The antibody gold complex was centrifuged for 40 min, the resultant precipitate was suspended in small amounts of PBS containing 1% BSA, the suspension was used for immunoblot analysis and electron microscopy.

For electron microscopy, a grid (nickel) was coated with a carbon film just before use. In the case of vertical dipping, the grid surface was rendered hydrophilic by treatment with electric discharge. After deposition of the LB film of PM fragments, the grid was treated with 0.1% BSA in order to avoid non-specific adsorption of a gold-labeled antibody on the grid surface in the absence of PM fragments. The grid was then incubated with the solution containing the antibody–Au conjugate, followed by shadowing it with platinum, and it was observed with an electron microscope (JEM 2000FX).

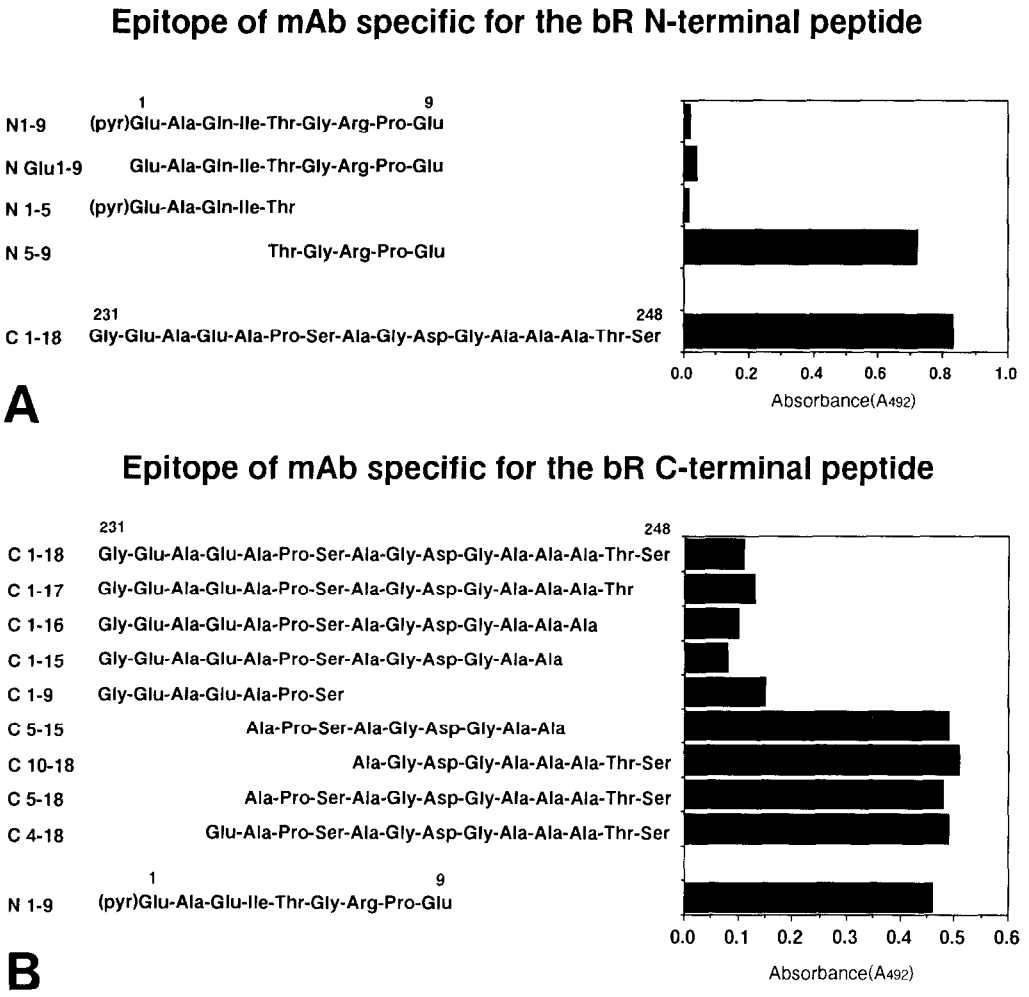


Fig. 1. Three and eight peptide analogs of varying lengths of the native BR-N and the BR-C peptides, respectively, were synthesized and examined with the anti-BR antibodies by competitive inhibition assay. Microtiter plates were coated with 50 μg/ml of BR in 0.1 M carbonate-buffered saline at pH 8.5. The plates were rinsed in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) prior to use. Competitive assays were carried out by preincubating serial concentrations (10^{-4}–10^{-8} M) of the synthetic peptide antigen with 1 μg/ml of IgG N7-3 or C3-2 in PBS for 2 h at 37°C, followed by adding 50 μl of the mixtures to the BR-coated plates. The plates were incubated for 1 h, washed in PBS containing 0.05% Tween-20 and treated with a secondary anti-mouse goat IgG conjugated with horse radish peroxidase (HRP) After 1 h, the plates were again washed in PBS and then developed with the peroxidase reaction to measure the absorbance at 492 nm. (A) The competition assay of BR-N peptide analogs with the N7-3 antibody (B) The competition assay of BR-C peptide analogs with the C3-2 antibody

3. RESULTS AND DISCUSSION

Monoclonal antibodies against BR have already been prepared by immunizing either intact BR or defined fragments to mice for studying the surface topography of BR [18,19]. The use of these antibodies confirmed the earlier conclusion that the carboxyl terminus is exposed to the cytoplasmic site.

To produce monoclonal antibodies capable of recognizing the respective sides of BR, we chose two peptides as antigens, one of which is 9 residues of (pyr)Glu-1 to Glu-9 (BR-N peptide) as an extracellular portion, the

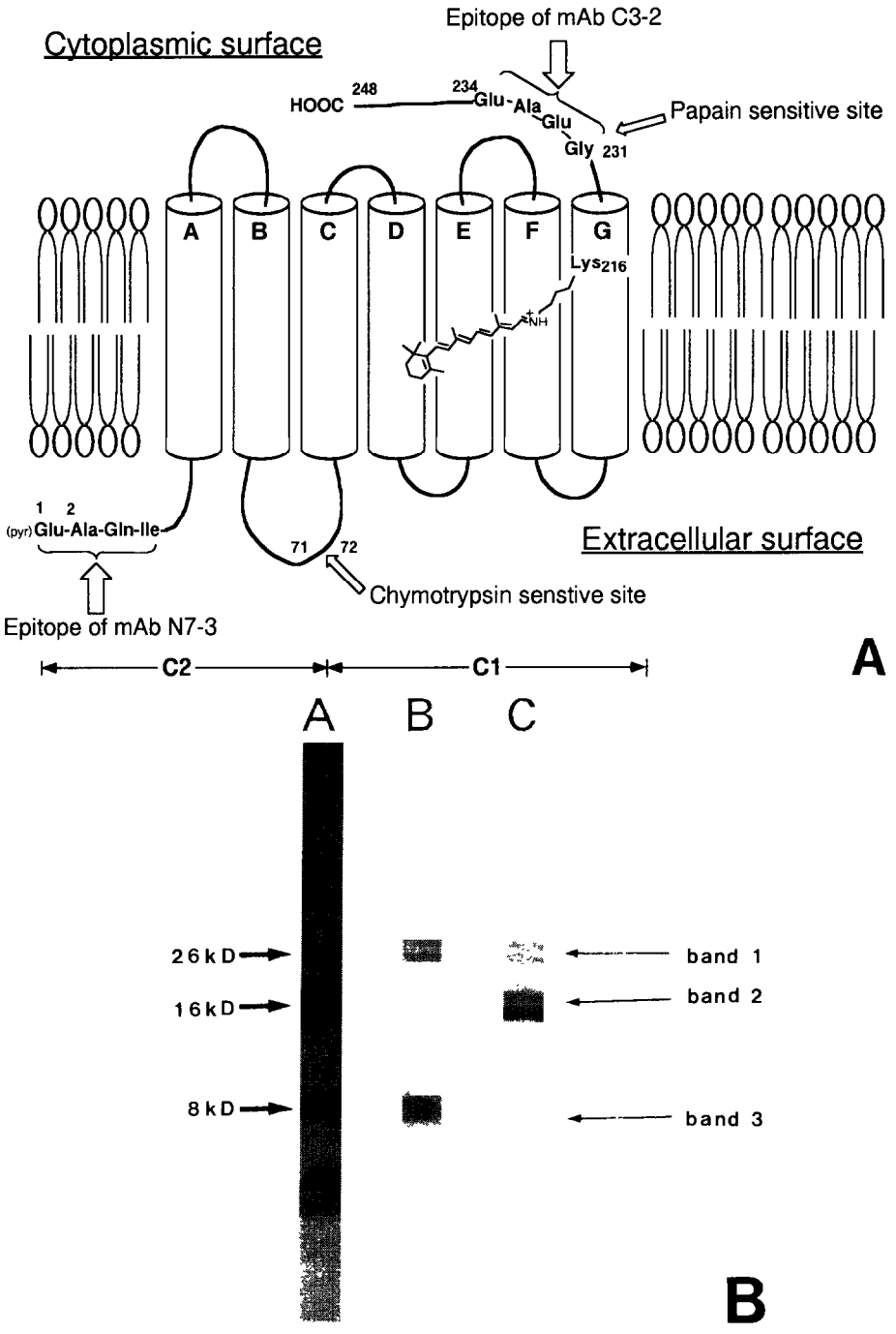
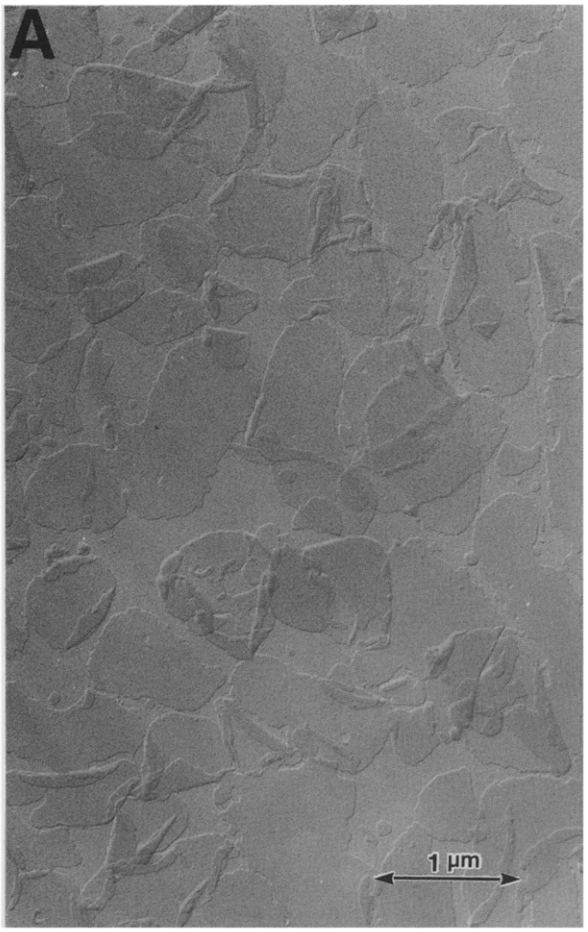


Fig. 2. (A) Secondary structure model for BR. Chymotrypsin cleaves BR into two fragments, C1 (72–248 amino acids) and C2 (1–71 amino acids). Papain removes about 17 amino acids from the COOH terminus of BR. (B) Immunoblot analyses of BR fragments cleaved with chymotrypsin. Products digested by chymotrypsin were subjected to SDS-PAGE (10%) and blotted to a PVDF membrane (Millipore) using a transblot apparatus. The filters were blocked with BSA and incubated with the respective gold-labeled antibody. Lanes: A, SDS-PAGE analysis of BR fragments, Coomassie Blue-stained gel; B, immunoblot from a duplicate gel, stained with the anti-BR-N antibody (N7-3, 5 nm colloidal gold labeled); C, stained with the anti-BR-C antibody (C3-2, 5 nm colloidal gold labeled). Bands 1, 2, and 3 represent unreacted BR, C1, and C2 fragments, respectively.

other 18 residues of Gly-231 to Ser-248 (BR-C peptide) as on the cytoplasmic portion. The two peptides were prepared with a peptide synthesizer and then conjugated with KLH as a carrier protein. After these conjugates were separately immunized in mice, seven stable hybridoma cell lines were established for the BR-N peptide and eight were established for the BR-C peptide through the conventional procedure. We selected the monoclonal antibodies secreted by these cells, N7-3(IgG1) for the BR-N peptide and C3-2(IgG2a) for the BR-C peptide, for further investigations because they have relatively high binding affinity.

In an attempt to determine the epitope in the amino acid sequence, we studied the binding capability of each antibody to several truncated peptide analogs by a competitive inhibition assay. The epitope of N7-3 was found to exist between (pyr)Glu-1 and Ile-4, that of C3-2 lying between Gly-231 and Glu-234 (Fig. 1A,B). Replacement of (pyr)Glu with Glu at the N-terminus did not affect the binding affinity with N7-3. Both epitopes are generally small (3 or 4 amino acid residues) in length, although Lerner et al. [20] suggested that monoclonal antibodies recognize a minimum of 7 residues. However, Hodges et al. recently reported that monoclonal antibodies directed to the cytoplasmic carboxyl terminus of bovine rhodopsin recognized residues confined to small linear epitopes ranging from 4 to 11 residues [21], which is consistent with our results.

The binding specificity of the two antibodies was examined using the immunoblotting technique (Fig. 2). Treatment of BR with chymotrypsin specifically cleaves at Phe-71 to form two fragments, C1 (72–246 amino acids) and C2 (1–71 amino acids) [22], which were located at the 16 kDa and 8 kDa regions, respectively, as determined by Coomassie blue-stained SDS-PAGE analysis (Fig. 2B, lane A). The bands were transferred to a PVDF (polyvinyliden difluoride) membrane and treated with a monoclonal antibody labeled directly with colloidal gold, either C3-2 or N7-3 (Fig. 2B, lanes B and C). The C2 part (containing the N-terminus) was stained with gold labeled N7-3 in lane B, while C1 part (containing the C-terminus) was stained with gold labeled C3-2 in lane C. In addition, BR prepared by papain digestion [23], which lacks approximately 17 amino acids from the COOH terminus, did react with N7-3, but not with C3-2 (data not shown). Hence, it was found that the two monoclonal antibodies had specificity sufficient to evalute the surfaces of the PM.



(For legend see facing page.)

First, we used ELISA to estimate the ratio of the surfaces (C-, or N-terminal side) of a PM monolayer film on a glass substrate prepared by the LB method [17]. Two types of PM-LB films, the so-called Z- and X-type films, were prepared by vertical and horizontal dipping of a glass plate, respectively. On the assumption that the C-terminal side of the PM film on the aqueous surface orients to the aqueous phase by hydrophilic interactions due to the asymmetry of surface charge densities [12], the C-terminus side of a PM-LB film should be exposed to the air on an X-film; on the other hand, the orientation of PM should be opposed on a Z-film. The films deposited on the glass plate were reacted with either N7-3 or C3-2 as a probe, and then incubated with an HRP conjugated goat anti-mouse antibody in order to determine the proportion of the respective surfaces. The ratio of the amounts of the C-terminus in the Z-film to that in the X-type film was 1:1.4 with the use of C3-2, whereas that of the N-terminus was 1:0.8 with N7-3. These results reveal that there is actually only a slight tendency for the the C-terminus to orient to the aqueous subphase. This fact conflicts with the hypothesis that the C-terminal side of PM exclusively orients to the water subphase.

Further, we have attempted to visualize the surface of PM using immuno-gold labeling and electron microscopy to determine the sidedness of the membrane precisely. The antibody–Au conjugate was prepared by mixing the colloidal gold solutions with either N7-3 or C3-2 (Fig. 2B). The two types of LB films of PM were prepared using the procedure described above. Fig. 3A shows the platinum-shadowed PM monolayer film transferred on to the grid by the horizontal attachment method prior to immune-gold staining. Each film on the grid was pre-treated with BSA and incubated with the solution containing the antibody–Au conjugate. After

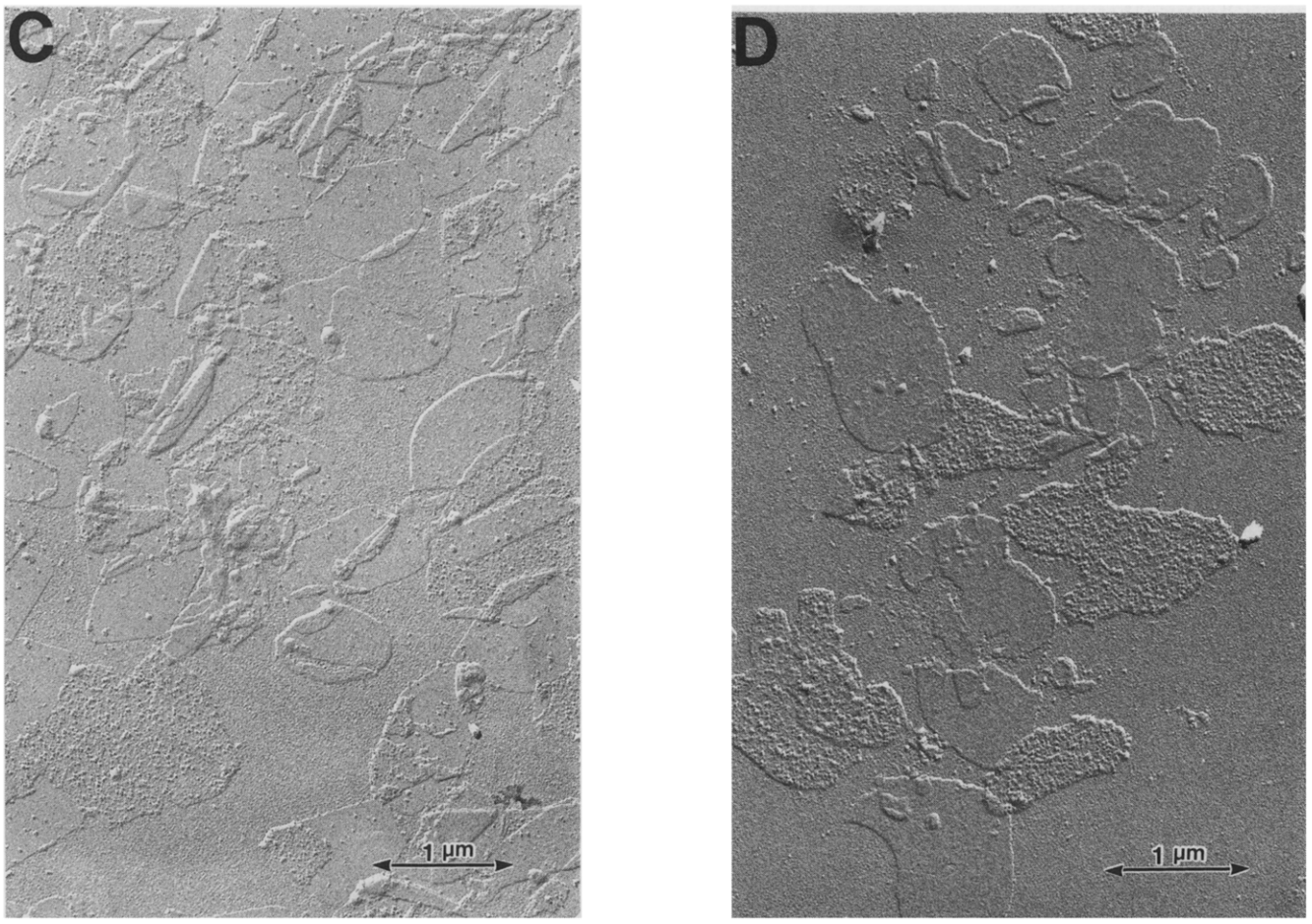


Fig. 3. Electron micrograph of a PM monolayer. PM fragments prepared by a LB method or spontaneous adsorption were deposited on an electron microscope grid and treated twice with BSA solution. It was then transferred to the labeling buffer containing N7-3–Au conjugate for 10 min at 37°C, rinsed in phosphate buffer and in pure water. After shadowing with platinum, it was observed with an electron microscope (A) A monolayer of PM on a grid prepared by the LB method without immuno-gold labeling. Neither the 'cracked' nor 'pitted' areas shown by Fisher et al. appear in the LB film [15]. (B) Immuno-gold labeling with N7-3–Au conjugate of the PM film transferred by horizontal dipping, (C) by vertical dipping and (D) of PM fragments adsorbed spontaneously on a grid in concentrated PM suspension Rough regions, which are covered with gold particles, indicate the extracellular side of PM.

platinum-shadowing treatment, the grid was subjected to electron microscope observation. The N7-3–Au conjugate clearly distinguished the surface morphology of PM where the extracellular surface is exclusively labeled with gold particles, while the C3-2–Au conjugate was somewhat less distinct than the N7-3–Au conjugate under various conditions. This difference is probably because the binding affinity of C3-2 is lower by one order of magnitude than that of N7-3. Fig. 3B and 3C compare the morphologies of the PM surfaces obtained by horizontal and vertical dipping, respectively, after reaction with the N7-3–Au conjugate. In each case, we conclude that the surface orientation of the PM monolayer is substantially random, which is in agreement with the results in ELISA.

As a reference, a PM film which underwent no specific treatment for orientation was prepared by allowing a grid for spontaneous adsorption of PM from a highly concentrated PM suspension. As shown in Fig. 3D, PM fragments were adsorbed on the grid leaving much vacancy because they were not compressed on the aqueous surface. Apparently, no orientation is recognized. This fact confirms that the LB film of PM fragments is randomly in orientation. These results ensure that the orientation of PM can hardly be controlled at hydrophobic/hydrophilic interfaces such as an air/water interface with the successful assistance of a difference in the surface charge densities.

In conclusion, the result presented in this study demonstrate that immuno-gold labeling techniques provide a very useful and direct means of detecting the sidedness of biological membrane proteins such as BR, and that controlling the orientation of PM fragments at the air/water interface is essentially difficult, so long as a neutral water is employed as an aqueous phase. Nevertheless, it is of interest that the LB film prepared by spreading PM at the air/water interface results in a photoelectric response which is highly rectified [10,24–26]. Mechanistic studies of photocurrents arising from randomly oriented PM fragments are currently being investigated.

Acknowledgements We thank Prof F. Tokunaga (Osaka Univ.), Dr. I. Itoh, and Dr. T. Miyasaka for helpful discussions and Dr. H Togashi for instruction of a method of producing monoclonal antibodies.

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