Role of Salt in Reconstituting Photocycle Behavior in Triton-Damaged Purple Membranes by Addition of Native Lipids

Salil Bose, Anup K. Mukhopadyay, Swetlana Dracheva, and Richard W. Hendler*

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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We demonstrated recently that damage to the normal bacteriorhodopsin (BR) photocycle caused by brief exposure to dilute Triton X-100, which removes lipids from the membrane, can be repaired by adding back an extract of purple membrane (PM) lipids (Dracheva; et al. *FEBS Lett.* **1996**, *386*, 209). It was shown that reconstitution with lipids required the presence of a high concentration of NaCl (∼0.5 M for half-maximal reconstitution). This paper shows that reconstitution can be achieved with divalent and trivalent cations at much lower concentrations than were required for NaCl (i.e. ∼7.5 mM for Ca^{2+} and ∼0.15 mM for La^{3+} for half-maximal reconstitution), indicating that negative surface charges must be neutralized to allow the added lipids to reach and be incorporated at the proper locus. We also report here that reconstitution in the absence of added salt can be accomplished by protons alone with an apparent p*K* near 5. The role of salt involves both charge-screening and specific binding. It was found that, contrary to the prediction of the Gouy−Chapmann equation, the pK_{app} for reconstitution rises with increasing salt concentration.

Introduction

The purple membrane (PM) of *Halobacterium halobum is* an excellent system for studying the role of surface charge in several aspects of bacteriorhodopsin (BR) behavior and function such as the kinetics of the photocycle, proton-pumping, and the blue-to-purple transition. The electric double-layer theory of charged surfaces, which is widely used in membrane research,[1−3] has been used to determine the surface charge density of PM.[4−8] From studies of pH-dependence and cation effects on the blue-to-purple transition, Stoeckenius and co-workers concluded that this phenomenon is controlled by a cation-mediated charge-screening according to Gouy−Chapman theory.[9,10] Balasov et al. reported a red shift of the PM absorption band at high pH and interpreted the effect as due to change in the surface potential mediated through charge-screening.[11] In addition to charge-screening, it is known that cations bind to specific sites of BR and lipids (refs 12−14 and references therein). Specific cation-binding has been implicated in a direct way to influence several properties of the purple membrane (PM) such as photochemistry and color.[15−17] From our studies on the reconstitution of normal BR structure and photocycle behavior in Triton-treated PM by the addition of PM lipids, we find support for both charge-screening and a direct binding role for cations.

We demonstrated recently that lipids of the PM are important in the kinetics, relaxation pathways, and control by actinic light of the BR photocycle.[18−20] Damages to the normal BR photocycle caused by brief exposure to dilute Triton X-100, which removes lipids from the membrane, can be repaired by adding back an extract of PM lipids.[18,19] For the successful reconstitution of normal photocycle activity by addition of lipids, it is essential that a high concentration of NaCl (>2 M) be present.[19] The purpose of the present paper was to explore the high salt requirement. Based on our findings that the requirement for NaCl can be satisfied by a much lower concentration of $CaCl_2$ and an even lower concentration of $LaCl_3$, we conclude that charge-screening of negative groups at the surface of the PM is essential for bringing the added PM lipids to the proper locus for reconstitution. We also report that successful reconstitution at very low levels of salt can be accomplished by titrating a group with an apparent p*K* ∼5. This finding, the fact that divalent Ca is more effective than divalent Mg in affecting reconstitution, and other observations discussed later suggest that other factors in addition to charge-screening are important for the activity of salt in the reconstitution procedure.

Experimental Procedures

Most of the experimental procedures used in this work have been described previously:[19] the isolation of PM, the treatment of PM with 0.1% Triton X-100, isolation of lipids from PM, and the repair of damaged BR photocycle activity by reconstituting Triton-treated PM with isolated PM lipids in the presence of salt. However, the phosphate buffer in the reconstitution medium was replaced by TRIS-acetate buffer to avoid formation and precipitation of Ca^{2+} and Mg^{2+} phosphates. The ultrafast multichannel spectrometer and kinetic analyses based on singular value decomposition (SVD) have also been previously described (see ref 19 for specific references). An important aspect of these studies was the definition and use of 13 quantitative parameters to measure the extent of damage to the native BR photocycle caused by exposure to Triton and the extent of recovery achieved by reconstitution under different conditions. Seven of these parameters are used in the present study for purposes of fitting experimental data to Gouy−Chapman and Henderson−Hasselbalch equations.

Figure 1 is provided to illustrate the nature of the Triton-induced damage to the photocycle and how the seven parameters measure the extent of damage and repair. The figure shows difference spectra for each kinetic transition involving the decay of the M intermediate (A_{max} at 412 nm) in the BR photocycle, resolved by the SVD analysis of multichannel data for the native, Triton-treated, and reconstituted membrane cases. The native membrane (panels A, B, and C) shows essentially two transitions with time constants (τ) of ∼2 and ∼6 ms. The faster is called M_f and the slower M_s. True M_f, seen in the native membrane, is characterized both by its τ = ∼2 ms and the fact that the

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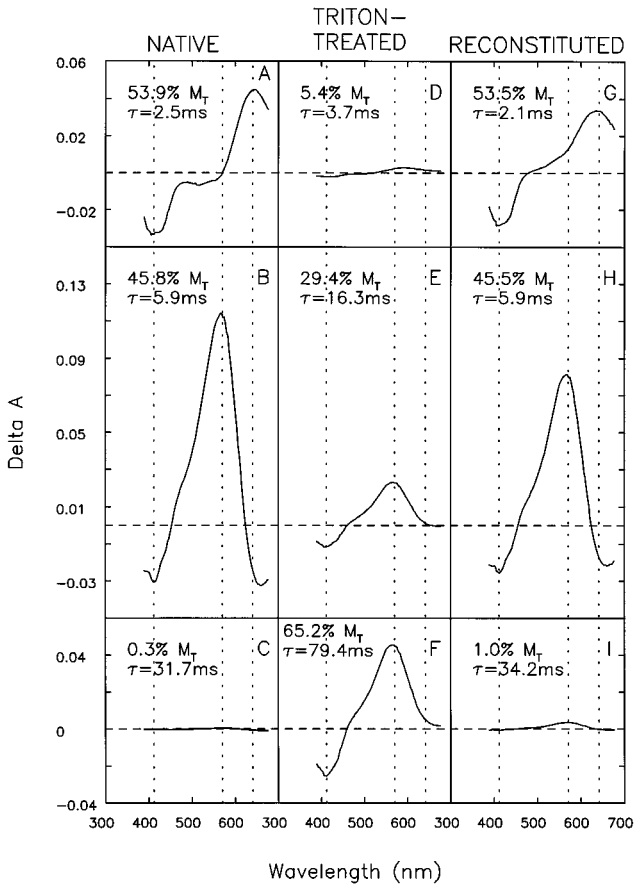

Figure 1. Kinetic transitions during decay of the M intermediates in the BR photocycle. SVD-deduced difference spectra for native (left-hand panels), Triton-treated (center panels), and reconstituted (right-hand panels) preparations of PM are shown. Each panel shows the percent mole fraction of fast M (M_f) to total M and the time constant (τ). See text for further details.

loss of absorbance at 412 nm in the difference spectrum is compensated by a gain in absorbance at 641 nm rather than at 570 nm. The A_{641} is characteristic of the O intermediate, while the A_{570} is characteristic of the ground state, BR. M_s, on the other hand, shows a decay of M compensated by a rise in BR (A_{570}). Under the conditions used for these experiments, M_f is present at a higher mole fraction than M_s. In the Triton-treated case (panels D, E, and F), M_f disappears, and two slower forms of M_s arise. Upon reconstitution of the Triton-treated membranes with an extract of PM lipids in the presence of high salt (panels G, H, and I), the native characteristics are restored. One of the quantitative parameters, average τ, quantifies the loss of true M_f and the appearance of slower forms of M intermediate. Average τ is the weighted average obtained by summing the product of the mole fraction of each form of M and its τ. Average τ is ~4.5 for native PM and about 60 for Triton-treated PM. The parameter fraction M_f is the mole fraction of M_f, which is ~0.5 in native and ~0.07 in Triton-treated PM. The position of A_{max} moves from ~641 nm in native (Figure 1A) to about 587 in Triton-treated (Figure 1D) PM. The presence of true M_f is also quantified by the amount of formation of O (A_{641}). One measure is the ratio of the increase at A_{641} to the total increase at A_{64l} plus A_{570}, which arise during the disappearance of the fastest form of M present. This parameter $\%A_{641}$ varies from ~80% in native to ~45% in Triton-treated PM. Another measure is the maximum formation of A_{64l} related to the total amount of M (A_{412}) that disappears ($\Delta O_M/\Delta M_T$). This parameter is ~0.08 in native and ~0.36 in Triton-treated PM. An additional measure of the presence of the native $M_f \rightarrow O$ decay pathway depends on the transience of the O intermediate which forms rapidly with the decay of M_f and then decays back to the ground state. This is quantified by the ratio of the peak absorbance at 641 nm, which occurs at ~6 ms, to the final absorbance at ~230 ms. This parameter ($\Delta O_M/\Delta O_T$) varies from ~1.2 in native to ~0.4 in Triton-treated PM. A final parameter used in these studies is the position of A_{max} in the light-adapted, resting membrane, which is at ~570 in native and ~562 nm in Triton-treated PM.

Another, very important, consequence of Triton-induced damage to PM is the loss of regulation by actinic light of the proportion of BR which cycles through the $M_f \rightarrow O$ pathway in relation to that which goes through M_s and bypasses the O intermediate. This is quantified by comparing the ratio of mole fraction for true M_f obtained with a low-energy activating laser pulse (2.7 pcp units) compared to a high-energy laser pulse (80 pcp units).[19] In native and reconstituted preparations the ratio is ~1.5. In Triton-treated preparations, the measure does not exist because the $M \rightarrow O$ pathway is lost and actinic light has little effect in altering mole fractions of the slower forms of M present.

TABLE 1: Regulation of Mole Fraction of M_f by Actinic Light in Native and Reconstituted PM

	mole fraction of M_f		ratio M_f
	high intensity[a]	low intensity	low *I*/high *I*
native PM (7)	0.48 ± 0.02	0.75 ± 0.03	1.56
Triton-treated (19)	*b*	0.05 ± 0.003[c]	
reconstituted by			
Na^{1+} (19)	0.51 ± 0.01	0.71 ± 0.01	1.40
Ca^{2+} (5)	0.52 ± 0.07	0.71 ± 0.07	1.37
La^{3+} (5)	0.43 ± 0.02	0.61 ± 0.02	1.44
H^{1+} (12)	0.49 ± 0.01	0.70 ± 0.01	1.43

[a] Low-intensity laser flash at 532 nm (2.7 pcp units) (Hendler et al.,1994). High-intensity laser flash at 532 nm (80 pcp units). [b] Signal too small to measure. The fastest species of M present, which is not true M_f. The numbers in parentheses represent the number of replicate experiments, and those in columns 2 and 3 show the averages ± standard error of the means.

Results

A summary of results is presented in Table 1 and Figure 2. The table shows the loss and recovery of the ability of actinic light to influence the mole fraction of M_f. In native PM, the mole fraction of true M_f (defined above) is close to 0.5 at pH 7.2 when the actinic laser flash is of high intensity (Figure 1) and about 0.8 when a low intensity laser flash is used to activate the photocycle.[20] Brief exposure of PM to 0.1% Triton X-100 destroys true M_f and removes the ability of actinic light to regulate the photocycle. Table 1 shows the recovery of the actinic light regulation by reconstitutions in the presence of NaCl, $CaCl_2$, $LaCl_3$, or H^+.

Figure 2 shows the cation concentration dependence of the percent repair of Triton-induced damage to the BR photocycle, as measured by the seven quantitative parameters discussed under Experimental Procedures. The range of magnitude for each of these parameters in native and Triton-treated PM is listed above. One of the most important points to note in this figure is the marked effect of the valence of the cation on the ability of added salt to support reconstitution for all of the tested parameters. For example, Ca^{2+} (open triangles) was about 160 times more effective than Na^+ (open squares), and La^{3+} (open diamonds) was about 50 times more effective than Ca^{2+}. The other most important observation was that in the absence of metal cation reconstitution can still be accomplished by lowering the pH of the reconstitution medium to 4 or below, the apparent p*K* being ~5. The relationship between the valence of the cation

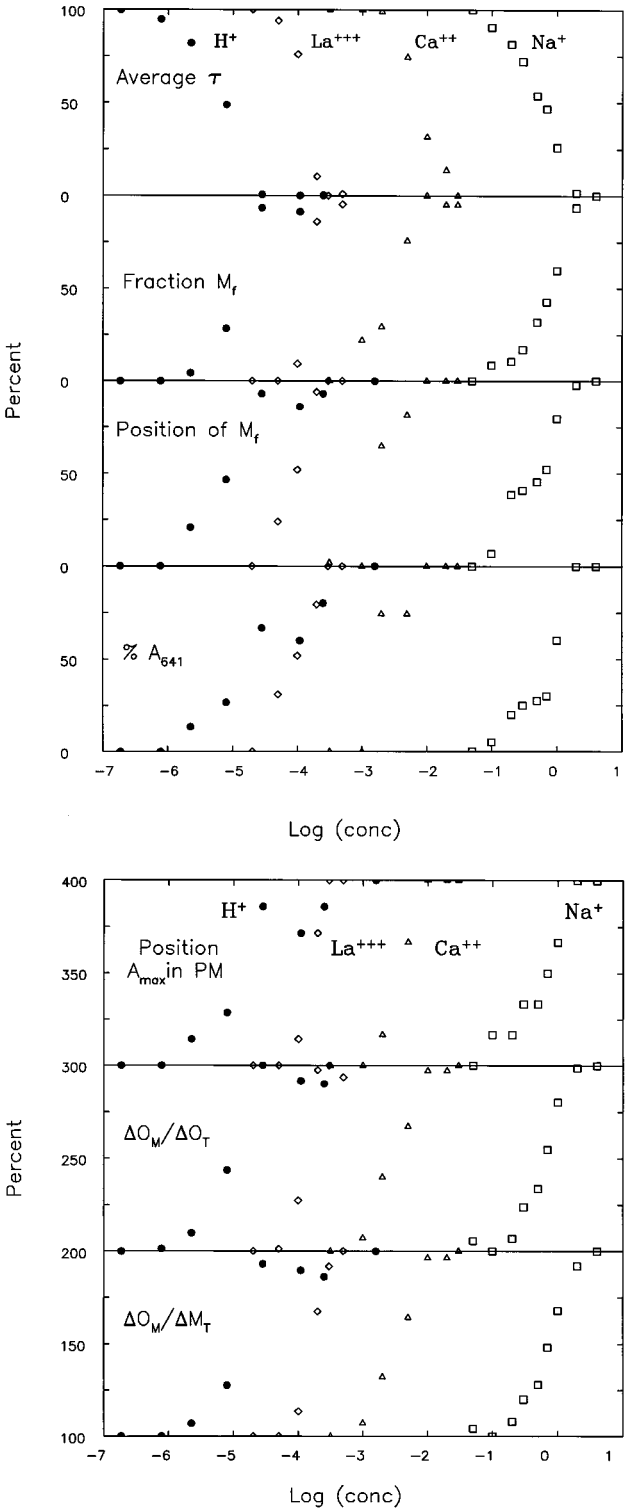

Figure 2. Normalized parameters of the BR photocycle vs the log of concentration of salt used in reconstituting Triton-treated PM with isolated PM lipids (as described in the text). The change in magnitude of each parameter was normalized to 100% using the lowest and highest values as 0 and 100%. Symbols: open squares, Na^{1+}; open triangles, Ca^{2+}; open diamonds, La^{3+}; closed circles, H^+. (A) The actual ranges were close to 5−52 ms for average τ, 0.07−0. 51 for fraction M_F, 587−640 nm for position of M_F, and 46−77% for % A_{641}. (B) The actual ranges were close to 563−569 nm for position of A_{max} in PM, 0.41−1.19 for $\Delta O_M/\Delta M_T$, and 0. 08−0.36 for $\Delta O_M/\Delta M_T$.

and the effective concentration of the salt indicates that screening of negative surface charge of the purple membrane in the Gouy−Chapman double layer is required for successful incorporation of the added lipids into the damaged membrane.

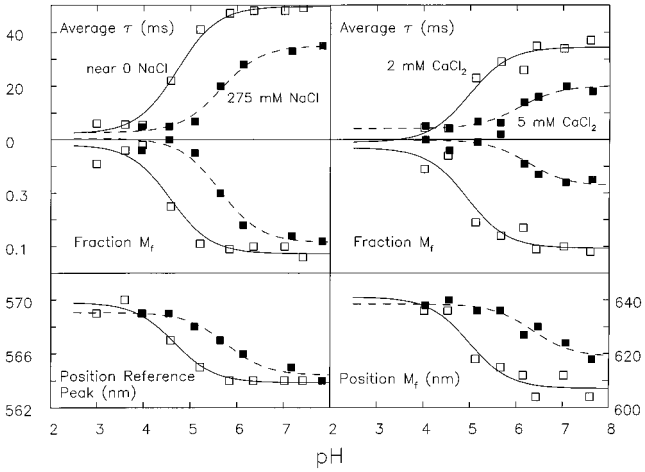

Figure 3. Influence of salt on titration curves of the efficiency for reconstituting the BR photocycle in Triton-treated PM as measured by some of the quantitative parameters shown in Figure 2. The left-hand panels show titrations for recovery of three of the parameters of reconstitution at two concentrations of NaCl, one near zero and the other at 275 mM. The right-hand panels show titrations carried out in the presence of 2 and 5 mM concentrations of $CaCl_2$. The lines drawn through the experimental points were obtained from curve-fitting using the Henderson−Hasselbalch equation.

If the Gouy−Chapman charge screening is solely responsible for reconstitution, one would predict that the pH-dependence of reconstitution would show a decrease in apparent pK with increasing cation concentration. This follows from the facts that because of the high negative charge at the membrane surface, the surface pH is $\psi_o/60$ units lower than in the bulk phase (ψ_o is surface potential) and that salt neutralizes ψ_o. This theoretical prediction has been experimentally confirmed for an amino acid residue of BR on the external surface of PM.[7] Our experimental results on the pK_{app} for reconstitution are opposite of this expectation, as shown in Figure 3. Using three of the quantitative parameters, it is seen that raising the NaCl concentration from near 0 to 275 mM causes the pK_{app} to rise ∼1.3 units (left-hand panels). Raising the $CaCl_2$ concentration from 2 to 5 mM increased the pK_{app} by about the same amount. According to the Gouy−Chapman equation, for a surface charge density of −1 per 300 Å^2 at 25 °C, increasing the NaCl concentration from 0.1 to 275 mM should have decreased ψ_o by 192 mV, causing a decrease in pK_{app} of 3.2 units. Raising the $CaCl_2$ concentration from 2 to 5 mM should have decreased ψ_o by 12 mV, causing a decrease in pK_{app} of ∼0.2 units.

A plot of pK_{app} for reconstitution vs salt concentration shows a positive slope which is steeper in the case of Ca^{2+} (closed triangles) than in the case of Na^+ (Figure 4). The experimental data differ from the predictions both qualitatively (rise in pK_{app} with increasing salt concentration) and quantitatively (the magnitude of change in pK_{app} for a particular change in salt concentration).

Discussion

Purple membrane has a very high negative surface charge density, σ, of ∼2 negative charges per BR molecule (see ref 12 for a review). Due to the high σ, counterions will be attracted to and concentrated in the vicinity of the membrane surface.[21] As a consequence, the magnitude of the negative surface potential, ψ_o, is decreased with increased salt concentration according to the Gouy−Chapman equation. Our results are consistent with the idea that reduction of surface potential is required in order to accomplish reconstitution. In other words, ψ_o must be decreased to allow the added lipids to reach and be

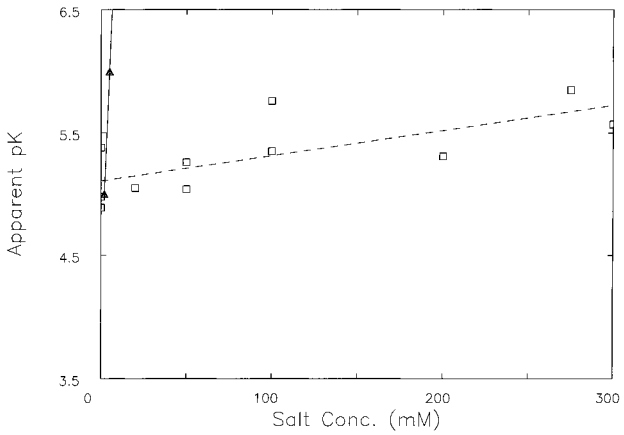

Figure 4. Apparent p*K*'s for reconstitution observed in the presence of various concentrations of NaCl (open square symbols) and $CaCl_2$ (solid triangles symbols). The straight lines are fitted curves.

TABLE 2: Concentration of Metal Cations for 50% Reconstitution and Corresponding Membrane Potential

	Na^+	Ca^{2+}	La^{3+}
conc (mM)	55	7.5	0.13
ψ_o (mV)[a]	−30	−61	−75

[a] ψ_o is calculated from the Gouy−Chapman equation.

incorporated at the proper locus with the proper orientation. If this is the only consideration, one would expect the degree of reconstitution to be correlated to the magnitude of the membrane potential. All cations of the same valence should be equally effective at all tested concentrations. Table 2 shows the concentrations of three metal cations for half-maximal reconstitution and the corresponding membrane potentials estimated from the Gouy−Chapman equation. The results show that although the ψ_o for Ca^{2+} and La^{+3} are close to each other, the ψ_o is lower in the case of Na^+. This suggests that other factors in addition to charge-screening are involved, such as specific binding of particular cations. It is known that cations bind at specific sites in BR with varying affinities and a varying number of binding sites.[12,22−27] Such direct binding could be important in at least two ways. The direct binding of a cation to a charged center would reduce the local charge more effectively than by the process of simple charge-screening in the surrounding medium. Because Ca^{2+} and La^{3+} are known to bind more effectively than Na^+, the actual ψ_o in the presence of Ca^{2+} and La^{3+} cations may be much less than the values shown in Table 2. As mentioned earlier, Ca^{2+} mediates reconstitution more effectively than Mg^{2+}, although both are bivalent cations. This result is consistent with the possible importance of specific binding, especially since Ca^{2+} has been reported to have greater binding affinity for BR than Mg^{2+}.[27] Another possibility is that specific lipid−metal−BR associations play a crucial role in the native membrane. The reconstitution results obtained with protons alone must be interpreted in terms of specific binding. Reconstitution in the absence of added metal cations was achieved with an apparent p*K* of 5 (i.e. 10^{-5} M), which is ∼5 orders of magnitude lower than found for Na^+.

Another observation that points to the importance of other factors in addition to simple charge-screening is the observed increase in the pK_{app} of reconstitution with increasing salt concentration. We have attempted to explain this observation using models with individual binding constants for protons and cations and constraints that favored the binding of lipid when either one or both ligands were bound or by invoking cooperative binding effects for the two ligands. None of the models we tested could be fitted by our data. Other possibilities under consideration involve a direct effect of particular cations on the p*K* of a group at a crucial location for lipid binding. Alternatively, the effect of particular cation-binding on the critical p*K* may be exerted indirectly by a mechanism involving conformational changes in the BR. The partial removal of lipids by detergents is known to decrease the number of metal cation binding sites.[14] A possibly related phenomenon to the one we are considering here was reported by Naito et al.[28] In detergent-solubilized BR monomers, the p*K*'s for the purple-to-blue spectral transition were shifted to higher values as the salt concentration was increased. An explanation for both of these unexpected findings should increase our understanding of membrane surface phenomena.

References and Notes

(1) McLaughlin, S. *Curr. Top. Membr. Transp.* **1977**, *9*, 71.
(2) Barber, J. *Biochim. Biophys. Acta* **1980**, *594*, 253.
(3) McLaughlin, S. *Ann. Rev. Biophys. Biophys. Chem.* **1989**, *18*, 113−136.
(4) Carmeli, C.; Quintanilha, A. T.; Packer, L. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 4707.
(5) Ehrenberg, B.; Ebrey, T. G.; Friedman, N.; Sheves, M. *FEBS Lett.* **1989**, *250*, 179.
(6) Koutalos, Y., Ebrey, T. G., Gilson, H. R.; Honig, B. *Biophys. J.* **1990**, *58*, 493−501.
(7) Alexiev, U.; Marti, T.; Heyn, M. P.; Khorana, H. G.; Scherer, P. *Biochemistry* **1994**, *33*, 298.
(8) Cao. Y.; Brown, L. S.; Sasaki, J.; Maeda, A.; Needleman, R.; Lanyi, J. K. *Biophys. J.* **1995**, *68*, 1518.
(9) Kimura, Y.; Ikegami, A.; Stoeckenius, W. *Photochem. Photobiol.* **1984**, *40*, 641.
(10) Szundi, I.; Stoeckenius, W. *Biophys. J.* **1989**, *56*, 369.
(11) Balasov, S. P.; Govindjee, R.; Ebrey, T. G. *Biophys. J.* **1991**, *60*, 475.
(12) Jonas, R.; Koutalos, Y.; Ebrey, T. G. *Photochem. Photobiol.* **1990**, *52*, 1163.
(13) Chang, C.-H.; Chen, J.-G.; Govindjee, R.; Ebrey, T. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 396.
(14) Griffiths, J. A.; Masciangioli, T. M.; Roselli, C.; El-Sayed, M. A. J. *Phys. Chem.* **1996**, *100*, 6863.
(15) Ariki, M.; Lanyi, J. K. *J. Biol. Chem.* **1986**, *261*, 8167.
(16) Jonas, R.; Ebrey, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 149.
(17) Zhang, N.; Sweetman, L. L.; Awad, E. S.; El-Sayed, M. A. *Biophys. J.* **1992**, *61*, 1201.
(18) Dracheva, S.; Bose, S.; Hendler, R. W. *FEBS Lett.* **1996**, *382*, 209.
(19) Mukhopadhyay, A. K.; Dracheva, S.; Bose, S.; Hendler, R. W. *Biochemistry* **1996**, *35*, 9245.
(20) Mukhopadhyay, A. K.; Bose, S.; Hendler, R. W. *Biochemistry* **1994**, *33*, 10889.
(21) Gueron, M.; Weisbach, G. *Biopolymers* **1980**, *19*, 353.
(22) El-Sayed, M. A.; Yang, D.; Yoo, S-K.; Zhang, N. *Isr. J. Chem.* **1995**, *35*, 465.
(23) Yang, D.; El-Sayed, M. A. *Biophys. J.* **1995**, *69*, 2056.
(24) Zhang, N.; El-Sayed, M. A. *Biochemistry* **1993**, *32*, 14173.
(25) Masuda, S.; Nara, M.; Tasumi, M.; El-Sayed, M. A.; Lanyi, *J. K. J. Phys. Chem.* **1995**, 99, 7776.
(26) Yoo, S-K.; Awad, E. S.; El-Sayed, M. A. *J. Phys. Chem.* **1995**, *99*, 11600.
(27) Griffiths, J. A.; King, J.; Yang, D.; Browner, R.; El-Sayed, M. A. *J. Phys. Chem.* **1996**, *100*, 929.
(28) Naito, T.; Kito, Y.; Kobayashi, M.; Hiraki, K.; Hamanaka, *T. Biochim. Biophys. Acta* **1981**, *637*, 457.