Photoexcitation of the O-Intermediate in Bacteriorhodopsin Mutant L93A

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ABSTRACT During the extended lifetime of the O-state in bacteriorhodopsin (bR) mutant L93A, two substates have been distinguished. The first O-intermediate (OI) is in rapid equilibrium with N and apparently still has a 13-*cis* chromophore. OI undergoes a photoreaction with a small absorbance change, positive charge transport in the pumping direction, and proton release and uptake. None of these effects was detected after photoexcitation of the late O (OII). The most likely interpretation of the effects seen is an accelerated return of the molecule from the OI- to the bR-state. However, with a lifetime $\approx$140 ms, the reaction cannot account for the observed high pumping efficiency of the mutant under continuous illumination. We suggest that OII corresponds to the O-intermediate with a twisted all-*trans* chromophore seen in the photocycle of wild-type bR, where the 13-*cis* OI-intermediate under the usual conditions does not accumulate in easily detectable amounts and, therefore, has generally been overlooked. Both the OI- and OII-decays are apparently strongly inhibited in the mutant.

INTRODUCTION

In the companion paper on the photocycle of bacteriorhodopsin (bR) mutant L93A (Tóth-Boconádi et al., 2003), we have shown that proton uptake, charge translocation, and absorbance changes in the second half of the cycle have slow components (350 and 1800 ms lifetimes) and are only completed in the last step of the cycle, when the ground-state bR is restored. These results contradicted earlier work by Subramaniam and collaborators who first studied this mutant and had concluded that charge transport is completed in the N-state (Subramaniam et al., 1991; Delaney et al., 1995; Delaney and Subramaniam, 1996; Subramaniam et al., 1997; Subramaniam et al., 1999). We also presented additional evidence for efficient proton pumping of this mutant in continuous light due to absorption of a second photon by the N-component of the N- first O-intermediate (OI) equilibrium, whereas Subramaniam and collaborators preferred the O-component as the source of accelerated transport. However, our findings did not exclude a photoreaction of O as another possible mechanism for increased pumping efficiency, which we investigate here. Earlier works on excitation of wild-type bacteriorhodopsin (WTbR) photocycle intermediates have been reviewed by Balashov (1995).

We present evidence for a previously unnoticed transition in the O-state, confirm an accelerated return of excited O to the ground state, but find it too slow to account for the efficient proton pumping of this mutant under continuous illumination.

MATERIALS AND METHODS

The materials and methods were essentially the same as in our companion paper in this issue (Tóth-Boconádi et al., 2003). Oriented samples of purple membrane in gels (Dér et al., 1985) or purple membrane-suspensions were used as indicated. Two lasers served as light sources for flash illumination: a frequency-doubled Nd-YAG laser (Surelite I-10, Continuum, Santa Clara, CA) and an excimer laser-driven dye laser (Lambda Physik, EMG 101 MSC, Göttingen, Germany) with dyes LC5530 fluorescein 27 (550 nm), LC6200 sulphorhodamine B (605 nm), and LC6500 DCM (650 nm) (Lambdachrom Laser Dye, Göttingen, Germany). The Nd-YAG laser provided the first flash and the excimer laser the second. Energy of the lasers varied between 1 and 2 mJ. In one experiment, a photographic flash lamp (Flash Gun P21*44, Hanimex, Hong Kong) illuminated the sample as first flash through water heat bath (the half-time of the flash was 0.65 ms). We used a 200-W tungsten lamp to illuminate the samples with continuous red light through heat and high pass filters ($\lambda > 650$ nm) and 550 or 625 nm interference filters for monitoring beams. A homemade time generator controlled the delay between flashes. Shutters (opening and closing time 7 ms) were used with quasicontinuous illumination and for green light the 514 nm line of an argon ion laser (Stabilite 2016, Spectra-Physics, Mountain View, CA). Light intensity was varied with neutral density filters and measured with a LI-250 light meter (LI-COR, Lincoln, NE).

RESULTS

The main problem in studying photoreactions, e.g., charge translocation, of a photocycle intermediate is the separation of its response from signals due to the unavoidable excitation of the residual ground state and possibly other intermediates generated by the first flash. The population of these species changes with the wavelength, duration, and intensity of the first flash and the delay times of the second. Complete separation can only be approximated. In L93A, the O-intermediate appears last with a relatively long rise time in a red-shifted N $\leftrightarrow$ O equilibrium when other intermediates, except for the small amount of N, have decayed and will contribute negligibly at the optimal excitation wavelength. We measured the electric responses to the first flash $A(\mathrm{bR})_{530}(t)$, to the second flash without the first flash $A(\mathrm{bR})_{650}(t,\infty)$ and together with the first flash $A_{\mathrm{D}}(t,T)$, where

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For a definition of the terminology used and a few other general points, see Footnote 1 and Notes 2, 3, and 4 in the companion paper (Tóth-Boconádi et al., 2003).

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t is the time in the photocycle and T is the time delay between the flashes. Thus

$$A_D(t,T) = A(\mathrm{bR})_{530}(t) + rA(\mathrm{bR})_{650}(t,T) + A(I)_{650}(t,T), \quad (1)$$

where the coefficient r gives the fraction not excited by the first flash ("residual ground state") and I denotes the intermediate. Subtracting the recorded $A(\mathrm{bR})_{530}(t)$ from $A_D(t,T)$, we obtain $A_M(t,T)$. To determine $A(I)_{650}(t,T)$, we should correct $A_M(t,T)$. The bR absorbance maximum for L93A is at 540 nm, and the O-maximum near 590 nm. We used 530 and 650 nm flashes and assumed that at short delays (T_0), when the N ↔ O equilibrium has not yet formed, the second flash excites the residual ground state and generates no significant currents from the earlier intermediates. Only at long delays do we take into account regenerated bR-state excitation. The time dependence for the correction then will be:

$$C(t,T) = rA(\mathrm{bR})_{650}(t,T_0) + ((A(\mathrm{bR})_{650}(t,\infty) - rA(\mathrm{bR})_{650}(t,T_0)) \times (1-\exp-(T-T_0)/\tau), \quad (2)$$

where τ is the lifetime for regeneration of bR. From the measured amplitudes $A_M(t,T)$ values of $C(t,T)$ are subtracted and the differences $A_0(t,T)$ are taken to represent the response to O-excitation.

The microsecond components of the electric signals for excitation of O and bR are very different. The bR signal is negative (Fig. 1, *line a*), the O-signal is positive (*line b*). We use the time integral for this first, fast current component as a measure for the amount of excited O and plot it as a function of the time delay between the flashes (Fig. 2 *A*, *circles*). At longer delay times, the values fall by increasing amounts below a curve showing the O-absorbance changes at 632 nm (*line a*). The current integral reaches negative values after 600 ms and continues to fall. We compared the shape of the current signal in the second region with that after single flash excitation and found no difference within error. Consequently we assigned this region to excitation of the residual bR-state and extrapolated it by fitting to shorter delay times (*line b* in Fig. 2 *A*). As expected, it falls to near zero values for the residual bR-excitation at short delays.

To check our assumption that there are no significant contributions from other intermediates, we repeated the experiment using 605 nm for the second flash (Fig. 2 *B*). In this case the bR-state must absorb more photons and increase any possible distortion of the results. The fitted and extrapolated b functions served as the integral of $C(t,T)$ functions for the fast signals. These values were subtracted from the integral of the measured fast signals (*solid circles* in Fig. 2, *A* and *B*). The differences are similar in both cases and confirm that these data points (*squares*) apparently represent the integral of the fast current signals generated by O-excitation. The corrected values follow O-absorbance (*lines a*) up to 80 ms and then drop to zero with a lifetime of 320 ± 30 ms. This value—within the accuracy of our data—matches the 360-ms decay components found in the current and absorbance signals after single flash excitation (Tóth-Boconádi et al., 2003). We conclude that O converts to a second O-state (OII) with slightly lower and/or shifted absorbance, and this OII upon excitation does not generate a detectable current within the time domain and sensitivity of our measurements.

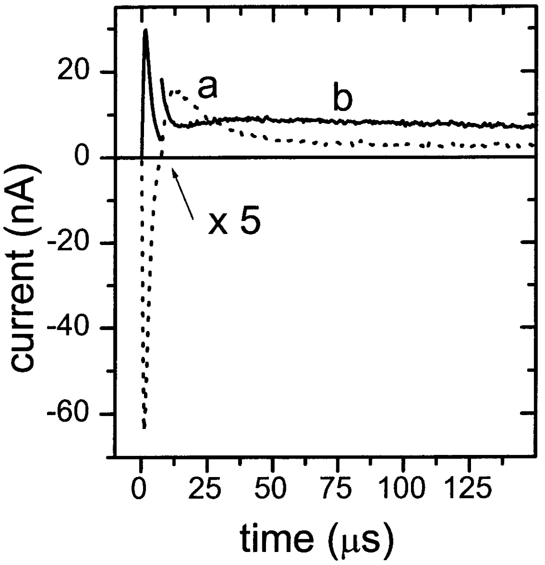

FIGURE 1 Electric signals excited with laser flash of 650 nm. (*a*) Single flash experiment, using only the second flash; (*b*) double flash experiment with a flash interval of 80 ms (Nd-YAG laser as first flash and dye laser of 650 nm as second). The recording was triggered in both cases with the second flash. Solution 50 μM $CaCl_2$, pH 7.5, 22°.

The O-current excited at a delay of 80 ms was determined using the complete correction method (Eqs. 1 and 2). It shows a small negative exponential with a time constant of 5.1 μs, followed by positive 0.5 and 9.6 ms components (Fig. 3 *A* and Table 1). Its time integral (Fig. 3 *B*) is positive, indicating charge transport in the pumping direction. The corresponding pH measurements detect a proton release with a lifetime of 11 ± 2 ms (Fig. 4), which, if it corresponds to the 9.6-ms current signal, must occur from the external surface. No current corresponding to the 143 ± 6 ms proton uptake component has been seen, probably because the charge movement is too slow and of small amplitude to be detected.

The correlated 625 nm and 550 nm absorbance changes for O-excitation (Fig. 5, *A* and *B*) reveal more details. They show a large, not time-resolved absorbance decrease followed by a rising 0.51 ms component (Table 1) of relatively large amplitude, which appears with opposite sign and small amplitude at 550 nm. Increasing small 2.1-ms components are seen at both wavelengths. The slower absorbance component with a measured lifetime of ≈140 ms at 625 nm (Fig. 6) and 130 ms at 550 nm (Fig. 7) corresponds

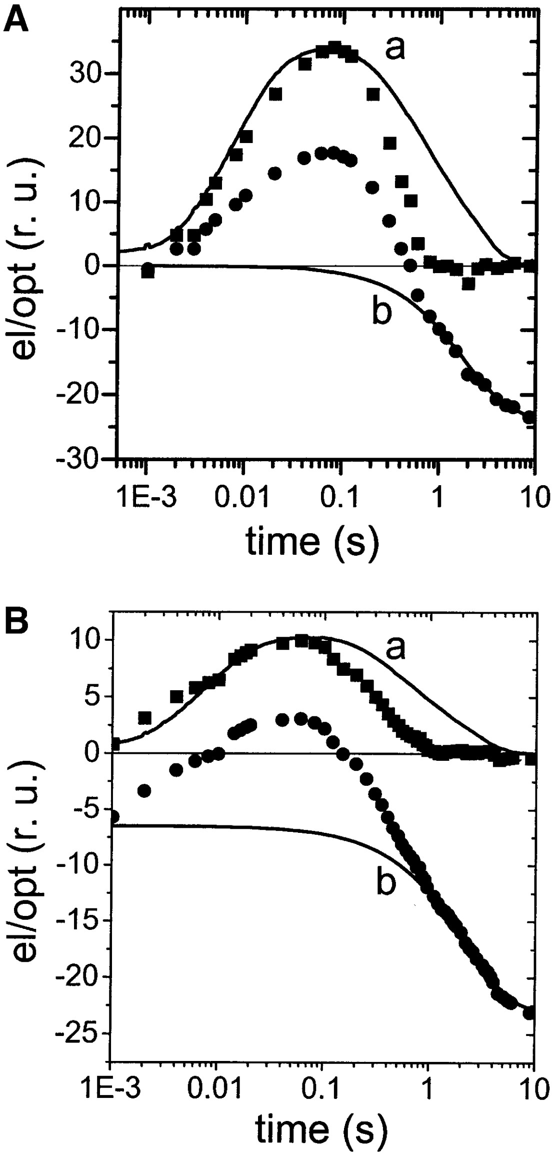

FIGURE 2 Electric currents and absorbance changes after flash excitation (*A*) at 650 nm and (*B*) at 605 nm. Lines (*a*), light absorbance changes at 632 nm; lines (*b*), fitted and extended functions for the recovery of bR; circles, current integrals of the first, fast electric signal for different delay times of the second flash; squares, the integrated values corrected for the contribution from the excitation of the ground state with functions *b* and scaled to lines *a*. Note that bR is represented by the integral value of the first negative signal. Medium conditions as in Fig. 1.

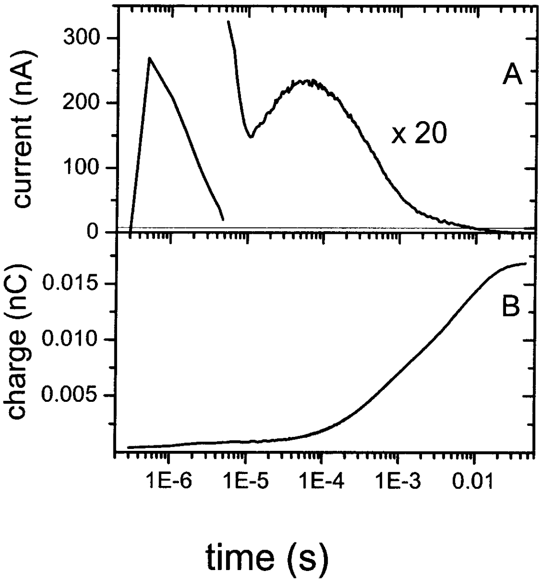

FIGURE 3 Electric response to 650-nm laser excitation of the O-intermediate. (*A*) Time dependence of the current; (*B*) its time integral. Lifetimes are given in Table 1. Conditions as in Fig. 1.

to the proton uptake signal and completes the O-photoreaction. The remaining components of 1050 and 1400 ms, respectively, obviously reflect the slow decay of residual O and N from the singly excited ground state. We attribute the decrease in lifetime compared to the experiments of Figs. 1 and 2 to heating of the sample by the strong flashes.

The proton release and uptake during the O-photoreaction could be due to a proton-pumping O-photocycle. The negative sign of the pyranine absorbance indicates that the protons are first released and then taken up. To decide whether or not it is a pumping cycle, we used quasicontinuous green and red double illumination. If an O-cycle existed, after shutting off the green light, the red light should continue to drive this cycle at least for a short time and result in a higher red-light current than produced by red illumination without green preillumination. Fig. 8 shows the result: The red-light current in the presence of green background light is nearly twice as large as without it; but most of the drop-off is as fast as after green illumination only (see Fig. 3 in the companion paper). The small, slower current decrease during the first second after green shut-off (*line a* in Fig. 8) apparently reflects the 140-ms thermal decay of the photoproducts from the

TABLE 1 Lifetimes of the electric signals, the absorbencies at 625 and 550 nm, and the acidity changes (pyranine signal at 450 nm) after excitation of the O-intermediate at 80 ms after the first laser flash

Signal	τ_1	τ_2	τ_3	τ_4
Electric	(−) 0.5 ± 0.1	(+) 9.6 ± 0.1		
Absorbance (625 nm)	(d) 0.51 ± 0.09		(r) 130 ± 15	(d) 1400 ± 200
Absorbance (550 nm)			(r) 140 ± 10	(d) 1050 ± 100
$[H^+]$ (pyranine)		(r) 11 ± 2	(d) 143 ± 5	

For electric signals, (+) and (−) indicate movement of positive charge in and against the pumping direction; (r) and (d) in front of the time values mean rise or decrease, respectively. Times are given in ms.

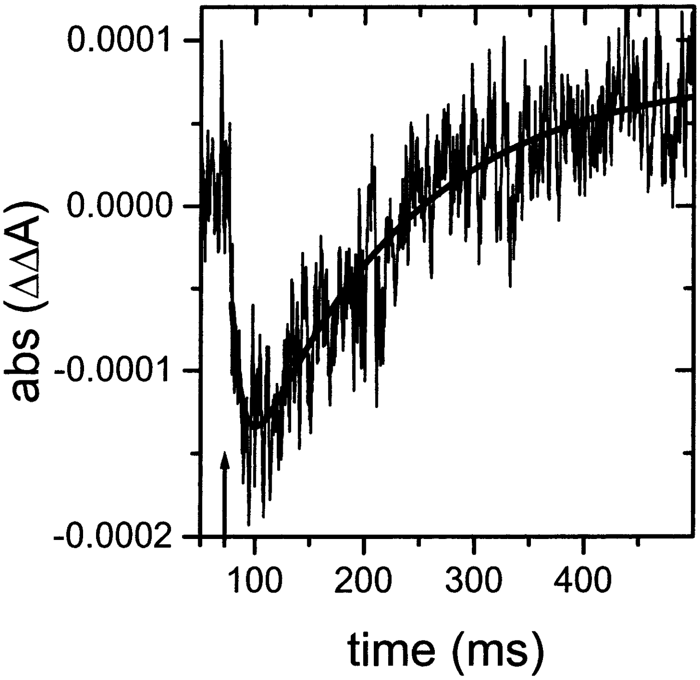

FIGURE 4 Proton release and uptake for excitation of the O-intermediate. First flash from Nd-YAG laser, second flash from dye laser at 605 nm with 80-ms delay. Solid line, absorbance difference curve, i.e., the pH changes. Arrow shows the time of second flash. Lifetimes are given in Table 1.

additional O generated by the green light. The small remaining difference to the red light current obtained without green background light (*line b*) is probably a heat effect due to the high intensity background light. The experiment thus rules out a proton-pumping O-photocycle (i.e., a cycle starting and ending in O), which should be sustained by the red light only.

This conclusion is further supported by a comparison of O-absorbance restoration at 625 and 550 nm. The flash-induced absorbance decrease at 550 nm (Fig. 7, *dashed lines*) has returned to the baseline within 1 s, whereas at 625 nm the O-absorbance (Fig. 6, *line b*) decays at the same rate as in singly excited O (*line a*). Apparently, the excited O-intermediate decays with a lifetime of ≈140 ms to either bR or N. A likely explanation for the observed absorbance and transient pH changes is a back reaction of OII to OI (see Discussion). The apparent overshoot of the pH recovery seen in Fig. 4 is consistent with this interpretation. Another possible cause is a transient pK change of a group near the cytoplasmic membrane surface, which may not be directly involved in the proton transport. In either case, however, we must assume the existence of a small and relatively slow current increase during the pH recovery, which is below the detection limit of our measurements.

DISCUSSION

We discuss our data using as a reference the WTbR photocycle model of Chizhov et al. (1992), which explicitly includes the possibility of "spectroscopically silent" intermediates. We also assume that, at least for ground-state excitation in a pumping mutant, significant changes occur only in the kinetics, not in the pathway of proton transport through the intermediate states of the WTbR photocycle. This is the most parsimonious basis for interpretation and we consider it also the most likely, because otherwise we have to assume the existence of substantial sections of alternative proton-pumping pathways in the molecule. If one amino acid in or near the WTbR pathway or bound water substitutes for the mutated amino acid, we would not consider that a change of pathways.

The earliest determination of O-chromophore structure in bR was based on resonance Raman spectra, which showed a twisted all-*trans* retinal configuration (Smith et al., 1983). However, at that time, existence of the N ↔ O equilibrium was not recognized and O-accumulating mutants were not available. The existence of a 13-*cis* O in the WTbR-cycle has been postulated by Milder (1991) based on the comparison with spectral shifts in model systems and actually been observed by absorption spectroscopy in WTbR (R. A. Bogomolni, unpublished). As outlined in the Introduction, Subramaniam and collaborators have shown that in L93A, an O-intermediate with a 13-*cis* chromophore accumulates because of hindered reisomerization. They convincingly argued that this effect is due to the missing interaction between the terminal methyl groups of leucine and the 13-methyl of retinal and that it is overcome under continuous illumination through absorption of a second photon in the O-state. They consequently proposed the following reaction sequence for late intermediates of L93A:

$$M \leftrightarrow N^{-1} \leftrightarrow N^{0} \leftrightarrow O \rightarrow bR,$$

where the superscripts reflect their erroneous assumption that charge transport and proton uptake are completed in the

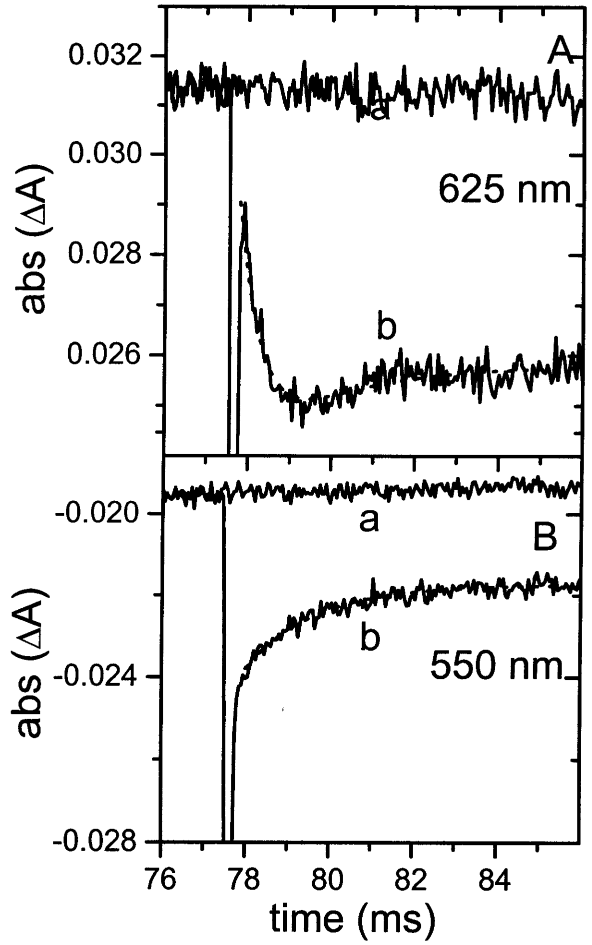

FIGURE 5 Time dependence of absorbencies at 625 nm and 550 nm in the ms time range for single (*lines a*) and double (*lines b*) flash excitation (Nd-YAG laser and dye laser 650 nm).

M → N ↔ O transition (Delaney et al., 1995, see companion paper). The twisted all-*trans* chromophore similar to that in WTbR was left out, because they assumed that excitation of O rapidly reisomerizes the retinal and suffices to return the molecule directly to the bR-state. Our experiment of Fig. 2, however, clearly shows the presence of two consecutive O-intermediates with relatively long lifetimes, of which the first, shorter-lived (OI) generates net positive charge transport when excited. We also find an optical transition, which corresponds to the decay time of the current-generating OI. We thus recognize two consecutive O-intermediates with similar red-shifted absorption spectra. The sequence of intermediates, which should also apply to WTbR, thus becomes:

$$\mathrm{M} \leftrightarrow \mathrm{NI} \rightarrow \mathrm{NII} \leftrightarrow \mathrm{OI} \leftrightarrow \mathrm{OII} \rightarrow \mathrm{bR}.$$

(We are not using the 0 and –1 superscripts used by Delaney and Subramaniam, which designate the change in total charge of the molecule, because our time-resolved protonation changes show that in the NII ↔ OI equilibrium, not all released protons have yet been taken up).

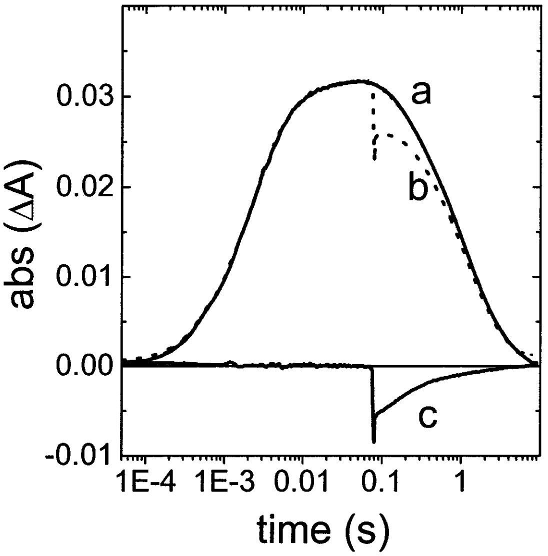

FIGURE 6 Time dependence of absorbencies at 625 nm for single (*solid line a*) and double (*dotted line b*) flash excitation (Nd-YAG laser and dye laser 650 nm). Line *c*: difference of *a* and *b*. Lifetimes in Table 1.

Since OI still has the 13-*cis* conformation, OII probably has the twisted all-*trans* chromophore seen in the WTbR cycle. Inclusion of the virtually irreversible MNI → NIIOI reaction in this scheme is based on the photocycle model of Chizhov et al. (1992, 1996). The rise of the NII ↔ OI equilibrium indicates the partial reprotonation of D96, and is reflected in the initial, rapid proton uptake shown in Fig. 2 of

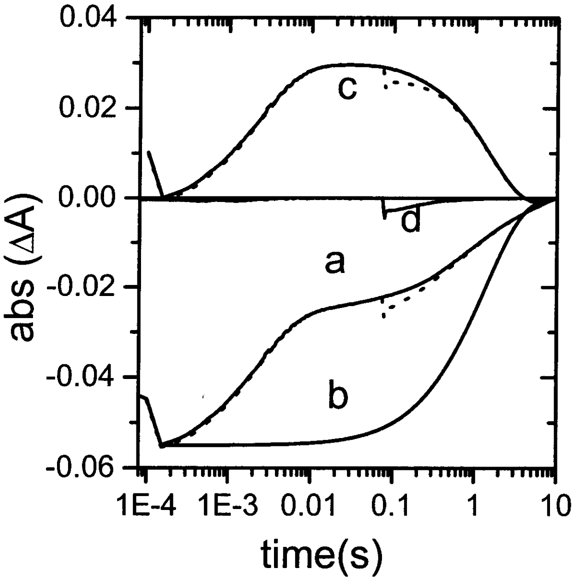

FIGURE 7 Time dependence of absorbencies at 550 nm for single (*solid line a*) and double (*dotted line a*) flash excitation (Nd-YAG laser and 650 nm dye laser). Line *b* represents the calculated recovery of the ground state. Lines *c* (*solid* and *dotted*) are the difference lines of *a* and *b*. Line *d* is the difference of solid minus dotted lines *c*, i.e., the absorbance change at 550 nm caused by the double flash. Lifetimes are in Table 1.

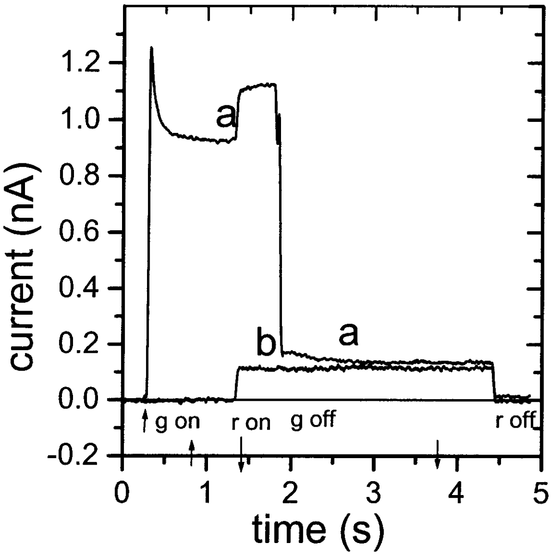

FIGURE 8 Line *a*, electric current with green light and red light added at up arrow, green turned off at down arrow. Line *b*, electric current for red illumination only. Intensities: 3.07×10^{17} photons cm^{-2} s^{-1} for green, 3.38×10^{17} photons cm^{-2} s^{-1} for red light (>640 nm). The spike on the descending part of line *a* is an artifact caused by the shutter.

the companion paper (Tóth-Boconádi et al., 2003). The rapid uptake amounts to ≈40% of the released protons, which satisfactorily agrees with the apparent pK_a 7.1 ± 0.2 for D96 in WTbR observed at this stage of the photocycle (Druckmann et al., 1993; Zscherp et al., 1999). We conclude that both the reisomerization of 13-*cis* to twisted all-*trans* retinal and the twisted all-*trans* transition to the planar conformation in bR are inhibited if leucine 93 is replaced by alanine.

We can estimate whether the quantity of detected protonation change caused by OI-excitation is significant. In our companion paper, we measured 0.15 nC as the transported charge and a maximal pyranine absorbance change of −0.004 for ground-state excitation (Figs. 1 and 2 in Tóth-Boconádi et al., 2003). The corresponding values for OI-excitation are 0.015 nC and –0.0002 absorbance change (Figs. 3 and 4). If we take into account that the currents and pH changes are small and measurements were made on gels and solutions, respectively, we find that the ratios of absorbance change and transported charge for OI- and bR-excitation are comparable.

The transient proton release upon excitation of OI can be understood as due to a back reaction from OII to OI. Though the recoveries of 550 nm and 632 nm absorbencies after excitation at 650 nm show 0.51 ms components of opposite sign at 550 and 632 nm, which would be consistent with the back reaction from OII to OI, the transient protons should then be released from the cytoplasmic surface. This assumption is incompatible with the positive 9.6-ms current corresponding to the 11-ms proton release. We assume that the small electric signal corresponding to the 140-ms proton uptake is buried in the noise and the same may be true for the uptake signal.

In the MNI → NIIOI transitions, more than 60% of the total charge transport occurs, and the connection of the Schiff's base switches from the outer to the inner membrane surface (Vonck, 2000, and Fig. 1 in the companion paper). It is therefore likely that in this transition also the path, by which an excited intermediate returns to bR, switches from backward to forward through the cycle. Consistent with this conclusion, we find scattered observations of O-like intermediates in photoreactions of N and O in the literature, e.g., (Ohtani et al., 1992; Váró and Lanyi, 1990).

This could resolve a problem caused by introduction of the MNI equilibrium: Our earlier conclusion that the cycle acceleration in continuous light is due to the N-component of the N ↔ O equilibrium was based on the action spectrum, which may not be able to distinguish between NI and NII. Showing that NI returns backward through the cycle will be an obvious test of the proposed model. The two-photon cycle is more complex than anticipated, which probably also causes the nonlinearity in Fig. 6 of the companion paper, and merits further investigation.

Finally, a word of caution: Delaney et al. (1995) have found that L93A bR in the light-adapted ground state contains ≈20% 13-*cis* retinal with, so far, unknown photoreactions. This is a frequently encountered problem when working with mutants. For lack of a solution, it has usually been ignored, as, for the time being, we have done here. However, if our concept of only kinetic changes holds, it could simply be a very rapid dark adaptation, or, more likely, some N intermediate.

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