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The binding site of the strongly bound Eu^{3+} in Eu^{3+}-regenerated bacteriorhodopsin

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A Scatchard plot for the strongly bound Eu^{3+} to deionized bacteriorhodopsin (bR) was made using a method based on measuring the concentration of unbound Eu^{3+} from its fluorescence intensity. The results suggest that the first mole of Eu^{3+} added to a mole of bR is strongly bound by displacing 2–3 protons. In order to reconcile this result with the previous time-resolved fluorescence studies on Eu^{3+}-regenerated bR, which showed the presence of 3 sites of comparable binding constants, one is forced to conclude that the emission from the strongly bound Eu^{3+} is completely quenched, e.g. by energy transfer to the retinal. For this to take place, the Eu^{3+} must be within a few Å from the retinal, i.e. within the retinal pocket (the active site). The possible importance of this conclusion to the deprotonation mechanism of the protonated Schiff base, the switch of the proton pump in bR, is discussed.

Cation binding; Bacteriorhodopsin; Eu^{3+} luminescence; Scatchard plot

1. INTRODUCTION

The retinylidene protein bacteriorhodopsin (bR), the other photosynthetic system besides chlorophyll, is a protein pigment in the purple membrane of *Halobacterium halobium* [1], arranged in clusters of 3 molecules in a two dimensional hexagonal lattice [2]. Light adapted bR contains an all-*trans* retinal which is covalently bound via a protonated Schiff base (PSB) linkage to the protein [3]. bR undergoes the following photochemical cycle [4] upon the absorption of visible light:

$$bR_{568} \xrightarrow[0.4\ ps]{h\nu} J_{625} \xrightarrow[5\ ps]{} K_{590} \xrightarrow[2\ \mu s]{} L_{550} \xrightarrow[60\ \mu s]{-H^+} M_{412} \xrightarrow[ms]{} N_{520} \longrightarrow O_{640} \longrightarrow bR_{568}$$

As a result of this cycle, protons are translocated from the inside to the outside of the cell membrane [5]. The proton gradient created across the membrane is then used to transform ADP into ATP in the final step of the photosynthesis of bR [4,6].

The role of metal cations in the function of bR has been the subject of several investigations. The removal of metal cations (e.g. by addition of H^+) is found to shift the absorption from 568 (purple bR) to 605 nm (blue or deionized bR (dIbR)) [7–10]. Furthermore, the formation of M_{412}, and thus the deprotonation of the PSB [9,11], as well as the 296 nm transient [12] are found to be inhibited even though the isomerization [13] as well as the K_{590} and L_{550}-type intermediates [13,14] are found to be formed. This suggested that metal cations are important for the deprotonation process [13].

Using ESR, the binding constants of Mn^{2+} and La^{3+} to blue membrane have been determined [15] by use of Scatchard plots. For Mn^{2+} at pH = 5, one very strongly bound ($K = 26 \times 10^6\ M^{-1}$), three strongly bound sites ($K = 2.0 \times 10^6\ M^{-1}$) and one moderately strongly bound ($0.6 \times 10^6\ M^{-1}$) [15] are found.

The color change upon deionization was attributed to cation binding and the dissociation state of the counter ions of PSB was directly linked to the occupancy of the binding sites of the cations [16–22]. Two proposed locations of the metal cations have been proposed. Because protons are released in roughly stoichiometric amounts, metal cations are proposed to be chelated to carboxylate groups [16,17]. Recently, due to observed effect of changing the lipids on the metal cation–proton equilibrium, metal cations are proposed to be physically held within the double layer of the surface membrane [23]. More recent work by Ebrey et al. [24] on the correlation of color change and the number of Ca^{2+} ions bound and the H^+ ions produced, strongly suggested the importance of one calcium ion in bR. From the recently proposed model for the structure of bR [25], Ebrey suggested that this Ca^{2+} ion might be located within the retinal pocket [24].

Using time-resolved fluorescence techniques, Corcoran et al. [26] were able to resolve 3 different emission sites for Eu^{3+}-regenerated bR containing 4 Eu^{3+} per bR. The intensity of each of the 3 sites, as well as the total intensity, is found to increase linearly with in-

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creasing the Eu^{3+}/bR ratio from 0 to 3. This suggests that the 3 sites have comparable binding constants. This does not agree with the result of having one very strongly bound and 3 strongly bound metal cation sites found for Mn [15], unless either Eu^{3+} and Mn^{2+} have different sites or else the emission from the very strongly bound Eu^{3+} in the Eu^{3+}-bR samples is completely quenched, as would be the case if it is very near the retinal [26]. In this latter case the observed emission would reflect mainly the equilibrium of involving the 3 strongly bound emitting sites in the range of the Eu^{3+} concentrations studied [26]. In order to distinguish between these 2 possibilities, we need to determine the Scatchard plot for the binding of Eu^{3+} to dIbR.

In the present work, the Scatchard plot for the Eu^{3+} binding to dIbR has been determined. We have determined the free Eu^{3+} concentration (in equilibrium with the bound ones) from the fluorescence intensity of its chelates. The results strongly suggest that as Eu^{3+} is added to dIbR, the first filled site (the one with the largest binding constant) contains only one Eu^{3+} per bR. The binding constant is found to be pH sensitive suggesting the displacement of 2–3 protons/bR. Combining this result with that of the previous time resolved studies [26] strongly suggests that the emission from the Eu^{3+} ion that first binds to dIbR is not observed in the time-resolved fluorescence experiments in regenerated Eu^{3+}-bR samples [26]. The most likely mechanism of its fluorescence quenching is by energy transfer. Since the emission bands are at 570–630 nm, the only energy acceptor in bR is the retinal. It is shown that for the transfer to occur, the Eu^{3+} has to be extremely near (within a few Å) to the retinal in the Eu^{3+}-regenerated bR system. The implication of this conclusion on the proposed mechanisms of the deprotonation of the PSB in bR is discussed.

1.1. *The method*

We need to determine the number of Eu^{3+} (n) in the strongly bound site with a binding constant K. The following equation [27] is used:

$$\frac{\nu}{L} = K(n-\nu)$$

where ν is the number of moles of bound Eu^{3+} per mole of bR, L is the concentration of unbound free Eu^{3+} in equilibrium with the bound ones. The plot of ν/L vs. ν is the Scatchard plot [27] which should give a straight line with a slope of $-K$ and an intercept that gives n. For any added concentration of Eu^{3+} to a sample of dIbR, if the concentration of the free Eu^{3+} (L) is determined, the moles of Eu^{3+} bound ν can be calculated. We have used chelating agents to enhance the fluorescence [28] of the free Eu^{3+} to determine its concentration after separating it from the bR and its bound Eu^{3+}. A mixture of the 2 chelating agents 2-thenoyltrifluoroacetone (TTA) and tri-n-octylphosphine oxide (TOPO) with polyoxyethylene isooctylphenol (Triton X-100) is used. This was found to give a tetrakis (eightfold) coordination [29], not interact with the solvent and have a much higher quantum efficiency, and therefore a higher fluorescence intensity.

2. EXPERIMENTAL

Halobacterium halobium was grown from master slants of ET1001 provided by Professor R. Bogomolni (University of California, Santa Cruz). bR was purified by using a combination of previously described methods [30,31]. The purple bR suspension was deionized by passing it through a cation exchange column (200–400 mesh hydrogen form AG50W-X8; Bio-Rad) [32]. Eu^{3+}-regenerated bR was prepared by adding $EuCl_3$ (99%; Sigma) standard solution with a micropipette to the dIbR. Eu^{3+}/bR ratios are expressed as moles of Eu^{3+} added to moles of dIbR. The latter was determined by using an HP 8415A Diode Array absorption spectrophotometer and an absorptivity of 60000 $cm^{-1} \cdot M^{-1}$ [32] at 605 nm. For samples where pH adjustment was done, the purple bR solution was passed through an anion exchange column (100–200 mesh hydroxide form AG1-X4; Bio-Rad) before passing them through the cation exchange column; thus counteracting the pH reducing effect of the second column. The Eu^{3+}-regenerated bR was allowed to equilibrate overnight, then centrifuged at 19000 rpm for 30 min using an SS-34 Sorvall rotor to remove the Eu^{3+}/bR complex (bR bound with Eu^{3+}) as well as the excess dIbR. The supernatant solution was then analyzed for free unbound europium. From the unbound Eu^{3+} concentration and the initial Eu^{3+} concentration, it was possible to determine the amount of bound Eu^{3+} by difference.

The unbound free Eu^{3+} in the supernatant solution was chelated with TTA and TOPO in aqueous 0.2% Triton X-100R solution at pH 3.6 [29]. The concentration of the Eu^{3+} is determined from its emission intensity and that of standard Eu^{3+} solutions chelated in the same manner. The standard solutions of europium were prepared by dissolving europium chloride (99%; Sigma) in doubly deionized water at pH 4, using hydrochloric acid. A TTA-TOPO reagent solution containing 5×10^{-3} M TTA and 5×10^{-4} M TOPO was prepared by dissolving the reagents (99%; Aldrich). A 0.1 M acetate buffer (pH 3.6) was used. To 0.5 ml of the supernatant sample solution or the europium standard solution, 0.3 ml of 0.1 M acetate buffer, 0.2 ml of the TTA-TOPO reagent solution, and 1.5 ml of doubly deionized water were added. Additional reagent was added to maximize the fluorescence.

A Fluorolog2 Series Fluorometer was used to excite the Eu^{3+} chelate at 346 nm and record the emission at 614 nm. The spectra of the Eu^{3+} chelate (see Fig. 1) were measured by using 1.0 cm polystyrene cuvets (Fischer Scientific) and a 90 degree collection angle. A $NaNO_2$ solution filter was used to reject light from the xenon lamp source.

3. RESULTS

The important results of the Eu^{3+} binding studies can be summarized as follows: (i) The technique of enhancement of Eu^{3+} by chelate formation [29] can be used for the determination of unbound Eu^{3+}. The calibration curve is shown in Fig. 2 which obeys Beer's law over the concentration range of 10^{-9}–10^{-5} M Eu^{3+}. These concentrations match the concentrations of unbound europium found in the supernatant solutions in equilibrium with the Eu^{3+}-regenerated bR. (ii) Figure 3 shows the Scatchard plots of the experiment performed at pH 5.3 and 5.6, respectively. The Eu^{3+} to

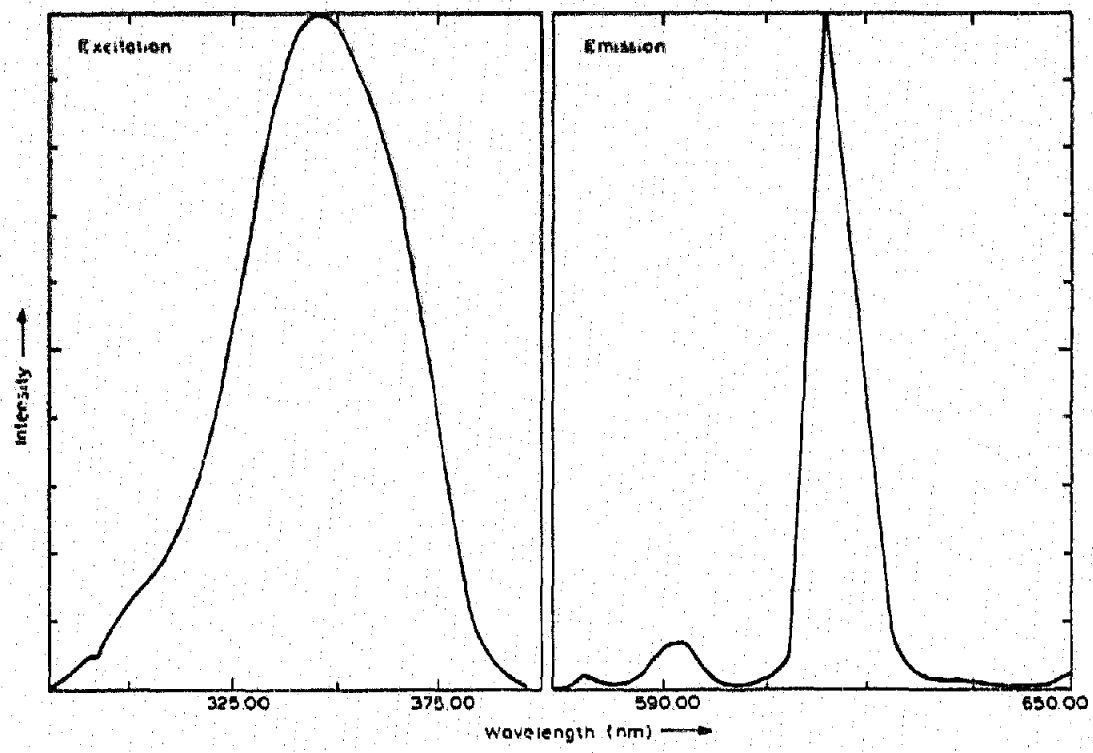

Fig. 1. The fluorescence (right) and excitation (left) spectra of Eu^{3+} TTA-TOPO chelate.

bR ratios varied from 0.2 to 1.0. The linear relationship (with correlation coefficients, R^2, of 0.99 and 0.97 at pH = 5.6 and 5.3, respectively) indicates only one type of site present in this ratio range. The binding constant given from the slope is $4-5 \times 10^7$ at pH 5.3 and 3×10^8 at pH 5.6. The x-intercepts, which give the number of europium ions bound in the site with this value of binding constant, are found to have a value of 0.75 and

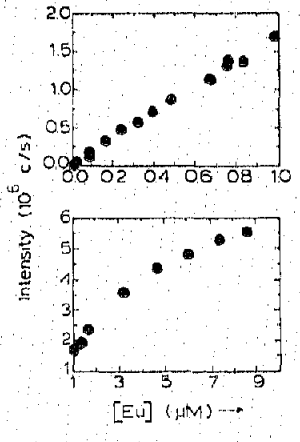

Fig. 2. The dependence of the fluorescence emission intensity of Eu^{3+} chelate on concentration for the standard solutions. From this plot and the intensity of chelated Eu^{3+} in the supernatant solutions, the concentrations of the free Eu^{3+} in equilibria with the bound Eu^{3+} in different regenerated Eu^{3+} bR solutions is calculated.

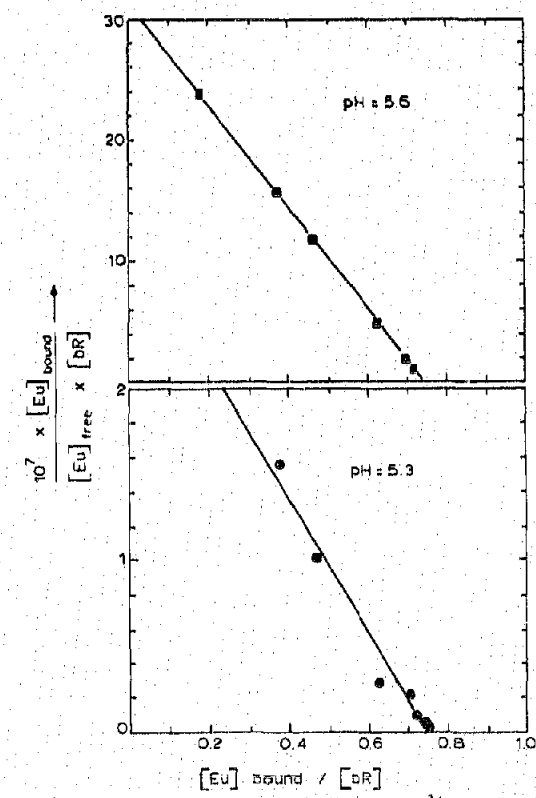

Fig. 3. The Scatchard plot for the binding of Eu^{3+} to deionized bR at pH = 5.6 and 5.3. From this plot, the binding constants as well as the number of Eu^{3+} ions in this strongly bound site are determined from the slopes and x-intercepts, respectively.

0.87, indicating that about one europium was bound to each bR in the strongly bound site. Non-Scatchard-like behavior was found at higher Eu^{3+}/bR ratios which is probably due to self aggregation of the bR and/or the presence of other weaker bound sites. In order to prevent self aggregation, polyacrylamide gels have been used previously. Unfortunately our fluorescence emission studies show that Eu^{3+} binds to the gel molecule itself, making the use of a gel to prevent aggregation at high Eu^{3+}/bR ratio impractical for this type of studies.

4. DISCUSSION

The slope from the Scatchard plot (Fig. 3) give the equilibrium constant for the reaction:

$$dlbR + Eu^{3+} = Eu^{3+} - bR \qquad K = \frac{[Eu^{3+} - bR]}{[dlbR][Eu^{3+}]} \qquad (1)$$

The value of the constant is found to be $4–5 \times 10^7\ M^{-1}$ at pH = 5.3 and $3 \times 10^8\ M^{-1}$ at pH = 5.6. Thus it is not a constant and suggests that a number of hydrogen ions (e.g. m) are part of the equilibrium. Thus the real equilibrium, K', should be written as:

$$dlbR + Eu^{3+} = Eu^{3+}bR + mH^+$$

$$K' = \frac{[Eu^{3+} - bR][H^+]^m}{[dlbR][Eu^{3+}]} = K[H^+]^m \qquad (2)$$

One can obtain m from the ratio of two K's (K_1/K_2) at 2 different hydrogen ion concentrations $[H^+]_1$ and $[H^+]_2$ from the following equation that is derived from expressions (1) and (2) above:

$$m = \log\frac{K_1}{K_2} \Big/ \log\frac{[H^+]_2}{[H^+]_1} \qquad (3)$$

With at least 30% uncertainties in the values of K, m is calculated to be between 2 and 3. This suggests that the binding site probably involves at least 2 carboxylate groups.

The above results suggest that the first strongest binding site contains one Eu^{3+} rather than 3. Previous time-resolved fluorescence results [26] on Eu^{3+}-regenerated bR containing 4 Eu^{3+}/bR showed the presence of only 3 or 4 Eu^{3+} ions in sites with different decay times but with not too different binding constants. If these 3 sites are the strongest bound, a Scatchard plot should have given an intercept of 3 or 4 and not only one. Since this is not observed, one is forced to propose that the time-resolved experiments did not detect the Eu^{3+} emission from the site with the highest affinity but only detected the emission from the 3 Eu^{3+} in the sites with the next-to-highest affinity. Unfortunately, due to aggregation and the lower sensitivity of the present studies, we could not go to higher concentrations to determine their binding constants.

Even though we do not observe the emission from the Eu^{3+} in the very strongly bound site, the filling of this site should affect indirectly (and thus weakly) the emission intensity of the 3 Eu^{3+} in the other type of sites as the Eu^{3+}/bR ratio changes from <1 to >1. This is because all 4 types of sites compete for the added Eu^{3+} ion. There are 2 reasons for not observing this effect in the Corcoran et al. work [26]. First, even though the binding constant of the very strongly bound site is 10 times larger than to the other 3 strongly bound sites, the affinity ratio for binding to these 2 different types of sites is reduced to 10:3, since only one Eu^{3+}, binds to the very strongly bound site and 3 binding to the second site. Secondly, the intensity of the bound Eu^{3+} in the 0–1 Eu^{3+}/bR ratio range is sufficiently weak (and thus the errors are large) that any small curvature in the plot of the intensity vs Eu^{3+}/bR ratio would not be detected.

The question is then raised as to why the emission from the Eu^{3+} ion in the highest affinity site was not observed, i.e. why was its emission quenched? It is known that retinal acts as an excitation energy sink for most of the tryptophan emission in bR [33–36]. Having the lowest electronic excitation energy in the system, retinal becomes the most viable candidate to accept the electronic excitation energy of the Eu^{3+} ions if they were both placed within coupling range of one another. How close does Eu^{3+} have to be in order for its emission to be completely quenched would depend on the mechanism of the coupling between the 2 electronic systems. There are 2 mechanisms possible, the dipolar (Forster type [37]) and the exchange (Dexter type [38]) mechanisms. For the exchange mechanism, orbital overlap is required and thus short separation distance (in the few Å range) is a must. Dipolar energy transfer can take place over distances as long as 50 Å but only if: (i) both the donor and the acceptor have large electronic transition dipole moments (i.e. have strong absorptions), and (ii) the emission frequency distribution of the donor (Eu^{3+}) overlaps well with that of the absorption of the acceptor. These 2 conditions lead to a reduction of the transfer probability in the Eu^{3+}-retinal system, thus requiring relatively short distances. First, while the $^7F_{0,1,2} \longleftarrow {}^5D_0$ emission of Eu^{3+} is embedded within the absorption band of retinal, its narrow band width makes the absolute value of the overlap to be small. In addition, while the electronic transition of retinal has a reasonable dipole moment, that of Eu^{3+} does not as it is inherently forbidden transition and is weakly induced by the crystal field at the binding site. The latter fact alone could reduce the *maximum* transfer distance from 50 Å to 20 Å. Since the emission is completely quenched, one would guess that Eu^{3+} must be within 10–15 Å from the retinal even if the long range dipolar mechanism was responsible for the Eu^{3+} fluorescence quenching. This suggests that, irrespective of the transfer mechanism, Eu^{3+} could very well be within the retinal pocket.

The fact that both Ca^{2+}, Mn^{2+} and Eu^{3+} all have one

very strongly bound site (and 3 strong sites) suggests a similar site for all. Using Henderson's model for the structure of bR [25], binding to carboxylate groups of aspartic acid residues 85 and 212 is an attractive possibility. The presence of the Ca^{2+} within the active site might provide the reason for the presence of 2 aspartate groups within the active site. The presence of water molecules in the retinal pocket bonded to the protonated Schiff base (PSB) as well as to the Ca^{2+} (or Mg^{2+}) could shield the repulsion between the positive charges on the PSB and that of the Ca^{2+} (or Mg^{2+}). The unusual infrared spectroscopic behavior of Tyr-185 as well as the charge perturbation of tryptophan 182 (leading to the 296 nm transient absorption [39]) might all be a result of the strong charge perturbation by this metal cation.

If the above conclusions are indeed correct, the presence of several positive charges (on the PSB, on the metal cations and on Arg-82), 2 negative charges (on the carboxylate of aspartic acids 85 and 212), a few water molecules and strong H-bonding with tyrosine 185 could all lead to highly inhomogeneous and anisotropic electric fields within the retinal pocket (the active site). The electric field at the PSB could thus be extremely sensitive to small changes in the coordinates of these different charged species and the few water molecules during the protein conformation changes occurring during the photocycle. This might make credible the previously proposed [40] mechanism of reducing the pK_a of the PSB that leads to its eventual deprotonation during the $L_{550} \longrightarrow M_{412}$ transformation by the electrostatic repulsion between its positive charge and an exposed positive field during this step in the photocycle. This positive field might very well be due to the rearrangement of the water molecules and the carboxylate groups in such a manner as to unshield the charge of the metal cation within the pocket (Ca^{2+} or Mg^{2+}).

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REFERENCES

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature (London) New Biol. 233, 149–152.
[2] Henderson, R. (1975) J. Mol. Biol. 93, 123–138.
[3] Oesterhelt, D. and Stoeckenius, W. (1971) Nature (London) New Biol. 233, 149–152; Bridgen, J. and Walker, I.D. (1976) Biochemistry 15, 792–798; Bayley, H., Huang, K.S., Radhakrishnan, R., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
[4] Lozier, R., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 215–278; Mathies, R.A., Cruz, C.H., Pollard, W.T. and Shank, C.V. (1988) Science 240, 777–779.
[5] Dencher, N. and Wilms, M. (1975) Biophys. Struct. Mech. 1, 259–271.
[6] Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 52, 587–619; Stoeckenius, W. (1979) Biochim. Biophys. Acta 505, 215–279.
[7] Oesterhelt, D. and Stoeckenius, W. (1971) Nature (London) New Biol. 233, 149–152.
[8] Moore, T.A., Edgerton, M.E., Parr, G., Greenwood, C. and Perham, R.N. (19) Biochem. J. 171, 469–476.
[9] Mowery, P.C., Lozier, R.H., Chae, Q., Tseng, Y.-W., Taylor, M. and Stoeckenius, W. (1979) Biochemistry 18, 4100–4107.
[10] Kimura, Y., Ikegami, A. and Stoeckenius, W. (1984) Photochem. Photobiol. 40, 641–646.
[11] Fischer, U. and Oesterhelt, D. (1979) Biophys. J. 28, 211–230.
[12] Dupuis, P., Corcoran, T.C. and El-Sayed, M.A. (1985) Proc. Natl. Acad. Sci. USA 82, 3662–3664.
[13] Chronister, E.L. and El-Sayed, M.A. (1987) Photochem. Photobiol. 45, 507–513.
[14] Kobayashi, T., Ohtani, H., Iwai, J., Ikegami, A. and Uchiki, H. (1983) FEBS Lett. 162, 197–200.
[15] Dunach, M., Seigneuret, M., Rigaud, J.-L. and Padros, E. (1987) Biochemistry 26, 1179–1186.
[16] Chang, C.-H., Jonas, R., Melchiore, S., Govindjee and Ebrey, T.G. (1986) Biophys. J. 49, 731–739.
[17] Ariki, A. and Lanyi, J.K. (1986) J. Biol. Chem. 261, 8167–8174.
[18] Chang, C.-H., Chen, J.G., Govindjee and Ebrey, T. (1985) Proc. Natl. Acad. Sci. USA 82, 396–400.
[19] Chronister, E.L., Corcoran, T.C., Song, L. and El-Sayed, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 8580–8584.
[20] Chronister, E.L. and El-Sayed, M.A. (1987) Photochem. Photobiol. 45, 507–513.
[21] Dunach, M., Seigneuret, M., Rigaud, J.-L. and Padros, E. (1987) Biochemistry 26, 1179–1186.
[22] Dunach, M., Seigneuret, M., Rigaud, J.-L. and Padros, E. (1986) Biosci. Rep. 6, 961–966.
[23] Szundi, I. and Stoeckenius, W. (1989) Biophys. J. 56, 369–383.
[24] Jonas, R. and Ebrey, T.G. (1990) Proc. Natl. Acad. Sci. USA 88, 149–153.
[25] Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckman, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
[26] Corcoran, T.C., Ismail, K.Z. and El-Sayed, M.A. (1987) Proc. Natl. Acad. Sci. USA 84, 4094–4098.
[27] Scatchard, G. (1949) Ann. NY Acad. Sci. 51, 660–691.
[28] For review see: (a) Crosby, G.A. (1966) Molecular Crystals; (b) Harrock, DeW. and Albin, M. (1984) in: Progress in Inorganic Chemistry (Lippard, S.J. ed.) Wiley, NY, 31, pp. 1–105.
[29] Taketatsu, T. and Sato, A. (1979) Anal. Chim. Acta 108, 429–432.
[30] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[31] Becker, B.M. and Cassim, J.Y. (1975) Prep. Biochem. 5, 161–178.
[32] Kimura, Y.A., Ikegami, A. and Stoeckenius, W. (1984) Photochem. Photobiol. 40, 641–646.
[33] Sherman, W.V. (1981) Photochem. Photobiol. 33, 367–371.
[34] Sherman, W.V. (1982) Photochem. Photobiol. 36, 463–469.
[35] Palmer, P.L. and Sherman, W.V. (1985) Photochem. Photobiol. 42, 541–547.
[36] Jang, D.-J., Corcoran, T.C. and El-Sayed, M.A. (1988) Photochem. Photobiol. 48, 209–217.
[37] Forster, Th. (1948) Ann. Phys. 2, 55–65; (1959) Disc. Farad. Soc. 27, 7–20.
[38] Dexter, D.L. (1953) J. Chem. Phys. 21, 836–843.
[39] Wu, S., Jang, D.-J., El-Sayed, M.A., Stern, L.J., Mogi, T. and Khorana, H.G. (1990) FEBS Lett. (submitted).
[40] (a) Hanamoto, J.H., Dupuis, P. and El-Sayed, M.A. (1984) Proc. Natl. Acad. Sci. USA 81, 7083–7087. (b) El-Sayed, M.A. (1988) Int. J. Quantum Chem. 22, 367–375.