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Melittin-Regenerated Purple Membrane

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Abstract—We have investigated the character of melittin-regenerated purple membrane. Adding melittin to blue membrane causes the color transition and partial regeneration of the photocycle and the proton pump. The reconstitution of bacteriorhodopsin by melittin is proved to be charge-dependent. In studying the location of melittin binding on the blue membrane, we suggest that melittin anchors on the membrane through both hydrophobic and electrostatic interactions. The electrostatic interaction is dominant. The binding sites for the electrostatic interaction should be on the surface of the membrane.

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Key words: bacteriorhodopsin, blue membrane, melittin, electrostatic interaction

Bacteriorhodopsin (bR) is the only protein in the purple membrane of *Halobacterium salinarum*; it contains a retinal molecule attached via a Schiff base [1]. Upon illumination, the protein undergoes a photocycle and pumps protons across the cell membrane [2, 3].

It has been found that well-washed native purple membrane (PM) contains $Ca^{2+}/Mg^{2+}/bR \sim 1 : 4 : 1$ [4]. The removal of these cations by deionization or acidification causes a color transition from purple ($\lambda_{max} = 568$ nm) to blue ($\lambda_{max} = 603$ nm). Blue membrane (BM) does not form the M_{412} intermediate and thus does not have proton pump function [4].

The spectroscopic property, photochemical activity, and proton pump of bR can be reconstituted by adding different metal cations to the deionized blue membrane suspensions [5-7]. This illustrates the importance of metal cations in the function of bR.

Then the question arises, which is the key factor for regeneration of PM from BM—the charge or the cation specific binding? Various studies [8-10] have shown some organic cations, such as Et_3N^+-$(CH_2)_4$-N^+Et_3 and Bu_4N^+, can regenerate PM from BM as well, and the bR regains not only purple color, but also the photocycle. It seems the ions that are able to regenerate PM from BM are all positive-charged whether metal cations or organic cations.

Thus, we investigated the reconstitution of bR by melittin (MLT). MLT is the main component of bee venom. It is a small linear polypeptide of 26 residues containing six positive charges [11]. Its sequence is: NH_3^+-GIGAVLK$^+$VLTTGLPALISWIK$^+$R$^+$K$^+$R$^+$QQ-CONH$_2$.

The amino-terminal region (residues 1-20) is predominantly hydrophobic, whereas the carboxy-terminal region (residues 21-26) is hydrophilic due to the presence of a stretch of positive-charged amino acids. This amphiphilic property makes MLT water-soluble, and yet it spontaneously associates with natural and artificial membranes.

Such an amphiphilic sequence of amino acids is characteristic of many membrane-bound peptides [12]. As a result, MLT is used as a convenient model for monitoring lipid–protein interactions in membranes [13-15]. Many previous studies have described the orientation of membrane-bound melittin without consensus [15, 16]. Most of these studies are concerning artificial membranes [17-25]. But there are a few studies on the interaction between MLT and cell membranes. The nature of protein–protein interactions between MLT and other proteins in cell membranes is poorly understood.

Our previous result, that the bacteriorhodopsin immobilization phenomenon depends on the melittin/protein ratio but not the melittin/lipid ratio, has focused on the region of protein–protein interaction between MLT and bR in the purple membrane, which is a kind of natural membrane, and shown that there is direct electrostatic interaction between MLT and bR by measuring the rotational diffusion [26].

Abbreviations: BM, blue membrane; bR, bacteriorhodopsin; MLT, melittin; NBS, N-bromosuccinimide; PM, purple membrane.

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The effect of melittin on purple membrane was also studied: the proton release and uptake rates are significantly decreased and the yields and rate of the slow-decaying component of M_{412} are also decreased, but the rate of the fast-decaying component of M_{412} has only slight changes [27]. In addition, melittin mutants with different positive charge also delay the slow-decaying component of M_{412} [28]. But there was no previous study on the effect of melittin on blue membrane. To cover this unexplored issue, we performed further studies here.

In this paper, we investigated how melittin affects the deionized BM. We found that adding melittin causes color transition of bR from blue to purple. The function of bR, including the photocycle and the proton pump, are partially regenerated. The MLT binds on the membrane via both hydrophobic and electrostatic interactions. The positive-charge-rich C-terminal of MLT binds on the surface of the membrane, and the hydrophobic N-terminal inserts shallowly into the membrane.

MATERIALS AND METHODS

Materials. Purple membrane was prepared from the *H. salinarum* R_1M_1 as described by Oesterhelt et al. and Xu et al. [29, 30]. The deionized membrane (BM) was prepared by passing the PM suspension through a well-washed cation-exchange Dowex AG-50W column. The prepared BM was always kept in polystyrene vessels in order to be free from metal ions. The bR concentration in the PM was determined by the absorption at 568 nm with a molar extinction coefficient $\varepsilon = 63{,}000\ M^{-1}\cdot cm^{-1}$ for its light-adapted state [31] and in the blue membrane at 603 nm with $\varepsilon = 55{,}000\ M^{-1}\cdot cm^{-1}$.

Melittin was purchased from Sigma (USA). To study the melittin-reconstituted purple membrane, it is prerequisite to avoid contamination of other cations. Melittin was purified on a C_{18} column by high-performance liquid chromatography, and then MLT was pooled, lyophilized, and dissolved in deionized water.

Site-specific mutants were a kind gift of Prof. Cherry (University of Essex, UK). They were stored as a lyophilized powder and were dissolved in double-distilled water before experiments and then directly added to the experimental samples.

N-Bromosuccinimide (NBS) was purchased from Sigma. No further purification was carried out. NBS was dissolved in water every time just before an experiment.

Pyranine, which was used as an excellent pH probe [32-34] for detection of proton pump function, was purchased from Sigma.

Methods. Bacteriorhodopsin was light adapted before experiments. Room temperature was used in all experiments. (We confirmed experimentally that in the course of MLT reconstituting bR, there is no observable difference at different temperatures.)

Absorption spectra were measured on a Hitachi U-3200 UV/Visible spectrometer.

The flash photolysis kinetic spectra were recorded on our homemade instruments. The sample was excited with a flash of about 40 μsec filtered to 500-600 nm. The continuous probe light was created with an 18 V/8 A lamp, and went through the sample cell in the vertical direction with two monochromators respectively before and after the sample cell. The probe wavelength of 412 nm was applied for M_{412}, 640 nm for O_{640}, and 444 nm for proton pump function. The final curve was the average of 100 flash curves.

Steady state fluorescence spectra were measured on a Hitachi F4500 spectrometer, the excitation wavelength being set at 295 nm.

RESULTS

Color transition of reconstituted bR. Figure 1 shows the absorption changes from 602 to 569 nm by adding MLT into the BM. Thus, we first of all confirmed that MLT can reconstitute BM to PM. When the molar ratio of MLT to bR reaches about 2 : 1, the absorption peak completes the movement from 602 to 569 nm. Adding more MLT causes neither further blue shift of the absorption nor absorption increase to the same level of the absorption of Mg^{2+} regenerated PM. That means this protein molecule with positive charges, like metal cations and some organic cations, can also reconstitute BM to PM, though there is difference between MLT reconstituted PM and native PM.

The MLT mutants with different positive charges were employed to change bR color. Figure 2 shows the

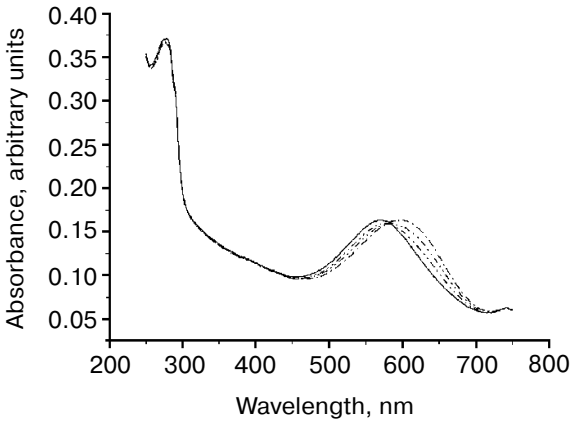

Fig. 1. Absorption spectra of the blue-to-purple conversion while adding MLT to BM with fully regenerated PM by Mg^{2+} as a control. Designations: BM (– · –); MLT reconstituted PM at molar MLT/bR ratio of 0.33 : 1 (– ·· –), 1 : 1 (– ··· –), and 2 : 1 (——). [bR] = 3 μM. The pH of the BM suspension is about 5.0 and the pH of the MLT reconstituted bR solution is about 4.5.

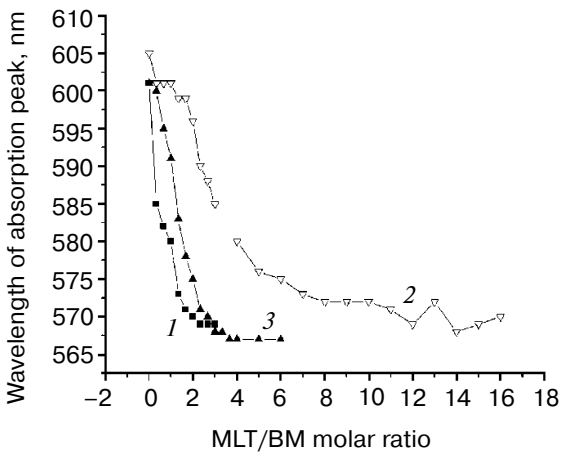

Fig. 2. Dependence of bR absorption peak wavelength on the molar MLT/BM ratio for three kinds of MLT: native MLT (*1*), MLT mutant that lacks four positive charges on its C-terminal (*2*), and MLT mutant that lacks one positive charge on its N-terminus (*3*). [bR] = 3 μM.

absorption peak of bR reconstituted by different MLTs with different positive charge. Besides native MLT, the two MLT mutants are one lacking four positive charges on the C-terminal (sequence: NH_3^+-GIGAVLK$^+$VLTT-GLPALISWIQQQQQQ-$CONH_2$) and the other lacking one positive charge on the N-terminus (sequence: NH_3^+-EIGAVLK$^+$VLTTGLPALISWIK$^+$R$^+$K$^+$R$^+$QQ-$CONH_2$). They can both restore the purple color of bR like the native MLT but have different efficiencies (shown in Fig. 2). The native MLT is the most efficient due to its large number of positive charges, while the one with four positive charges missing on the C-terminal is the least efficient. The different efficiencies confirmed the charge dependence of the restoration of purple bR color. Furthermore, the different efficiencies suggest different contribution to the reconstitution of the MLT charges: as Fig. 2 shows, to move the absorption peaks to 570 nm, 2 molecules of MLT or 7/3 molecules of the MLT mutant lacking one positive charge on N-terminus or 12 molecules of the MLT mutant lacking four positive charges on C-terminal is needed per molecule bR. This means the charge on the N-terminus works as 1/7 MLT, the charge on Lys7 works as 1/42 MLT, the four charges on C-terminal work as 5/6 MLT overall. The contribution of the charge in the middle of the molecule is negligible, and the charges on two terminals are critical.

Regeneration of function of reconstituted bR. Before description of the function of MLT reconstituted bR, it should be established whether the bR reconstituted by MLT is homogeneous or not. In Fig. 1, the isosbestic point was found at 585 nm. The existence of an isosbestic point suggests that there are only two retinal configuration components [35], and in other words, only one conformation of bR is created apart from the conformation of bR in BM. Thus, only one kind of function of MLT reconstituted bR should come into later discussion.

We used the flash kinetic spectra to study the M_{412} and O_{640} intermediates and the proton pump function of MLT-reconstituted bR. For comparison, experimental studies under the same conditions were performed for the native bR.

In the native PM, bR should release H^+ to the solution first and then take up another H^+ during the photocycle. Pyranine was used to reflect the change of pH in the solution. The absorption of pyranine at 444 nm should decrease when the solution becomes more acidic. We compared the absorption change of the native bR with and without pyranine; the difference between these two conditions should be attributed to the pH-introduced absorption change of pyranine.

The results are shown in Figs. 3-5. The lifetimes of the MLT-reconstituted bR and the native bR and the ratios of the yields between them are shown in the table. The decay rates of M_{412} and O_{640} are reduced comparing with the native bR. Their yields cannot be restored to the same level of those of the native PM by adding more MLT to the system. The difference between the rates of their yields, which is about 8%, should be attributed to the residue of native bR in the BM, which is about 8% (shown in Fig. 4) too. The proton pump function is also regenerated: the decay rate is expected to be reduced, but the yield is low. Nevertheless, we are still able to conclude that the reconstitution of MLT to BM can regenerate part of the function of bR.

Study of location of MLT binding in BM. To characterize the location of MLT in MLT-reconstituted bR, the fluorescence of the MLT–BM system was monitored. A tryptophan residue can reveal whether it is in a hydrophilic or hydrophobic environment: when its fluorescence peak is about 350 nm, the environment is

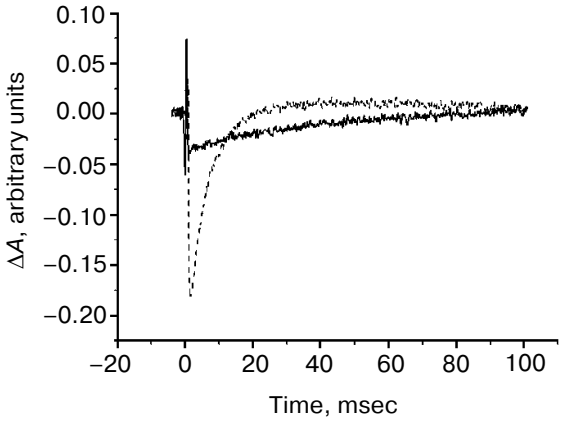

Fig. 3. Proton pump signal of native bR (dashed line) and MLT-reconstituted bR (solid line). The lines have been smoothed (20 points for average). [bR] = 8 μM; [pyranine] = 400 μM. pH values of native bR solution and MLT reconstituted bR solution are 6.0.

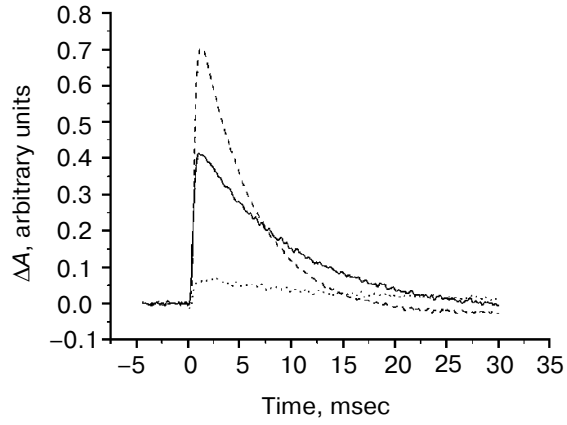

Fig. 4. Regeneration of the M intermediate of MLT-reconstituted bR. Designations: native PM (dashed line); BM (dotted line); MLT reconstituted PM at MLT/bR molar ratio of 2 : 1 (solid line). The lines have been smoothed (20 points for average). [bR] = 3 μM.

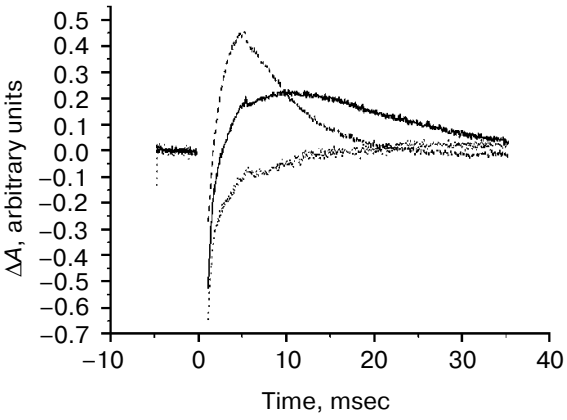

Fig. 5. Regeneration of the O intermediate of MLT-reconstituted bR. Designations: native PM (dashed line); BM (dotted line); MLT-reconstituted PM at MLT/bR molar ratio of 2 : 1 (solid line). The lines have been smoothed (five points for average). [bR] = 15 μM.

hydrophilic; when the peak blue-shifts, the environment has become hydrophobic.

Since several Trp residues in bR are also fluorescent [36], we must separate the fluorescence contribution of MLT from that of bR. We quench the fluorescence of MLT thoroughly by adding an exactly equal amount of NBS [37, 38], and ensured the quenched MLT can reconstitute bR (data not shown). Then we got the fluorescence change contributed only from bR in the quenched MLT-reconstituted bR. The oxidation by NBS causes the quenching of Trp19 in MLT to be more hydrophilic, but it has nothing to do with the bR fluorescence during the reconstitution. We assumed that the fluorescence contribution from bR is exactly the same for the MLT-reconstituted bR and the quenched MLT-reconstituted bR. Subtracting it from the total fluorescence change (actually, comparing with the fluorescence change caused by adding MLT, the fluorescence change of bR is negligible), we got the fluorescence change contributed only by MLT in the MLT-reconstituted bR (shown in Fig. 6). The peak of the contribution of MLT in the MLT-reconstituted PM is about 350-360 nm, which means the Trp19, the single intrinsically fluorescent residue of MLT [39], is in a hydrophilic region after the reconstitution of bR by MLT [40-42].

We also selected another MLT mutant, which has Trp1 instead of Trp19 (sequence: NH_3^+-WIGAVLK$^+$VL-TTGLPALISLIK$^+$R$^+$K$^+$R$^+$QQ-CONH$_2$) to undergo the analysis described above. The peak of the fluorescence change contributed only by the MLT mutant is obviously

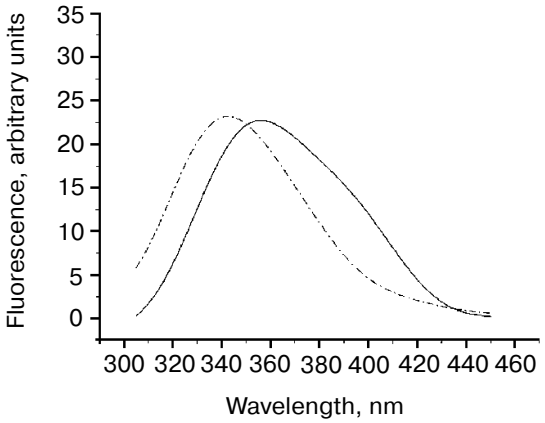

Fig. 6. Fluorescence of native MLT (dash-dot line) and fluorescence change contributed by only MLT in MLT-reconstituted bR (solid line). The excitation wavelength was 295 nm. The lines have been smoothed (20 points for average). [bR] = 3 μM.

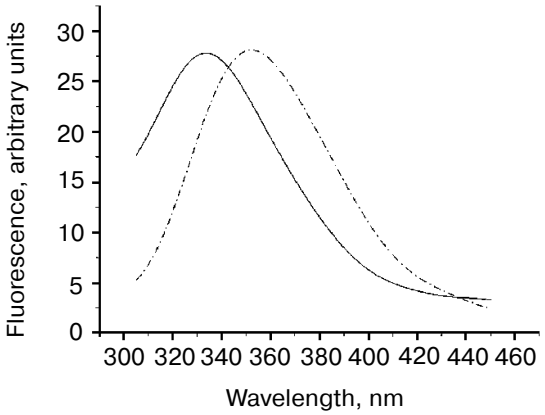

Fig. 7. Fluorescence of the Trp1 MLT mutant (dash-dot line) and the fluorescence change contributed by only this MLT mutant in MLT-reconstituted bR (solid line). The excitation wavelength was 295 nm. The lines have been smoothed (20 points for average). [bR] = 3 μM.

Lifetimes of the MLT-reconstituted bR and the native bR and their yield ratio

Object	Lifetime, msec		Yield of MLT-reconstituted bR/yield of native bR, %
	MLT-reconstituted bR	native bR	
M_{412}	9.55 ± 0.01	5.29 ± 0.004	58.44
O_{640}	66.04 ± 3.28	6.28 ± 0.02	50.99
Proton pump	43.86 ± 0.37	5.92 ± 0.01	20.90

blue-shifted (shown in Fig. 7), which means the Trp1 occupies a hydrophobic region in the MLT-reconstituted bR.

DISCUSSION

First of all, we confirmed that there is an interaction between MLT and the blue membrane. MLT can reconstitute the BM to the PM. The reconstitution is charge-dependent. As shown in Fig. 2, the native MLT, with maximal positive charges, is the most efficient, while the MLT mutant with four positive charges missing on the C-terminal, with the minimal positive charge, is the least efficient. Previous studies [43-45] have shown that to convert BM to PM about 2 Tb^{3+}/bR, 5-6 Mg^{2+}/bR, or >1000 Na^{+}/bR is needed (molar ratios are given). These results suggest that the manners of interaction are various between different kinds of ions and BM. Despite this, each kind of positively charged ion (except Hg^{2+} and Pt^{4+} [5, 46], as far as we observed), including MLT and its mutants, causes a charge-dependent interaction in the BM-to-PM reconstitution. This is a confirmation that electrostatic interaction induces the reconstitution from BM to PM by MLT.

The results show the function of bR, the photocycle and the proton pump, is also able to be regenerated with the electrostatic interaction between MLT and the membrane. We suggest that the positive charges of MLT change the surface potential, thus influence the apparent pK_a of Asp85, which is consist with previous studies [47-52]. Though the function is regenerated, the regenerated function is not the same as that of the native bR: the yields and the decay rates of the photocycle and the proton pump are reduced. We suggest that the incomplete regeneration yields should be attributed to the difference between MLT and other cations: considering the bulk and configuration of MLT, the positive charges of MLT might be difficult to bind on the membrane surface as well as the other small positively charged ions. Consequently, the surface pH of MLT regenerated PM is not as high as those of PM regenerated by other cations. It should be closer to or even lower than the pK_a (5.8) of the group releasing a proton in the M intermediate [53]. This low value of the surface pH results in inhibition of rapid H^+ release [54]. Consequently the relaxation rate of the M intermediate and the accumulation and relaxation rates of the O intermediate are slowed. At low pH values [55, 56], the order of proton release and uptake can be reversed. Slow accumulation and relaxation of O intermediate could be due to slowing of H^+ uptake in accord with the slower relaxation of the M intermediate and the slow phase of H^+ uptake registered by the pyranine signal. Furthermore, there is hydrophobic interaction between MLT and the membrane, which is likely to inhibit the proton pump as a previous study suggested [44]. In contrast to the alkali and alkaline-earth metal cations that can thoroughly regenerate the PM (data not given), lanthanide cations, such as Tb^{3+} [45], La^{3+} [4], Eu^{3+} [5, 57] reconstituted bR had a long lifetime M intermediate. These studies gave a possible interpretation that lanthanide cation binding inhibits the photocycle, and the inhibition caused by the lanthanide cation binding is due to the change in the spacing of the lipid head groups or the mobility of phospholipid molecules. Similarly, previous studies [57, 58] showed that the removal of 75% of the lipid from bacteriorhodopsin decreases the efficiency and rate of deprotonation of the protonated Schiff base, and the same phenomenon was observed by adding Triton X-100, suggesting the importance of the lipid environment in PM. So it is likely that MLT acts similarly to lanthanide cations to inhibit the photocycle, and the MLT binding involves the membrane lipids too.

There is hydrophobic interaction besides the electrostatic interaction in the system of constitution BM to PM by MLT. From the fluorescence spectrum of the Trp19 in native MLT and Trp1 in this kind of MLT mutant, we conclude that the N-terminal of MLT inserts into the hydrophobic region of the membrane, but at least from Trp19 the positive-charge-rich C-terminal still remains in the hydrophilic region. The previous model of the interaction between MLT and native bR [59] suggested the N-terminal of MLT is in the hydrophilic region, binding on the surface of the membrane. This characterization modifies this model in the point that the N-terminal of MLT inserts into the hydrophobic region of the membrane.

In contrast to a previous study [60] in which the author claimed that the hydrophobic terminal of MLT would span the bilayer, an EPR result (not presented) suggests that MLT is not inserted deep into the membrane in this MLT–BM system. The order parameter (S) of 16-doxyl acid labeled PM, BM, and MLT-reconstituted PM is 0.22071, 0.32708, and 0.16044, respectively. The increase in S after membrane deionization should be attributed to the influence of conformation change of bR, which makes the lipid molecules much less mobile. The fact that largest order parameter is of blue membrane

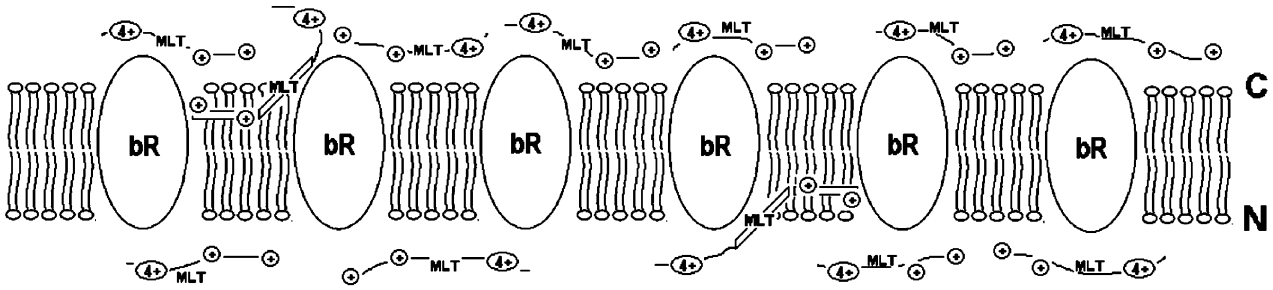

Fig. 8. Sketch showing proposed nature of binding of MLT on the membrane.

consists with a previous study [61] that indicated the tilted helices enlarge the trimer in the blue membrane. It is reasonable to accept that the lipids are tightly packed by the enlarged trimers. But the conformational change theory cannot explain why the *S* of MLT reconstituted PM is less than that of native PM if we suppose MLT reaches the depth characteristic of 16-doxyl acid and crowds the surrounding of the hydrophobic terminal of the lipid molecule. So we prefer the interpretation that the N-terminal of MLT is not inserted into the membrane deep enough to affect the hydrophobic terminal of the lipid molecule. This characterization confirms a previous study [28].

We have known the rough location of MLT on BM: the N-terminal of MLT inserts shallowly into the membrane, leaving the positive-charge-rich C-terminal on the membrane surface. We describe the probable location of MLT binding on the membrane (Fig. 8) [60].

The MLT mutant missing one positive charge on the N-terminus can induce the reconstitution when the remaining active charges (four charges on C-terminal) are not inserted into the membrane. As the N-terminal of this MLT mutant without the charge has only hydrophobic interaction on the membrane, the reconstitution should be performed by the four charges on the C-terminal (we have deduced above that the contribution of Lys7, the charge in the middle of the molecule, is negligible). The location of the MLT should be on the hydrophilic surface of the membrane. This conclusion does not conflict with the fact that the MLT mutant missing the four positive charges on C-terminal can also reconstitute bR. In this case, the MLT mutant can bind with BM not through hydrophobic interaction but through electrostatic interaction with the surface, and reconstitute bR from BM to PM. This means that the percent of MLT inserted in the membrane is not a constant. Between the hydrophobic interaction and the electrostatic interaction, the latter is dominant.

We can give some detailed description about the probable location of MLT binding on the membrane (Fig. 8). We have shown in Fig. 2 that the reconstitution of bR required 2 molecules of native MLT or 7/3 molecules of the MLT mutant lacking one positive charge on the N-terminus per bR molecule. If we assume that 100% of this MLT mutant inserts its charge-lacking N-terminal into the membrane, then we get 28/3 positive charges per molecule of bR needed for reconstitution. As we have discussed above, the electrostatic interaction between MLT and bR is dominant, so we conclude that 1 molecule of MLT insert its N-terminal in the membrane and 5 molecules of MLT bind on the surface of the membrane for per bR trimer. The N-terminals inserted into the membrane form α-helices and the residues in water forms random coils. A study [62] estimated the asymmetric charge distribution on the two sides of the blue membrane: the ratio of the negative charges between the cytoplasmic side and the extracellular side of the blue membrane is about 1.4-1.7 at pH 4-5. Then we can roughly evaluate that there are about 3.5-3.8 molecules MLT binding on the cytoplasmic side of the membrane and 2.2-2.5 molecules of MLT binding on the extracellular side of the membrane.

In summary, the following conclusions of the reconstitution of MLT to BM emerge from the present results and those of the literature. (i) MLT-reconstituted bR completes the color transition from blue to purple. The reconstitution is charge-dependent. (ii) The function of MLT-reconstituted bR, including the photocycle and the proton pump, are partially regenerated. (iii) Some N-terminal of MLT inserts shallowly into the membrane with hydrophobic interaction and the positive-charge-rich C-terminal is left on the membrane surface binding with electrostatic interaction. Other MLT molecules bind on the membrane surface with electrostatic interaction. The electrostatic interaction is dominant.

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