Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1044-1050

BACTERIORHODOPSIN: PHOTOCHEMISTRY AND ENERGY CONVERSION

Béla Karvaly

Department of Chemistry, University of California,
Los Angeles, California 90024, U.S.A.

Received March 7,1980

SUMMARY. A new, dynamic functional structure and photochemical mechanism are presented for bacteriorhodopsin, in which the azomethine (Schiff-base) linkage is deprotonated and exists in a tautomeric form in the non-photolyzed molecule. When the retinal chromophore in trans conformation absorbs photon, the azomethine linkage can interact with the ε-amino group of the neighboring lysine via a nucleophilic reaction. Thus, the first photochemical intermediate is an energy-rich double-protonated conjugate acid of an amidine base, which then undergoes deprotonation and hydrolysis, while the retinal becomes temporarily detached from the lysine residues. The cycle is completed when the deprotonated azomethine linkage is spontaneously reformed. The model clarifies most experimental observations.

INTRODUCTORY REMARKS

The traditional picture of bacteriorhodopsin (BR)[1] (and visual chromoproteins) is a retinal pigment chromophore bound to (bacterio-)opsin via a protonated Schiff-base (SB). The response of BR to light is thought to involve the reversible deprotonation of the protonated SB linkage with ensuing a vectorial proton translocation through the lipoprotein matrix [1-3] (Fig. 1). This, of course, assumes *a priori* that the SB is indeed protonated in the non-photolyzed molecule. The light-induced deprotonation of the SB is for several reasons inadequate to account for the results of numerous experiments accumulated in recent years. Without recourse to completeness, the most striking (and sufficiently documented) findings which deserve particular attention in this respect are the following:

(1) Under physiological conditions[2] one photolyzed BR molecule ejects more than one (probably two) proton into the aqueous phase when the purple membrane fragments are suspended in the solution [4,5]. In reconstituted (liposome) systems, the specific proton release may apparently amount to as much as thirty [6].

(2) Of the numerous photochemical intermediates revealed under various experimental conditions, apparently only a few (bR_{570}, K_{610}, L_{550}, M_{410}

1 — ABBREVIATIONS: BR - bacteriorhodopsin; bR_{570}, K_{610}, L_{550}, M_{410}, N_{530} and O_{640} are its intermediates in the light-adapted (proton-pumping) photochemical cycle; SB - Schiff-base.

2 — Here and hereafter, one means by physiological conditions those conditions (in particular, ionic strength) which are close to that required to maintain the normal functional structure of BR in membrane patches at room temperature.

0006-291X/80/0801044-07$01.00/0

and, perhaps, N_{530} and O_{640}) seem to participate in the proton-pumping photocycle [7]. K_{610}, L_{550} and M_{410} exhibit spectroscopic degeneracy, i.e. species with identical spectroscopic properties show different kinetic patterns [7-10].

(3) Both the rate of formation and the rate of decay of the long-lived intermediate M_{410} (in which the SB is supposedly deprotonated) exhibit marked enhancements in alkaline media [7,11,12].

(4) The resonance Raman spectra of BR and its intermediates, in which molecular interactions inherently related to or coupled to the vibrational modes of the retinal chromophore are selectively enhanced, (a) are hardly compatible with those of any known retinal SB model compounds; (b) report unusually low C=N stretching vibrational energies (especially for K_{610}); (c) show anomalous deuterium effects [13-16].

In suspension samples and under physiological ionic strengths the specific proton release exceeds one, indicating that the simple deprotonation of a singly protonated SB can not directly supply the protons in the required amount. Results obtained with liposomes, where compartmentalization is ensured, were explained with a non-specific release of loosely bound protons by the lipoprotein matrix. This has, however, neither firm theoretical basis nor experimental verification and its relation to the photochemistry of BR would also need clarification. Otherwise, one could conclude that the proton pump has nothing to do with the multiproton production and a similar non-specific proton release could be induced by other means as well.

The spectroscopic degeneracy indicates that the intermediates in question may occupy two, nearly identical energy states which are separated by fairly high barriers.

The pH-dependence of the kinetic data mentioned under (3) can be comprehended in such a manner that the thermal formation process of M_{410} ($K_{610} \rightarrow M_{410}$) and its spontaneous decay process ($M_{410} \rightarrow bR_{570}$) are both base-catalyzed. This could be the case for a simple deprotonation process of the originally protonated SB, but it certainly stands contradictory to normal logic if applied to reprotonation.

An analysis of photochemical and spectroscopic data suggest that not only a simple SB, but also other covalent bonds containing $\rangle C{=}N-$ linkage may occur during the photochemical cycle.

These representative examples of inconsistencies of the prevailing models which start with the protonated state of the SB, make it timely to reconsider the possible elementary events involved in the molecular mechanism of BR. Thus, the first and the most important question that arises is: Is the SB, in fact, protonated in the non-photolyzed BR molecule ? In the following, a different

molecular approache will be briefly outlined. It includes some elements of the interpretations of other investigators [17,18]. The unique feature of this dynamic model is that it is energetically feasible, chemically consequent and explains almost all experimental findings in one consistent theory. Here, we restrict ourselves mainly to the description of the suggested mechanism.

THE MODEL

The retinal chromophore is bound to the protein apparently at Lys-41[3] via an azomethine[4] (>C=N-) linkage [19-22]. This Lys residue separates a predominantly hydrophilic polypeptide section (— Ser-36 — Asp-37 — Pro-38 — Ala-39 — Lys-40 —) and a completely hydrophobic polypeptide sequence (— Phe-42 — Tyr-43 — Ala-44 — Ile-45 —) [20-22][5]. Since Lys-40 and Lys-41 are included in a helical part of the protein, their side chains are directed towards a relatively empty space, supposedly within the hydrophobic protein moiety. The ε-amino nitrogen of Lys-40 is hybridized in an sp^3 fashion, whereas that involved in the azomethine linkage is of sp^2 character and its non-bonded electrons delocalize into the π-electron system of retinal. For these reasons alone the free ε-amino group must be a significantly stronger base than the azomethine group and, will thus be the first site of protonation. One may conclude then that the azomethine linkage is not necessarily protonated.

Because of the dominance of hydrophobic amino acids and of the extraordinary compactness of the protein structure in BR, it is plausible to assume that, in light-adapted form and under physiological conditions:

(1) neither the primary amine (Lys-40) nor the azomethine linkage (Lys-41) is normally protonated in the non-photolyzed chromoprotein;
(2) the two nitrogen bases on the flexible side chains of Lys-40 and Lys-41 have their relative positions preserved by hydrogen bonds; and
(3) these nitrogen bases might not experience very different local environments.

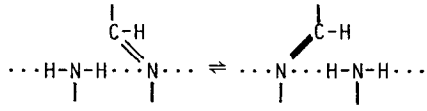
---

3 — Prior to sequential analysis, the retinal was irreversibly fixed to the protein by chemical treatment which could perturb the microenvironment of retinal. Therefore, the preferred binding site of retinal in the native non-photolyzed BR molecule can be either Lys-40 or Lys-41. This will make, however, no difference in the following.

4 — As will be seen immediately, it is not desirable to call the C=N linkage a Schiff-base in the present context, since this overemphasizes its basic character. Therefor, its use will be intentionally avoided hereafter.

5 — The slight differences reported by different authors [20-22] in the amino acid sequences of these sections have no influence on the conclusions.

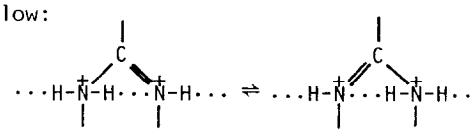
Then, in the trans retinal conformation (and only in this case), the α-carbon of retinal is shared by the two almost equivalent nitrogens in such a manner that a tautomeric equilibrium is established[6,7]:



(I)

Due to the possibility of slight local asymmetry the two possible tautomeric forms do not necessarily occur with the same probability, i.e. the equilibrium in (I) may favor one of the tautomeric forms to some appreciable extent. These two tautomeric bond forms of retinal can be understood as *syn* - *anti* geometric isomers. They may be energetically somewhat different, but the barrier separating them is thermally 'transparent' at room temperature [24]. This dynamic mode of bonding reduces the double-bond character of the carbon - nitrogen linkage and is manifested by a charge polarization along the bond, due to mixing with single bond.

When the chromophore absorbs a visible photon a large dipole (of the order of magnitude 10 D) immediately develops along the long axis of the conjugated polyene chain, pointing towards the β-ionone ring [25]. This greatly labilizes the attached proton and permits the involvement of the α-carbon in further chemical interactions. With the adjacent non-protonated ε-amino group (a strong nucleophile) at hand, a rapid nucleophilic substitution reaction takes place, beginning with the departure of the proton on the α-carbon. This is associated with concomitant translocation of protons along the hydrogen bonds and electron transfer reactions. (All these processes occur on a subpicosecond time scale.) The result is shown below:



(II)

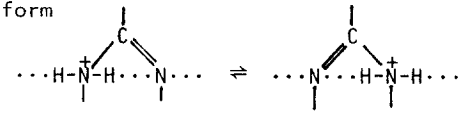
Thus, a high-energy protonated amidine derivative (the double-protonated conjugate acid of an amidine base) is formed, which exhibits also tautomerism. This rather significant change in the molecular architecture is easily seen in the absorption and resonance Raman spectra, and the corresponding intermediate is identified as K_{610}. One is forced to conclude then that the drastic bathochromic shift observed when bR_{570} converts to K_{610} is primarily due to the protonation

6 — In a recent paper Harris et al. [23] exclude the possible involvement of Lys-40 in the photochemical and proton-pumping processes. Repeated washing of purple membrane patches after incubation with dansyl-Cl seems, however, inadequate for the complete removal of non-specifically bound (lipid-embedded and adsorbed) label. Therefore, the labelling of Lys-40 could easily take place during the cleavage procedure.

7 — In the chemical formalism only the groups involved in the primary reactions will be denoted. Dots indicate the possible hydrogen bonds.

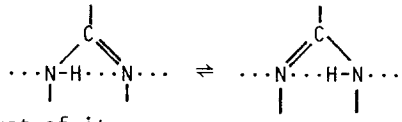
of the azomethine link in the amidine group rather than changes only in retinal conformation or in secondary interactions between retinal and the protein moiety (see also ref. [26]).

The above light-induced rearrangement certainly initiates subsequent transitions in the protein conformation. The new conformational states provide the favorable conditions for the step-by-step deprotonation and make it possible for hydrolyzing water molecules (perhaps, with the delicate assistance of the hydrophilic peptide sequence) to approach the deprotonated amidine linkage. The partially deprotonated form



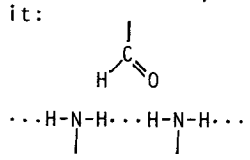
(III)

is assigned to L_{550}, while M_{410} is either the completely deprotonated amidine base



(IV)

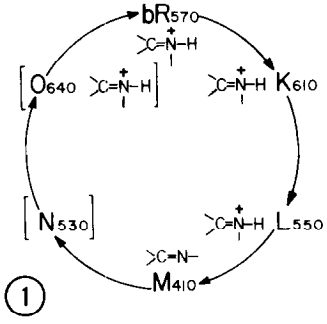
or the hydrolysis product of it:



(V)

provided that the deprotonation step, but not the hydrolysis, is rate limiting. This is possible, because all elementary steps involved in the straight-forward synthesis of the amidine linkage are reversible [27]. Thus, there is a detachment of the chromophore and lysines somewhere around M_{410}, while the terminal aldehyde group on the former and the primary amine groups on the later are restored. Accordingly, the significantly blue-shifted absorption spectrum of L_{550} is primarily attributed to the deprotonation of the azomethine link. Similarly, the further blue-shift accompanying the evolution of M_{410} is predominantly due to the loss of the amide proton (and subsequent hydrolysis).

In the recovery phase $M_{410} \rightarrow bR_{570}$, there are probably two hidden intermediates, N_{530} and O_{640} [7], which can be trapped only under special conditions. Their existence, however, imparts to the photochemical cycle spectroscopic symmetry with respect to the $bR_{570} - M_{410}$ axis (Fig. 1). This may well be the indication that the reformation of the functional linkage (I) involves intermediates which are structurally (and also, spectroscopically) closely related to those which occur in the thermal decomposition pathway $K_{610} \rightarrow M_{410}$. Due to the lack of sufficient details on the properties of N_{530} and O_{640} the exact nature of the chromophore - protein covalent bonds is uncertain in these cases. It is, however, very likely that both of them contain an azomethine link. In the case of N_{530} the azomethine nitrogen is probably not protonated, but it may be tempora-



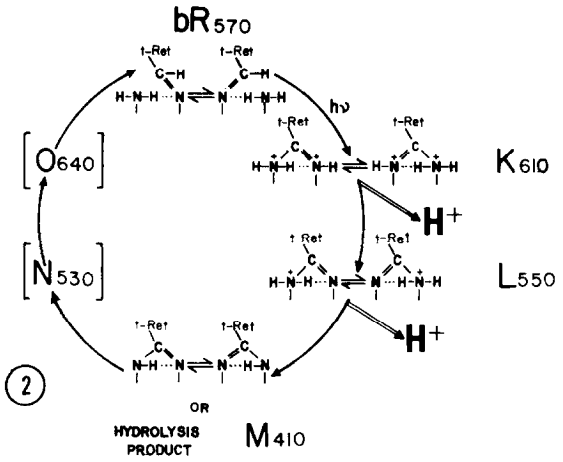


Figure 1. The photochemical cycle of bacteriorhodopsin based upon simple Schiff-base deprotonation.

Figure 2. The proton-pumping photochemical cycle of bacteriorhodopsin, based upon non-protonated Schiff-base (in the non-photolyzed native state) and the formation of energy-rich amidine derivatives (in photolyzed state). t-Ret denotes trans-retinal. For details see the text.

rily protonated in O_{640}. The deprotonation of O_{640} would then occur during the transition $O_{640} \rightarrow bR_{570}$, when the structural restoration accompanies drastic changes in molecular order [28]. This would be consistent with the proposed model (Fig. 2).

The dynamic functional structure and photochemical mechanism outlined above state that upon light excitation a reactive carbon is formed, which then interacts with a nearby nucleophile and thus, yields energy-rich intermediates. When these intermediates are formed, BR may function as a molecular light-energy converter and an energy storehouse. The origin of protons released during the first half of the photochemical cycle is, in the end, the excess proton production within the hydrophobic nest of retinal, which is due to the deprotonation of the photogenerated conjugate acid formed without the direct involvement of water at this stage. The labile protons of this acid originate from the retinal - protein complex itself. The protons are regained, however, during a hydrolysis process. Apparently, photoexcited BR molecules do not split water directly, but rather shift its thermal dissociation by eliminating protons. Therefore, there is a special role of compartmentalization in its function. It can be shown that when the physiological protein structure is partially impaired (e.g. due to low ionic strength, etc.), only one ejectable proton is produced in the course of a single photochemical cycle, even though the energy converting mechanism is almost the same as above. Finally, it is believed that some reconsideration in the photochemistry and photophysics of visual chromoproteins might also be needed.

One is aware of the lack of unambiguous and direct evidence for the proposed mechanism. Nevertheless, its compability with most experimental findings and preliminary studies along this line, lend it substantial support. Full details of the model and treatment of some particular questions (significance of isomeric forms, cooperativity, electric and magnetic field-effects, etc.) will be published elsewhere.

ACKNOWLEDGEMENT. The author is grateful to Professor M. A. El-Sayed for the hospitality, encouragement and stimulating discussions during the maturation period of this work. Special thank is due to Dr. A. R. Burns and Mr. J. M. Fukumoto for reading the manuscript and correcting its language and style. Mr. Fukumoto's illuminating comments and criticism were especially of great value and are acknowledged. This work was supported by the U.S. Department of Energy, Office of Basic Energy Sciences.

REFERENCES

[1] Oesterhelt, D. & Stoeckenius, W. (1971) Nature (New Biol.) 233, 149-152.
[2] Oesterhelt, D. & Hess, B. (1973) Eur. J. Biochem. 37, 316-326.
[3] Oesterhelt, D. & Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2853-2857.
[4] Hess, B. & Kuschmitz, D. (1978) in: Frontiers of Biological Energetics, Vol. I: Electrons to Tissues, Eds. Dutton, P. L., Leight, J. & Scarpa, A. pp. 257-264, Academic Press, New York, N.Y.
[5] Ort, D. R. & Parson, W. W. (1979) Biophys. J. 25, 341-353.
[6] Eisenbach, M., Garty, H., Bakker, E. P., Kemperer, G., Rottenberg, H. & Caplan, S. R. (1978) Biochemistry 17, 4691-4698.
[7] Stoeckenius, W., Lozier, R. H. & Bogomolni, R.A. (1978) Biochim. Biophys. Acta 505, 215-278.
[8] Vsevolodov, N. N., Kostikov, A. P. & Rikhireva, G. T. (1974) Biofizika (Russian) 19, 942-946.
[9] Edgerton, M. E. & Greenwood, C. (1979) Biochem. Soc. Trans. 7, 1075-1077.
[10] Slifkin, M. A. & Caplan, R. S. (1975) Nature 253, 56-58.
[11] Hess, B. & Kuschmitz, D. (1977) FEBS Letters 78,57-60.
[12] Hurley, J. B., Becher, B. and Ebrey, T. G. (1978) Nature 272, 87.
[13] Marcus, M. & Lewis, A. (1978) Biochemistry 17, 4722-4735.
[14] Terner, J., Hsieh, C.-L. & El-Sayed, M. A. (1979) Biophys. J. 26, 527-541.
[15] Terner, J., Hsieh, C.-L., Burns, A. R. & El-Sayed, M. A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3046-3050.
[16] Terner, J., Hsieh, C.-L., Burns, A. R. & El-Sayed, M. A. (1979) Biochemistry 18, 3629-3634.
[17] Blondin, G. A. & Green, D. E. (1975) Chem. & Engng. News 53(45), 26-42.
[18] Crespi, H. L. & Ferraro, J. R. (1979) Biochim. Biophys. Res. Commun. 91, 575-582.
[19] Bridgen, J. & Walker, I. D. (1976) Biochemistry 15, 792-798.
[20] Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Kiselev, A. V. & Lobanov, N. A. (1979) FEBS Letters 100, 219-224.
[21] Walker, J. E., Carne, A. F. & Schmitt, H. W. (1979) Nature 278, 653-654.
[22] Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K. & Biemann, K. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5046-5050.
[23] Harris, G., Renthal, R., Tuley, I. & Robinson, N. (1979) Biochem. Biophys. Res. Commun. 91, 926-931.
[24] Tokunaga, F., Iwasa, T. & Yoshizawa, T. (1976) FEBS Letters 72, 33-38.
[25] Mathies, R. & Stryer, L. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2169-2173.
[26] Favrot, J., Vocelle, D. & Sandorfy, C. (1979) Photochem. Photobiol. 30, 417-421.
[27] Shriner, R. L. & Neumann, F. W. (1944) Chem. Revs. 35, 351-425.
[28] Ort, D. R. & Parson, W. W. (1979) Biophys. J. 25, 355-364.