Thermal Equilibration between the M and N Intermediates in the Photocycle of Bacteriorhodopsin

S. Druckmann,* M. P. Heyn,‡ J. K. Lanyi,§ M. Ottolenghi,*‖ and L. Zimanyi§¶

*Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; ‡Biophysics Group, Freie Universität Berlin, D-1000 Berlin 33, Germany; and §Department of Physiology and Biophysics, University of California, Irvine, California 92717 USA

ABSTRACT The stages in the photocycle of bacteriorhodopsin (BR) involving the M and N intermediates are investigated using a double pulse excitation method. A first (cycling) pulse at 532 nm is followed, with an appropriate time delay, by a second pulse (337, 406, 446, or 470 nm) which induces the M → BR back-photoreaction. After depletion by the second pulse a repopulation of M in the millisecond range is observed which is interpreted in terms of a thermal N → M relaxation. It is thus concluded that a (thermal) M ↔ N equilibrium accounts for the biphasic decay of M in the BR photocycle. Other models for this stage of the light-driven proton-pump are therefore unnecessary.

INTRODUCTION

The photocycle of the light-driven proton pump bacteriorhodopsin (BR) in *Halobacterium halobium*, describes the reactions after photoexcitation of the retinal chromophore which, over a complex series of steps, result in the translocation of a proton across the cytoplasmic membrane of halobacteria and in the recovery of the initial state (Mathies et al., 1991; Rothschild, 1992; Lanyi, 1992; Oesterhelt, 1992). The spectroscopically identified intermediates of the photocycle, J, K, L, M, N, and O, and substates of some of these, appear to rise and decay in a roughly sequential manner. An exact description of the kinetic scheme which connects these intermediates is essential to the understanding of the proton transport mechanism. However, as widely recognized (e.g., Nagle et al., 1982; Varo and Lanyi, 1991a; Lozier et al., 1992) there are serious problems in directly testing kinetic schemes, because the absorption spectra of many of the intermediates overlap, and some of their relaxation constants are too close to one another.

The reactions associated with the M intermediate involve changes in the protonation of the Schiff base linkage between the retinal and the protein and are an essential part of the complex proton translocation mechanism (see Ebrey (1993), for a recent review). Specifically, the pathway from M to BR has been particularly controversial. There are a number of proposed schemes which view the photocycle in fundamentally different ways. They pertain to the relaxation kinetics of the M intermediate, which is described approximately by two time constants. The biphasic M decay is attributed either (*a*) to the single sequence M ↔ N ↔ O (Otto et al., 1989; Ames and Mathies, 1990; Gerwert et al., 1990; Varo and Lanyi, 1991a, 1991b; Souvignier and Gerwert, 1992), i.e., to a significant N to M back-reaction, or (*b*) to two distinct M states which decay unidirectionally in a parallel fashion (Hanamoto et al., 1984; Diller and Stockburger, 1988; Dancshazy et al; 1988; Bitting et al., 1990; Fukuda and Kouyama, 1992a,b; Tokaji and Dancshazy, 1992a, 1992b). The proposed origins of the two kinetic components in models with two parallel M states include heterogeneity in the initial BR state (Hanamoto et al., 1984; Diller and Stockburger, 1988; Dancshazy et al., 1988; Kouyama et al., 1988), branching at an earlier time in the photocycle (Sherman et al., 1979; Butt et al., 1989; Drachev et al., 1992), cooperativity which arises from photoexcitation of neighboring BR molecules (Tokaji and Dancshazy 1991, 1992b), or a photoreaction of the N intermediate which generates the slower decaying M (Kouyama et al., 1988; Fukuda and Kouyama, 1992b).

The simpler sequential model is supported by self-consistency of the pH and temperature dependencies of the calculated amplitudes and rate constants of the reactions (Otto et al., 1989; Ames and Mathies, 1990; Varo and Lanyi, 1990; Varo and Lanyi, 1991b). However, since the two kinds of models are kinetically equivalent such arguments cannot be conclusive. In this report we examine the existence of the N to M back-reaction, which is the critical difference between the two kinds of models, by a direct approach based on the back photoreaction of M (Kalisky et al., 1978, 1981). Since equilibria can be distinguished from unidirectional reactions by perturbation kinetics, our methodology is to produce M with a first flash, and partially photodeplete it during its decay with a second flash (Lozier et al., 1978; Druckmann et al., 1992). Analysis of the data shows repopulation of the depleted 410-nm band of M, characteristic of its nonprotonated Schiff base linkage. The reaction, which takes place in the millisecond range, is accompanied by a corresponding absorbance decay around 550 nm. These phenomena are in keeping with a thermal N ⇄ M equilibrium as an essential basic feature of the BR photocycle.

The two pulse excitation technique has been recently described (Druckmann et al., 1992). The bR photocycle is

Received for publication 26 March 1993 and in final form 9 June 1993.

‖To whom inquiries should be addressed.

¶Permanent address: Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary H-6701.

0006-3495/93/09/1231/04 $2.00

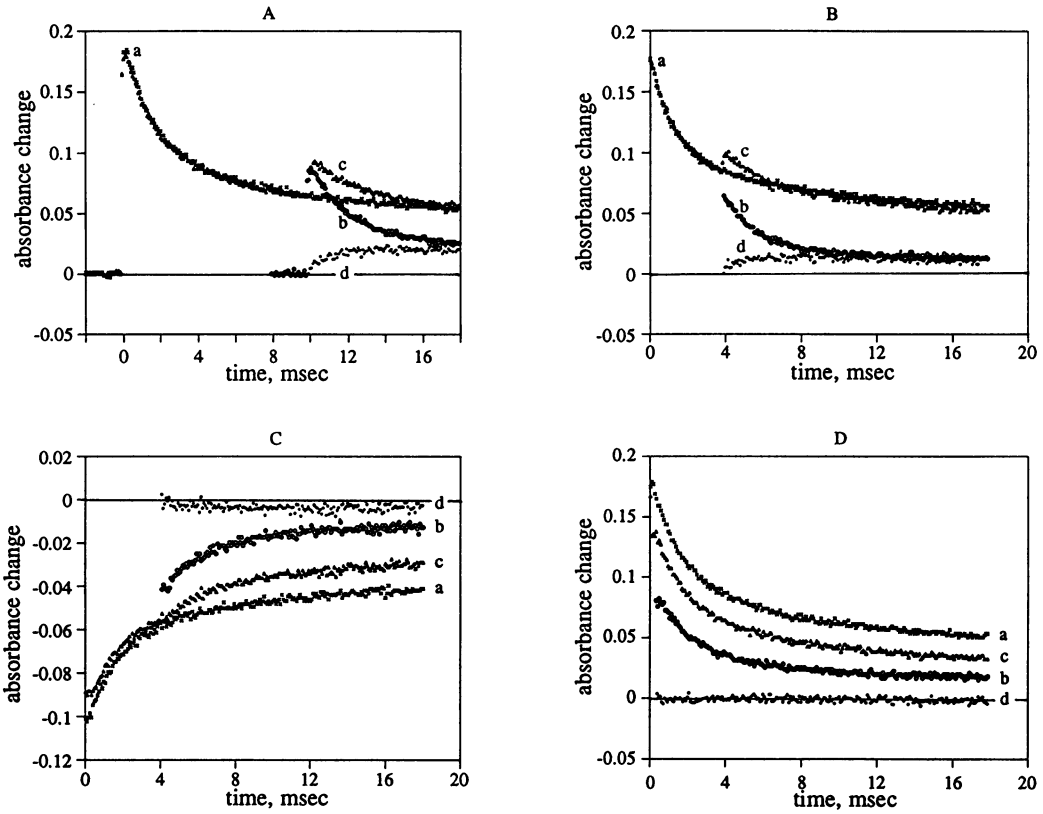

FIGURE 1 Characteristic traces (*a–c*) at pH 9.4 of double pulse experiments, and the resulting relaxation kinetics (*d*) following the photodepletion of the M intermediate in the BR photocycle. *A*, *B*, and *D* show the absorbance changes for the characteristic 410-nm band of M, while *C* shows the changes at 580 nm, where N and BR absorb. First (cycling) pulse at λ_1 = 532 nm. Second (back-reacting) pulse at λ_2 = 406 nm (*A*) or λ_2 = 337 nm (*B–D*), fired with a time delay Δt. (*a*) ΔD_1, first pulse only. (*b*) ΔD_2-second pulse only. (*c*) $\Delta D_{1,2}$, both pulses superimposed. (*d*) ΔD_P (see text). (Note that *A* shows signals including the pre-(laser) trigger traces, while these are not shown for *B–D*.) f_1 (see text) is determined by the magnitude of ΔD_1 relative to the absorbance of the solution. f_2 (see text) was estimated from the initial amount of bleaching of the band by the second pulse, namely, by: $f_2 = (\Delta D_{1,2} - f_1\Delta D_2)/\Delta D_1$. (*A*) λ_2 = 406 nm, monitoring at 410 nm, Δt = 10 ms, f_1 =0.75, f_2 = 0.32. (*B*) λ_2 = 337 nm, monitoring at 410 nm, Δt = 4 ms, f_1 = 0.75, f_2 = 0.57. (*C*) λ_2 = 337 nm, monitoring at 580 nm, Δt = 4 ms, f_1 = 0.75, f_2 = 0.4. (*D*) λ_2 = 337 nm, monitoring at 410 nm, Δt = 250 μs, f_1 = 0.75, f_2=0.48. BR concentrations were 10–20 μmol/liter. Each sample contains 100 mM NaCl, 50 mM buffer, for pH 7 phosphate buffer, and for pH 9.4 carbonate buffer. All experiments were carried out at room temperature (20°C).

driven by exposing purple membrane suspensions to the (first) 532 nm, ~10 ns, pulse of a Nd/YAG laser. Subsequently, the second pulse (337 nm, ~1 ns from a PRA N_2 laser, or 406, 446, and 470 nm from a PRA N_2/dye laser) is fired with a tunable time delay, Δt, in the range 200 μs < Δt < 10 ms. When analyzing the net effects of the second pulse in the double pulse experiments, one should bear in mind that the second pulse is absorbed not only by the M intermediate, but also by (noncycling) BR. One should also consider the contribution of the absorbance changes induced by the first pulse. We thus proceed by denoting the absorbance changes induced by the first pulse only, by the second pulse only and by both pulses superimposed, as ΔD_1, ΔD_2 and $\Delta D_{1,2}$ respectively. Accordingly, any net perturbation of the photocycle induced by the depletion (back-photoreaction) of M, which we denote as ΔD_P, will be given by

$$\Delta D_P = \Delta D_{1,2} - \Delta D_2 \cdot f_1 - \Delta D_1 \cdot f_2 \qquad (1)$$

where f_1 denotes the fraction of BR molecules which have not been photocycled by the first pulse and f_2 denotes the fraction of the M intermediate which has not back photoreacted by the second pulse. Bleaching of the 410-nm M band due to the back-photoreaction is completed after ~0.5 μs (28, 31). At this time, which we define as t = 0, ΔD_P is zero

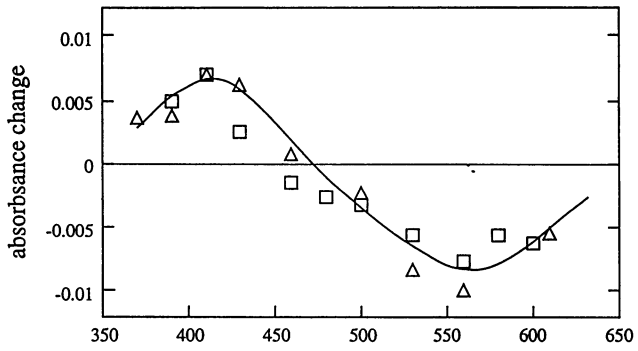

FIGURE 2 Wavelength dependencies of ΔD_P^{max}. λ_2 = 337 nm, Δt = 4 ms. △, results measured at pH 7; □, results measured at pH 9.4. Data at pH 7 have been scaled to fit those at pH 9.4. The continuous line is the (scaled) M-N difference spectrum based on the spectra of N and M taken from Varo and Lanyi (1991a).

TABLE 1 Relative efficiency of the M regeneration reaction D_P^{max} for a variety of (second pulse) excitation wavelengths

	B/I		ϵ_M	$\Delta D_P^{max}/B$		$\Delta D_P^{max}/I$		ϵ_N
λ	pH 7	pH 9.4	$\times 10^{-4}$	pH 7	pH 9.4	pH 7	pH 9.4	$\times 10^{-4}$
nm			$M^{-1}\ cm^{-1}$					$M^{-1}\ cm^{-1}$
337	0.09 ± 0.02	0.13 ± 0.02		1.5 ± 0.3	1.25 ± 0.2	0.15 ± 0.03	0.16 ± 0.03	
406	1.30 ± 0.20	1.00 ± 0.20	4.48	0.70 ± 0.2	1.00 ± 0.2	0.98 ± 0.20	1.00 ± 0.20	1.07
446	0.32 ± 0.06	0.40 ± 0.08	2.60	0.55 ± 0.2	0.75 ± 0.2	0.18 ± 0.04	0.32 ± 0.06	0.70
470	0.19 ± 0.04	0.22 ± 0.04	0.69	0.60 ± 0.2	1.3 ± 0.2	0.12 ± 0.02	0.27 ± 0.06	0.75

B represents the relative efficiency of M depletion (bleaching) induced by the second pulse, while I is the relative intensity of the latter measured by ΔD_2. Values are in arbitrary units, scaled to those at 406 nm, pH 9.4 which are taken as unity. ϵ_M and ϵ_N are the extinction coefficients of M and N, respectively (see Varo and Lanyi, 1991a; 1991b).

by definition, while the subsequent time evolution of ΔD_P should reflect the relaxation effects induced by the photodepletion of M. Note that $\Delta D_2 \cdot f_1$ corrects for the effects due to bR molecules driven into the photocycle by the second pulse, while $\Delta D_1 \cdot f_2$ represents the contribution of the M population which has not been depleted by the second pulse.

Fig. 1, *A* and *B*, show characteristic traces at pH 9.4 of ΔD_1, ΔD_2, $\Delta D_{1,2}$, and ΔD_P at 410 nm, for a Δt = 10 ms or Δt = 4 ms delay between the 532-nm cycling pulse and the back-reacting, 406- or 337-nm pulse, respectively. It is evident that ΔD_P increases with time after the bleaching of M, reaching a maximum (ΔD_P^{max}). The effect is reversed at 580 nm (Fig. 1 *C*) and is not observed for Δt = 250 μs, when ΔD_P = 0 at all photocycle times (Fig. 1 *D*). The rise of ΔD_P is followed by a slow decay which is evident in *B* (but not in *C* where the signal-to-noise ratio is relatively low). Analogous phenomena are also observed at pH 7. The above data are consistent with a relaxation reaction in which the 410-nm absorption of M is regenerated at the expense of bleaching at 580 nm. In Fig. 2 we plot $\Delta D_P{}^{max}$ at both pH 7 and 9.4 as a function of λ and compare the results with the normalized M-N difference spectrum (Varo and Lanyi, 1991b). The fact that the two curves are identical within the limits of our experimental accuracy, along with the absence of a relaxation for short time delays (e.g., Δt = 250 μs), when N is not yet generated (Varo and Lanyi, 1991a), is in keeping with a net N → M relaxation process in which the M ⇄ N equilibrium is re-established for the photodepleted M population.

The above conclusion is based on the assumption that the observed repopulation of M induced by its photodepletion, is due to thermal re-equilibration from N. The possibility that this N → M transition is due primarily to a photoreaction of N (Kouyama et al., 1988; Fukuda and Kouyama, 1992b), induced by the second pulse, is excluded by the data presented in Table 1. The table compares the relative amplitude of the relaxation reaction (ΔD_P^{max}) for four wavelengths of the back-photoreacting (second) pulse, namely: 337, 406, 446, and 470 nm, which span the M absorption band. The first (B/I) column in Table 1 shows that the bleaching of the M absorption fairly follows the relative values of the M extinction coefficient. The second column ($\Delta D_P^{max}/B$) shows that the M refill effect is essentially proportional to the amount of M which back-photoreacted. A tendency to somehow higher values at 470 and 337 nm may be attributed to a contribution of the N → M photoprocess (Kouyama et al., 1988; Fukuda and Kouyama, 1992b), but it cannot account for more than ~20% of the M refill effect. The conclusion that the present M-repopulation effect is due to the N → M thermal (rather then photochemical) reaction is also reflected by the third ($\Delta D_P^{max}/I$) column in Table 1. Since the N extinction coefficient is essentially constant in the 337–470-nm range, a photochemical N → M process would have yielded an almost constant $\Delta D_P^{max}/I$ value. This is obviously not the case. Thus, our present observations indicate a relative inefficiency of the N → M photoconversion with λ = 337–470 nm. This is in keeping with a recent study (Brown et al., submitted for publication)[1] of the N → M photoprocess in the T46V mutant of BR, showing that the yield of the photo reaction, when induced by blue light excitation, is very small compared to that produced by yellow-green wavelengths. As to the possibility that the observed M ⇄ N equilibrium represents a photoequilibrium induced by the monitoring beam (Kouyama et al., 1988; Fukuda and Kouyama 1992b) rather than a thermal reaction, this alternative was excluded by running our experiments with variable monitoring light intensities. Intensity changes of up to a factor of ~30 did not have any measurable effect on ΔD_P.

For the thermal N ⇄ relaxation process we find at pH 9.4 $k_r = k_{NM} + k_{MN} = (1 \pm 0.5 \times 10^3\ s^{-1})$. Within experimental accuracy this value is the same as the M ⇄ N equilibration rate (k_r = 0.5–0.7 × $10^3\ s^{-1}$) predicted by the sequential model for the M↔N↔O transition (Varo and Lanyi, 1990; 1991a; Zimanyi et al., submitted for publication). We conclude that the thermal N to M back reaction accounts satisfactorily for the M decay kinetics, thus rendering unnecessary other approaches to the mechanism of the decay of the M intermediate in the BR photocycle.

This work was supported by grants (to M. Ottolenghi) by the U.S.-Israel Binational Science Foundation and (to J. K. Lanyi) from the National Institutes of Health (GM 29498) and the Department of Energy (DE-FGO3-86ER 13525).

[1] Brown, S. L., L. Zimanyi, R. Needleman, M. Ottolenghi, and J. K. Lanyi. Photoreaction of the N intermediate, and its relationship to the decay kinetics of the M intermediate. Submitted for publication.

REFERENCES

Ames, J. B., and R. A. Mathies. 1990. The role of back-reactions and proton uptake during the N→O transition in bacteriorhodopsin's photocycle: a kinetic resonance Raman study. *Biochemistry.* 29:7181–7190.

Bitting, H. C., D.-J. Jang, and M. A. El-Sayed. 1990. On the multiple cycles of bacteriorhodopsin at high pH. *Photochem. Photobiol.* 51:593–598.

Butt, H.-J., K. Fendler, A. Der, and E. Bamberg. 1989. Temperature jump study of charge translocation during the bacteriorhodopsin photocycle. *Biophys. J.* 56:851–859.

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

Diller, R., and M. Stockburger. 1988. Kinetic resonance Raman studies reveal different conformational states of bacteriorhodopsin. *Biochemistry.* 27:7641–7651.

Drachev, L. A., A. D. Kaulen, and A. Y. Komrakov. 1992. Inter-relations of M-intermediates in bacteriorhodopsin photocycle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 313:248–250.

Druckmann, S., N. Friedman, J. K. Lanyi, R. Needleman, M. Ottolenghi, and M. Sheves. 1992. The back photoreaction of the M intermediate in the photocycle of bacteriorhodopsin: mechanism and evidence for two M species. *Photochem. Photobiol.* 56:1041–1047.

Ebrey, T. G. 1993. Light energy transduction in bacteriorhodopsin. *In* Thermodynamics of Membranes, Receptors, and Channels. M. Jackson, editor. CRC Press, Chicago. pp. 353–387.

Fukuda, K., and T. Kouyama. 1992a. Formation and decay of bacteriorhodopsin's N intermediate: softening the protein conformation with alcohols affects intra-protein proton transfer and retinal isomerization. *Photochem. Photobiol.* 56:1057–1062.

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

Gerwert, K., G. Souvignier, and B. Hess, 1990. Simultaneous monitoring of light-induced changes in protein side-group protonation, chromophore isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy. *Proc. Natl. Acad. Sci. USA.* 87:9774–9778.

Hanamoto, J. H., P. Dupuis, and M. A. El-Sayed. 1984. On the protein (tyrosine)-chromophore (protonated Schiff base) coupling in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 81:7083–7087.

Kalisky, O., U. Lachish, and M. Ottolenghi. 1978. Time resolution of a back photoreaction in bacteriorhodopsin. *Photochem. Photobiol.* 28:261–263.

Kalisky, O., M. Ottolenghi, B. Honig, and R. Korenstein. 1981. Environmental effects on formation and photoreaction of the M412 photoproduct of bacteriorhodopsin: implications for the mechanism of proton pumping. *Biochemistry.* 20:649–655.

Kouyama, T., A. Nasuda-Kouyama, A. Ikegami, M. K. Mathew, and W. Stoeckenius. 1988. Bacteriorhodopsin photoreaction: identification of a long-lived intermediate N (P, R350) at high pH and its M-like photoproduct. *Biochemistry.* 27:5855–5863.

Lanyi, J. K. 1992. Proton transfer and energy coupling in the bacteriorhodopsin photocycle. *J. Bioenerg. Biomembr.* 24:169–179.

Lozier, R. H., W. Niederberger, M. Ottolenghi, G. Sivorinovsky, and W. Stoeckenius. 1978. The photocycle of light- and dark-adapted bacteriorhodopsin. *In* Energetics, and Structure of Halophilic Microorganisms. S. R. Caplan, and M. Ginzburg, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. pp. 123–141.

Lozier, R. H., A. Xie, J. Hofrichter, and G. M. Clore. 1992. Reversible steps in the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 89: 3610–3614.

Mathies, R. A., S. W. Lin, J. B. Ames, and W. T. Pollard. 1991. From femtoseconds to biology: mechanism of bacteriorhodopsin's light-driven proton pump. *Annu. Rev. Biophys. Biophys. Chem.* 20:491–518.

Nagle, J. F., L. A. Parodi, and R. H. Lozier. 1982. Procedure for testing kinetic models of the photocycle of bacteriorhodopsin. *Biophys. J.* 38: 161–174.

Oesterhelt, D., J. Tittor, and E. Bamberg. 1992. A unifying concept for ion translocation by retinal proteins. *J. Bioenerg. Biomembr.* 24:181–191.

Otto, H., T. Marti, M. Holz, T. Mogi, M. Lindau, H. G. Khorana, and M. P. Heyn. 1989. Aspartic acid-96 is the internal proton donor in the reprotonation of the Schiff base of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 86:9228–9232.

Rothschild, K. J. 1992. FTIR difference spectroscopy of bacteriorhodopsin: toward a molecular model. *J. Bioenerg. Biomembr.* 24:147–167.

Sherman, W. V., R. R. Eicke, S. R. Stafford, and F. M. Wasacz. 1979. Branching in the bacteriorhodopsin photochemical cycle. *Photochem. Photobiol.* 30:727–729.

Souvignier, G., and K. Gerwert. 1992. Proton uptake mechanism of bacteriorhodopsin as determined by time-resolved stroboscopic FTIR spectroscopy. *Biophys. J.* 63:1393–1405.

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

Tokaji, Z., and Z. Dancshazy. 1992a. Kinetics of the N intermediate and the two pathways of recovery of the ground-state of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 311:267–270.

Tokaji, Z., and Z. Dancshazy. 1992b. Light density controls the mechanism of the photocycle of bacteriorhodopsin. *In* Structures, and Functions of Retinal Proteins. J. L. Rigaud, editor. John Libbey Eurotext Ltd., Paris. 175–178.

Varo, G., and J. K. Lanyi, 1990. Pathways of the rise and decay of the M photo intermediate of bacteriorhodopsin. *Biochemistry.* 29:2241–2250.

Varo, G., and J. K. Lanyi. 1991a. Kinetic and spectroscopic evidence for an irreversible step between deprotonation and reprotonation of the Schiff base in the bacteriorhodopsin photocycle. *Biochemistry.* 30:5009–5015.

Varo, G., and J. K. Lanyi. 1991b. Thermodynamics and energy coupling in the bacteriorhodopsin photocycle. *Biochemistry.* 30:5016–5022.

Zimanyi, L., Y. Cao, R. Needleman, M. Ottolenghi, and J. K. Lanyi. Pathways of proton uptake in the bacteriorhodopsin photocycle. Submitted for publication.