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PHOTOSELECTION AND AGGREGATION IN PURPLE MEMBRANE OF *HALOBACTERIUM HALOBIUM*

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Summary

Preparations of the purple membrane of *Halobacterium halobium* suspended in dilute buffer and basal salt have been examined by circular dichroism spectroscopy and correlation analysis of scattered laser light. Dark adapted samples suspended in basal salt show photoselection when examined by circular dichroism. This was confirmed by irradiation with plane polarized light. Light-adapted samples or dark-adapted samples suspended in dilute buffer did not show this phenomenon. The reaction responsible for photoselection was shown to be the light induced *cis-trans* isomerization of bacteriorhodopsin. The stability of the induced anisotropy was due to aggregation in the basal salt suspensions which occurred despite little or no visual indication. This aggregation was confirmed by correlation analysis of scattered laser light.

Introduction

Studies on basal salt suspensions of the purple membrane of *Halobacterium halobium* have held a central role in the investigation of the function of this energy transformation system [1]. Circular dichroism measurements of the purple membrane in dilute buffer demonstrated chromophore-chromophore interactions [2—4], indicating a highly ordered state of the pigment within the membrane, a result corroborating independent studies based on the use of electron scattering techniques [5]. Independent investigations carried out in this laboratory [6] were in general agreement with the finding of chromophore-chromophore interactions. We present here a new aggregation phenomena of the membrane system as indicated by photoselection as well as light scattering fluctuation spectroscopy [7].

Materials and Methods

Dichroism measurements were made on a Jobin Yvon Dichrograph III coupled to a Nicolet Model 1074 Instrument Computer through a 100X linear

amplifier and a Rockland Model 1020F active RC filter. Background scans were digitally subtracted from the spectra to obtain a flat baseline. Typical measuring conditions were 25°C, 20 Å bandwidth program, 1 nm/s scan, $10 \cdot 10^{-6}$ extinction units/mm scale, 1 Hz low pass filter and 1 cm optical path.

The purple membrane was prepared as given in [1,8] and suspended in basal salt solution [1] or in 50 mM phosphate buffer at pH 6.9. The protein concentration ranged from $1.4 \cdot 10^{-5}$ M to $2.5 \cdot 10^{-5}$ M, based on a molar extinction coefficient of 63 000 $M^{-1} \cdot cm^{-1}$ at 570 nm [1]. The dark adapted samples were held in darkness for 12 h at room temperature, or in some cases at 4°C, in stoppered cuvettes prior to agitation and insertion in the spectrometer for measurement. Light-adapted samples were irradiated for 10 min with light from a 250 W Fiber optics light source (Schott-Mainz LK150B) protected with a 560 nm high-pass glass filter (Eppendorf 105600). After bleaching in the measuring cuvette with shaking, the samples were immediately inserted into the spectrometer for measurement.

A Polaroid filter was placed in the holder on the "sample side" of the Eppendorf filter. The flexible head of the light source was mounted in a fixed position 2 cm from the sample cuvette in the dichrograph and removed during measurements. Between 500 and 700 nm the polaroid filter showed a relatively flat absorption spectrum with an extinction coefficient of approx. 0.5. Polarization irradiation was for 1 min at the lowest setting of the lamp power source. Matching irradiation without the polarizer was for 30 s at the same power setting.

Light scattering studies of aggregation were conducted with a Malvern System 4300 Correlation Spectrometer using a Spectra Physics 125A, 50 mW He-Ne laser emitting 632.8 nm light as the radiation source. The high scattering intensity of these samples and long sampling times required the use of neutral density filters to reduce laser beam intensity and/or the Malvern Clipping Counter Model RR 95 to extend the clip level. Calculations of correlation time, diffusion constant and extrapolation to zero scattering angle for measurement of rotational diffusion were done according to a method described elsewhere [9].

Results

Measurement of the circular dichroism spectra of the light- and dark-adapted purple membrane, suspended in basal salt as well as dilute phosphate buffer, reveals the composite spectrum given in Fig. 1 with a slightly asymmetric bilobe centered at 570 nm, a negative maximum at 312 nm and a positive peak complex in the region 265—275 nm, confirming results published elsewhere [2]. In an analysis of the ionic strength effect on these phenomena it was found that only in the dark-adapted condition in basal salt, the circular dichroism spectra changed slightly during the course of a scan. At lower scan speeds, successive scans of the dark-adapted sample generated a family of curves with isosbestic points at 540, 445 and 390 nm as shown in Fig. 2. This phenomena is not observed at low ionic strength. It is reversible by shaking in the dark and is thus dependent on the spatial arrangement of the membrane particles.

In order to exclude settling of the membrane a sample was allowed to stand

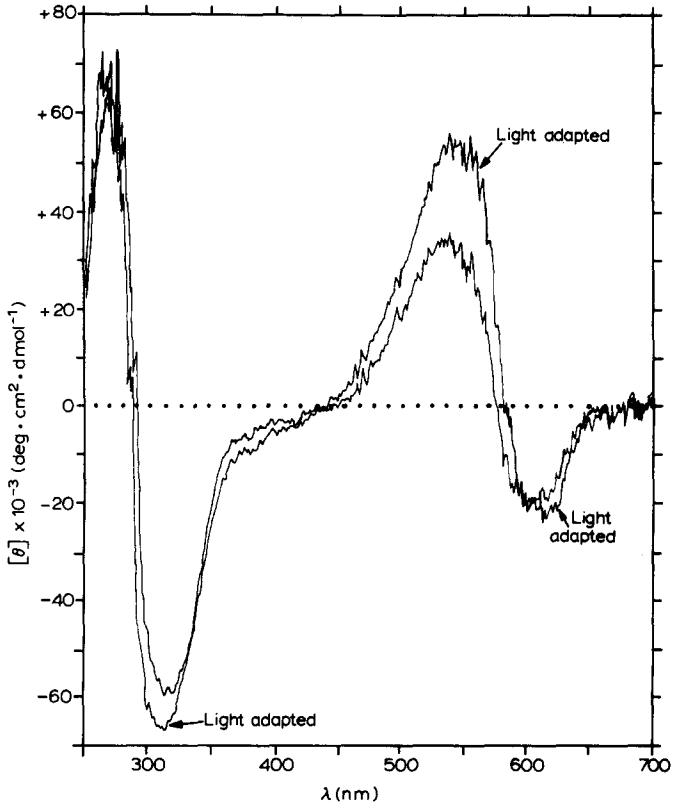

Fig. 1. Circular dichroism spectrum of light- and dark-adapted purple membrane suspensions in basal salt. The scan speed was 1 nm/s.

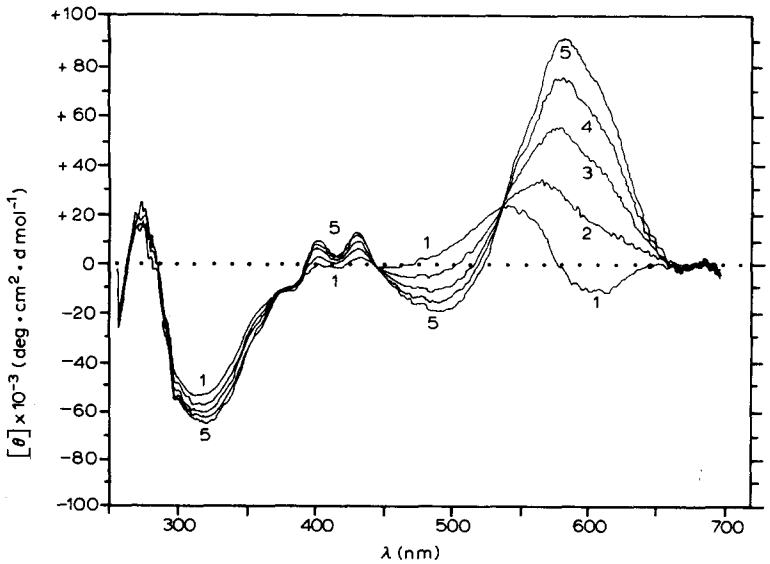

Fig. 2. The development of a "curve family" during successive scans in the circular dichrograph. Each complete scan required 15 min. The back scan and waiting periods were accomplished with the sample completely in the dark. Each fresh scan was started precisely 20 min after the beginning of the preceding scan.

in the spectrometer for over 1 h before the first measurement was taken. The curve then obtained was the typical bilobe curve (curve 1 in Fig. 2) leading to the family formation on successive scanning. By variation of the time of measurement and thus the exposure of the sample to the measuring light beam, it was shown that light is the causative factor of the family curve formation. By varying the initial wavelength at which the spectral scans were carried out, curve family development was shown in the spectral range between 400 and 650 nm.

Since the light effect is induced by circularly polarized light of the measuring beam, the question was raised whether plane polarized light would also induce this phenomenon. When the sample was irradiated through a Polaroid filter (vertically polarized) for 1 min and immediately scanned in the CD spectrometer the envelope of a typical curve family as shown in Fig. 3a was found indicating that curve family formation was dependent on the presence of an ordered light source. Here again the phenomenon was reversible with agitation and was not observed in the light-adapted state of the purple membrane. On rotating the axis of polarization 90°, an inversion of the envelope curve of Fig. 3a, giving the curve of Fig. 3b, was recorded. This result was not obtained in a normal light-adapted purple membrane suspension. Irradiation of a dark-adapted sample with an equivalent amount of non-polarized light produced normal light adaptation effect. This is not shown in a diagram since at the scale used for demonstration of photoselection effects, there is no apparent difference between light-adapted and dark-adapted curves. In either filter orientation, extended irradiation (30 min) resulted in the replacement of the family or inverted family curve envelope with a normal light-adapted purple membrane spectrum of the type seen in Fig. 1. Both experiments show the dependence of this spectral behavior upon an anisotropic light source as exemplified by plane or circularly polarized light. These results clearly indicate that the curve family effect results from photoselection.

Photoselection in the circular dichroism measurement can be detected under the following conditions: the time lapse betwee the production of photoselection and the detection of the photoselected population must not be greater than either the life-time or rotational relaxation time of the photoselected molecules. Thus, it implies a relatively restricted rotation of the chromophore. Since the development of the curve family effect was dependent on the ionic strength of the medium, the question was whether the membrane particles change their state of aggregation as a function of the ionic strength. An analysis of the diffusion coefficient of the particles was carried out in low ionic strength (dilute buffer) and in high ionic strength (basal salt) using the technique of digital autocorrelation of intensity fluctuations in scattered laser light [10]. If the particle dimension is comparable to the wavelength of the laser light, rotational as well as translational diffusion affects the spectrum. Using a thin disk with a diameter of approx. 0.5 μm as a model of the membrane fragment, the dominant term of the correlation function should be [9]:

$$g(t) = e(-(k^2 D_T + 6D_R)t)$$

where D_T = translational diffusion coefficient, D_R = rotational diffusion coefficient, $k = 4\pi\, n\sin(\theta/2)/\lambda$, n = refractive index of the solution, θ = scatter-

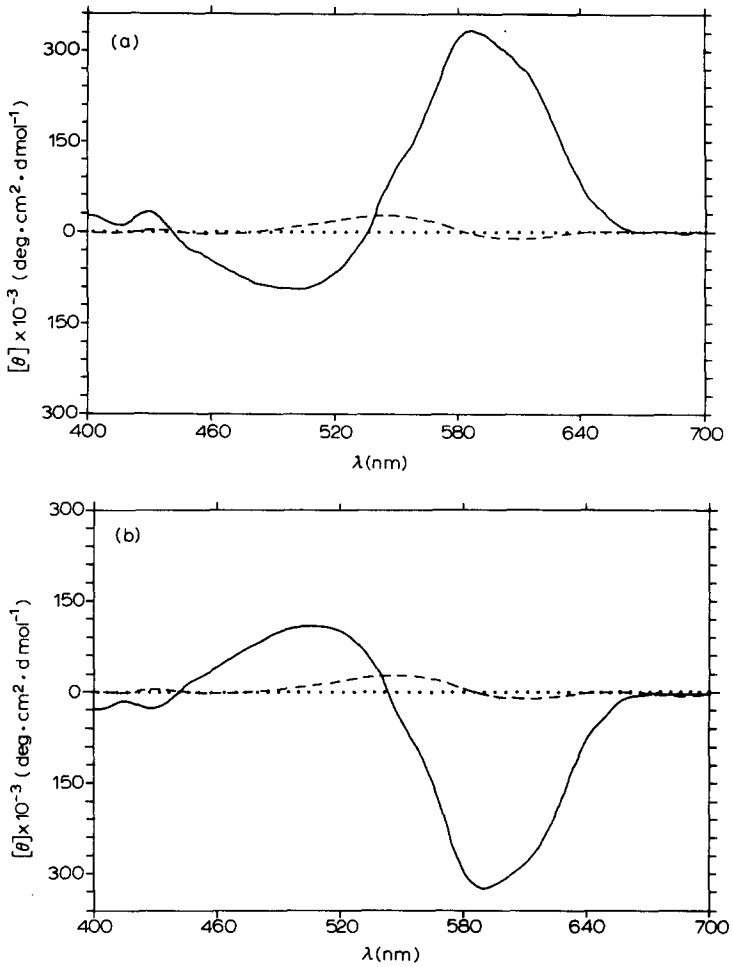

Fig. 3. (a) The circular dichroism spectrum of dark-adapted purple membrane (dashed line) compared with the spectrum of the same sample after 30 s illumination with plane polarized light (vertical axis of polarization). (b) The circular dichroism spectrum of dark-adapted purple membrane (dotted line) compared with the spectrum of the same sample after 30 s ilumination with plane polarized light (horizontal axis of polarization).

ing angle and λ = laser wavelength.

In dilute buffer the scattering spectrum of the purple membrane can be represented by a single exponential function. A typical correlation decay curve measured at 90° with the intensity fluctuation spectrometer was obtained as shown in the insert of Fig. 4. The correlation rate plotted as a function of $\sin^2\theta/2$ (Fig. 4, curve a) follows the relationship:

$$\Gamma = k^2 D_T + 6D_R$$

From the slope and the intercept one obtains:

$$D_T^{20} = 3.65 \pm 0.05 \cdot 10^{-9}\ \mathrm{cm^2/s}$$

$$D_R^{20} = 10.4 \pm 0.2\ \mathrm{s^{-1}}$$

The rotational diffusion coefficient is in agreement with the value obtained

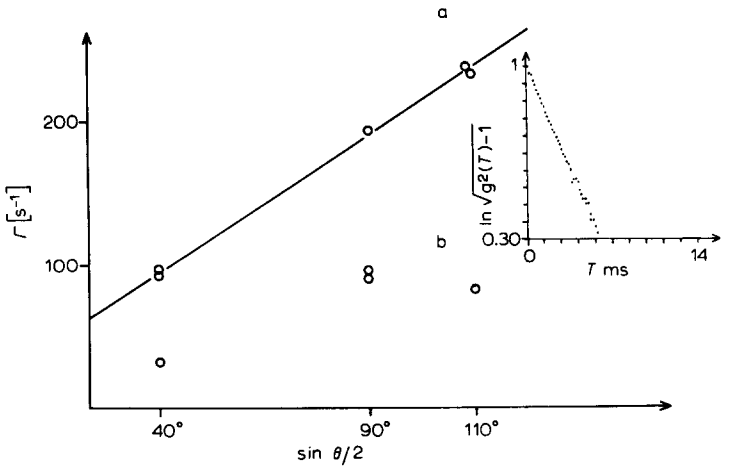

Fig. 4. The correlation time of purple membrane suspensions as a function of the scattering angle. Curve a suspended in dilute buffer, curve b, suspended in basal salt. Insert: A plot of the log of the correlation function versus time for purple membrane.

by transient linear dichroism measurements of purple membrane at low ionic strength and with semiquantitative estimates based on the known dimensions and shape of the membrane fragments [11]. Thus, the purple membrane fragments studied in dilute buffer can be aggregated only to a small extent, if at all.

In the case of the basal salt suspension (Fig. 4, curve b) the angular dependency of the correlation time is distinctly non-linear. In fact the zero angle intercept and hence the rotational correlation time would be expected to be much smaller than that found in the case of the dilute buffer suspension, indicating slower translational and rotational movements of the membrane particles over the time of the measurements.

Discussion

The development of a family of curves during repeated dichroic measurements of visual pigment preparations has been observed before in bovine rhodopsin fixed in glycerol-glasses at 77 K [12]. First identified as the development of a series of transient intermediates stabilized at the low temperature of the measurements, only several years later was the phenomenon correctly identified as photoselection by the original authors [13]. This particular example caused some problems of interpretation for investigators attempting to study the transient intermediates of rhodopsin [14] by dichroic methods even after they were aware of the possibility of this systematic error.

Although photoselection in circular dichroism measurements in rigid media is commonplace, its occurrence in a liquid, non-viscous sample at room temperature is rare if not unique. It is, therefore, important to establish the mechanism reponsible for this "curve family effect".

Spectral anomalies arising during dichroic measurements as a result of interaction between a specimen and polarized light are based on some form of polarization in the inducing radiation, a photoreaction producing a stable or

metastable product and a rigid support medium to prevent thermal dissipation of the resulting anisotropy [15]. The ordered structure results from the requirement that the plane polarization of the light must be parallel to the electrical vector of the chromophore to result in photoactivation. Clearly, then either plane or circularly polarized light will induce an ordered structure which will cause linear dichroism and wavelength-dependent birefringence interpreted by the CD spectrometer as circular dichroism [16].

The stability of these photoselection curves over several hours indicates that the induced photochemical change involves a relatively stable photoproduct. The photochemical cycle of purple membrane in the light-adapted state [17,18] and that of purple membrane containing only 13-*cis* retinal [18] involve two sequences of transient intermediates which recycle to the original chromophores. All these intermediates have very short half-lives and are thus excluded from consideration.

Since it is the dark-adapted form which undergoes photoselection, the difference between light-adapted and dark-adapted purple membrane must be considered. Light-adapted purple membrane has all of its retinal present as the all-*trans* isomer [19,20]. Although dark-adapted purple membrane was earlier considered to have its retinal chromophore totally in the 13-*cis* configuration [19], recent information establishes it as a mixture of about 50% 13-*cis* isomer and 50% all-*trans* isomer [20]. Although indirect evidence suggests that there may be slight, as yet undefined, differences in protein conformation between light and dark adapted forms or purple membrane [33,34], aside from the differing *cis-trans* isomer composition, no difference in molecular structure between the light- and dark-adapted forms has been reported. The shift in absorption maximum from 560 nm in dark-adapted purple membrane to 568 nm in the light-adapted form has been shown to be entirely a result of isomer composition [18].

While light-adaption and hence the photoinduced *cis* to trans conformation change of the individual retinal molecules is rapid [18], the conversion of light-adapted to dark-adapted purple membrane is relatively slow. Half-times for this reaction have been reported with values ranging from 20 min (at 35° C) to 40 min (at 23° C) [8,21]. In considering the apparent identity between light- and dark-adapted states and differential isomer content, this is equivalent to the statement that the photoinduced conversion of 13-*cis* retinal to all-*trans* retinal is much more rapid than the thermal conversion of the *trans* to *cis* isomers. Throughout these discussions, the retinal is considered to be bound into the purple membrane via a Schiff base to a lysine [22].

Irradiation with polarized light causes conversion of dark-adapted purple membrane to light-adapted purple membrane to occur in an ordered fashion, thus inducing photoselection. At the molecular level, only appropriately oriented 13-*cis* isomers are converted to all-*trans* isomers. Initially, the patterning thus developed is lost against the random background of mixed *cis* and *trans* retinal isomers, but as more oriented *cis-trans* conversion occurs, the patterning dominates the random background and manifests itself as photoselection. This is an extrememly powerful effect as is shown by the relative amplitude of photoselection and pure circular dichroism curves for purple membrane (Fig. 3a and b). The equivalent amount of non-polarized light

produces only normal light adaptation effects which are nearly two orders of magnitude smaller than the corresponding photoselection effects.

The ordered conversion of 13-*cis* retinal to all-*trans* retinal by polarized light is thus specifically responsible for the development of photoselection in dark-adapted purple membrane. This is confirmed by two observations. First, "curve family" development does not occur in light-adapted purple membrane in which all the 13-*cis* retinal has been transformed to all-*trans* retinal, excluding the possibility of pattern formation due to selective conversion. Second, extended illumination with plane polarized light results in eventual loss of the spectral "family curve" feature resulting in the development of a normal light-adapted CD spectrum.

This family curve conversion to a normal light-adapted spectrum occurs because incomplete polarization and scattering depolarization of the irradiating light causes random orientation of some of the photoadapted molecules. The large excess of polarized light results in rapid initial development photoselection. The concentration of randomly oriented photoproducts builds up more slowly, but the initial ordering is eventually submerged in the background of transformed chromophores (30 min).

The apparent spatial stability of the purple membrane in basal salt suspension despite the low viscosity of the medium and the lack of visual indication of extensive aggregation required more direct proof. In dilute buffer the spectrum of the purple membrane can be represented by a single Lorentzian curve (Fig. 4, insert) which persists even at low scattering angles. This yields a rotational diffusion coefficient of $10.4\ s^{-1}$ (Fig. 4, curve a). This value is far too low to permit observation of photoselection effects, and this is in agreement with the results obtained in dichroism measurements in dilute buffer. The standard method of preparation ensures that the particles will have a relatively homogeneous size distribution (0.5 μm diameter) in dilute buffer suspension [22].

In basal salt an increased scattering intensity and a lower translational diffusion coefficient indicate aggregation. The rotational diffusion is too small to give a significant contribution to the spectrum. As a consequence of the large aggregate size, the correlation function is not a pure exponential but tends to be Gaussian. Therefore, the correlation rate does not vary linearly with k^2.

We may assume that the orientation of the assembled aggregates is essentially random. Were this is not the case, linear dichroism effects would be observed in light-adapted purple membrane in basal salt suspension. While such a result clearly indicates that extensive aggregation has occurred and is thus in agreement with the overall findings of the dichroism measurements, an extension of the limits of the rotation time into the region of 1 h would be desireable. Unfortunately, the ability to make such an estimate is limited by the experimental error inherent in making extrapolations from large particles of indefinite structure.

The purple membrane is 75% protein and 25% lipid, of which 71% is either phosphatidyl or sulfato lipid. Structural studies suggest that a significant portion of the membrane surface is protein, although diet confirmation is lacking [17]. Thus an explanation for aggregation of purple membrane in high ionic strength media may be sought in part from the aggregation properties of

proteins and the surface behavior of membranes with hydrophylic surfaces.

Intramolecular secondary bonding, in large part responsible for the compact structure of proteins may act intermolecularly to produce association between protein molecules [23]. This is seen, for example, in both insulin, [24] and β-lactoglobin [25] as a function of pH. While hydrogen ion concentration of the membrane preparations is not directly altered, the large change in ionic strength produces effects equivalent to those caused by pH changes. The change in membrane surface potential produced by ionic strength changes can markedly alter the ionization of groups attached to the surface [26]. Increasing ionic strength tends to reduce the apparent pK of surface-attached ionizable groups. If one considers the protein portion of the membrane surface as a special case, the same result is obtained. The alteration of pK in soluble proteins as a function of ionic strength is well established and an exhaustive discussion of the interaction of side-chain group ionization, ionic strength and pH has been presented [27]. Specifically, in the case of purple membrane, pH titration at high ionic strength has shown major changes in pK of the various substituents: the pK of the acidic groups tending to become smaller in agreement with theoretical predictions for acidic groups attached to membranes [28] and the pK of basic groups remaining unchanged. This suggests the possibility for attractive interactions between groups of differing charge. Such interactions would be facilitated since the diffuse counter layer which tends to shield membrane groups from external charge interactions is drastically reduced in thickness at high ionic strength [26].

These mechanistic considerations completely ignore possible structural or charge assymetry in the purple membrane. X-ray diffraction investigations are not consistent with a symmetrical membrane and suggest that there may be more lipid on one side than the other [29,32]. Field orientation experiments [30] and field jump measurements [11,31] demonstrate the dipolar nature of the purple membrane particles though at the present time it is impossible to say whether a permanent or induced dipole is being observed. While at low ionic strength the diffuse counter ion layer can be quite thick and might tend to shield local differences in charge distribution or geometry, marked compression of this layer at high ionic strength would permit local charge differences to be more easily sensed and permit interaction between specific features on adjacent surfaces. Thinking in terms of surface potential, which behaves inversly to the thickness of the diffuse counter ion layer [26], low ionic strength would produce increased surface potential tending to separate adjacent surfaces and prevent local feature interaction. This clarifies membrane behavior at ionic strength approaching zero where very thick counter ion layers and their meaning are hard to visualize. Thus, lack of purple membrane aggregation in pure water does not contradict this mechanistic interpretation of events. Such a picture suggests that compression of the diffuse charge layer or reducing of surface potential could be sufficient in itself to cause aggregation without excluding the possibility that aggregation might be enhanced by ionic strength-induced pK and membrane surface changes.

References

1 Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316—326
2 Heyn, M.P., Bauer, P.J. and Dencher, N.A. (1975) Biochem. Biophys. Res. Commun 67, 897—903
3 Becher, B. and Cassim, J.Y. (1975) 19th Meet. Biophys. Soc., Philadelphia, Abstr. W-PM-F2 and W-PM-F3
4 Becher, B. and Ebrey, T.G. (1976) Biochem. Biophys. Res. Comun. 69, 1—6
5 Unwin, P.N.T. and Henderson, R. (1975) J. Mol. Biol. 94, 425—440
6 Reed, T. and Hess, B. (1976) Minisymposium on Light Energy Transducing Systems 67th Meet. Am. Soc. Biol. Chem., San Francisco, Abstr. 45
7 Reed, T. and Hess, B. (1976) FEBS Symposium on Biochemistry of Membrane Transport, Zürich, Abstract, p. 116
8 Oesterhelt, D., Meentzen, M. and Scuhmann, L. (1973) Eur. J. Biochem. 40, 453—463
9 Aragon, S.R. and Pecona, R. (1977) J. Chem. Phys. 66, 2506—2516
10 Chu, B. (1974) Laser-Light Scattering, pp. 202—204, Academic Press, New York
11 Shinar, R., Druckmann, S., Ottolenghi, M. and Korenstein, R. (1977) Biophys. J. 19, 1—5
12 Horwitz, J. and Heller, J. (1977) Biochemistry 10, 1402—1409
13 Heller, J. and Horwitz, J. (1973) in Biochemistry and Physiology of Visual Pigments (Langer, H., ed.), pp. 57—68, Springer Verlag, Berlin
14 Yoshizawa, T. and Horiuchi, S. (1973) in Biochemistry and Physiology of Visual Pigments (Langer, H., ed.), pp. 68—81, Springer Verlag, Berlin
15 Weigert, F. (1920) Ann. Phys. 4, 63, 681
16 Disch, R.L. and Sverdlik, D.I. (1969) Anal. Chem. 41, 82—86
17 Henderson, R. (1977) Annu. Rev. Biophys. Bioeng. 6, 87—109
18 Sperling, W., Carl, P., Rafferly, Ch. N. and Dencher, N.A. (1977) Biophys. Struct. Mech. 3, 79—94
19 Jan, L.Y. (1975) Vision Res. 15, 1081—1086
20 Pettei, M.J., Yudd, A.P., Nakanishi, K., Hensleman, R. and Stoeckenius, W. (1977) Biochemistry 16, 1955—1959
21 Ebrey, T.G., Becher, B., Mao, B., Kilbrink, P. and Honig, B. (1977) J. Mol. Biol. 112, 377—397
22 Oesterhelt, D. and Stoeckenius, W. (1971) Nat. New Biol. 233, 148—152
23 Tanford, C. (1967) Physical Chemistry of Macromolecules, pp. 519—523, John Wiley and Sons Inc., New York
24 Townend, R. and Timasheff, S.N. (1956) Arch. Biochem. Biophys. 63, 482—487
25 Gutfreund, H. (1952) Biochem. J. 50, 564
26 Träuble, H. (1977) Membrane Electrostatics, in Structure of Biological Membranes, Proc. of Nobel-Symposium 34 (Abrahamsson, S. and Pascher, J., eds.), pp. 509—550, Plenum Publ. Comp., New York
27 Edsall, J.T. and Wyman, J. (1958) Biophysical Chemistry, Vol. 1, pp. 512—549, Academic Press, New York
28 Hess, B. (1977) Ionic Control of Proton and Electron Transfer, presented at Lindstrom-Lang Conference on Biochemical Control Mechanisms of Respiration at the Molecular and Tissue Level, Espoo, Finland, August 1977
29 Blaurock, A.E. (1975) J. Mol. Biol. 93, 139—158
30 Eisenbach, M., Weissman, C., Tanny, G., and Caplan, R.S. (1977) FEBS Lett. 81, 77—80
31 Hess, B. and Kuschmitz, D. (1976) Coupling of the Photochemical Bacteriorhodopsin Cycle and the Proton Release and Binding in Halobacterium halobium. Post Congress Bioenergetics Workshop on Electrontransport Oxidative Phosphorylation-Photophosphorylation, Hamburg, Abstr.
32 Blaurock, A.E. and King, G.I. (1977) Science 196, 1101—1104
33 Konishi, T. and Packer, L. (1977) FEBS Lett. 80, 455—458
34 Konishi, T. and Packer, L. (1976) Biochem. Biophys. Res. Commun. 72, 1437—1442