Dimeric-like Kinetic Cooperativity of the Bacteriorhodopsin Molecules in Purple Membranes

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ABSTRACT The kinetics of the absorption changes accompanying the photocycle of bacteriorhodopsin (BR) strongly depend on the intensity of the exciting short laser pulse. The decrease in the flash intensity dependence of the M kinetics after different extents of bleaching of the purple membranes by hydroxylamine proves the existence of a cooperative interaction between the photocycling BR molecules. The yield of the slow component of the M decay (M_s) is a quadratic function of the extent of the fraction cycling. The slope of the relative weight of M_s versus the fraction cycling is 0.5. This slope indicates a dimeric-like cooperative interaction, although the structural units of the purple membranes are the trimers of the BR molecules. For the most probable cooperative mechanism an asymmetric trimeric interaction is suggested, which accounts for the apparently dimeric features. A photocycling molecule may influence only one of its two neighbors in the trimer. From this asymmetric feature a deformative interaction is expected to be the cooperative mechanism, which would be an allosteric regulating mechanism in the purple membrane.

INTRODUCTION

Cooperativity of the excited BR molecules arranged in the trimeric structure of the purple membrane has been postulated again recently (Danshina et al., 1992). Although a few previous reports had demonstrated that the kinetics of BR photocycle strongly depend on the intensity of the exciting flash and suggested the existence of a kinetic cooperativity of the BR molecules (Korenstein et al., 1979; Ohno et al., 1981), its importance was not recognized, and its existence has been questioned (Xie et al., 1987; Dancsházy et al., 1988).

Various findings have been published in the literature, which were also explained by cooperative interactions of the BR molecules (Marinetti, 1988; Czégé and Reinish, 1991). Moreover, the role of the flash intensity in the efficient formation of the N (P, R) intermediate has also been briefly mentioned (Drachev et al., 1986).

Recently, we demonstrated that either the light-induced heterogeneity of the BR ground-state or cooperativity is necessary for the description of the BR photocycle (Tokaji and Dancsházy, 1991).

The present paper will provide strong evidence that the actinic light density dependence of the photocycle kinetics originates in a cooperative interaction of the neighboring BR molecules and suggest a model based on the trimeric structure of the purple membrane.

MATERIALS AND METHODS

The preparation of the sample and the absorption kinetic measuring system were essentially the same as described in (Tokaji and Dancsházy, 1991). The exciting flash was provided by a flash-lamp-pumped dye laser (Carl Zeiss, Germany, 1-μs half duration, Rhodamine 6G dye, λ = 590 nm). The data were collected by a transient recorder with a logarithmic time base, described previously in Gárgyán et al. (1982).

The fraction cycling was measured (at 412 nm) by the M forming efficiency of a second flash (Danshina et al., 1992, Tokaji and Dancsházy, 1991) provided by a nitrogen laser (JATE, Hungary, 10-ns half duration) pumped dye laser (Rhodamine 6G).

The decaying part of the absorption kinetic data was fitted with two exponential components (M_f and M_s) by a computer program (based on the least square method).

In the calculations equal quantum yields are assumed for the molecules forming M_f and M_s (i.e., lack of cooperativity is assumed for the quantum yields). This assumption is in accordance with the previous finding that the saturation of the total M yield versus the flash intensity very well fits to the theory assuming the same quantum yield for all of the BR molecules (Nagle et al., 1983).

The purple membranes were incorporated in a 1.5-mm-thick slice of 10% polyacrylamide gel, with absorbance indicated in the figures. Partial photobleaching of the purple membranes by hydroxylamine was carried out in gel at pH 7 by the method described by Ebrey et al. (1977). After the treatments the samples were washed in distilled water for 12 h, and then incubated in 30 mM universal buffer (citric acid, monopotassium sulfate, borate, diethyl barbiturate) with 1 M NaCl. The treatment practically did not influence the lifetimes of M_f and M_s.

RESULTS AND DISCUSSION

The relative weight of the slow component of the M decay strongly depends on the fraction cycling as it can be seen in Fig. 1, *a* and *b*. The lifetimes of the two components (M_f and M_s) are independent of the actinic light density in accordance with (Tokaji and Dancsházy, 1991). At very weak excitation the relative weight of M_s is only about 15%. At higher actinic light densities it increases linearly with the fraction cycling with a slope of 0.5. This linear dependence of the relative weight of M_s is equivalent with a quadratic dependence of the yield of M_s versus the fraction cycling.

The actinic light density dependence of the M kinetics can be decreased if a considerable amount of the BR molecules are bleached by hydroxylamine, as it can be seen in Fig. 2. We have recently shown that either light-induced heteroge-

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Abbreviations used: BR, bacteriorhodopsin; M_f and M_s, rapidly and slowly decaying components/forms of the M intermediate; β, the fraction cycling; P_0, P_M, P_D, P_T, the probability of the occupancy of the cooperative unit with no, 1, 2, or 3 cycling molecules, respectively.

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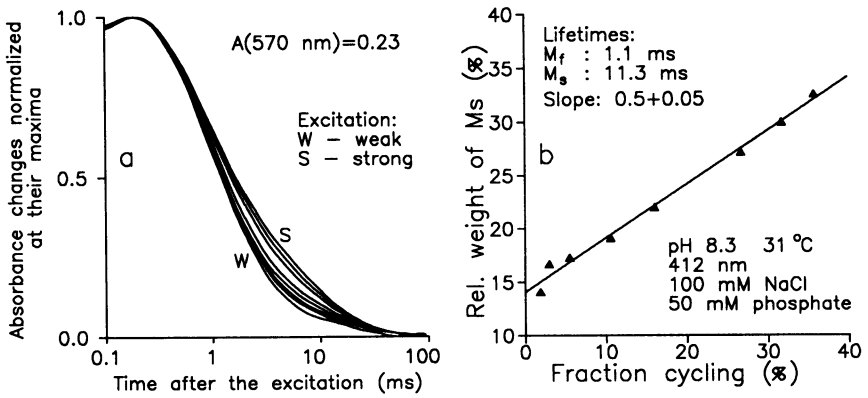

FIGURE 1 Actinic light density dependence of the M kinetics (*a*) original data traces, (*b*) the relative weight of M_s versus the fraction cycling.

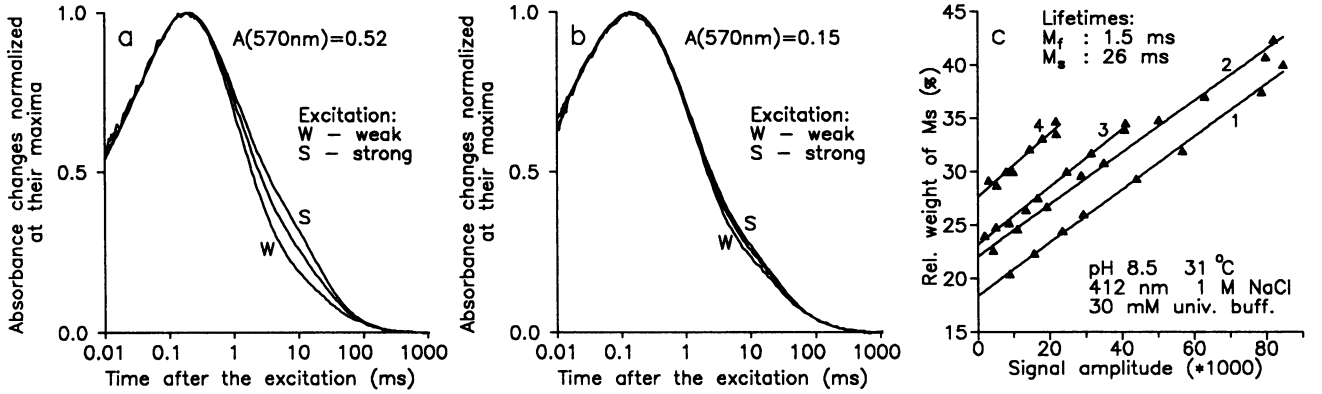

FIGURE 2 Influence of the partial bleaching of the BR sample (A = 0.55) on the actinic light density dependence of the M kinetics. (*a* and *b*) The actinic light density dependence of the sample incubated in hydroxylamine in the dark for 12 h (nonbleached; referred as *trace 2* in *c*) and a strongly bleached sample (referred to as *trace 4* in *c*), respectively. (*c*) The relative weight of M_s versus the signal amplitude obtained at different flash intensities. In *c*, *1* refers to the untreated sample, *2* to a sample incubated in hydroxylamine in the dark for 12 h, and *3* and *4* to two samples bleached in different extents. The absorbances of the samples at 570 nm are: *1*, 0.55; *2*, 0.52; *3*, 0.27; *4*, 0.15.

neity or cooperativity is responsible for the actinic light density dependence of the M kinetics (Tokaji and Dancsházy, 1991). From these two possibilities this experiment supports cooperativity, since the phenomenon is sensitive to the concentration of the functional BR molecules in the purple membranes.

An independent indication for the cooperativity of the BR molecules is the fact that the actinic light intensity dependence of the M kinetics disappears by the solubilization of the purple membranes by Triton X-100 published recently by Danshina et al. (1992) and also found by us (data not shown). However it should be noted that the solubilization seriously alters the kinetics of the photocycle (Danshina et al., 1992) in contrast to the method applied in the present study.

The highest actinic light densities were the same in all cases shown in Fig. 2 *c*, but the more bleached sample gave the smaller signal amplitude and the smaller change in the relative weight of M_s. In this representation the lines fitted to the data points (*solid lines* in Fig. 2 *c*) are parallel to each other within the experimental error, indicating that the changes in the relative weight of M_s, for a given change in the actinic light density, are proportional to the remaining absorption of the sample. Consequently, if the bleaching of the BR molecules is not cooperative, the course of the photocycle is altered either by the average concentration of the (not bleached and) photocycling BR molecules in the purple membrane (in a statistical way), or exclusively by a particular one (and not more) of the neighbors of the cycling molecule (dimeric-like cooperativity).

From the opposite point of view, as the same conclusion can be drawn for the cooperativity from the fact that the yield of M_s is a quadratic function of the fraction cycling (see Fig. 1. and text), the data shown in Fig. 2 *c* indicate the lack of cooperativity of the bleaching.

Note that the relative weight of M_s at very weak exciting flashes is on one hand not zero, probably mainly due to some kind of heterogeneity of the BR molecules (Dancsházy and Tokaji, 1993; Fukuda and Kouyama, 1992), and on the other hand it usually slightly increases in the bleached samples compared to the nonbleached ones (see Fig. 2 *c*). This additional increase in the relative weight of M_s may originate in a small amount of such conformations (i.e., in heterogeneity) of the bleached molecules which are detected by the neighboring and cycling molecules as if this part of the bleached molecules would also be cycling. Moreover, incubation in hydroxylamine in the dark for only 20 min or for

12 h does not decrease the absorption of the sample appreciably, but also causes a small (irreversible) increase in the relative weight of M_s. This, and the detectable difference between Figs. 1 and 2 *c* (*line 1*) in the relative weight of M_s at infinitesimal flash intensities indicate some importance of the solvent composition used for the incubation of the purple membranes, also mentioned by Kouyama et al. (1988).

The possible contribution of the optical thickness of the sample to the changes in the relative weight of M_s had been excluded previously (Tokaji and Dancsházy, 1991). However this result was confirmed for the present study in separate control measurements in which the absorbance of the sample (at 570 nm) was 0.15 and 0.05 before and after the bleaching, respectively.

The unit in the crystalline structure of the purple membranes is the trimer of the BR molecules, and this is also the most probable cooperative unit. However, first let us consider a dimeric interactive unit with a rule that a cycling BR becomes M_s only if its counterpart (which remains M_f) is also cycling. The possible states of the dimer are shown in Fig. 3 *a*. From simple calculations the average number of the cycling molecules is 2β in the dimer, and the relative weight of M_s is $\beta/2$ (see Table 1, column 1) in agreement with the results, shown in Figs. 1 and 2 (β is the fraction of the photocycling BR molecules).

The different possible states of a trimer are shown in Fig. 3 *b*. The average number of the cycling molecules in the trimer is 3β. The simple mechanisms in which M_s could be formed are the following: (*a*) If two or three molecules are cycling in the trimer, all of them become M_s (suggested by Ohno et al. (1981)). (*b*) If more than one molecules are cycling, only the first one remains M_f, the second (and third) become M_s. (*c*) M_s is produced only if all the three molecules of the trimer are cycling.

For these cases the expected functions of the relative weights of M_s versus β are listed in Table 1 (columns 2, 3, and 4, respectively). This indicates that none of these simple cooperative mechanisms are in accordance with the experimental results.

Up to the present we assumed that the cooperative interaction is symmetric, i.e., the excited molecule is able to alter both its left and right neighbors. In fact this assumption is not trivial, and not essentially true. For example, a deformative interaction could lead to a cooperativity in which the mol-

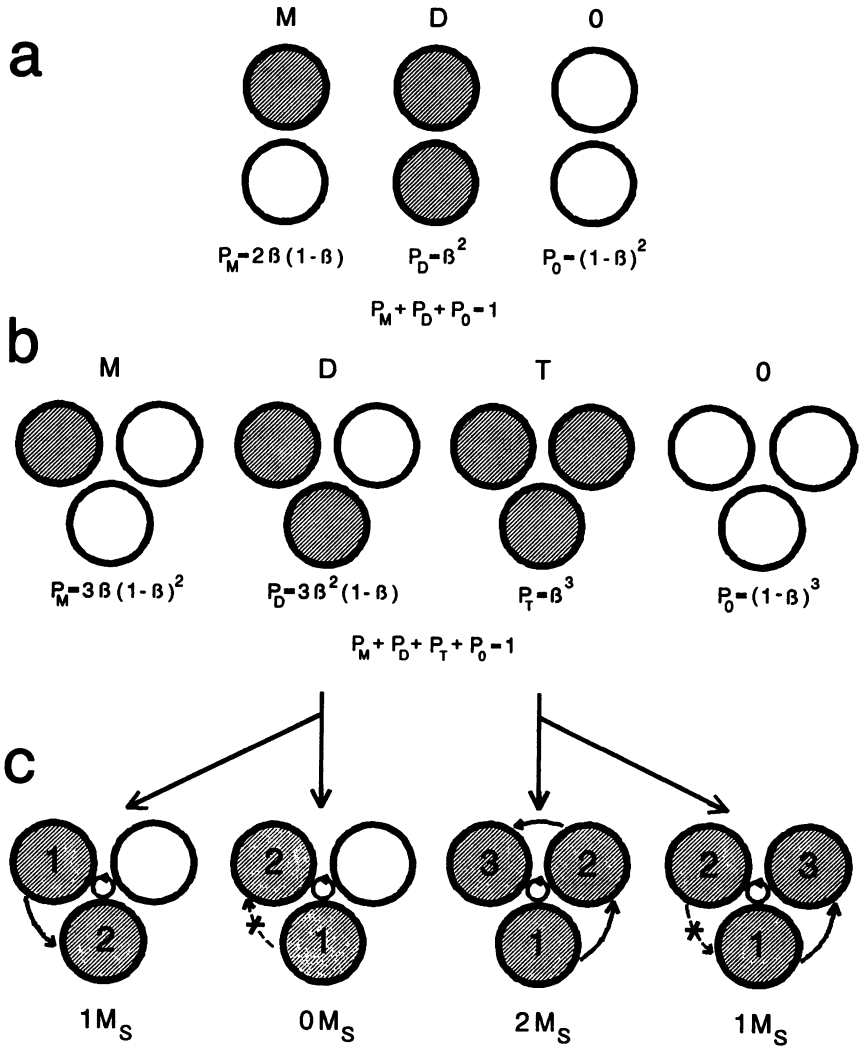

FIGURE 3 The excited states of (*a*) a dimer and (*b*) a trimer. (*c*) Representation of the sequential asymmetric cooperative interaction, in which passage from one photocycle state to the next causes unidirectional interaction with one of the neighbors, but only if that neighbor is in the first state. The numbers in *c* indicate the decay sequence of the molecules, and the centered circular arrows the possible direction of the interaction. The solid and dashed arrows indicate the successful or unsuccessful alteration of the photocycle of the neighboring, and cycling BR molecule.

TABLE 1 The expected slope of the relative weight of M_s (M_s/M) versus the fraction cycling (β) at different cooperative interactions.

	Type of cooperativity				
	Dimeric	Trimeric (*a*)	Trimeric (*b*)	Trimeric (*c*)	Trimeric sequential asymmetric
n	2β	3β	3β	3β	3β
M_s per cycling pair	1	2	1	0	0 or 1 (average = 0.5)
M_s per cycling trimer		3	2	1	1 or 2 (average = 1.5)
M_s/M	$\beta/2$	$2\beta - \beta^2$	$\beta - \beta^2/3$	$\beta^2/3$	$\beta/2$
Slope of M_s/M	1/2	$2 - 2\beta$	$1 - 2\beta/3$	$2\beta/3$	1/2

n is the average number of the photocycling molecules in the cooperative unit.

ecules could have sensitive and unsensitive parts, or oppositely the molecules could have (cooperatively) effective and ineffective parts. In other words we may assume that not both of the left and right segments of the BR molecule (in a trimer) are sensitive/effective for the M_s production.

In such a (nonstatistical) cooperativity, if the decay sequence of the molecules is unimportant, two excited molecules in one trimer would result 1 M_s, while, if all three are excited, they all would become M_s. The slope of the relative weight of M_s would be 1 versus β. If the interaction is statistical with a probability of 0.5, or rather if the decay sequence of the molecules of the trimer becomes important during a certain transition of the photocycle for the appearance of the cooperative interaction, as shown in Fig. 3 *c*, the expected slope drops to the experimentally measured value of 0.5 (see Table 1). This transition could be the L → M decay, as the actinic light density has detectable influence on the photocycle kinetics only after the end of this process (see, e.g., Fig. 2 *a*).

In conclusion the cooperativity resulting in the shift of the relative weight of M_s is probably trimeric, and consequently, if it is not a statistical process, it is asymmetric, and depends on the transition sequence of the involved molecules. Since the possible cooperative mechanisms are usually symmetric (excitonic; electric interactions through the bulk phase or in the surface of the purple membrane, etc.) except the ones connected to the motions of certain parts of the protein, this latter one is considered to be the most probable mechanism.

The present conclusion is in accordance with the latest structural studies (Subramaniam et al., 1993) on native BR in which, besides the motion of helix G, the only other considerable structural change is asymmetric and located to helix B in the M state. Helix B is at the border of two BR molecules and that is why this conformation change could be responsible for the asymmetric cooperative interaction suggested in this paper.

The suggested cooperativity would be an allosteric regulating mechanism of the purple membrane resembling in many respect to the other one (Ackers et al., 1992) extensively studied in the case of the hemoglobin tetramers. The importance of the sequentiality and the symmetry property of these two cooperative mechanisms seems to be common features, and perhaps these features can be of importance in a part of other allosteric regulations, too.

Finally, as the purple membrane contains uniform BR molecules, while a hemoglobin tetramer contains two chemically different types (α and β) of the hemoglobin molecules, and as BR can be easily studied by various spectroscopic methods, by further supporting results to the present work the purple membrane may become another or possibly better prototype of the allosteric regulating mechanisms.

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REFERENCES

Ackers, G. K., M. L. Doyle, D. Myers, and M. A. Daugherty. 1992. Molecular code for cooperativity in hemoglobin. *Science (Wash. DC).* 255: 54–63.

Czégé, J., and L. Reinisch. 1991. Photodestruction of bacteriorhodopsin. *Photochem. Photobiol.* 53:659–666.

Dancsházy, Z., and Z. Tokaji. 1993. Actinic light density dependence of the photocycle of bacteriorhodopsin. *Biophys. J.* In press.

Dancsházy, Z., R. Govindjee, and T. G. Ebrey. 1988. Independent photocycles of spectrally distinct forms of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 85:6358–6361.

Danshina, S. V., L. A. Drachev, A. D. Kaulen, and V. P. Skulachev. 1992. The inward H^+ pathway in bacteriorhodopsin: the role of M_{412} and $P(N)_{560}$ intermediates. *Photochem. Photobiol.* 55:735–740.

Drachev, L. A., A. D. Kaulen, V. P. Skulachev, and V. V. Zorina. 1986. Protonation of a novel intermediate P is involved in the M-bR step of the bacteriorhodopsin photocycle. *FEBS (Fed. Eur. Bioch. Soc.) Lett.* 209: 316–320.

Ebrey, T. G., B. Becher, B. Mao, P. Kilbride. 1977. Exciton interactions and chromophore orientation in the purple membrane. *J. Mol. Biol.* 112:377–397.

Fukuda, K., and T. Kouyama. 1992. Photoreaction of bacteriorhodopsin at high pH: origins of the slow decay component of M. *Biochemistry.* 31: 11740–11747.

Gárgyán, J., D. Kuschmitz, H. Schlüter, P. Klein, and Z. Imre. 1982. A log time scale recorder averager. *Aspects Mol. Bioenerg.* 363:543.

Korenstein, R., B. Hess, and M. Markus. 1979. Cooperativity in the photocycle of purple membrane of Halobacterium halobium with a mechanism of free energy transduction. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 102:155–161.

Kouyama, T., A. Nasuda-Kouyama, A. Ikegami, M. K. Mathew, and W. Stoeckenius. 1988. Bacteriorhodopsin photoreaction: identification of a

long-lived intermediate N (P, R^{350}) at high pH and its M-like photoproduct. *Biochemistry.* 27:5855–5863.

Marinetti, T. 1988. Nonproton ion release by purple membranes exhibits cooperativity as shown by determination of the optical cross-section. *Biophys. J.* 54:197–204.

Nagle, J. F., S. M. Bhattacharjee, L. A. Parodi, and R. H. Lozier. 1983. Effect of photoselection upon saturation and dichroic ratio in flash experiments upon effectively immobilized systems. *Photochem. Photobiol.* 38:331–339.

Ohno, K., Y. Takeuchi, and M. Yoshida. 1981. On the two forms of intermediate M of bacteriorhodopsin. *Photochem. Photobiol.* 33:573–578.

Subramaniam, S., M. Gerstein, D. Oesterhelt, and R. Henderson. 1993. Electron diffraction analysis of structural changes in the photocycle of bacteriorhodopsin. *EMBO J.* 12:1–8.

Tokaji, Z., and Z. Dancsházy. 1991. Light-induced, long-lived perturbation of the photocycle of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem Soc.) Lett.* 281:170–172.

Xie, A. H., J. F. Nagle, and R. H. Lozier 1987. Flash spectroscopy of purple membrane. *Biophys. J.* 51:627–635.