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Discussion* to II. Light-Induced Conformational Changes of the Rhodopsin Molecule

Reported by J. Wyman** (Chairman), N. A. Dencher***, and K. Hamacher***

To the question of conformational changes of vertebrate rhodopsin upon bleaching, M. Chabre made the following comment:

Small but definite conformation changes upon bleaching have been observed for rhodopsin in intact rod outer segment disc membrane and also for delipidated rhodopsin in detergent micelles.

– By X-ray diffraction on intact retina [1] and on isolated rod outer segment preparations oriented in magnetic fields [2], a structural change is observed on the cytoplasmic side of the disc membrane.

– Neutron diffraction on isolated rod outer segments [3] confirmed the occurrence of this structural change upon bleaching and allowed to identify it unambiguously as being due to an outward shift of the protein mass from the hydrophobic center of the membrane toward the cytoplasm. But this shift is very small: if the whole protein mass is shifting, it amounts to less than one Ångström. The data do not allow to determine whether this is the case, or whether a larger displacement occurs for only a part of the protein (unfolding).

– The kinetics of exchange of labile protons inside a protein is highly dependent on the conformation of the protein. Osborne [4] has recently studied these kinetics, both in intact membrane and in delipidated detergent micelles. For dark adapted rhodopsin, the results are quantitatively similar for the two preparations, although in both cases the kinetics are sensitive to buffer changes in the aqueous solution. This is a good indication that native rhodopsin is not much denatured in such detergent preparations. Upon bleaching a small change is observed for rhodopsin in the membrane, indicating that part of the protein has been made more accessible to the protons in water. But for the detergent preparation, a very large change occurs, all

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the exchangeable protons becoming accessible to the solution; this is characteristic of a large unfolding of the protein resulting from denaturation.

– Neutron small angle scattering [5] on rhodopsin detergent micelles confirmed also that a large perturbation occurs upon bleaching such preparations. This causes an aggregation of the micelles. One observes first a dimerisation followed by a slow aggregation.

– The diamagnetic asymmetry of the rods, which allows their orientation in magnetic fields, is due to the great number of rhodopsin molecules aligned in the same direction within one rod. It is therefore directly related to the conformation and orientation of the protein. Upon bleaching, a 6% change in the diamagnetic asymmetry if observed [6].

– The linear dichroism in the UV is related to the orientation of chromophoric groups in the proteins (tyrosines and tryptophanes). Recent measurements of linear dichroism [7] on intact rods oriented in a magnetic field have shown that a large signal is observed around 290 nm and that this signal is clearly modified upon bleaching.

References

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3. Saibil, H., Chabre, M., Worcester, D.: Nature **262,** 266–270 (1976)
4. Osborne, H. B.: FEBS Lett. **67,** 23–27 (1976)
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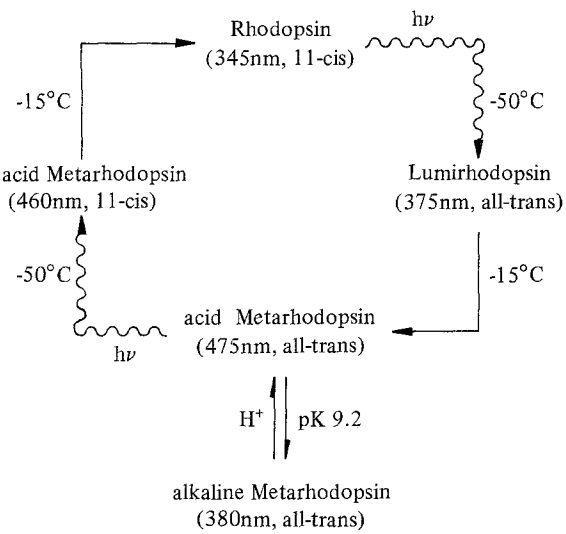
6. Chabre, M.: In preparation
7. Chabre, M., Breton, J.: In preparation

Besides these biophysical techniques suitable for detecting small or localized changes in protein conformation also chemical techniques can be used to show conformational changes at the membrane level. N. Virmaux reported that in studying the interaction of the anionic detergent sodium dodecylsulfate (SDS) with the retinal outer segment (ROS) membranes they found that below the critical micellar concentration (CMC) SDS cannot extract rhodopsin molecules from the ROS membranes kept in the dark but does extract the opsin molecules of the light-exposed membranes. After addition of 11-cis retinal to the bleached membranes SDS below the CMC cannot extract again the regenerated molecules. Rhodopsin molecules can be extracted only by SDS above the CMC. In conclusion the two major conformations of the protein moiety of the rhodopsin molecules have different behavior toward the same detergent.

In contrast to vertebrate rhodopsin both invertebrate rhodopsin and bacteriorhodopsin do not release retinal from the protein moiety upon illumination. Whether changes in the protein conformation occur during photolysis of invertebrate rhodopsin was discussed by R. Paulsen:

In the previous discussion on the photolysis of vertebrate rhodopsin the accumulation of distinct intermediates at low temperatures has been attributed partly to the fixation of the protein structure in a certain conformation which cannot change unless the temperature is raised by some degree. In invertebrates, rhodopsin is converted by light at physiological temperatures finally into a thermostable metarhodopsin. The formation of intermediates during the conversion of rhodopsin into

metarhodopsin has been demonstrated by low temperature experiments with cephalopod rhodopsin [1] and with insect visual pigment [2]. Our studies on the UV-visual pigment of the insect *Ascalaphus* indicate that conformational changes occur during both the conversion of rhodopsin into metarhodopsin and the photoregeneration of rhodopsin from metarhodopsin. The reaction sequence established so far for the digitonin extracted pigment can be outlined as follows:



In the rhodopsin state this visual pigment has 11-cis retinal as chromophoric group and absorbs maximally at shorter wavelenghts than the chromophore itself.

Illumination of rhodopsin with UV light at −50° C produces a bathochromic shift in the spectrum and increases the maximum absorbance. On warming the extract to −15° C metarhodopsin is formed in the dark. Therefore, we assume that the intermediate accumulating at −50° C is lumirhodopsin having the chromophore in the all-trans configuration but with a protein conformation different from that of metarhodopsin. Long wavelength illumination of acid metarhodopsin at −50° C shifts the spectrum to shorter wavelenghts by about 15 nm and decreases the maximum absorbance. This intermediate is converted into rhodopsin on warming the extract in the dark to −15° C. The chromophoric group of this photoproduct, produced during photoregeneration of rhodopsin, must already be in the 11-cis configuration while the protein part should still have a conformation similar to metarhodopsin. Thus changes in the chromophore configuration apparently induce changes in the conformation of invertebrate rhodopsins.

References

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2. Hamdorf, K., Paulsen, R., Schwemer, J.: Photoregeneration and sensitivity control of photoreceptors of invertebrates. In: Biochemistry and physiology of visual pigments (ed. H. Langer), pp. 155–166. Berlin-Heidelberg-New York: Springer 1973

In bacteriorhodopsin either 13-cis or all-trans retinal$_1$ is bound to a lysine residue of the protein via a Schiff base and not 11-cis retinal as in rhodopsin. F. Tokunoga, R. Crouch and T. Ebrey have shown that non-physiological analogues of retinal form pigments when reacted with the apoprotein (chromophore-free protein) of the purple membrane of *Halobacterium halobium*. Both the all-trans and 13-cis isomers of a retinal analogue having an elongated chain with an extra double bond formed pigments which were unlike the native all-trans and 13-cis retinal$_1$ based pigments. The new pigments were not interconvertible with each other and were unstable against hydroxylamine. Moreover, the ability of the extended length retinal to form pigments contrasts with its non-reactivity with opsin (apoprotein of rhodopsin), suggesting a less stringent binding site for the purple membrane chromophore. All-trans retinal$_2$ also combined with bleached purple membrane to form a pigment absorbing at c. 590 nm.

The question has been raised by J. Wyman whether the rhodopsin system can be regarded as an allosteric one. Although, for the present at least, the answer to this question must, he fears, remain somewhat inconclusive, partly due the nature of the concepts involved, the following reflections bear closely on the subject. They are also relevant to the larger question as to how far the system can be assimilated to a functioning enzyme.

In the dark, when the system is at equilibrium, or at least in a steady state close to equilibrium, the visual substance is almost wholly in the form of rhodopsin. On the other hand, in the presence of light the rhodopsin is, to a greater or lesser extent depending on the strength of the light, converted into an excited state which passes quickly through a series of other forms not present in appreciable amounts in the dark (batho-, lumi-, and the metarhodopsins). Subject to the restriction of one step transitions, the system may be represented by the following network:

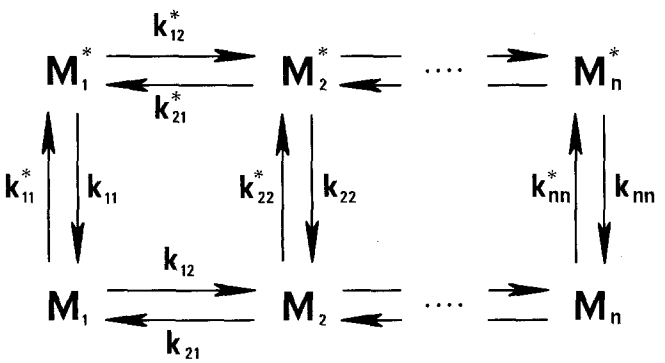

Fig. 1

Here the k's stand for kinetic constants and the stars for excited states. (k^*_{11}, k^*_{22}, ..., k^*_{nn} are of course light dependent.)

The kinetic equations for this system may be written as

$$\begin{aligned}
\dot{M}_1 &= -(k^*_{11} + k_{12})\, M_1 + k_{11}\, M^*_1 + k_{21}\, M_2 + 0 \\
\dot{M}^*_1 &= k^*_{11}\, M_1 - (k_{11} + k^*_{12})\, M^*_1 + k^*_{21}\, M^*_2 + 0 \qquad (1) \\
&\vdots \\
\dot{M}^*_n &= 0 + k^*_{(n-1)n}\, M^*_{n-1} + k^*_{nn}\, M_n - (k^*_{nn} + k_{n\,(n-1)})\, M^*_n
\end{aligned}$$

where the dot signifies a time derivative. It will be seen that this set of equations satisfies the condition for the existence of a steady state, namely that the sum of the coefficients of any M in any column of the determinant constructed from the equations be equal to zero [see Wyman, PNAS **72**, 3983–3987 (1975)]. The system will therefore be asymptotically stable, i.e. it will approach a steady state from any given starting point by a transient process involving a number of relaxation times one less than the total number of forms ($M_1, M_1^* \ldots M_n^*$). These relaxation times will be given by the solution of the secular equation constructed from the matrix of the coefficients of the M's in Equation (1). It will be noted that in the above scheme we have assumed that there is no direct return from M_n or M_n^* to M_1 or M_1^*. Actually the same result would hold if there were such a direct transition.

As the result of a light flash the rhodopsin system will be perturbed, with the appearance of new forms associated with the excited state. Then, after the flash is over, it will start relaxing to the steady state in the dark (if all the light is removed) or (if not) to that corresponding to some other level of illumination. The limiting rate of this relaxation process will be determined by the longest member of the spectrum of relaxation times. It is generally supposed that the act of seeing is associated with the liberation of a transmitter substance (possibly Ca ion) from one or more of the excited states. If the liberation of the transmitter were the result of a change of conformation rather than a direct effect of the light, as seems not unreasonable to suppose, then the system could properly be regarded as an allosteric one, light playing the role of a control ligand in the more familiar type of allosteric system. It must be admitted however that apart from some results on birefringence there is not very much direct evidence for any such drastic conformational change in the protein moiety of the macromolecule as is observed in unquestioned allosteric systems like hemoglobin. On the other hand, the prostetic group retinal attached to the protein passes from a cis to a trans configuration. Rhodopsin is not an oligomeric protein.

Of course, in actual life as we look round upon a changing scene, constantly shifting our point of fixation, the rhodopsin system never has time to reach a steady state. Thus the visual experience is the result of a sequence of incompleted transients[1]. Indeed, if the system were to reach a steady state it might be thought that we should cease to see, the eye being completely adapted. Since relaxation in the light (as determined from the secular equation) may be expected to be much faster than in the dark, this explains why there should be no appreciable time lag involved in the ordinary seeing process, although adaptation to the dark is a slow process. (Remember that in a simple one step transition the relaxation time is given by the sum of the forward and backward velocities.) Might it not be possible to study relaxation in the light by exploring the frequency at which the flicker of an alternating light is just discernable?

There are two objections which might be raised against this scheme. In the first place it takes no account of a possible association or dissociation of the rhodopsin molecule in its various transitions. Actually it is now established that the final step of the visual process involves a splitting of the visual substance into retinal and the apoprotein opsin, from which the original rhodopsin is later reconstituted, possibly

[1] In a broad sense is this not true of most if not all forms of experience and indeed of history itself?

correlated with the uptake of transmitter, in the return half of the cycle, i.e. relaxation in the dark. In the second place it makes no distinction between the liganded and unliganded forms, transmitter being a ligand. In the argument given for the existence of a steady state in the reference cited above it is assumed that the ligand is held at constant activity; actually, in the visual system the transmitter is present in constant amount, rather than at constant activity. Now it is easy to see that when the equations are reformulated to take account of these two considerations they are no longer linear, with the result that the solution becomes complicated, if indeed an analytical solution is possible at all. Nevertheless, without attempting to find an exact solution but looking at things from a qualitative point of view in the manner introduced by Poincaré and Liapounov for such cases, we might expect that the situation will remain qualitatively the same, i.e. there will still be a steady state (a critical point in mathematical language) which will always be approached by some kind of relaxation process, however complex. If this is so, then in its general outlines the picture sketched above will still apply; and it may be pointed out once again that if we compare this picture with that of more familiar allosteric systems such as hemoglobin and other working proteins, of which the basic feature is a set of ligand linked conformational changes, then in this special case light is playing the role of a control ligand. Moreover, in so far as the sequence of events involves a change of molecular weight – a splitting of rhodopsin into retinal and opsin – we are confronted by a phenomenon reminiscent of the reversible dissociation of hemoglobin (at high dilution) induced by the binding of oxygen. (This extreme type of ligand linked conformational change has been called "polysteric" and requires a somewhat more involved formulation than that applicable to a "classic" allosteric system [see Colosimo, Brunori, and Wyman, J. Mol. Biol. **100,** 47–57 (1976)].

The above discussion was written on the basis of the facts (as I understand them) relating to the vertebrate visual system. The invertebrate system described in the communication by K. Hamdorf appears to be quite different. In that system, and apparently in some other invertebrate systems, there are two pigments in photo equilibrium with one another (or at least related by a steady state under illumination). These two pigments, denoted by P and M, are characterized by quite different but overlapping spectra as shown in Figure 2. Of the two only *P* seems to liberate the transmitter substance involved in seeing. On the basis of the spectral properties

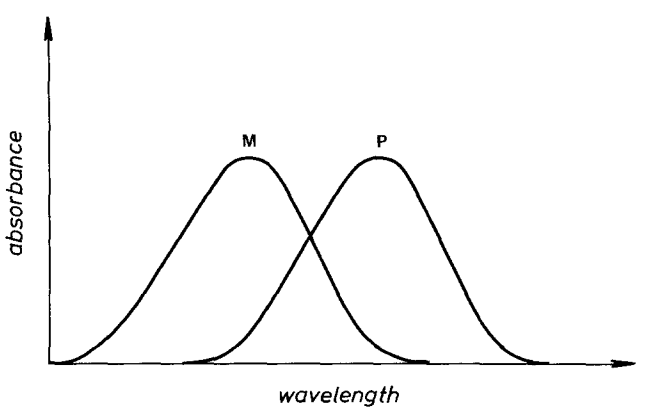

Fig. 2

shown in the figure it has been supposed that a light stimulus shifts the balance between P and M without any change of molecular weight, and that this shift, possibly associated with the liberation of a transmitter, is what produces vision. This interpretation, if correct, would clearly justify regarding this system also as an allosteric one.

Another side of the versatile rhodopsin system is seen in the photosynthetic activity of the purple membrane of the halobacteria described by W. Stoeckenius. Here rhodopsin plays the role of a polyfunctional macromolecule (enzyme) which, circulating round a closed network in a steady state, acts as a transducer, making the free energy derived from one chemical reaction, or from light, available to drive another. Once again we are confronted by an allosteric type of behavior.

In conclusion I might point out that all that has thus far been said relates to the single rhodopsin molecules. Actually these molecules in situ form part of a "community" of molecules comprising the membrane in which they are located. The question arises whether or not this "community" of molecules shows any significant cooperativity (or integration), the excitation of one rhodopsin molecule modifying the sensitivity of others linked to it. If there were such cooperativity, within the membrane, it might well represent an allosteric phenomenon at a higher level. That there is indeed cooperativity, in the most general sense, in the seeing process is clearly brought out by the observations described in the communication by W. A. H. Rushton, but at what level of organization it occurs remains an open question, indeed a fundamental one to any understanding of the way in which the nervous system functions.