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ACTIVE SITE STRUCTURE OF BACTERIORHODOPSIN AND MECHANISM OF ACTION

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SUMMARY. Pressure experiments with freeze-dried bacteriorhodopsin indicate that water is an essential part of the chromophore. This observation is combined with already known information on (a) the pH dependence of proton pumping, (b) the secondary protein-chromophore interaction with lysine-40, and (c) the proton transfer in the initial photochemical step to give a detailed structure of the active site and a mechanism for proton pumping which is consistent with the bacteriorhodopsin polypeptide sequence.

The bacteriorhodopsin of *Halobacterium halobium* is a light-driven proton pump (1). When bacteriorhodopsin (bR) absorbs light, it is photochemically transformed in a few picoseconds to an intermediate observed at 610 nm (K_{610}) with concomitant proton transfer (2,3). Subsequent reactions proceed thermally to the formation of species absorbing at 550 nm (L_{550}) in 2 μsec, and at 412 nm (M_{412}) in 40 μsec. Recently Lewis and coworkers (4) have observed an intermediate between L and M, here called "X". Reformation of the original complex is then slow, as the complete cycling time at physiological temperatures is about 10 msec. A proton will appear in the external medium in about 30-50 μsec.

We have subjected freeze-dried bacteriorhodopsin to high pressure in a diamond anvil cell (5). Figure 1 shows the response of bR to the application of pressure. As pressure is increased in the range of 0 to 20 kbar, the absorption maximum blue-shifts from 540 nm (freeze-dried bR under no pressure) to about 500 nm. An absorption

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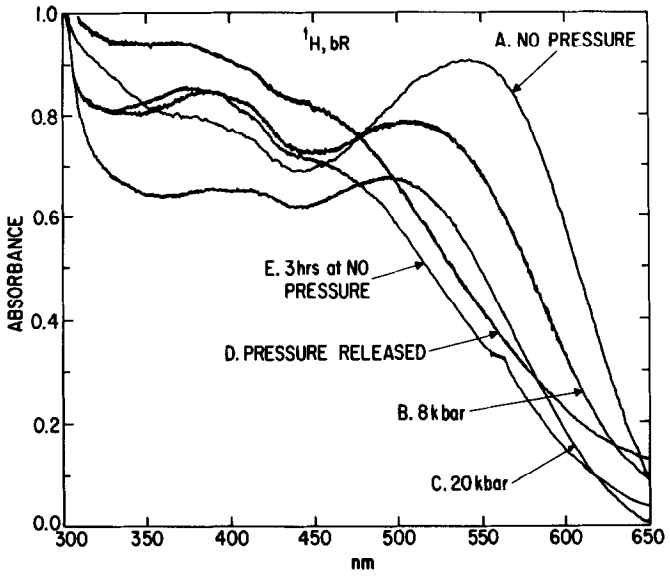

Figure 1. Optical spectra of freeze-dried natural hydrogen abundance (^{1}H) bacteriorhodopsin (bR) showing the sequential color changes involved in going from the starting material at no pressure, blue-shifted bR at pressure, and bleached bR upon release of pressure. After standing three hours under ambient conditions, the bleached bR showed a slight recovery of absorption at 560 nm. Bleached bR is also observed when the entire sequence of pressure application and release is conducted in the dark.

maximum grows in at 390-400 nm. Upon total release of pressure (from a minimum of ∿3 kbar), there is instant, extensive, and often total bleaching of the sample. Absorption appears at 370 nm at the expense of the band at 500 nm.

Similar results were obtained in a parallel experiment done with bacteriorhodopsin exchanged with D_2O for eight hours at an average of about 50°. The partially deuterated material consistently showed more color to the eye and also more absorption at the red end of its spectrum. We observed little or no reversibility with all samples allowed to stand (*in situ* after pressure release) up to 24 hours at ambient conditions (very low relative humidity). However, as shown in Fig. 2, if the bleached sample is kept at high relative humidity at room temperature, recovery of color is complete (6).

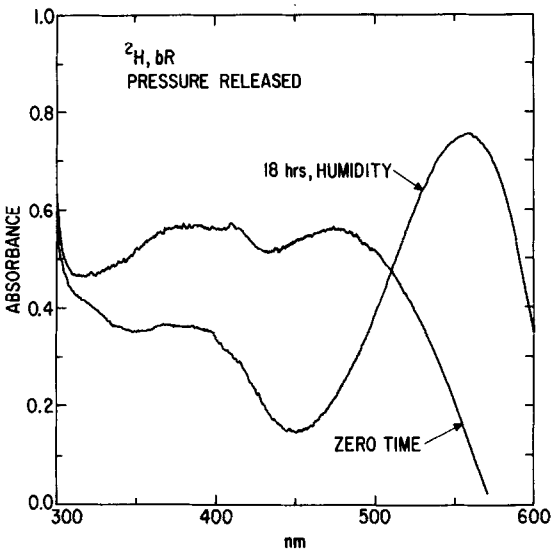

Figure 2. Bacteriorhodopsin recovers its natural 560 nm absorption when the pressure-bleached material is exposed to 100 percent relative humidity at room temperature. This experiment was done with fully deuterated (^{2}H) bR. The same result obtains with ^{1}H material.

Studies of the high pressure denaturation of protein in solution are consistent with this result (7). Pressures above 3 kbar cause denaturation. Suzuki and Taniguchi (7) point out that high pressure favors the disruption of hydrophobic and ionic bonding and the formation of hydrogen bonds. Although pressure-denatured bR appears to be renatured by hydration, the observed pressure-release effect may be a specific one not related to overall protein hydration. Our results indicate that high pressure distorts the region about the chromophore, causing the release of water (8). Upon release of pressure, this water is no longer available to restore this region of the molecule to its native conformation, so relaxation to a bleached form takes place.

The possible effects of dehydration on bR films has been discussed in detail by Korenstein and Hess (9), who measured the decay kinetics of the M_{412} intermediate using air-dried films of bacteriorhodopsin. The concluded that the photochemical reaction "is only

influenced by the microenvironment of the reaction system" and suggest that the state of hydration is a conformation-controlling event. When bacteriorhodopsin is dried there is a progressive blue shift in color and when the dried material is subjected to pressure there is a further progressive blue shift due, presumably, to a general redistribution of water under pressure, a result in agreement with the observations of Korenstein and Hess. Because of the bleaching upon release of pressure, we suggest that a water molecule is involved in the native structure of the active center.

DISCUSSION

The molecular mechanism underlying the proton pumping function of bacteriorhodopsin is of fundamental importance in the area of bioenergetics. In a recent publication, Lewis *et al.* (2) presented data suggesting such a molecular mechanism. They proposed that light causes the deprotonation of an amino acid side chain of high pK (arginine); the deprotonated amino acid is then reprotonated by the Schiff base complex. We suggest here a detailed mechanism which appears consistent with existing data and results presented here.

Figures 3 and 4 describe in detail our suggestion concerning the mechanism of this proton pumping. This scheme is consistent (a) with the pH dependence of proton pumping [pumping takes place between pH 2.5 and 12.5] which implicates carboxyl and guanidino groups (2), (b) with the evidence for a secondary protein-chromophore interaction [the hydrogen bond from the Schiff base to the ε-amino group of lysine-40] (2), (c) with the observation of proton transfer in the initial photochemical step (3), (d) with the subsequent kinetics and appearance of a proton in the external medium, and (e) with the present observation that water is an essential part of the active center. A space-filling (CPK) model is not

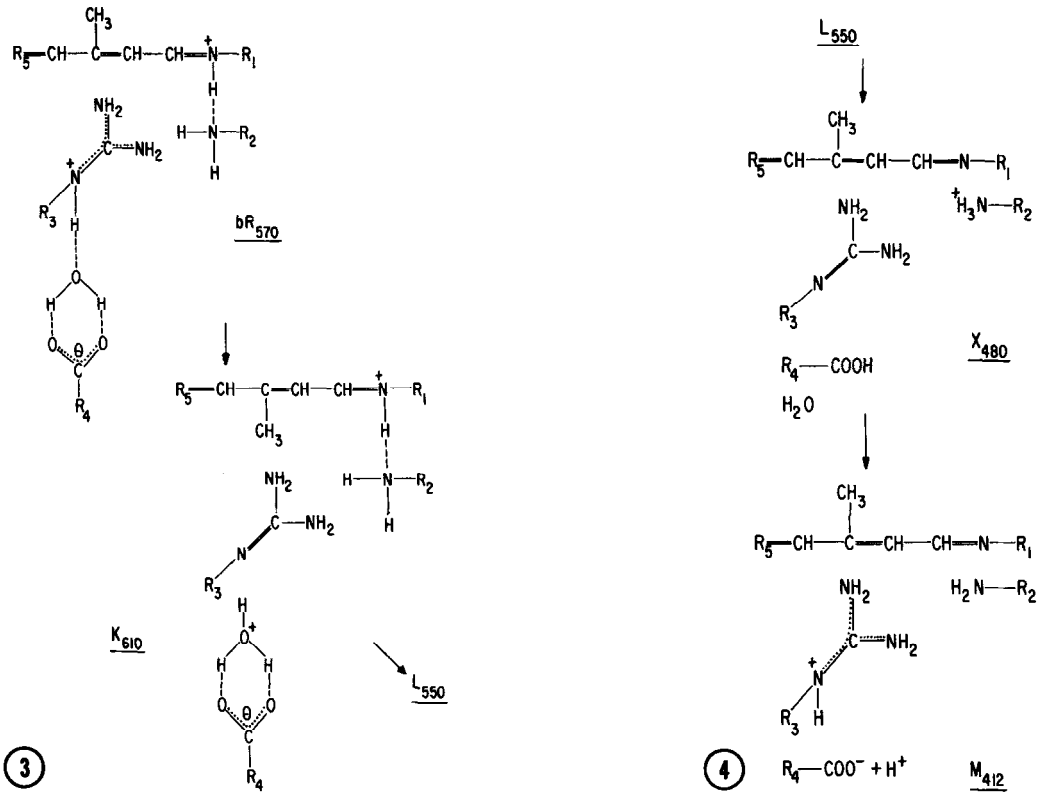

Figure 3. A proposed mechanism for proton pumping by bacteriorhodopsin. Structure bR_{570} represents the resting state. R_1, R_2, R_3 and R_4 indicate surrounding protein, and R_5 is the remainder of the retinylidene group. A secondary interaction (hydrogen bond) between the Schiff base and a lysine ε-amino group is shown, the guanidinylium group is in a planar configuration, and there is a salt bridge from a carboxyl group to the guanidinylium group. It has been shown (10) that the favored structure of the guanidinylium group has the positive charge at the NH position. In K_{610}, the methyl group at position 13 of the retinylidene is given a "cisoid" conformation, which severely crowds the guanidinylium group, destroying its planarity. The reduced basicity of the non-planar guanidinyl group leads to the formation of H_3O^+ bound to the carboxyl group, while the guanidino group goes to an unprotonated state. Form L is not shown in detail, as the change from K would involve only a relaxed retinylidene and perhaps slightly changed protein positions.

Figure 4. Intermediate X is formed when the Schiff base proton is transferred to the ε-amino group of lysine-40. The guanidino group is unprotonated, and the carboxyl group may ionize or give up water and then ionize. Intermediate M forms when the guanidino group takes the proton from the lysine-40. Ionization of the carboxyl group is complete; the system is ready to accept a proton from the cytoplasm and reform bR_{570}.

inconsistent with this scheme. Data implicating a tyrosine residue in the proton pumping action (11) could mean that this group is involved in reprotonation of the Schiff base.

In Fig. 3 we show a possible mechanism for proton transfer in the photochemical step. The conformation change in the retinylidene group that drives the proton transfer is still an open question. We have shown in Fig. 3 a rotation of the methyl group at position 13, but the thrust of our argument is that the light-induced conformation change distorts the planarity of the guanidinylium group and decreases its basicity. If this conformation change localizes any positive charge near the NH group of the guanidinylium ion (for example, a sudden polarization (12) in a 90° twisted excited state), proton transfer could be further facilitated. Intermediate K is then an unprotonated guanidino group, a carboxyl "salt", a still-intact secondary Schiff base interaction with the ε-amino group of lysine-40, and a conformationally changed retinylidene group. Intermediate L is similar to K, but there will be relaxation of the retinylidene, there could be some movement in the protein due to charge redistribution, and there could also be some ionization of the carboxyl "salt."

Intermediate X is shown in Fig. 4. The retinylidene is in the relaxed conformation assumed in intermediate L, the ε-amino group of lysine-40 has accepted a proton from the Schiff base, and the carboxyl group is in the process of giving up its proton to the external medium.

Once the proton originally associated with the arginine side chain is removed, a second proton is in a position to cascade from the Schiff base (least basic) through the ε-amino group of lysine-40 to the arginine (most basic) (13). Intermediate M is formed on transfer of the proton of lysine-40 to the guanidino group, the ionization of the carboxyl group is complete, and the system is poised to reform bR_{570}. This mechanism may be an example of the entatic state described by Vallee and Williams (14), whereby the

destablized state (K) is favored by a number of favorable interactions elsewhere in the protein.

The recently published sequence (15) of the bacteriorhodopsin polypeptide is consistent with the model presented here. The polypeptide chain structure can be fitted to the helical array determined by Henderson and Unwin (1), such that Asp-96 and Arg-224 form a salt bridge and the Arg-224 is in a position to interact with the retinal moiety. In addition, Tyr-26 is positioned to act as a proton donor to the Schiff base, as proposed by Konishi and Packer (11).

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REFERENCES

1. Osterhelt, D., and Stoeckenius, W. (1978) Proc. Natl. Acad. Sci. USA 70, 2853-2857; Henderson, R., and Unwin, P. N. T. (1975) Nature 257, 28-32; Stoeckenius, W., Lozier, R. H., and Bogomolni, R. R. (1978) Biochim. Biophys. Acta 505, 215-278.
2. Lewis, A., Marcus, M. A., Ehrenberg, E., and Crespi, H. L. (1978) Proc. Natl. Acad. Sci. USA 75, 4642-4646.
3. Peters, K., Appleburg, M. L., and Rentzepis, P. M. (1977) Proc. Natl. Acad. Sci. USA 74, 3119-3123.
4. Lewis, A., personal communication.
5. Ferraro, J. R., and Basile, L. J. (1974) Appl. Spectr. 28, 505-516.
6. We have attempted to denature bR by warming the freeze-dried material in vacuum ($\sim 10^{-4}$ Torr) for up to one hour at 80-85°, but observed no marked color change.
7. Suzuki, K., and Taniguchi, Y. (1972) in The Effect of Pressure on Organisms, Symposia of the Soc. for Exptl. Biol. (Academic Press, New York), Vol. 26, pp. 103-124; Visser, A. J. W. G., Li, T. M., Drickamer, H. G., and Weber, G. (1977) Biochemistry 16, 4879-4882; Murphy, R. B. (1978) Experientia 34, 188-189.
8. The slight deuterium isotope effect involving exchangeable hydrogen probably indicates that the compression of the hydrophobic region about the chromphore is resisted by the hydrogen bonding in the protein.

9. Korenstein, R., and Hess, B. (1977) Nature 270, 184-186.
10. Yavari, I., and Roberts, J. D. (1978) Biochem. Biophys. Res. Comm. 83, 635-640.
11. Konishi, T., and Packer, L. (1978) FEBS Lett. 92, 1-4.
12. Salem, L. (1979) Accts. of Chem. Res. 12, 87-91.
13. Favrot, J., Sandorfy, C., and Vocelle, D. (1978) Photochem. Photobiol. 28, 271-272.
14. Campbell, I. D., Dobson, C. M., and Williams, R. J. P. (1978) Molecular Movements and Chemical Reactivity, in Adv. Chem. Phys., R. Lefever and A. Goldbeter, Eds. (Interscience), pp. 55-95.
15. Orchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Kiselev, A. V., and Lobanov, N. A. (1979) FEBS Lett. 100, 219-224.