Pure & Appl. Chem., Vol. 67, No. 1, pp. 149–155, 1995.
Printed in Great Britain.

On the molecular mechanisms of the rapid and slow solar-to-electric energy storage processes by the other natural photosynthetic system, bacteriorhodopsin

M. A. El-Sayed[1]*, Jennifer Griffiths[1], Li Song[1], and Nancy Zhang[2]

[1]School of Chemistry and Biochemistry
Georgia Institute of Technology, Atlanta, Ga. 30332-0400

[2]Department of Pharmacy,
University of Southern California, Los Angeles, CA 90033

Abstract

Upon the absorption of solar energy by retinal in bacteriorhodopsin (bR), a very rapid and highly specific photoisomerization of the retinal around the C_{13} -C_{14} bond takes place. This is followed by the formation of a number of intermediates resulting from conformational changes of the protein around the retinal which leads to the deprotonation of the protonated Schiff base of the retinylidene system. This is the switch of the proton pump which leads to the last step in the storage of solar energy in the form of electric energy by this photosynthetic system. The removal of metal cations from bR is found to inhibit the deprotonation process.

In the present paper we summarize the results of our studies and the others regarding two important questions in the conversion process: 1) what is(are) the molecular mechanism(s) of the protein catalysis of the photoisomerization process and 2) what is the role of metal cations in the deprotonation process of the protonated Schiff base (the switch of the proton pump)? In order to answer the first question, the results of the subpicosecond photoisomerization rate of retinal in bR and in a number of its relevant mutants are discussed in terms of the steric and electronic factors. In an effort to answer the second question, we discussed the results of the binding studies of Ca^{2+} to bR, to its mutants and to bR after its C-terminus is cleaved. From these results and the results of Roux et al. on the ^{31}P NMR of Nd^{3+} regenerated bR, we concluded that one or two metal cations strongly bound to the protein but not on the surface, are functionally important. The model in which these metal cation(s) control the pK values of Aspartic acids in the 85 and 212 positions and that of the protonated Schiff base (PSB) during the photocycle is discussed.

I. Introduction

Bacteriorhodopsin (bR) is one of the two natural photosynthetic systems, the second being chlorophyll. While photosynthesis using the latter system is as old as life on earth (~ 3 billion years) and utilizes electron pumps, bR photosynthesis is not as old and uses proton pump.

The proton pump creates proton gradients that are used both by the cell for ATP synthesis and other transport processes (for recent reviews, see references 1-5). bR is the only protein found in the purple membrane, PM, of *Halobacterium Salinarium* . This is a single cell (few microns in length) light-utilizing bacterium which was first discovered in 1971 by Oesterhelt and Stoeckenius[6]. It contains toretinal as a chromophore which is covalently bound via a Schiff base (-CNH-) linkage the ε-amino group of the lysine 216 residue in the protein. Upon absorbing a photon, it cycles through a number of intermediates occurring on different time scales before returning to its original form bR_{570} (i.e. having its retinal absorption maximum at 570 nm):

$$\underset{}{\overset{h\nu}{bR_{568}}} \overset{}{\sim\sim\sim>} \underset{0.2ps}{(bR)^*} \overset{}{\text{-----}>} \underset{0.5\ ps}{I_{460}} \overset{}{\text{----}>} \underset{\sim 3ps}{J_{625}} \overset{}{\text{-----}>} \underset{2\mu s}{K_{610}} \overset{}{\text{-->}} \underset{70\mu s}{L_{550}} \overset{-H^+}{\text{--->}} \underset{ms}{M_{412}} \overset{+H^+}{\text{--->}} N \text{ --> } O \text{ --> } bR_{568}$$

In the $L_{550} \rightarrow M_{412}$ step, a proton is transferred from the protonated Schiff base to aspartate D85[7]. As a result, protons are pumped across the cell to its outside surface, establishing a pH gradient used by the

organism for metabolic processes such as ATP synthesis[8]. The protons are ejected from the cell at a rate comparable to that for the formation of the M_{412} intermediate[9]. This intermediate is the only one in which the Schiff base is unprotonated. Consequently, many studies have inferred that the deprotonation of the protonated Schiff base (PSB) is closely associated with the proton pump mechanism. Preceding M_{412} formation (or PSB deprotonation) is the formation of the early intermediates J_{625}, K_{610} and L_{550}. The retinal in bR_{570} is in the all-trans form, whereas in K_{610} it has a distorted 13-cis conformation[10]. In the L_{550} form, the isomerization is complete[10]. The pK_a value of the PSB is 13.3 in bR_{570}[11], yet it deprotonates during the L_{550} --> M_{412} step of the cycle even if the pH of the medium is lowered to below 4, suggesting a large change in its pK_a value has taken place during the photocycle[12].

The absorbed solar energy is thus converted into electrical energy in two important processes. First is the very rapid photoisomerization of the retinal which separates the positive charge on the protonated Schiff base (PSB) and its counter ions (aspartate D85 and D212). A few intermediates later (on the microsecond time scale) the PSB is deprotonated which "switches on" the proton pump responsible for the creation of the proton gradient. As will be discussed in section IIIa, metal cations are found to be required for the deprotonation process.

In section II of this paper, we summarize the results of our efforts in the study of the rapid primary retinal photoisomerization process. Using subpicosecond time-resolved Raman spectroscopy, we have previously shown that the rapid process observed previously by optical spectroscopy occurring in 450 fs is indeed an isomerization process. Following the rate of photoisomerization in different mutants in which individual (one at a time) charged or hydrogen bonded residues are replaced by neutral or non hydrogen bonding residues, we have found that the negative charges on D85 and D212 are important in the catalysis of the photoisomerization of retinal in the protein. We discuss the possible molecular mechanism that could be responsible for this catalysis.

In section III of the paper, we discuss the different models of binding of metal cations in bR and summarize our results as well as those of others on the binding and the structural studies. From the integrated results we conclude that the functionally important one or two metal cations are bound within the protein and not to the surface. Possible mechanisms for their involvement in the deprotonation process are qualitatively discussed.

II. Protein Catalysis of Retinal Photoisomerization:

Kaiser and coworkers, and Mathies and coworkers determined that the observed red shift in the retinal absorption maximum from 568nm to 625 nm occurs[13] in about 500 fs. This was assigned to the photoisomerization of the retinal chromophore from the all-trans to the 13-cis isomer. Using subpicosecond time resolved resonance Raman technique primarily developed earlier in this laboratory[14], it was shown[15] that the retinal in the intermediate that absorbs at 625nm has a different conformation than that of the all-trans retinal present in the parent molecule. This supports the assignment of earlier transient optical absorption measurements[13] that the photoisomerization process takes place in 500 fs. In methanol, the protonated Schiff base is found to photoisomerize in less than 5 ps[16]. In addition, it was found that the photoisomerization of the PSB (formed from all-trans retinal and n-butylamine) occurs predominately along the C_{11}-C_{12} bond to produce the 11-cis retinal isomer[17]. These observations suggest that the protein catalyzes the photoisomerization process and makes it highly specific around the C_{13} - C_{14} bond. In order to identify the mechanism by which this specific catalysis takes place, we have studied the photoisomerization rate in a number of bR mutants[18] in which charged or hydrogen bonding amino acid residues within the retinal pocket are individually replaced by neutral and non-hydrogen bonding residues. The studied mutants are D85N, D212N, D115N, R82Q, and Y185F (Abreviations for the amino acid residues are: D, Asp; F, Phe; N, Asn; Q, Gln; R, Arg; and Y, Tyr).

Figure 1 gives the excited state decay of the retinal in bR and all these mutants. From this figure, it is clear that D85N has the longest decay time followed by R82Q and D212N. The decay time of Y185F and D115N in which the replaced residues are neutral did not change greatly. Unlike the light-adapted PM in which retinal is present as all trans, the retinal in these mutants is present as a mixture of the all-trans and 13-cis isomers. Thus each decay curve is deconvoluted into two components, one for the all-trans to 13-cis photoisomerization and the other one for 13-cis to all-trans photoisomerization. The observed decay times in D85N are then longer than that in bR by a factor of 4 in one component and as high as 20 in the other.

Since the observed decay of the excited-state is a result of photoisomerization and nonradiative processes back to the ground state of bR (internal conversion), a change in the rate of either processes could lead to the observed increase in the decay times. However, the observed lengthening of the excited-state lifetime cannot be a result of inhibiting the internal conversion process alone without a decrease in the

photoisomerization rate. An isomerization quantum yield of 0.65 and a lifetime of 0.5 ps of the excited-state in bR corresponds to a photoisomerization lifetime of 0.77 ps
and an internal conversion time of 1.4 ps. Thus the longest decay time that should have been observed if the internal conversion process is the only process that is inhibited in D85N should have been 0.77 ps. The fact that we observed an increase in the lifetime from 0.5 ps to 2ps or 10 ps suggests that both the photoisomerization and the $S_1 \rightarrow S_0$ internal conversion processes must have been greatly inhibited in the mutants in which the charged residues were replaced by neutral ones. One can then conclude that the Aspartates 85 and 212 as well as Arginine 82 in the protein catalyze both the photoisomerization process around the C_{13}-C_{14} bond as well as the internal conversion process from the retinal lowest excited singlet state to its ground state. The observed effect of positively charged Arginine is known to insure that Aspartate 85 is in the ionized form[19]. This leads to the conclusion that the negative charge on Aspartate 85 is the most effective in the catalysis process. This is followed by Aspartate 212. It is interesting that removing metal cations by ion-exchange of PM, which leads to the protonation of D85, is found[20] to give a decay curve very similar to D85N, since in both types of bR the residue in the 85 position is neutral.

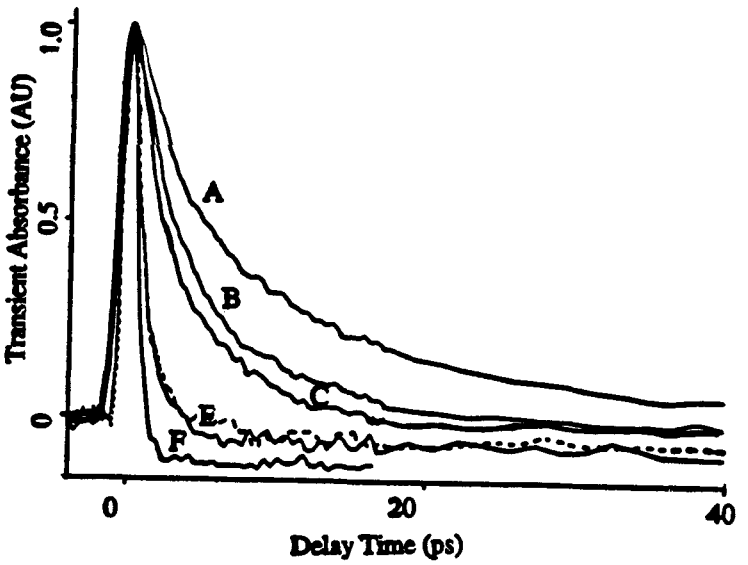

Figure 1. The subsicosecond decay of the excited-state absoprtoipn of retinal in bR at pH 5.8 (F) and in some of its mutants: D85N at pH 5.8(A); R82Q at pH 4.4 (B); D212N at pH 5(C) ; D115N at pH 5.8 (solid curve, E); Y185F at pH 5.8 (dotted curve, E). The replacement of only charged residues removes the protein catalysis for the photoisomerization process.

There are at least two factors that can make the photoisomerization process of retinal in bR so rapid: steric and electronic. It is possible that the retinal in the pocket is highly strained due to anisotropic repulsive forces between specific atoms on the retinal (e.g. the hydrogens on the CH_3 groups) and on the side chain groups on the amino acid residues within the retinal pocket. This together with the demands of the formation of the hydrogen bond network around the PSB gives rise to the steric factors. Upon reaching the excited state, rotation around the C_{13}-C_{14} bond could relieve and relax the "loaded retinal spring". The electronic factor comes in by reducing the barrier to rotation around the C_{13}-C_{14} bond in the excited state.

The contribution of Aspartates 85 and/or 212 to the specific catalysis of the photoisomerization process could be via both the steric and the electronic factors. Either or both aspartates could "tighten up" the retinal pocket so as to induce the necessary anisotropic potential with specific repulsions at the atoms needed to load the "retinal spring" thus contributing to the steric effect. Both aspartates could also contribute to the electronic factor by reducing the barrier to rotation around the C_{13}-C_{14} bond in the excited state by decreasing its bond order from 2 in the ground state to near 1 in the excited state. As we proposed earlier[18], the valence bond method could give a simple way by which this can be visualized. In the electronic ground state, it is believed[21] that the NH^+ group of the protonated Schiff base is H-bonded to Aspartate 212 via a H_2O molecule. This helps to localize the positive charge on the nitrogen. The excited state can be described by a linear combination of resonance structures in which the positive charge is located on the different odd numbered carbon atoms (e.g. C_{15}, C_{13}, C_{11},....) along the retinal chain. The distance between C_{13} and the closest oxygen atom of Aspartate 85 and 212 (the oxygen that is not H-bonded to the nitrogen) calculated from the data of Henderson et al.[22]are 0.456 and 0.416 nm, respectively. The dielectric constant within the protein is very small and if there is no intervening groups within these small distances its value is expected to be near that in vacuum. This suggests that a strong coupling will take place

between the O^- atoms on aspartes 85 and 212 and the positive charge on C_{13} in the retinal excited state. Since these distances are much smaller than that between the O^- of D85 or D212 and C_{11}, C_9, C_7, etc., it suggests that the stabilization of the structure with the positive charge on C_{13} is largest and hence the contribution of this structure to the excited state wave function is the largest. Since in this structure the C_{13}-C_{14} bond is single (instead of a double in the ground electronic state), it suggests that a large reduction in the isomerization barrier around this bond takes place upon photoexcitation. This could be the molecular origin of Asp85 and 212 catalysis of the photoisomerization around the C_{13}-C_{14} bond in bR. The fact that the photoisomerization around this bond (C_{13}-C_{14}) is expected to be faster than that around C_{11}-C_{12}, C_9-C_{10} etc. explains the specificity of the photoisomerization process. Recent studies in our laboratory suggest that by replacement of either Asp85 or Asp 212 other isomers such as 11-cis and 9-cis are present in light adapted D85 and D212 mutants[23] which are not found in wild type bR.

Once the barrier to rotation around the C_{13}-C_{14} bond is reduced, rotation both by π (to give the 13 cis isomer) and by 2π (to go back to the all trans configuration) becomes possible. This could explain the observation that both the rate of retinal photoisomerization as well as the rate of the internal conversion processes are catalyzed by the protein in bR as a result of both steric and electronic factors.

III. Metal Cations in bR control color and function:

a. Previous studies:

Well-washed PM[24] contains ~ 3-4 moles of Mg^{2+} and ~ 1 mole of Ca^{2+} per mole of bR at a bulk pH of ~ 5.5. Removal of these cations from PM by ion exchange[24,25] or acidification[6,26,27] causes a color transition from purple to blue. While photoisomerization of the retinal takes place in blue bR, it does not form the M_{412} intermediate, and thus does not pump protons[24]. Different models have been proposed regarding the binding of metal cations and how they control the color of purple membrane. Szundi and Stoeckenius[28-30] treated the metal ions as free positive charges distributed uniformly on the membrane surface which regulate the surface pH via the Gouy-Chapman effect. In this model the removal of the cations increases the negative surface charge density, which in turn lowers the surface pH. This pH change causes the protonation of the aspartate counter ion(s) which affects the retinal color. Several other groups[31-37] emphasize the existence of specific chemical binding between some of the metal cations and the negatively charged groups (e.g. carboxylate groups of Asp and Glu side chains) within the protein.

A good correlation[12] has been observed between the efficiency of the deprotonation of the PSB and the extent of the conversion of the purple color of PM to the blue bR. Thus the deprotonation mechanism of the PSB during the photocycle is somehow connected to the structure of the retinal cavity of bR prior to the absorption of the photon[12]. The surface metal binding controls the surface pH which in turn could control the protonation state of the proton acceptor[38] (D85). The removal of the metal cations decreases the surface pH and thus could protonate the proton acceptor (D85) and thus inhibits the proton transfer process in the $L_{550} \rightarrow M_{412}$ step in the photocycle. On the other hand, if the functionally important metal cation is bound within the protein it could internally control (decrease) the pKa value of D85 to insure that it is deprotonated for accepting the proton during the cycle. It can also change the pKa value of the PSB during the photocycle to make it a proton donor in the L550-->M412 step. This makes the proton transfer process in the L550 $\rightarrow$ M412 step thermodynamically and kinetically favorable. Thus in order to determine the exact role of the metal cation in the deprotonation process, it is important to determine the location of the functionally important metal cations: is it on the surface thus controlling the surface pH and the protonation state of the proton acceptor, or is it within an interaction distance inside the protein thus changing the pKa of the PSB (the proton donor) during the photocycle and controlling the pKa of the proton acceptor (D85) to keep it unprotonated before and during the photocycle?

Various methods such as electron spin resonance spectroscopy[31,33] for Mn^{2+}, filtration techniques[32] for Ca^{2+} and Hg^{2+}, steady-state fluorescence methods[39] for Eu^{3+} and potentiometric titrations using specific Ca^{2+} electrodes[34] have been employed to measure the binding constants between deionized bR and different cations. All of these studies reached the conclusion that there are more than two classes of cation binding sites in bR, with different binding affinities and therefore different binding environments.

Several attempts have been made to locate the metal cations through x-ray diffraction and electron diffraction techniques. Studies by Stroud and co-workers on bR films[40,41] with Pb^{2+} substituted for the wild type cations indicate that up to four distinct binding sites are located on the alpha helices of the protein. Due to the inherent nature of the 2D structure of purple membrane, however, the results of Stroud cannot localize the binding sites in the third dimension, i.e., along the alpha helices. Unfortunately, these results

could not be duplicated[42]. Engelhardt, *et al.* [43] have investigated the binding environment of the metal cations with EXAFS. These results[43] are consistent with an octahedral geometry for the metal ions with 5.8± 1.5 ligands. The authors[43] suggest that the metal cation is bound to the carboxylate groups, although the data is not sufficient to distinguish between nitrogen and oxygen ligands.

b. The distribution of metal cations

If it is assumed that the binding sites of calcium in bR can be separated into classes in which the binding affinity is mostly the same within a class and that these binding sites are non interacting, then there is a relation[44,45] between the number of binding sites (Ca^{2+} metal cations),n, with the same affinity (association) equilibrium constant, K, and the average number of metal cations per bR initially added, v:

$$v/c = K(n-v)$$

where n is the number of sites of metal cations having the same binding affinity and c is the free cation concentration. A Scatchard plot[44,45] of v/c versus v gives straight lines with a slope of -K and an x intercept giving the value of n. Regions of this plot will be linear if the binding affinity of the different classes is sufficiently separated in magnitude.

The observed Scatchard plots in our laboratory by Zhang, *et al.*[34] clearly shows linear regions which correspond to two binding sites with equilibrium constants that differ by a factor of 4. At pH 4, $K_1 = 0.6 \pm 0.05 \times 10^6\ M^{-1}$ and $K_2 = 0.15 \pm 0.01 \times 10^6\ M^{-1}$. Upon further calcium addition, it appears that there are four to six lower affinity binding sites as indicated on the Scatchard plot with $K_3 = 4.0 \pm 0.6 \times 10^3\ M^{-1}$.

The binding experiments on wild type bR do not give any insight into the location of the metal cation, i.e., are they on the surface of the membrane or bound within the protein. To further characterize the location of these binding sites observed for the deionized wild type bR, similar experiments were performed on mutants[46] of bR by replacement of charged (D85N, D212N, and R82Q/D85N) and H-bonding (Y185F and R82Q/Y185F) residues in the retinal pocket by neutral non-hydrogen bonding ones. It is observed[46] that in all mutants there are still two high affinity binding sites. However, replacement of the charged residues is found in all cases to reduce the value of the affinity constants of both of these binding sites (K_1 and K_2) by approximately the same factor. D212N gives the largest reduction of 1/15 for K_1 and K_2. In contrast, replacement of the H-bonding residue (Y185F) is found to have a greater effect on K_2 than on K_1, indicating that the first high affinity binding sites is not influenced as much as the second high affinity binding site by this mutation. Finally, it is observed[46] that the mutants of both the charged residue and the H-bonding residue show no effect on the binding affinity of the 4-6 weakly bound sites. These results strongly suggest that the charged aspartate residues are involved in the binding of the calcium cation in the two high affinity sites and therefore these high affinity sites are located *within* the protein. The minimal effect of mutation on the binding constant of the 4-6 low affinity sites suggests that these sites are not located near the retinal pocket and are possibly located on the surface of the membrane protein.

The location of the weakly bound sites has been further investigated in our laboratory[47] by performing the identical calcium binding experiment on wild type bR in which the C-terminus has been cleaved. The C-terminal has a number of charged glutamate and aspartate groups[48] and is believed[49] to cover part of the cytoplasmic surface that could offer binding sites for surface metal cations. The Scatchard plot for this modified bR[47] showed no significant change in the value of K_1 and K_2, i.e, no effect on the high affinity binding sites. This supports the previous conclusion that these two high affinity sites are located within the protein. There is, however, a significant decrease in the number of the low affinity binding sites from 4-6 down to one.

In conclusion our results of the calcium binding experiments[46,47] suggest that there are two strong binding sites located within the protein. The results indicate that these two high affinity sites are stabilized by the negative charges of Asp85 and Asp 212 located near the retinal pocket. In addition, there are several weaker metal cation binding sites which appear to be located mostly on the cytoplasmic surface of the protein.

^{31}P NMR experiments by Roux, *et al.*[50] have shown that the titration of the blue membrane with divalent cations resulted in an increase in the chemical shift anisotropy of the phospholipid phosphate groups. Furthermore, when a paramagnetic cation e.g. Nd^{3+}, is used for the regeneration of purple bR, broadening of the ^{31}P peak is observed. One important aspect of their results is that the broadening of the ^{31}P NMR peak, which is indicative of close interaction between the phosphorous nucleus and the perturbing paramagnetic Nd^{3+} cation, occurs strongly only after a cation to bR ratio of two has been added. This can be explained by our conclusions given above. Since the two high affinity binding sites are located within the protein, binding of Nd^{3+} to these sites is expected to have no significant electronic perturbation on the ^{31}P peak of the phospholipid head groups due to the large spatial separation. However, the further addition of

metal cations at or above a 2:1 ratio, the weak binding sites located on the surface begin to be occupied. This would place the Nd^{3+} ions in close proximity to the lipid head groups and thus could result in perturbation of the observed ^{31}P NMR band shape. Thus some of the surface sites must involve the PO^{2-} of the head groups of the lipids as well as the glutamates and aspartates of the C-terminus.

IV. The location of the functionally important metal cations:

Figure 2 in reference[12] shows a strong correlation between the deprotonation efficiency of the PSB and the extent of the conversion of the purple color of bR into the blue color of deionized bR. This suggests that the initial structure of the retinal cavity (and thus its color) dictates whether upon going thorough the photocycle the protonated Schiff base will deprotonate or not. In order to assign which site is functionally important, we need to determine the binding constant of the metal ion that changes the color of blue bR into purple by use of optical spectroscopy. If it is equal to the value determined potentiometrically for K_1 or K_2 then it can be concluded that the important cation resides within the protein. If on the other hand it is found to equal to K_3, then it is located on the surface.

The binding constant, K_s, of the metal cation that converts blue bR into purple bR is that for the equilibrium:

$$bbR + Ca^{2+} = PbR \ldots\ldots \qquad K_s = \frac{[PbR]}{[bbR][Ca^{2+}]}$$

The concentrations of the blue bR [bbR] and the purple bR [PbR] are determined optically and the free Ca^{2+} concentration is determined potentiometrically[34]. From these experiments[34] carried out on a series of solutions at different stages of conversion, K_s has been determined and found to be $1.6 \times 10^5\ M^{-1}$. This is very similar to K_2 ($4.0 \times 10^5\ M^{-1}$) obtained by potentiometric titration, suggesting that binding of the second affinity site is most important to the color change as well as to the function. Previous studies[36] based on the observed changes in optical spectra had already concluded that filling the second site converts blue bR to purple bR.

The fact that the second site begins to fill after the first one cannot eliminate the possible importance of the first site to the color change and function. In other words, is the metal cation in the first site needed together with that in the second site for the color change and function or is it a "spectator" and only after the first site is filled, the second site, which is the only one that is important, begins to fill. The answer cannot be derived from the results obtained so far. In any case, since both the first and second binding sites are bound to the protein, the conclusion is reached that the metal cation(s) that are important to the function are protein, and not surface, bound.

From the above, one metal cation (in the second high affinity site) or two (in the two high affinity sites) bound to the protein are important to the function of bR. The simplest model, the electrostatic model, by which these protein bound cations could catalyze the deprotonation process was proposed previously[12, 51]. During the photocycle, the reaction path brings the electrostatic coupling between the metal cation and the PSB in the $L_{550} \rightarrow M_{412}$ step to a certain strength to make the proton transfer process between the PSB and D85 free energy and kinetically favorable. The coupling between the metal cations, D85 and D212 could control the pKa values of the latter acids to assure that they remain deprotonated during the cycle. Thus, the presence of the high affinity metal cations increases both the acidity of the proton donor (the PSB) and the basicity of the proton acceptor, rendering the proton transfer process thermodynamically as well as kinetically favorable.

Acknowledgment: The authors would like to thank Professor J. Lanyi for supplying the mutants that were necessary for performing the previous work that was already jointly published with Professor Lanyi. It is the integration of those results with these of others that lead to some of the new conclusions in this work. For this we thank authors to whose work we have referred in this article. The authors would also like to thank the Department of Energy (Grant No. DE-FG03-88ER-13828) for the support of this research.

1. W. Stoeckenius, R. Lozier and R. A. Bogomolni, Biochem. Biophys. Acta 505, 215(1979).
2. R.A. Mathies, S.W. Lin, J.B. Ames and W.T. Pollard, Ann. Rev. Biophys. Biophys.Chem. 20, 491 (1991).
3. M. A. El-Sayed, Acc. Chem. Res. 25, 279(1992).
4. J. K. Lanyi, J. Bioenerg. Biomembr. 24, 169 (1992); J. K. Lanyi, Biochim. Biophys. Acta 1183, 241 (1993).
5. K. J. Rothschild, J. Bioenerg. Biomembr. 24, 147 (1992).
6. D. Oesterhelt and W. Stoeckenius, Nature, 233, 149 (1971).
7. M. S. Braiman et al., Biochemistry 27, 8516(1988); H. T. Otto et al., Proc. Natl. Acad. Sci. USA 86, 9228 (1990); H. K. Butt et al., EMBO Journal 8, 1657 (1989).
8. R. H. Lozier, W.Niederberger, R. A.Bogomolni, S.-B.Hwang and W. Stoeckenius, Biochim. Biophys. Acta 440, 545(1976).; D. R. Ort and W. W. Parson, J. Biol. Chem. 253, 6158(1978).

9. N.Dencher and M. Wilms, Biophys. Struct. Mech. 1, 259(1975); J. W. Belliveau and J. K.Lanyi, Arch. Biochem. Biophys. 178, 308(1977)
10. J.Terner, C.-L. Hsieh, A. R. Burns and M. A. El-Sayed, Proc. Natl., Acad. Sci. USA 76, 3046(1979); G. Eyring, B. Curry, A. Broeck, J. Lugtenberg and R. Mathies, Biochemistry 21, 384(1982).
11. S. Druckman, M. Ottolenghi, A. Pande, J. Pande and R. H. Callender, Biochemistry 21, 4953(1982); M. Sheves, A. Albeck, N. Friedman and M. Ottolenghi, Proc. Natl. Acad.Sci. USA 83, 3262(1986).
12. E. L. Chronister, T.C. Corcoran, L. Song and M. A. El-Sayed, Proc. Natl. Acad. Sci. USA, 83, 8580(1986).
13. M. C. Nuss, W. Zinth, W. Kaiser, E. Kolling and D. Oesterhelt, Chem. Phys. Lett. 117, 1(1985); H. Polland et al., Biophys. J. 49, 651 (1986); J. Dobler, W. Zinth, W. Kaiser and D. Oesterhelt, Chem. Phys. Lett. 144, 215 (1988); R. A. Mathies, C. H. Brito Cruz, W. T. Pollard, C. V. Shank, Science 240, 777 (1988).
14. J. Terner, J. D. Stong, T. G. Spiro, M. Nagumo, M. Nicol and M.A. El- Sayed. Proc. Natl. Acad. Sci. USA. 78, 1313 (1981); J. Terner, T.G. Spiro, M. Nagumo, M. F. Nicol and M. A. El-Sayed, J. Am. Chem. Soc. 102, 3238 (1980); J. Terner and M. A. El-Sayed, Acc. Chem. Res. 18, 331(1985).
15. R. van den Berg, D. J. Jang, H. C. Bitting, M. A. El-Sayed, Biophys. J. 58, 135 (1990).
16. H.Kandori and H. Sasabe. Chem. Phys. Lett. 216, 126(1993).
17. K. A. Freedman and R.S. Becher, J. Am. Chem. Soc. 108 1245(1986); Y. Koyama, K. Kubo, M. Komori, H. Yasuda and Y. Mukai Photochem. Photobiol. 54 433(1991).
18. L. Song, M.A. El-Sayed and J. K. Lanyi. Science 261, 891 (1993).
19. L.S. Brown, L. Bonet, R. Needleman, J. K. Lanyi Biophys. J. 65, 124(1993);S. P.Balashov, R. Govinjee, M. Kono, E. Imasheva, E. Lukashev, T. G. Ebrey, R. K.Crouch, D. R. Menick and Y. Feng Biochemistry 32 10331(1993).
20. T. Kobayashi, M. Terauchi, T. Kouyama, M. Yoshizawa and M. Taiji,SPIE J. 1403, 407 (1990).
21. K. J. Rothscild, M. S. Braiman, Y. W. He, T. Marti and H. G. Khorana, J. Biol. Chem. 265, 16985 (1990).
22. R. Henderson et al.,J. Mol. Biol. 213, 899 (1990) .
23. L. Song, Difei Yang and M.A. El-Sayed 1994 to be published.
24. C. H. Chang, J. G.Chen, R. Govindjee and T. G. Ebrey, Proc. Natl. Acad.USA 82, 396 (1985).
25. Y. Kimura, A. Ikegami and W. Stoeckenius, Photochem. Photobiol. 40, 641(1984).
26. T. Kobayashi, , H. Ohtani,, J. Iwai,, A. Ikegami and H. Uchiki, FEBS Lett. 62, 197(1983).
27. U. Fisher and D. Oesterheldt, Biophys J. 28, 211 (1979).
28. I. Szundi and W. Stoeckenius, Biophys. J. 56, 369 (1989).
29. I. Szundi and W. Stoeckenius, Proc. Natl. Acad. Sci. USA. 84, 3681(1987).
30. I. Szundi and W. Stoeckenius, Biophys. J. 54, 369(1988).
31. M. Dunach, M. Seigneuret, J. L. Rigaud and E. Padros, Biochemistry 26, 1179 (1987).
32. M. Dunach, M. Seigneuret, J. L. Rigaud and E. Padros,J. Biol. Chem. 263, 17378 (1988).
33. M. Dunach, E. Padros, M. Seigneuret and J. L. Rigaud, J. Biol. Chem. 263, 7555 (1988).
34. Y. N. Zhang, L. L. Sweetman, E. S. Awad, and M. A. El-Sayed, Biophys. J. 61, 1201 (1992).
35. C. H. Chang, R. Jonas, S. Melchiore, R. Govindjee and T.G. Ebrey, Biophys. J. 49, 731(1986).
36. M. Ariki and J.K. Layni, J. Biol Chem. 261, 8167(1986).
37. T. C. Corcoran, K. Z. Ismail and M. A. El-Sayed, Proc. Natl. Acad. Sci. USA. 84, 4094(1987).
38. (a) G. Metz, F. Siebert and M. Engelhard, FEBS Lett. 303, 237(1992). (b) K. Fahmay, O. Weidlich, M. Engelhard, J. Tittor, ,D. Oesterhelt , F. Siebert, Photochem. Photobio. 56, 1073 (1982).
39. L Sweetman and M.A. El-Sayed FEBS Lett. 282 436 (1991).
40. A. K. Mitra and R. M. Stroud, Biophys. J. 57, 301 (1990).
41. N. V. Katre,, Y. Kimura, & R. M. Stroud, Biophys. J. 50, 277 (1986).
42. C. Dudda, Ph.D. thesis, Freie Univeritaet Berlin.
43. M. Englehard, ,B. Hess, M. Chance and B. Chance, F.E.B.S Letter 222, 275 (1987) .
44. G. Scatchard, Ann. NY Acad. Sci. 51, 660 (1949).
45. C. Tanford, in Physical Chemistry of Macromolecules, Wiley, New York, 1961,ch. 8.
46. Y.N. Zhang, M.A. El-Sayed, M. L. Bonet, J. K. Lanyi, M.Chang, B. Ni and R.Needleman, Proc. Natl. Acad. Sci. USA, 90, 1445 (1993).
47. N. Zhang and El-Sayed, M.A. Biochemistry 32, 14173(1993).
48. M.-J. Liao, andH.G. Khorana, J. Biol. Chem. 259, 4194(1984).
49. R.Renthal, N. Dawson, J. Tuley and P. Horowitz, Biochemistry 22, 4 (1983).
50. M. Roux, M. Seigneuret, and J.L. Rigaud, Biochemistry 27, 7009(1988).
51. J. H. Hanamoto, J. P. Dupuis and M. A. El-Sayed, Proc. Natl. Acad. Sci. U.S.A. 81, 7083 (1984); M. A. El-Sayed, in Biophysical Studies of Retinal Proteins, (T. G. Ebrey et al. ed.), University of Illinois Press, Urbana-Champaign, P.174; M. A. El-Sayed, Int. J. Qurantum Chem. 22, 367(1988).