Spudich, J. L., & Bogomolni, R. A. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 193–215.
Steiner, M., Oesterhelt, D., Ariki, M., & Lanyi, J. K. (1984) *J. Biol. Chem. 259*, 2179–2184.
Stoeckenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem. 51*, 587–616.
Stoeckenius, W., Lozier, R. H., & Bogomolni, R. A. (1978) *Biochim. Biophys. Acta 505*, 215–278.
Taylor, M. E., Bogomolni, R. A., & Weber, H. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6172–6176.
Tittor, J., Oesterhelt, D., Maurer, R., Desel, H., & Uhl, R. (1987) *Biophys. J. 52*, 999–1006.
Tsuda, M., Hazemoto, N., Kondo, M., Kamo, N., Kobatake, Y., & Terayama, Y. (1982) *Biochem. Biophys. Res. Commun. 108*, 970–976.
Weber, H. J., & Bogomolni, R. A. (1981) *Photochem. Photobiol. 33*, 601–608.
Zimânyi, L., & Lanyi, J. K. (1987) *Biophys. J. 52*, 1007–1013.
Zimânyi, L., & Lanyi, J. K. (1989) *Biochemistry 28*, 1662–1666.

Transient Spectroscopy of Bacterial Rhodopsins with an Optical Multichannel Analyzer. 2. Effects of Anions on the Halorhodopsin Photocycle[†]

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ABSTRACT: We find that the photocycle of halorhodopsin (HR) in the presence of nitrate (but not chloride) consists of two parallel series of reactions. The first is essentially the same as that which occurs in the presence of chloride: $HR \xrightarrow{h\nu} HR_K \rightarrow HR_{KL} \rightarrow HR_L \leftrightarrow HR_O \rightarrow HR$. The second photocycle, however, which we describe as $HR \xrightarrow{h\nu} HR'_K \rightarrow HR_{KO} \rightarrow HR_O \rightarrow HR$, seems characteristic of what one would observe in the absence of chloride. Absorption spectra are calculated for all species but HR_K and HR'_K, which occur at shorter times (<60 ns) than we can resolve. At nitrate concentrations between 0.1 and 1 M, the proportion of HR which enters the first kind of photocycle increases in such a way as to suggest that nitrate can substitute for chloride, but much less effectively. At lower anion concentrations, the two photocycles are independent of one another, but at higher concentrations, they interact; i.e., the reaction $HR_{KO} \rightarrow HR_O \rightarrow HR_L$ can be observed. Thus, HR_O must be common to the two photocycles. Kinetic fitting of the time dependence of HR_L and HR_O at different chloride concentrations provides evidence for the participation of chloride in the interconversion of HR_L and HR_O. The results are consistent with a model in which the photoreaction is influenced by the binding of an anion (either chloride or nitrate) to site II in HR: when an anion is bound, the HR_K-initiated HR_L-type photocyle is observed, but when the site is not occupied, the HR'_K-initiated HR_O-type photocycle is seen.

Halorhodopsin (HR),[1] a retinal protein in the cytoplasmic membrane of extremely halophilic bacteria (Lanyi, 1986), is a light-driven pump for chloride ions. In the presence of chloride, absorption of light by the *all-trans*-retinal-containing chromophore of this pigment generates a transient bathochromic photoproduct (Polland et al., 1985; Franz, 1988), similar to the K intermediate of BR (Becher et al., 1978; Iwasa et al., 1980; Shichida et al., 1983). Relaxation back to HR is over several milliseconds; the ensuing sequence of thermally driven reactions (photocycle) should be regarded as the reaction cycle which drives chloride across the membrane. There is evidence with detergent-solubilized HR (Polland et al., 1985; Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986; Tittor et al., 1987; Zimânyi et al., 1989) that the photocycle in the presence of chloride includes the following reactions:

$$HR{\cdot}Cl^- \xrightarrow{h\nu} HR_K{\cdot}Cl^- \rightarrow HR_{KL}{\cdot}Cl^- \rightarrow HR_L{\cdot}Cl^- \leftrightarrow HR_O \rightarrow HR \leftrightarrow HR{\cdot}Cl^-$$

The intermediates in this scheme are very similar (Zimânyi et al., 1989) to those in the photocycle of BR; the most conspicuous difference between the two pigments is that in HR a deprotonated intermediate is not part of the reaction sequence (Hegemann et al., 1985). For the translocation of chloride, the critical step may be the reversible equilibrium of $HR_L{\cdot}Cl^-$ with HR_O (Oesterhelt et al., 1985), driven in favor of chloride release by the subsequent step, $HR_O \rightarrow HR$, which must represent the (irreversible) reisomerization of the retinal. Although evidence for this is still lacking, it seems likely that the chloride released at this step is the transported chloride. The $HR \leftrightarrow HR{\cdot}Cl^-$ equilibrium has been described before on the basis of a small red-shift in the absorption maximum of HR, which accompanies the binding of chloride to the so-called site II in the protein (Schobert et al., 1986). Under physiological conditions (i.e., high NaCl concentrations), this equilibrium is driven far in favor of chloride uptake, and it is assumed to occur in the photocycle, as written above, in order

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[1] Abbreviations: HR, halorhodopsin; BR, bacteriorhodopsin; HR_K (referred to elsewhere as HR_{600}), HR'_K (elsewhere HR_{600}), HR_{KO}, HR_{KL}, HR_L (elsewhere HR_{520}), and HR_O (elsewhere HR_{640}), photointermediates of HR, as described in the text; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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to balance the earlier chloride release. The HR → HR·Cl$^-$ step is likely to be the uptake of the transported chloride, since the selectivity of site II, which is involved in this binding reaction, agrees with the specificity of the transport (Schobert & Lanyi, 1982; Bamberg et al., 1984; Hazemoto et al., 1984). Unfortunately, there are no results as yet which would link events in chloride transport to the HR photocycle in the way events in proton transport have been linked to the BR photocycle [e.g., see Lozier et al. (1976), Li et al. (1984), and Grzesiek and Dencher (1986)].

Given the uncertainties over the specific step(s) in the HR photocycle where the anion translocation occurs, it is important to understand the nature of the photoreaction of HR in the absence of a transportable anion: we expect that those steps which involve the binding or the translocation of the anion would be absent or altered under these conditions. It has been thought that nitrate was not a transported anion, since in the presence of nitrate illumination of cell envelope vesicles (Schobert & Lanyi, 1982) produced no detectable electrogenic transport and HR-containing planar films (Bamberg et al., 1984) did not generate a sustained electrical potential. Thus, nitrate seemed to be a suitable anion for studying the alternative photocycle (illumination experiments in the absence of both chloride and nitrate are difficult to do, since under these conditions the chromophore of HR is irreversibly destroyed by light). Early evidence indicated that with nitrate alone HR_O is produced, but HR_L is not (Lanyi & Vodyanoy, 1986). Recently, the HR photointermediates in 1 M chloride and 1 M nitrate were compared (Tittor et al., 1987), and while the participation of HR_O in the photocycle with 1 M nitrate was confirmed, HR_L was also identified under these conditions. The appearance of the latter was attributed to chloride contamination of the nitrate. To us, the results raised the question, however, as to whether nitrate might be a transportable anion after all, and if it is, what the photocycle in the genuine absence of transport would be like. With these questions in mind, we reexamined the photoreaction of HR under these conditions, utilizing the gated optical multichannel analyzer described in the preceding paper (Zimânyi et al., 1989). Our results indicate that at high concentrations of nitrate two photocycles occur in parallel, one essentially like that in chloride, and another which might be the nontransporting photocycle. Chloride contamination does not account for the results. Thus, site II, which is linked to transport and the chloride-type photocycle (Schobert et al., 1986), may indeed bind nitrate as well as chloride, although with lower affinity. Thus, the kind of anion bound to site II might be less important in directing the course of reactions after the absorption of light than whether the site contains a bound anion or not.

MATERIALS AND METHODS

The preparation of HR, the experimental conditions, the operation of the Princeton Instruments optical spectroscopic multichannel analyzer (OSMA), and the analysis of the data were essentially as described in the preceding paper (Zimânyi et al., 1989). Besides NaCl or $NaNO_3$, the buffers used contained 25 mM MES, pH 6.0. When these salts were used at concentrations lower than 100 mM, 400 mM Na_2SO_4 was also present in the buffers.

Determination of the adventitious chloride content in a 1 M solution of $NaNO_3$ (Fisher Scientific, certified A.C.S.) was with a chloride-specific electrode.

RESULTS

Spectral Transitions of the HR Photocycle in 1 M $NaNO_3$. Difference spectra were obtained for HR in the presence of nitrate at increasing time delays after the actinic flash, as reported earlier in the presence of chloride (Zimânyi et al., 1989). The data obtained under these conditions were analyzed in the same way as for chloride; the fact that we could refer to the spectrum of HR_L, which is well described from experiments with chloride (Tittor et al., 1987; Zimânyi et al., 1989), gives us reason to be confident of the validity of the analyses. Important differences were seen between the results in 1 M nitrate at 3 °C and at room temperature; we describe first the data at 3 °C.

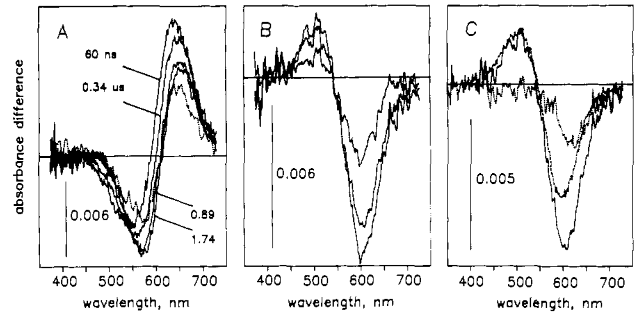

FIGURE 1: Flash-induced difference spectra of HR in the 60 ns–1.74 μs time domain, measured in 1 M nitrate buffer at 3 °C. Absorption of HR was 0.25. (A) Measured difference spectra at the times indicated (—). A scaled difference spectrum, measured at 60 ns in 1 M chloride (Zimânyi et al., 1989), is also included (···). (B) Net difference spectra, constructed by subtraction of the 60-ns nitrate spectrum from each of the later spectra in (A). (C) Comparison of the 0.89-μs net difference spectrum from (B) (—) with a scaled $HR_{KL} \rightarrow HR_L$ net difference spectrum (- - -) measured in 1 M chloride (Zimânyi et al., 1989). The third spectrum (···) is the difference between these two. The latter corresponds to the $HR_{KO} \rightarrow HR_O$ transition.

Figure 1A shows measured difference spectra in the 60 ns–1.74 μs time domain. For comparison, a 60-ns difference spectrum in 1 M NaCl at 3 °C, scaled to the same amount of photoconversion, is also included (dotted line). It is apparent that the 60-ns difference spectra differ in shape and magnitude when measured in nitrate and in chloride, arguing for the presence of different photoproducts at this time delay under the two conditions. The transition in Figure 1A, in the time period after 60 ns, is better seen when the first trace is subtracted from each of the later traces (Figure 1B). The net difference spectra indicate the disappearance of a red-absorbing species and the appearance of a blue-absorbing species, similar to what was recorded with chloride for the $HR_{KL} \rightarrow HR_L$ transition (Zimânyi et al., 1989), but the spectra do not quite agree under the two conditions. This is apparent from Figure 1C, where the 0.89-μs net difference spectrum in Figure 1B, for example, is compared with a net difference spectrum for a matched sample in 1 M chloride, scaled according to the amount of HR_L produced at later times in the two photocycles. The difference between the spectra in nitrate and in chloride consists of a simple negative peak between 550 and 650 nm. Thus, it would appear that the transition in Figure 1B can be described as the sum of the $HR_{KL} \rightarrow HR_L$ reaction and a second reaction which is not seen in chloride (Zimânyi et al., 1989). It will be shown below that the latter is a reaction which can be described as $HR_{KO} \rightarrow HR_O$, where HR_{KO} is the immediate precursor of HR_O, produced faster than 60 ns from a primary photoproduct, analogously to HR_{KL}.

At delay times between 2.8 and 54 μs, the difference spectra (Figure 2A) reveal that the $HR_{KL} \rightarrow HR_L$ component of the transition is finished, leaving what appears to be the second, apparently somewhat slower, component as the only observable change (compare the net difference spectrum in Figure 2A with the subtracted spectrum Figure 1C). When both of these

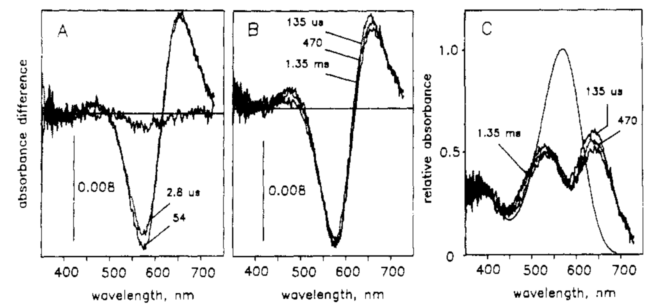

FIGURE 2: Flash-induced difference spectra of HR in the 2.8 μs–1.35 ms time domain, measured in 1 M nitrate buffer at 3 °C. (A) Measured difference spectra at 2.8 and 54 μs, as indicated. The third spectrum is the difference between these two (–•–). (B) Measured difference spectra at 135 and 470 μs and at 1.35 ms, as indicated. (C) Absolute spectra for the mixtures of photoproducts at 135 and 470 μs and at 1.35 ms, as indicated. For comparison, a spectrum for HR in these experiments is also included. The transition is the $HR_O \rightarrow HR_L$ reaction.

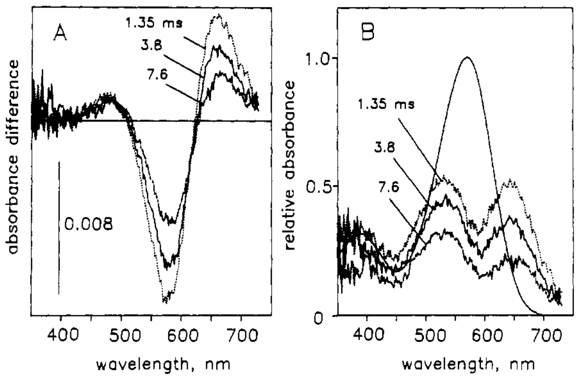

FIGURE 3: Flash-induced difference spectra of HR in the 1.35–7.6-ms time domain, measured in 1 M nitrate buffer at 3 °C. (A) Measured difference spectra at times, as indicated. (B) Absolute spectra for the mixtures of photoproducts at times, as indicated. For comparison, a spectrum for HR in these experiments is also included. The transitions are the $HR_L \leftrightarrow HR_O \rightarrow HR$ reactions.

transitions are completed, i.e., at 54 μs, the flash illumination will have produced a mixture of HR_L and HR_O.

At still longer delay times, between 135 μs and 1.35 ms (Figure 2B), there is absorbance decrease in the red region, which is accompanied by absorbance rise in the blue. The net difference spectra for this transition (not shown) reveal it to be the *reverse* of the $HR_L \rightarrow HR_O$ reaction observed in chloride in this time time domain (Zimányi et al., 1989). Since we wish to emphasize that the absolute amount of HR_L increases during this process, beyond what had been produced from HR_{KL}, we show this transition instead as a set of absolute spectra for the mixture of intermediates (Figure 2C). Thus, unlike in chloride-containing buffer, where the $HR_O \rightarrow HR_L$ reaction can be inferred only from the fact that HR_L equilibrates with HR_O (Tittor et al., 1987), in nitrate-containing buffer at 3 °C this reaction is directly observed.

The recovery of HR begins at delay times beyond 1.35 ms (Figure 3A). In 1 M chloride, HR_L and HR_O were seen to equilibrate at this point of the photocycle; i.e., they disappeared as a constant mixture as HR was regenerated (Tittor et al., 1987; Zimányi et al., 1989), but in nitrate at 3 °C, this is not so: it is evident from Figure 3B that HR_O decays more rapidly than HR_L. Thus, if the rate of the $HR_L \rightarrow HR_O$ reaction is v_1 and those of the $HR_O \rightarrow HR_L$ and $HR_O \rightarrow HR$ reactions are v_2 and v_3, respectively, then $v_2, v_3 > v_1$. In 1 M chloride (Zimányi et al., 1989), in contrast, $v_1, v_2 > v_3$.

At room temperature (22 °C), the initial transitions in the microsecond time domain are completed at earlier times than

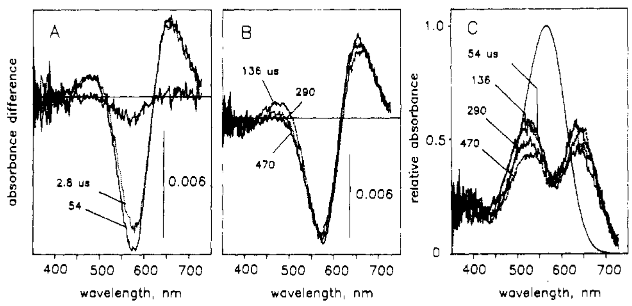

FIGURE 4: Flash-induced difference spectra of HR in the 2.8–470-μs time domain, measured in 1 M nitrate buffer at 22 °C. Absorption of HR was 0.205. (A) Measured difference spectra at 2.8 and 54 μs, as indicated. The third spectrum is the difference between these two (–•–). The transition is the $HR_{KO} \rightarrow HR_O$ reaction. (B) Measured difference spectra at 136, 290, and 470 μs, as indicated. (C) Absolute spectra for the mixtures of photoproducts as 54, 136, 290, and 470 μs, as indicated. For comparison, a spectrum for HR in these experiments is also included. The transition is the $HR_L \rightarrow HR_O$ reaction.

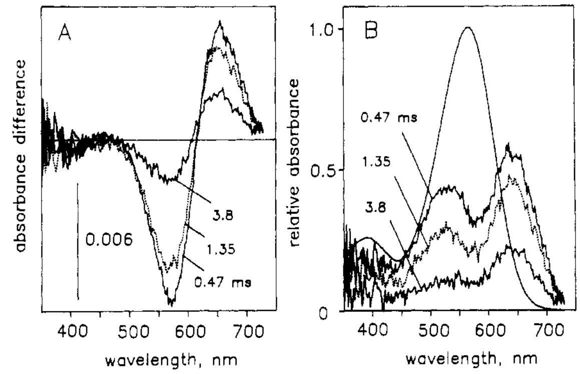

FIGURE 5: Flash-induced difference spectra of HR in the 0.47–3.8-ms time domain, measured in 1 M nitrate buffer at 22 °C. Same sample as in Figure 4. (A) Measured difference spectra at times, as indicated. (B) Absolute spectra for the mixtures of photoproducts at times, as indicated. For comparison, a spectrum for HR in these experiments is also included. The transitions are the $HR_L \leftrightarrow HR_O \rightarrow HR$ reactions.

at 3 °C, but the difference spectrum between 2.8 and 54 μs (Figure 4A) indicates that the $HR_{KO} \rightarrow HR_O$ reaction is still observable. At later times, between 136 and 470 μs (Figure 4B), absorption in the blue is seen to decrease and absorption in the red to rise; net difference spectra (not shown) suggest it to be the $HR_L \rightarrow HR_O$ reaction seen in chloride (Zimányi et al., 1989). As in Figure 2C for 3 °C, we show this process at 22 °C as a set of absolute spectra for the mixture of the photointermediates at these times (Figure 4C), where it is evident that unlike at 3 °C, under these conditions it is the amount of HR_O which rises while that of HR_L decreases.

Figure 5A shows difference spectra at 22 °C, at delay times between 0.47 and 3.8 ms, where the process of the recovery of HR is under way. Here (Figure 5B), as at 3 °C (Figure 3B), the ratio of HR_L and HR_O changes with time, but in the opposite direction: at 22 °C, it is HR_L which disappears more rapidly rather than HR_O. Thus, at this temperature, $v_1, v_3 > v_2$.

Intermediates and Kinetics of the HR Photocycle in 1 M NaNO₃. The spectra of the species which account for the spectroscopic changes in Figures 1–5 could be reconstructed, using spectra for HR and two of the intermediates of the photocycle in 1 M chloride (Zimányi et al., 1989). The events in the first few microseconds seem to contain partly the transformation of HR_{KL} into HR_L, as indicated by a difference spectrum which resembles this transition (Figure 1B,C). The amount of HR_{KL} can be estimated from spectra at later times,

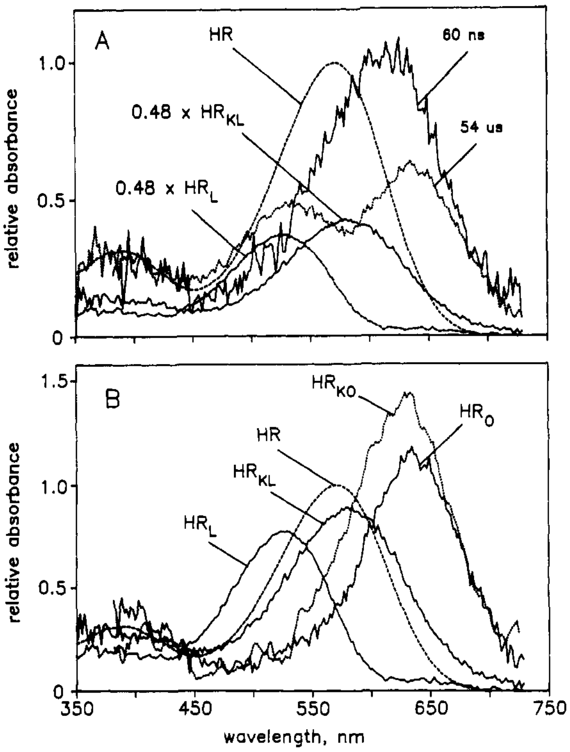

FIGURE 6: Calculated absolute spectra for the intermediates of the two HR photocycles in 1 M $NaNO_3$. The method of calculation was described previously (Zimânyi et al., 1989). Extinctions are given relative to the extinction of light-adapted HR [also shown (---)]. (A) Spectra for mixtures of intermediates at 60 ns and 54 μs, reconstructed from experiments in 1 M nitrate at 3 °C, and 48% of spectra for HR_{KL} and HR_L, reconstructed from experiments in 1 M chloride at 3 °C (Zimânyi et al., 1989). Details are given in the text. (B) Calculated absolute spectra for HR_{KO}, HR_O, HR_{KL}, and HR_L, as indicated.

from the amount of HR_L (cf. below). At 3 °C, this constitutes 48% of the photocycling fraction. Accordingly, 48% of a normalized spectrum for HR_{KL} (determined in the presence of 1 M chloride) was subtracted from the spectrum calculated for the mixture of the species at 60 ns in 1 M nitrate (Figure 6A). The resulting spectrum, normalized and labeled HR_{KO} (Figure 6B), is the spectrum of the precursor of HR_O, which we postulate coexists with HR_{KL} at the earliest times in our measurements. Its absorption maximum is at 628 nm; this species, like HR_{KL}, has not been observed before. In a similar way, the spectrum of HR_O was calculated by subtracting 48% of the HR_L spectrum at a time (54 μs) where only HR_O and HR_L exist (Figure 6A,B). The validity of this subtraction is shown by the following: (a) in the resulting HR_O spectrum, the region near 500–550 nm is smooth; (b) a similar spectrum for HR_O is obtained when the measurements are in 100 mM nitrate (not shown), where the amount of HR_L is much less and the amount of HR_O is more; and (c) the HR_O absorption maximum (at 635 nm) and amplitude relative to that of HR (1.16) agree well what we find (Zimânyi et al., 1989) for HR_O in 1 M chloride (638 nm and 0.96) and moderately well with the earlier published spectrum (Tittor et al., 1987) for this intermediate (640 nm and 0.79).

The difference between the spectra of HR_{KO} and HR_O in Figure 6B is a negative peak between 550 and 650 nm, i.e., essentially what is seen as the difference spectrum in the 2.8–54-μs time domain (Figures 2A and 4A). This was the basis for the identification of that transition as the $HR_{KO} \rightarrow HR_O$ reaction.

The kinetics during the first few microseconds after the flash are difficult to resolve, because the $HR_{KL} \rightarrow HR_L$ and $HR_{KO} \rightarrow HR_O$ reactions occur together in this time domain. We estimate from the traces that at 3 °C the former reaction has a half-time of about 0.4 μs, i.e., similar to that seen in chloride (Zimânyi et al., 1989), and the latter is 3–5 times slower. Events at later times consist of the $HR_L \leftrightarrow HR_O \rightarrow HR$ reactions, shown on a 3-decade logarithmic time scale at 3 °C and at 22 °C (Figure 7). It is important to note that at 3 °C HR_L is produced in two ways: the majority at microsecond delay times from HR_{KL}, and a small but detectable minority at millisecond delay times (Figure 7, upper panel) from HR_O. Conversely, at 22 °C, HR_O is produced in two ways: the majority at microsecond delay times from HR_{KO}, and a minority at millisecond delay times (Figure 7, lower panel) from HR_L. Thus, under these conditions, the two photocycles cross over in the millisecond time range. Interestingly, the ratio of HR_L and HR_O is somewhat temperature dependent even before the time range of the $HR_L \leftrightarrow HR_O$ interconversion (Figure 7). The reason for this may be temperature-dependent conformational perturbation of the HR, either by the detergent or by the 1 M nitrate, which would change the binding constant for nitrate and shift the ratio of the two parallel photocycles.

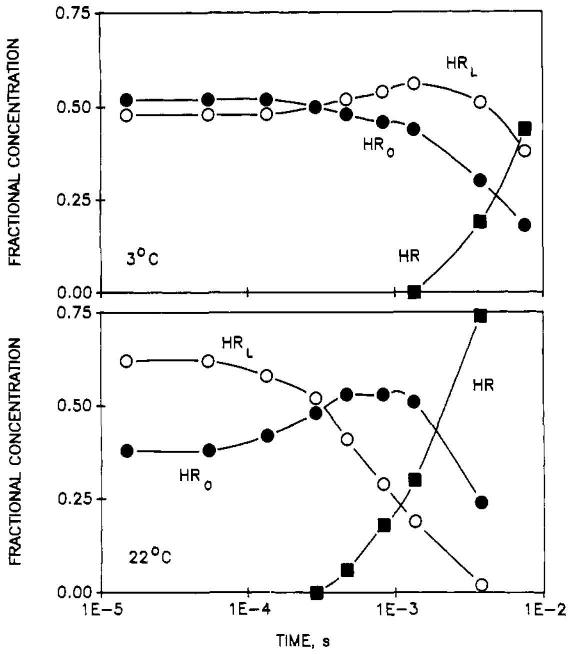

FIGURE 7: Kinetics of HR transformations in 1 M $NaNO_3$ at 3 and 22 °C. Fractional concentrations for HR_L (○), HR_O (●), and HR (■) are plotted against delay time after the flash.

Yield of HR_L as a Function of Chloride and Nitrate Concentrations. The proportion of HR which enters the HR_L-type photocycle depends on the kind of anion present and its concentration. At high chloride concentrations, the majority of HR produces HR_L (Schobert et al., 1983; Lanyi & Vodyanoy, 1986; Tittor et al., 1987; Zimânyi et al., 1989), while in nitrate mixtures of the HR_L and HR_O type photocycles are seen. Figure 8A shows reconstructed spectra for the photoproduct mixtures at 136 μs after the flash (before the $HR_L \leftrightarrow HR_O$ reaction would take place) at various nitrate concentrations. It is evident from the spectra that increasing the nitrate concentration from 0.1 to 1 M causes a progressive shifting of the mixture from HR_O toward HR_L. In Figure 8B, the fractional concentrations of HR_L at 136 μs are given at various nitrate and chloride concentrations. The chloride dependency curve is different from earlier data of this kind, derived from single-wavelength measurements (Tittor et al., 1987), because in Figure 8B the amounts of HR_L are those produced initially from HR_{KL}, while in the published plot the

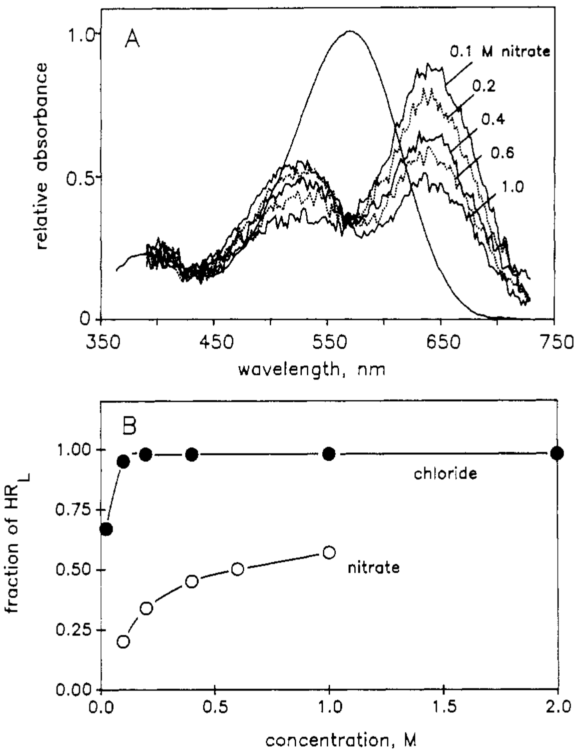

FIGURE 8: Dependence of the amount of HR_L, produced in the initial part of the photocycles, on the concentration of chloride and nitrate. (A) Reconstructed spectra for the mixture of intermediates at 136 μs, in 0.1, 0.2, 0.4, 0.6, and 1.0 M $NaNO_3$/25 mM MES, pH 6.0, as indicated. The spectra are normalized for the photocycling fraction. The spectra show the proportion of HR_L and HR_O produced initially in the photocycles and were roughly the same at delay times somewhat before and after 136 μs. A spectrum for HR is included for comparison. (B) Fractional concentration of HR_L at 136 μs as a function of chloride (●) and nitrate (○) concentration. The data for nitrate are from (A) and for chloride from analogous experiments. The point for the lowest chloride concentration (24 mM) was obtained in the presence of 76 mM nitrate as well, since below 100 mM chloride or nitrate HR is not photostable. Correction for the effect of nitrate in this sample would not change the chloride curve significantly.

maximal amounts, wherever they occur in the photocycle, are given. As expected, the curve in Figure 8B is consistent with the determined K_M of site II for chloride, which is 10–40 mM under various conditions (Schobert & Lanyi, 1982; Bamberg et al., 1984; Hazemoto et al., 1984; Steiner et al., 1984). The nitrate dependency curve in Figure 8B suggests a higher K_M for this anion, and a limitation of the maximal amount of HR which will produce HR_L. The reasons for the latter are not clear.

Previously it was thought that the production of HR_L is characteristic of the chloride-type photocycle only (Schobert et al., 1983; Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986), and observations of this intermediate in 1 M nitrate were attributed to chloride contamination of the nitrate (Tittor et al., 1987). If the observed HR_L originated from chloride contamination, the appearance of approximately half as much HR_L in 1 M nitrate as in 1 M chloride would only be possible if the nitrate contained about 25 mM chloride (as shown in a section below, where roughly this condition was created). In our measurements, the concentration of chloride in 1 M nitrate was determined to be 0.5 mM. Thus, we conclude that chloride is responsible for the results; i.e., the HR_L-type photocycle can be elicited by nitrate also, although at higher concentrations. This implies that site II has some, although lower, affinity for nitrate also. Preliminary results[2] indicate that, previous reports notwithstanding, *H. halobium* envelope vesicles containing halorhodopsin do transport nitrate to some extent.

Chloride-Dependent Equilibria in the HR Photocycle. The $HR_L \leftrightarrow HR_O$ reaction was postulated on the basis of the equilibration of these two intermediates before the recovery of HR (Oesterhelt et al., 1985; Lanyi & Vodyanoy, 1986; Tittor et al., 1987). Evidence in support of the participation of chloride in the reaction was given from the kinetics (Lanyi & Vodyanoy, 1986) and the maximum absorbance changes (Tittor et al., 1987) measured at single wavelengths at various chloride concentrations. Since we can now calculate the amounts of intermediates more reliably from their difference spectra, we reexamined the chloride dependency of this step in the HR photocycle. The general solution of the kinetic differential equations for the reaction $HR_L \leftrightarrow HR_O \rightarrow HR$ gives the time-dependent functions:

$$HR_L(t) = \frac{HR_L{}^0(q + p + k_3) + k_2}{2p}\exp(q + p)t - \frac{HR_L{}^0(q - p + k_3) + k_2}{2p}\exp(q - p)t \quad (1)$$

$$HR_O(t) = \frac{(1 - HR_L{}^0)(q + p) + k_1}{2p}\exp(q + p)t - \frac{(1 - HR_L{}^0)(q - p) + k_1}{2p}\exp(q - p)t \quad (2)$$

$$HR(t) = 1 - HR_L(t) - HR_O(t) \quad (3)$$

$$p = \tfrac{1}{2}[(k_1 + k_2 + k_3)^2 - 4k_1k_3]^{1/2} \quad (4)$$

$$q = -\tfrac{1}{2}(k_1 + k_2 + k_3) \quad (5)$$

where $HR_L(t)$, $HR_O(t)$, and $HR(t)$ are the transient fractional concentrations of HR_L, HR_O, and that portion of HR which entered the photocycle, respectively, $HR_L{}^0$ is the fractional concentration of HR_L produced initially from HR_{KL}, estimated from spectra at delay times after the production of HR_L but before the $HR_L \leftrightarrow HR_O$ reaction, and k_1, k_2, and k_3 are rate constants for the $HR_L \rightarrow HR_O$, $HR_O \rightarrow HR_L$, and $HR_O \rightarrow HR$ reactions, respectively. In the presence of chloride, $HR_L{}^0$ is nearly 1 (Zimányi et al., 1989), but at various nitrate concentrations, it assumes values between 0 and 1 (Figure 8).

Figure 9 shows measured fractional concentrations for the above three species at 22 °C as functions of time after the flash, in buffers containing 0.1, 0.4, and 1 M NaCl. At each concentration, lines according to eq 1–5 were fitted to the points, with rate constants k_1, k_2, and k_3 of 160, 1000 $[Cl^-]$, and 900 s^{-1}, respectively. The kinetic model predicts well the observed slowing of the decay of HR_L and the recovery of HR, as well as the decrease in the transient accumulation of HR_O, with increasing chloride concentration. The linear dependence of k_2 on chloride concentration indicates that the $HR_O \rightarrow HR_L$ reaction is second-order, as expected from the model where this reaction represents uptake of chloride (Oesterhelt et al., 1985). The rate constants agree, within a factor of 2–3, with previously published values (Lanyi & Vodyanoy, 1986). The dissociation constant for $HR_L{\cdot}Cl^-$ is given by $k_1[Cl^-]/k_2$, whose value is 160 mM. Previously given dissociation constants range from 100 mM (Tittor et al., 1987) to 450 mM (Lanyi & Vodyanoy, 1986).

Data for the three intermediates, obtained at 22 °C in several nitrate-containing buffers, could be fitted also with eq 1–5 (not shown). For this anion, k_2 was found to be much smaller than k_1, and the dissociation constant for $HR_L{\cdot}NO_3^-$, accordingly, is very high (>10 M). At 3 °C, the rate constants

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[2] A. Duschl, L. Zimányi, and J. K. Lanyi, unpublished results.

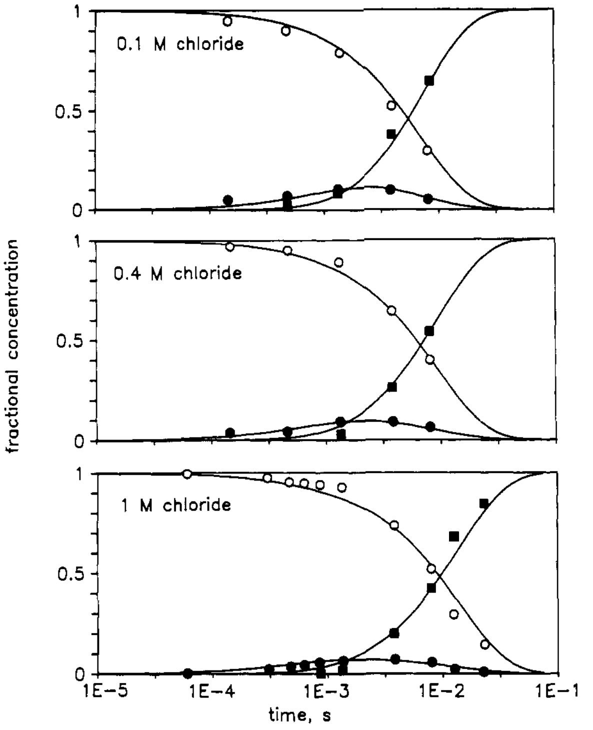

FIGURE 9: Kinetics of HR_L, HR_O, and HR at 22 °C at various chloride concentrations. The fractional concentrations of HR_L (○), HR_O (●), and the photocycling portion of HR (■) are plotted vs the logarithm of time at 0.1, 0.4, and 1 M chloride concentrations, as indicated. The kinetic model, $HR_L \leftrightarrow HR_O \rightarrow HR$, yielded eq 1-5, which were used to produce the lines fitted for the points. Details are explained in the text.

Table I: Half-Times for the $HR_{KL} \rightarrow HR_L$ Reaction, Measured at 3 °C at Various Chloride Concentrations

[chloride] (mM)	$t_{1/2}$ (μs)	[chloride] (mM)	$t_{1/2}$ (μs)
0.5[a]	~0.4	100	0.77
12[b]	0.56	1000	0.83
24[b]	0.75	4000	0.87

[a] Chloride contamination in 1 M $NaNO_3$. The value given for $t_{1/2}$ is approximate, because the reaction overlaps with the $HR_{KO} \rightarrow HR_O$ transition. [b] Also present in the buffer: nitrate in amounts to provide a total of 100 mM monovalent anion concentration, and 400 mM Na_2SO_4.

changed considerably (cf. Figure 7 and its description).

Because the rise time of HR_L decreased by as much as 2 orders of magnitude with increasing chloride concentration, Tittor et al. (1987) proposed that the formation of this intermediate involves the uptake of chloride. To balance this uptake, they also included in their photocycle scheme a chloride dissociation step during the formation of HR_K. In our kinetics analysis of the rise of HR_L [as in Zimányi et al. (1989)], we find only a very minor chloride dependency for the $HR_{KL} \rightarrow HR_L$ reaction half-time (Table I), and in the opposite direction. According to our results, therefore, the participation of chloride in the steps between HR and HR_L is not necessary to invoke.

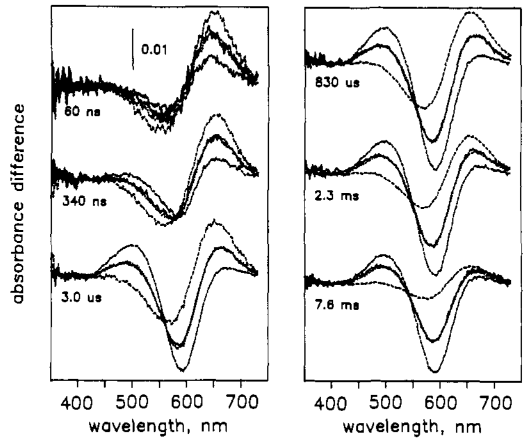

FIGURE 10: Comparison of HR difference spectra at various delay times, from 60 ns to 7.6 ms, in 100 mM NaCl, 100 mM $NaNO_3$, and 24 mM NaCl plus 76 mM $NaNO_3$. In addition to chloride and nitrate, the buffers contained 400 mM Na_2SO_4 and 25 mM MES, pH 6.0. At each delay time, as indicated, four spectra are given: one at 100 mM chloride (-··-), another at 100 mM nitrate (---), a third at 24 mM chloride plus 76 mM nitrate (—), and a fourth constructed from 54% of the chloride trace plus 46% of the nitrate trace (···).

HR Photocycle in Mixtures of Chloride and Nitrate. The results suggest the existence of two separate photocycles, with dominant intermediates HR_L and HR_O, respectively, which proceed independently up to the millisecond time range, where they can interact with one another. This hypothesis was tested by determining difference spectra in mixtures of chloride and nitrate. Results of an experiment with HR in 24 mM NaCl plus 76 mM $NaNO_3$ are shown in Figure 10. Controls were with HR in 100 mM NaCl and in 100 mM $NaNO_3$. These concentrations are sufficient for chloride to elicit the HR_L-type photocycle, but not for nitrate to do the same (cf. Figure 8). Furthermore, these concentrations are too low to drive the $HR_O \rightarrow HR_L$ reaction, which would link the two photocycles together. As shown in Figure 10, all experimental traces over a time scale of 60 ns-7.6 ms are virtually completely predicted by a linear combination of the two control traces, composed of 54% of the chloride trace and 46% of the nitrate trace. At a lower concentration of chloride, 12 mM NaCl plus 88 mM $NaNO_3$, the same kind of result was obtained, but the measured spectra agreed with a linear combination of the control traces which contained 35% of the chloride trace and 65% of the nitrate trace. The model of two parallel photocycles is thereby confirmed, and the results are consistent with the earlier suggestion (Lanyi & Vodyanoy, 1986; Tittor et al., 1987) that one of these originates from HR with site II occupied (by chloride in this case), while the other from HR with this site unoccupied.

DISCUSSION

The spectra and their analyses suggest that in 1 M nitrate the HR photocycle can be described by two parallel sets of reactions: one, which is essentially identical with that seen in 1 M NaCl, consisting of $HR \rightarrow HR_{KL} \rightarrow HR_L \leftrightarrow HR_O \rightarrow HR$, and another which consists of $HR \rightarrow HR_{KO} \rightarrow HR_O \rightarrow HR$. Primary photoproducts, detected with picosecond (Polland et al., 1985; Franz, 1988) or low-temperature (Zimányi & Lanyi, 1989) spectroscopy, which should be designated as HR_K and HR'_K, respectively, are assumed to be in these sequences also, but they will have decayed by 60 ns after the flash. The HR_L-type photocycle is characteristic of HR in the presence of a site II anion like chloride, while the HR_O-type photocycle is produced when site II is not occupied, i.e., at lower nitrate concentrations. The interconversion of HR_L and HR_O, particularly where the amount of HR_L increased while HR_O, produced directly from HR_{KO}, decreased (Figure 2C), strongly suggests that HR_O is common to the two cycles. It is a fortuitous but fortunate circumstance that in 1 M nitrate the rate constants of the reactions change in such a way with temperature that both the $HR_L \rightarrow HR_O$ reaction (at 22 °C) and its reverse (at 3 °C) are directly observable. The linear chloride dependency for the rate constant of the $HR_O \rightarrow HR_L$ reaction (Figure 9) confirms that association and dissociation of chloride take place during the interconversion of these two species.

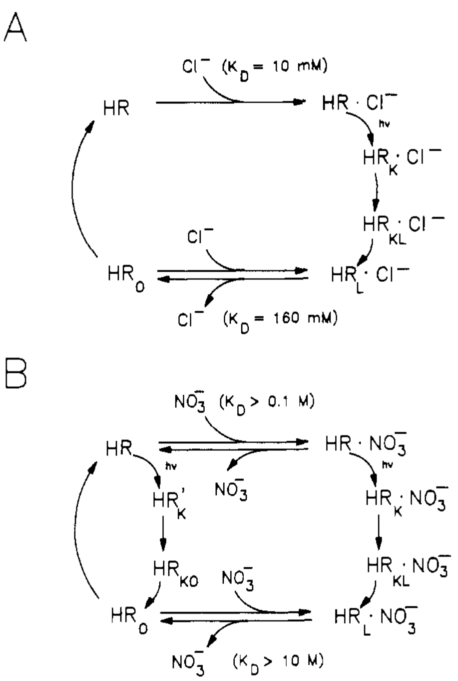

FIGURE 11: Model for the HR photoreactions in 1 M NaCl (A) and in 1 M $NaNO_3$ (B). Evidence for the existence of HR_K and HR'_K is from picosecond spectroscopy (Polland et al., 1985; Franz, 1988); the former was seen also at 110 K (Zimânyi & Lanyi, 1989). HR_L and HR_O have been observed before but are described in more detail in this and the preceding (Zimânyi et al., 1989) paper; HR_{KO} and HR_{KL} have not been reported before. Where an anion is shown with HR, the binding is presumed to be at site II (Schobert et al., 1986).

Low-temperature spectra (Zimânyi & Lanyi, 1989) support the absorption maximum for HR_K, reported (Tittor et al., 1987; Franz, 1988) to be at 598 nm. The identity and spectrum of the other initial photoproduct (HR'_K) present problems, however. Tittor et al. (1987) cited results (Franz, 1988) for the initial HR photoproduct in 1 M nitrate, determined at 100 ps, which gave its wavelength maximum also near 600 nm (in fact, our results suggest that this should be a mixture of HR_K and HR'_K). It seems likely that the species HR_{KO} at the beginning of our measurements (Figure 6B) is the intermediate which follows HR'_K, analogously to the HR_K–HR_{KL} sequence for HR in 1 M NaCl (Zimânyi et al., 1989) and to the intermediates for BR in this time range (Shichida et al., 1983; Milder & Kliger, 1988). However, the 100-ps spectrum was reconstructed (Franz, 1988) from a difference spectrum very similar to ours at 60 ns, but by determining the light flux and using 0.3 for the quantum yield, which was measured[3] to be equal to that in 1 M chloride. The latter, in turn, had been determined from the rate at which deprotonated HR was produced in the presence of azide [as in Hegemann et al. (1985)]. We find that our spectral reconstructions demand using photocycling fractions in nitrate which are only about 50% those in chloride, consistently with earlier single-wavelength measurements (Lanyi & Vodyanoy, 1986). If we use higher photocycling fractions in our calculations, the spectra for HR_L and HR_O in 1 M nitrate are unacceptably distorted [and no longer agree with those published by Tittor et al. (1987)]. Using the lower figure for the photocycling fraction in evaluating the picosecond data, however, would produce a wavelength maximum sufficiently shifted to the red to raise questions about (a) the wavelength maximum of HR'_K and (b) the possibility that what we designate as HR_{KO} might be actually the first photoproduct. Nevertheless, until these questions can be decided, we accept, provisionally, the model where HR'_K is the precursor of HR_{KO}.

Our complete model is shown in Figure 11A,B, where reactions observed in 1 M chloride are compared with those observed in 1 M nitrate. Although the scheme in Figure 11 is not radically different from those proposed earlier for HR, it represents changes in that (a) the emphasis now is on the occupancy of site II in determining the kind of photoreaction to be expected, rather than the kind of anion present, (b) the HR_L-type photocycle contains the newly described (Zimânyi et al., 1989) HR_{KL} intermediate, and likewise the HR_O-type photocycle contains a new intermediate HR_{KO} (this paper), (c) there is now good evidence that the HR_O produced from HR_L is identical with the species produced from HR_{KO}, and (d) the interconversion of HR_L and HR_O in chloride and nitrate is now supported by direct evidence. Since occupancy of site II is related to the appearance of HR_{KL} and HR_L, we conclude that it is the reactions of these species which must be involved in the anion translocation.

Registry No. Cl^-, 16887-00-6; NO_3^-, 14797-55-8.

REFERENCES

Bamberg, E., Hegemann, P., & Oesterhelt, D. (1984) *Biochim. Biophys. Acta 773*, 53–60.

Becher, B., Tokunaga, F., & Ebrey, T. G. (1978) *Biochemistry 17*, 2293–2300.

Franz, A. (1988) Ph.D. Dissertation, Technical University, Munich, Federal Republic of Germany.

Grzesiek, S., & Dencher, N. A. (1986) *FEBS Lett. 208*, 337–342.

Hazemoto, N., Kamo, N., Kobatake, Y., Tsuda, M., & Terayama, Y. (1984) *Biophys. J. 45*, 1073–1077.

Hegemann, P., Oesterhelt, D., & Steiner, M. (1985) *EMBO J. 4*, 2347–2350.

Iwasa, T., Tokunaga, F., Yoshizawa, T., & Ebrey, T. G. (1980) *Photochem. Photobiol. 31*, 83–85.

Lanyi, J. K. (1986) *Annu. Rev. Biophys. Biophys. Chem. 15*, 11–28.

Lanyi, J. K., & Vodyanoy, V. (1986) *Biochemistry 25*, 1465–1470.

Li, Q., Govindjee, R., & Ebrey, T. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7079–7082.

Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S., & Stoeckenius, W. (1976) *Biochim. Biophys. Acta 440*, 545–556.

Milder, S. J., & Kliger, D. S. (1988) *Biophys. J. 53*, 465–468.

Oesterhelt, D., Hegemann, P., & Tittor, J. (1985) *EMBO J. 4*, 2351–2356.

Polland, H. J., Franz, M. A., Zinth, W., Kaiser, W., Hegemann, P., & Oesterhelt, D. (1985) *Biophys. J. 47*, 55–59.

Schobert, B., & Lanyi, J. K. (1982) *J. Biol. Chem. 257*, 10306–10313.

Schobert, B., Lanyi, J. K., & Cragoe, E. J., Jr. (1983) *J. Biol. Chem. 258*, 15158–15164.

Schobert, B., Lanyi, J. K., & Oesterhelt, D. (1986) *J. Biol. Chem. 261*, 2690–2696.

Shichida, Y., Matuoka, S., Hidaka, Y., & Yoshizawa, T. (1983) *Biochim. Biophys. Acta 723*, 240–246.

Steiner, M., Oesterhelt, D., Ariki, M., & Lanyi, J. K. (1984) *J. Biol. Chem. 259*, 2179–2184.

Tittor, J., Oesterhelt, D., Maurer, R., Desel, H., & Uhl, R. (1987) *Biophys. J. 52*, 999–1006.

Zimânyi, L., & Lanyi, J. K. (1989) *Biochemistry 28*, 1662–1666.

Zimânyi, L., Keszthelyi, L., & Lanyi, J. K. (1989) *Biochemistry* (preceding paper in this issue).

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[3] J. Tittor, personal communication.