Evidence for Long Range Allosteric Interactions between the Extracellular and Cytoplasmic Parts of Bacteriorhodopsin from the Mutant R82A and Its Second Site Revertant R82A/G231C*

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Evidence is presented for long range interactions between the extracellular and cytoplasmic parts of the heptahelical membrane protein bacteriorhodopsin in the mutant R82A and its second site revertant R82A/G231C. (i) In the double mutants R82A/G72C and R82A/A160C, with the cysteine mutation on the extracellular or cytoplasmic surface, respectively, the photocycle is the same as in the single mutant R82A with an accelerated deprotonation of the Schiff base and a reversed order of proton release and uptake. Proton release and uptake kinetics were measured directly at either surface by using the unique cysteine residue as attachment site for the pH indicator fluorescein. Whereas in wild type proton uptake on the cytoplasmic surface occurs during the M-decay (τ ~ 8 μs), in R82A it occurs already during the first phase of the M-rise (τ < 1 μs). (ii) The introduction of a second mutation at the cytoplasmic surface in position 231 (helix G) restores wild type ground state absorption properties, kinetics of photocycle and of proton release, and uptake in the mutant R82A/G231C. In addition, kinetic H/D isotope effects provide evidence that the proton release mechanism in R82A/G231C and in wild type is similar. These results suggest the existence of long range interactions between the cytoplasmic and extracellular surface domains of bacteriorhodopsin mediated by salt bridges and hydrogen-bonded networks between helices C (Arg-82) and G (Asp-212 and Gly-231). Such long range interactions are expected to be of functional significance for activation and signal transduction in heptahelical G-protein-coupled receptors.

Allosteric interactions are well known and of great importance for water soluble enzymes, where ligand or substrate binding to a specific site alters the conformation and changes the affinity at a different site of the protein (for reviews see Refs. 1 and 2). For membrane-bound G-protein-coupled receptors analogous effects are expected to be of considerable functional significance. For this class of proteins ligand binding occurs mostly on the extracellular surface resulting in the active form of the receptor with binding and activation of the G-protein at the opposite cytoplasmic surface of the protein. The membrane protein bacteriorhodopsin (bR)1 shares the heptahelical bundle motif with G-protein-coupled receptors. In this report, we present evidence for long range interactions between the extracellular and cytoplasmic parts of bR based on evidence from the mutant R82A and its second site revertant R82A/G231C.

Bacteriorhodopsin acts as a light-driven proton pump in the plasma membrane of the archaea bacterium Halobacterium salinarium. A retinylidene chromophore is bound via a protonated Schiff base linkage to Lys-216. Upon flash excitation bR undergoes a cyclic photoreaction with distinct spectroscopic intermediates and proton translocation from one side of the membrane to the other (for reviews see Refs. 3–5). In the first half of this photocycle, the Schiff base becomes deprotonated during the transition to the M-intermediate, and in the same time range a proton is released at the extracellular surface (6). During the second half of the photocycle, the Schiff base is reprotonated from Asp-96 (7), which is located in the cytoplasmic part of the protein, and a proton is taken up from the cytoplasm. In the proton release pathway, Asp-85 has been identified as the primary acceptor of the proton from the Schiff base by site-directed mutagenesis and Fourier transform infrared difference spectroscopy (8). Arg-82 is located one helix turn away from Asp-85, facing toward the proton channel (9, 10). Steady-state and time-resolved UV/Vis absorption (11–13) and Fourier transform infrared (14) spectroscopy results indicate that Asp-85, Glu-204, Glu-194, and Arg-82 interact via a direct or indirect coupling of their pK values. Proton release to the extracellular medium may be facilitated by a hydrogen-bonded network between these residues (10, 13, 15, 16). In the three-dimensional structure of the unphotolyzed bR, a defined density for the arginine 82 side chain, has recently been resolved by x-ray crystallography at a resolution of 2.5 (17) and 2.3 Å (10). The proposed movement of this side chain toward the extracellular surface upon formation of the M-intermediate, based on computer calculations (18), is not yet established. However, recent, time-resolved electrical measurements support this proposal (19).

To further understand the mechanism of proton transport, we have studied the mutant R82A. In R82A an accelerated deprotonation of the Schiff base and a reversed order of proton

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The abbreviations used are: bR, bacteriorhodopsin; wt, wild type; DMPC, 1,2-dimyristoyl-sn-glycerol-3-phosphatidylcholine; BMF, 5-(bromomethyl)fluorescein; CHAPS, 3-[[(cholamidopropyl)dimethylammomio]-1-propanesulfonate; MOPS, 4-morpholinopropanesulfonic acid; A160C (G72C, G231C), mutant with alanine (glycine) in position 160 (72, 231) replaced by cysteine; R82A, mutant with arginine in position 82 replaced by alanine; A160C-MF(AF) (G72C-MF(AF) or G231C-MF(AF)), mutant A160C (G72C or G231C) with (methyl)- or (acetamido)fluorescein, respectively, bound to cysteine.

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release and uptake, detected with pH indicator dyes in the aqueous solution, were already observed (20, 21). Moreover, in R82A the pK_a of Asp-85, which controls the purple to blue transition, is increased by about 5 pH units compared with wild type (wt) (22). These dramatic changes are because of the replacement of the positively charged arginine by the neutral alanine.

We have used time-resolved absorbance spectroscopy in combination with pH-sensitive dyes (pyranine in the aqueous bulk phase and fluorescein derivatives attached at specific sites on the protein surface) to detect the kinetics of the photocycle and of proton release and uptake. To detect proton concentration changes separately on each surface of the protein, the attachment site (single cysteine residue) for the pH indicator dye at the surface of the mutant protein R82A was varied. We have used the single cysteine residue in position 72 at the extracellular surface and in the positions 160 and 231 at the cytoplasmic surface as attachment sites. Fig. 1 shows a tertiary structural model of bR based on x-ray crystallographic data (23). The positions of the residues Gly-72, Arg-82, Ala-160, and Gly-231 are indicated. Unexpectedly, the cysteine mutation in position 72 at the extracellular surface (EF loop), has on the other hand still all the altered properties of the single mutant R82A. The reversion effect observed only at position Gly-231 suggests an interaction between helices C (Arg-82) and G (Gly-231). The cysteine double mutant R82A/A160C, also with a cysteine mutation on the cytoplasmic surface (EF loop), has on the other side no visible effect on the conformation of bR in the visible absorbance spectrum of bacteriorhodopsin (21), (22).

**RESULTS**

**Characterization of the Unphotolyzed Single and Double Mutants (Purple to Blue Transition)—**The position of the maximum of the visible absorbance spectrum of bacteriorhodopsin reflects the charge distribution in the vicinity of the retinal-diene chromophore. Fig. 2 shows the absorption spectra of wt bR and the double mutants. Wild type (—), and the mutants G231C (---), R82A (---), and R82A/G231C (dotted line) are measured in 150 mM KCl at pH 6.

**EXPERIMENTAL PROCEDURES**

1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) and Tris-(hydroxy-methyl)-aminomethane (Tris) were obtained from Sigma. Ethylenediaminetetraacetic acid and 1,4-dithio-octoic acid were from Fluka. 8-Hydroxy-1,3,6-pyrene-trisulfonic acid trisodium salt (pyranine) was from Serva. Sephadex G-25 (fine) was from Amersham Pharmacia Biotech, 5-(iodoacetamido)fluorescein and 5-(bromomethyl)fluorescein (BMF) were from Molecular Probes.

The preparation and expression of the bR mutants A160C and G231C in H. salinarium, in which cysteine replaces Ala-160 and Gly-231, respectively, and the preparation of R82A, in which alanine is substituted for arginine in position 82, has been reported (25, 26). The bR double mutants R82A/A160C and R82A/G231C were prepared by following the described procedures (26) except that the synthetic restriction fragments bPbP-SphI and SphI-NotI were used, respectively, to construct the mutant genes.

Solubilization of bR membrane fragments and regeneration of bR in DMPC/CHAPS micelles were performed as described (27). The regeneration procedure was modified according to Ref. 28. After regeneration the retinal band is shifted back completely to 550 nm, resulting in about 96–100% regeneration, using an estimated extinction coefficient of ε_{500} ~ 56,000 M^{-1} cm^{-1}. Labeling with 5-(iodoacetamido)- and 5-(bromomethyl)-fluorescein and the determination of the labeling stoichiometry were performed as described (25, 28).

Flash spectroscopy and data analysis with a sum of exponentials were performed as described elsewhere (7). The excitation was with 10 ns pulses of 3–6 mJ of energy at 590 or 500 nm. Under these conditions about 15% of bR are cycling. Typically 30–50 time traces were averaged for the kinetics of the M-intermediate and 70–100 for the dye kinetics.

Proton release and uptake were detected in the aqueous bulk medium of the purple membrane suspension, containing 4–15 μM bR in 150 mM KCl, by calculating the difference of the measured flash-induced absorbance changes at 450 nm between samples with and without 10 mM Tris or MOPS buffer, pH 7.3, in 150 mM KCl at 22 °C (25, 29).

Titration experiments and analysis were performed as described in (30). To obtain the apparent pK_a, the measured absorbance changes were fitted with the Henderson-Hasselbalch equation.

\[
\Delta A = \Delta A_{max}/(1 + 10^{pK_a - pH})
\]

\[
\Delta A_{max} = n \times \Delta A_{max}/(1 + 10^{pK_a - pH})
\]

where \(\Delta A\) is the maximal absorbance difference, \(n\) is the number of protons involved in the transitions and \(pK_a\) is the midpoint of the titration.

The data plotted in Fig. 4 were fitted with the following equation described by Balashov et al. (21),

\[
\Delta A = a/(1 + e^{-b(\gamma)})
\]

with \(a = 1 + 10^{pK_a - pK_2}, b = 1 + 10^{pK_a - pK_2}, \) and \(\gamma = 10^{pK_a - pK_2}r\).
in the unphotolyzed state and that of the mutants R82A, G231C, and R82A/G231C in 150 mM KCl at pH 6. The chromophore absorbance maxima (λ_{max}) of wt, G231C, and R82A/G231C are nearly identical (568, 568, and 567 nm, respectively, in the light-adapted form). In the single mutant R82A, the Δabsorbance at 628 nm is red shifted about 40 nm compared with wt to 602 nm. In the mutant R82A/G231C, the additional mutation G231C at the cytoplasmic surface (C terminus) restores the wt absorbance spectrum and, therefore, presumably the charge distribution in the vicinity of the chromophore.

The absorption spectrum of unphotolyzed bR is characterized by a purple form with a λ_{max} of 568 nm around neutral pH, as shown above, which changes to a blue form at acidic pH with a λ_{max} of about 602 nm. In wt the pK_{a} of this so-called purple to blue transition is about 2.6 (in 150 mM KCl). It has been shown that this transition reflects the protonation state of the primary proton acceptor Asp-85 (22, 31). The purple to blue transition is directly affected by the proton concentration at the protein surface, which depends on the surface charge (30, 32). Therefore, the apparent pK_{a} of this transition is strongly dependent on ionic strength. We have compared the pK_{a} values of the purple to blue transition in the various mutants at a given salt concentration. The pH titration curves of wt and the different mutants in 150 mM KCl are shown in Fig. 3. The absorbance changes at 628 nm are plotted as a function of pH. The data were fitted with Equation 1. In wild type the Hill coefficient of n ~ 1.6 indicates a cooperative effect in the phototransformation/deprotonation reaction of the Asp-85 carboxyl group (33). In the bR mutant R82A, the pK_{a} of the purple to blue transition is shifted from 2.6 to 7.3 (n = 0.8). In the double mutants R82A/G231C and R82A/A160C, the pK_{a} of the purple to blue transition are 7.2 and 7.3, respectively (data not shown), i.e. similar to that of the single mutant R82A. In the double mutant R82A/G231C, however, in which the second mutation is located at the cytoplasmic surface at the end of helix G, the pK_{a} of the purple to blue transition is shifted completely back to the wild type value, even to a somewhat lower number (pK_{a} = 2.1, n = 0.9). The single mutant G231C has a pK_{a} of 2.7 (n = 1.1), similar to that of wild type. Note that for all mutants the n value is close to 1 (0.8–1.1) and clearly smaller than for wild type (1.6). The pK_{a} and n values are summarized in Table 1.

For wt and the mutant R82A/G231C the pH titration was also performed in the regenerated form in CHAPS/DMPC micelles. Doing the titration with bR micelles has the advantage of having a higher fraction of the blue state at alkaline pH values than is the case with membranes. pH-dependent absorption spectra of wt and the mutant were recorded between pH 1 and 9.5. In Fig. 4 the fraction of the blue membrane of the double mutant is plotted versus the pH. The titration data were analyzed according to the model proposed by Balashov et al. (21) using Equation 2. The scheme in the inset of Fig. 4 describes the coupling between the pK_{a} values of residues Asp-85 and another group denoted XH (probably Glu-204) and their dependence on the ionization state of each of the residues. The pK_{a} values of wt and the double mutant R82A/G231C are presented in the scheme. The comparison of the pK_{a} values clearly shows that in the double mutant R82A/G231C the pK_{a} of Asp-85 in the presence of the protonated group XH is shifted back from 6.9 in R82A (pK_{a} in micelles (30)) to 3.0; i.e. lower than the wild type value (pK_{a} = 4.7). This should be compared with the Asp-85 pK_{a} value of 2.6 and 2.1 in membranes for wt and R82A/G231C, respectively (Table 1). These results indicate that the electrostatic environments for these residues (Asp-85 and XH) differ in the double mutant and in wt. Also the pK_{a} of Asp-85 in the presence of the deprotonated group X' (like in the M-state), pK_{a} = 5.8, differs slightly from the wild type value (pK_{a} = 6.3). Similar shifts are observed for the pK_{a} of XH in the double mutant R82A/G231C, i.e. the pK_{a} of XH in the presence of the deprotonated Asp-85 is 7.5 for wild type and 6.7 for the double mutant. The corresponding numbers in the membrane fragments are 9.6 and 9.0, respectively.

The kinetics of light-dark adaptation of the chromophore in the unphotolyzed state of bR is also controlled by the protonation state of Asp-85 (12). In the light-adapted state the retinylidene chromophore is 100% in the all trans conformation. In the dark adapted state, the chromophore is in an equilibrium between the all trans (34%) and 13-cis (66%) configurations (34). In wt-bR the pH dependence of the dark adaptation rate and the fraction of molecules in the blue state were shown to be the same (12). We have measured the dark adaptation rate for wt, R82A, G231C, and R82A/G231C at selected pH values (data not shown). wt and the single mutant G231C have similar values of k_{DA} ~ 2.3–2.5 × 10^{-4} s^{-1} at pH 6. In the double mutant R82A/G231C a slightly higher value of 3.3 × 10^{-4} s^{-1} (pH 6) was observed. The fastest rate was observed for R82A with k_{DA} ~ 120 × 10^{-4} s^{-1} at pH 7.3. In wt the fastest rates are
measured at acidic pH, where also the highest fraction of blue membrane was observed. Therefore, the faster rate for R82A, \( k_{DA} \approx 120 \times 10^{-4} \text{s}^{-1} \), compared with wt, \( k_{DA} \approx 2.3 \times 10^{-4} \text{s}^{-1} \), and the close values for wt, G231C, and R82A/G231C are in qualitative agreement with the results obtained from the blue to purple chromophore titration.

**Photocycle Kinetics**—The formation and decay of the M-intermediate represent the deprotonation and reprotonation, respectively, of the Schiff base during the photocycle. We believe that a low \( pK_a \) of the proton acceptor Asp-85 and a wild type-like dark isomerization rate are prerequisites for wild type like photocycling and proton pumping (for further details see “Discussion”). Because, as shown in the previous section, these two prerequisites are fulfilled in the double mutant R82A/G231C, one may expect a restoration of wild type kinetics of the photocycle and of proton release and uptake. In Fig. 5 the time courses of the M-intermediate (absorbance changes measured at 410 nm) are shown for wild type, R82A, and R82A/G231C measured at the same protein concentrations in 150 mM KCl at pH 7.3 and 22 °C. The amplitudes of the M-intermediates are normalized to the wild type value, which was set to 1. For R82A and R82A/G231C the amplitude scaling factors were 5 and 1.15, respectively. The apparent time constants and amplitudes for the kinetics of the M-rise and decay are given in Tables II and III. The transient absorption spectra of Fig. 5 show that in comparison to wild type the rise of M in R82A is accelerated by approximately a factor of 10, whereas the decay is faster by less than a factor of 2. In the double mutant R82A/G231C on the other hand, the kinetics of the rise of M are virtually identical to that of wild type, and the decay is delayed by about a factor of 2. The major effect in R82A, the greatly altered rise of M, is thus restored in the double mutant. Fig. 6 shows the photocycle kinetics at three selected wavelengths (410, 570, and 650 nm) of wild type and the mutants G231C and R82A/G231C. These three wavelengths were chosen because they are diagnostic for the kinetics of the M intermediate.

| Table I | Proton Transfer and Mutation Effects in Bacteriorhodopsin |
|---------|--------------------------------------------------------|
| \( pK_a \) values and Hill coefficients (n) of the blue to purple transition of bR wild type and various mutants |
| wt      | 2.6 | 1.6 |
| G231C   | 2.7 | 1.1 |
| R82A    | 7.3 | 0.8 |
| R82A/G72C | 7.2 | 0.8 |
| R82A/A160C | 7.5 | 0.8 |
| R82A/G231C | 2.1 | 0.9 |

**FIG. 5.** Time course of the flash-induced M-intermediate of wild type bR and the mutants R82A and R82A/G231C. Kinetic absorbance changes were measured at 410 nm. The amplitude of the wt absorbance change was normalized to 1. The scaling factors for the mutants are given in the figure. Conditions: 150 mM KCl, pH 7.3, at 22 °C. The excitation was at 590 nm with flashes of about 6 mJ.

**Kinetics of M formation and proton release**

The M-formation is measured as the absorbance increase at 410 nm in 150 mM KCl, pH 7.3, at 22 °C. The relative contributions of the three rise components are given in percentages in parentheses following the rise times. When two components occur in the H+ release, the times are separated by a slash. \( \lambda_{\text{total}} \) gives the total amplitude as a percentage of the wild type amplitude of the M-intermediate. The proton release is detected with fluorescein bound to the cysteine at the indicated position at the protein surface and with pyranine in the aqueous bulk phase. A gives the amplitude of the proton signal as percent of the wild type amplitude of the proton signal.

| Table II | Proton transfer and mutation effects in bR |
|---------|------------------------------------------|
| M-rise  | \( \tau_1 \) \( \mu s \) \( \tau_2 \) \( \mu s \) \( \tau_3 \) \( \mu s \) \( A_{\text{total}} \% \) | Proton release |
| wt      | 1.2 (7) | 41 (44) | 138 (49) | 100 |
| G72C    | 1.1 (6) | 45 (42) | 152 (52) | 0.071 | 0.85 |
| R82A    | 0.42 (59) | 8.6 (36) | 20 |
| R82A/G72C | 0.7 (33) | 9.7 (34) | 18 |
| R82A/A160C | 0.3 (61) | 5.4 (39) | 18 |
| R82A/G231C | 1.0 (11) | 33 (39) | 136 (55) | 79 |
| wt      | 1.2 (7) | 41 (44) | 138 (49) | 100 |
| G72C    | 1.1 (6) | 45 (42) | 152 (52) | 0.071 | 0.85 |
| R82A    | 0.42 (59) | 8.6 (36) | 20 |
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| R82A/A160C | 0.3 (61) | 5.4 (39) | 18 |
| R82A/G231C | 1.0 (11) | 33 (39) | 136 (55) | 79 |

\( A_{\text{total}} \) | Surface | Bulk | \( A_{\text{total}} \) % of Awt. | % of Awt. |
|------------------|---------|-------|----------------|--------|
| wt               | 0.78    | 100   | 0.071         | 0.85   |
| G72C             | 0.73    | 10.0  | 20–30         | 10–15  |
| R82A             | 1.4/14  | 10.0  | 10–15         | 75–85  |
| R82A/G231C       | 0.075   | 0.58  | 75–85         |        |

*The data for G72C, a wild type-like bR cysteine mutant, are from Alexiev et al. (25).
ent, 89 mOD for wild type and 70 mOD for R82A/G231C (amplitudes from a multiexponential fit; the apparent amplitudes in Fig. 7 are approximately 74 and 64 mOD, respectively). The overall conclusion, however, deduced from the multiexponential fits of the time traces shown in Fig. 7 is that the pH dependence of the M-decay kinetics in the double mutant and wild type is quite similar. The time constants of the fastest two components of the M-rise of R82A/G231C and wild type are identical in the observed pH range. Both the maximum amplitude and the $pK_a$ of the slowest M-rise component of the double mutant, however, are different from wt. Fig. 8, inset, shows the amplitudes in mOD of the slowest M-rise component as a function of pH. After normalizing at pH 7, the amplitudes are replotted as the fraction of protonated Schiff base remaining (see "Results"). $f$, wild type; $E$, R82A/G231C.

Fig. 8. pH dependence of the amplitude of the slowest component $t_3$ in the M-rise. The data are from Fig. 7. The pH dependence was fitted with Equation 1. The $pK_a$ values are 9.6 for wt and 9.1 for R82A/G231C. It was shown previously, that this $pK_a$ value represents the $pK_a$ of the terminal proton release group (X'H) in the unphotolyzed state (13). The lower $pK_a$ of the terminal proton release group in the double mutant compared with wt is in agreement with the results for the group X'H from the pH titration of the unphotolyzed protein ($pK_a = 9.6$ and 9.0 for wt and R82A/G231C, respectively, in bR membrane fragments) described in the previous section.

Kinetic Isotope Effects in the Photocycle of Wild Type and R82A/G231C—Kinetic isotope effects because of H/D exchange allow insight into the nature of proton transfer steps inside the protein (36). We have performed photocycle measurements of wt, R82A, and R82A/G231C in H$_2$O and D$_2$O at selected wavelengths. Fig. 9 shows the time traces at 410 nm for the two mutants (A and B) and wt (C) in 150 mM KCl and pH/pD 8. The measuring conditions were chosen such that the mutant R82A is more than 50% in the purple state. The isotope effects on the kinetics of the main M-rise components are smaller for R82A (A) than for R82A/G231C (B) and wt (C). For a better comparison of the multiexponential M-rise kinetics (see Table II) we have fitted the data with a Gaussian distribution of exponentials. This distribution of time constants obtained in H$_2$O ($\tau_{\text{H},G}$) is centered at 5.2, 145, and 121 $\mu$s for R82A, R82A/G231C...
G231C, and wt, respectively; the corresponding values in D$_2$O (t$_{D,O}$) were determined to be 12.5, 1041, and 835 ms. The values of t$_{D,O}$/t$_{H,O}$ are 2.4 for R82A, and 6.9 and 7.2 for wt and R82A/G231C, respectively. The data thus indicate a similar proton release mechanism for wild type and the double mutant.

Proton Release and Uptake Kinetics—Proton concentration changes at the protein surface and in the aqueous bulk phase were detected with the surface bound dye fluorescein and the bulk pH indicator pyranine, respectively. Acetamido- or methyfluorescein was covalently bound to single cysteine residues introduced by site-directed mutagenesis at position Gly-72 (BC loop) on the extracellular side and at position Ala-160 (EF loop) and Gly-231 (C-terminal tail) on the cytoplasmic side of bacteriorhodopsin (Fig. 1) both in the single cysteine mutants and in the double mutants R82A/G72C, R82A/A160C, and R82A/G231C. Using the method described under “Experimental Procedures,” on average 0.65–0.95 mol of BMF or 0.5–0.7 mol of 5-iodoacetamido fluorescein were normally incorporated/mmol of mutant bR. In wild type bR, which lacks cysteine, less than 5 mol% fluorescein was bound under these conditions. A major change in the reactivity of the dye was observed for the cysteine residue in position 291. In the single cysteine mutant G231C, a labeling stoichiometry of only 0.15–0.2 mol 5-iodoacetamido fluorescein/mmol bR was obtained, whereas in the double mutant R82A/G231C an approximately 3-fold higher stoichiometry (0.55–0.7) was observed under identical conditions.

In Fig. 10 the kinetics of the rise and decay of the M-intermediate (upper panel) are compared with the kinetics of proton release and uptake, detected in the aqueous bulk phase (lower panel) of R82A (Fig. 10A) and at the protein surface (Fig. 10, B–D, lower panel) of the three double mutants R82A/G72C, R82A/A160C, and R82A/G231C, respectively. All measurements were performed with membrane fragments under the same conditions (150 mM KCl, pH 7.3, and 22 °C). All proton release and uptake times as detected by fluorescein and pyranine for wt and the mutants are collected together with the time constants and amplitudes for the kinetics of M in Tables II and III. Note from Fig. 10 and Tables II and III that in contrast to the double mutant R82A/G231C, the photocycles and the proton signals, measured with pyranine, of R82A/G72C and R82A/A160C are similar to that of the single mutant R82A. Five exponentials are required to fit the time traces for M in R82A, R82A/G72C, and R82A/A160C. Seven exponentials are required for R82A/G231C. The main time constants are marked by vertical arrows in the figures. The main proton signal components are also marked by arrows, pointing down for proton release and pointing up for proton uptake. The two major M-rise time constants are similar in R82A, R82A/A160C (cysteine mutation on the cytoplasmic surface), and R82A/G72C (cysteine mutation on the extracellular surface) with $\tau_1 = 420$ ns and $\tau_2 = 8.6$ ms, $\tau_1 = 300$ ns and $\tau_2 = 5.4$ ms, and $\tau_1 = 710$ ns and $\tau_2 = 9.7$ ms, respectively. In R82A/G231C (cysteine mutation on the cytoplasmic surface) on the other hand, the M-rise time constants are $\tau_1 = 1.0$ ms, $\tau_2 = 33$ ms, and $\tau_3 = 136$ ms and are virtually identical to those of wt type.

The same pattern of results was obtained for the proton signal measured with pyranine in the aqueous bulk phase (Fig. 10A, lower panel, and Tables II and III). In all three mutants R82A, R82A/G72C, and R82A/A160C proton uptake precedes proton release. Proton uptake was measured with a time constant of about 7 ms and the release with a time constant of about 10 ms in R82A. For R82A/G72C and R82A/A160C the same results were obtained (Tables II and III). With R82A/G231C on the other hand, proton release occurred first (0.58 ms) followed by proton uptake (11.8 ms), and these values are virtually identical to those obtained with wild type (Tables II and III).

With the surface-bound dye fluorescein at position A160C at the cytoplasmic surface, two proton uptake components were detected for R82A/A160C with time constants $\tau_1 < 1$ ms (~40%) and 150 ± 15 ms (~60%) (Fig. 10C, lower panel). The proton uptake with the two time constants was reproducible as verified in three independent experiments. The proton uptake measured with the dye bound at position G72C on the extracellular surface was fitted with two time constants of $\tau_1 = 84 ± 10$ ms (~50%) and $\tau_2 = 185 ± 12$ ms (~50%) (Fig. 10B, lower panel). For both double mutants, the proton uptake time constants detected at the protein surface are in the ms time range and thus at least 50 times faster than those detected in the bulk phase (milliseconds). Moreover, they clearly precede the normal H$^+$ uptake time in wild type, detected at the protein surface (ms time range), also by more than one order of magnitude. The main proton release component at the surface of R82A is about 1 ms ($\tau = 0.73$ ms for R82A/G72C and $\tau_1 = 1.4$ ms for R82A/A160C) or slower ($\tau_2 = 14$ ms for R82A/A160C). Our experimental data thus clearly show that in the mutant R82A the release is slowed down, and the uptake is accelerated compared with wt (proton release $\tau = 71$ ms and proton uptake $\tau = 8$ ms, as measured with the wt-like bR-mutant G72C-AF (25)).

In contrast to the double mutants R82A/G72C and R82A/A160C, which have photocycles similar to that of the single mutant R82A, the photocycle of R82A/G231C is similar to wt as described in the preceding two sections. It is thus not very surprising that the kinetics of the proton signals detected at the protein surface are also similar to wild type (Fig. 10D, lower panel, and Tables II and III). In particular the order is such that proton release (75 ms) precedes uptake (6 and 10.8 ms).
The reversed order of proton release and uptake in R82A and R82Q, as detected with the pH indicator dye pyranine in the aqueous bulk phase, was first reported by Otto et al. (20) using DMPC/CHAPS micelles. The effect of the arginine 82 to alanine mutation on dark adaptation, proton release, and photochemical cycle in membrane fragments was analyzed by Balashov et al. (21). These authors confirmed the reversed order of proton release and uptake observed in Ref. 20 using the same pH indicator dye, obtaining times of 8 ms (uptake) and 30 ms (release). Our measurements of the proton transfer kinetics in the aqueous bulk phase in the single mutant R82A also give proton uptake kinetics in the same time range of $t' \approx 7$ ms (Table III and Fig. 10A). The proton release kinetics is somewhat faster (10 ms compared with $\sim 30$ ms) as described in Ref. 21, and this difference may be because of the different salt concentrations used in the experiments (150 mM and 2 M salt, respectively). The analysis of the kinetics of proton concentration change measured with pyranine in the aqueous bulk phase is complicated by the fact that it represents the kinetics of the $H^+$ transfer between the protein surface and the bulk phase (6, 25, 28, 37, 38). Surface attached pH indicators dyes at both the extracellular and cytoplasmic protein surface overcome this limitation (25, 37, 39).

We therefore constructed double mutants with the R82A substitution as well as another residue at either surface mutated to cysteine as an attachment site for the pH indicator dye. We chose G72C at the extracellular surface and A160C at the cytoplasmic surface. Both single cysteine mutants exhibit photocycle kinetics like wild type, with minor changes in the second half of the photocycle for A160C (25). The photocycles of R82A, R82A/G72C, and R82A/A160C are very similar. The M-rise was fitted with two time constants, $t_1$; $300–700$ ns and $t_2$; $7$ ms (Table III and Fig. 10). The solid lines represent multieponential fits. The $H^+$ release and uptake times obtained from the fits are marked with arrows pointing down for release and pointing up for proton uptake. Conditions are as in Fig. 5.

**DISCUSSION**

**Kinetics of Proton Release and Uptake Detected at the Surface of the bR Mutant R82A**—The reversed order of proton release and uptake in R82A and R82Q, as detected with the pH indicator dye pyranine in the aqueous bulk phase, was first reported by Otto et al. (20) using DMPC/CHAPS micelles. The effect of the arginine 82 to alanine mutation on dark adaptation, proton release, and photochemical cycle in membrane fragments was analyzed by Balashov et al. (21). These authors confirmed the reversed order of proton release and uptake observed in Ref. 20 using the same pH indicator dye, obtaining times of 8 ms (uptake) and 30 ms (release). Our measurements of the proton transfer kinetics in the aqueous bulk phase in the single mutant R82A also give proton uptake kinetics in the same time range of $\tau \approx 7$ ms (Table III and Fig. 10A). The proton release kinetics is somewhat faster (10 ms compared with $\sim 30$ ms) as described in Ref. 21, and this difference may be because of the different salt concentrations used in the experiments (150 mM and 2 M salt, respectively). The analysis of the kinetics of proton concentration change measured with pyranine in the aqueous bulk phase is complicated by the fact that it represents the kinetics of the $H^+$ transfer between the protein surface and the bulk phase (6, 25, 28, 37, 38). Surface attached pH indicators dyes at both the extracellular and cytoplasmic protein surface overcome this limitation (25, 37, 39). We therefore constructed double mutants with the R82A substitution as well as another residue at either surface mutated to cysteine as an attachment site for the pH indicator dye. We chose G72C at the extracellular surface and A160C at the cytoplasmic surface. Both single cysteine mutants exhibit photocycle kinetics like wild type, with minor changes in the second half of the photocycle for A160C (25). The photocycles of R82A, R82A/G72C, and R82A/A160C are very similar. The M-rise was fitted with two time constants, $t_1$; $300–700$ ns and

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**TABLE III**

Kinetics of M-decay and proton uptake

The conditions are as given in Table I. The main decay components are listed. The relative contributions of the decay components are given in percentages following the decay time constants. When two components occur in the $H^+$ uptake, the times are separated by a slash.

| bR          | $t_1$  | $t_2$  | $t_3$  | Proton uptake |
|-------------|--------|--------|--------|---------------|
|             | ms     | ms     | ms     | Surface       |
|             |        |        |        | Bulk          |
| wt          | 0.7 (15)| 2.1 (53)| 6.2 (32) | 8.7 |
| G72C*       | 2.4 (34)| 4.0 (45)| 9.0 (21)| 7.9 |
| R82A        | 0.69 (35)| 2.9 (55)| 12 (7)  | 0.084/ 7 |
| R82A/G72C   | 0.68 (20)| 4.3 (60)| 18.0 (20)| 0.185 |
| R82A/A160C  | 0.7 (24)| 4.8 (64)| 24.5 (12)| 0.001/ 7 |
| R82A/G231C  | 2.1 (30)| 7.8 (57)| 33.2 (11)| 0.15 |
|             |        |        |        | 6/10.8 11.8 |

* The data for G72C, a wild type-like bR cysteine mutant, are from Alexiev et al. (25).
constant at the cytoplasmic surface of the proton release channel close to the extracellular surface, affects the removal of a charged residue can often be compensated by a second mutation, which removes the opposite charge at a nearby position and therefore restores the electrostatic environment in the mutants indicated). In addition, the pK_a of Asp-85 is down shifted by 0.5 pH units compared with wt, and (iv) the amplitude of the M-rise and of the proton signal are about 80% of the wt amplitude.

The second site revertants of mutants containing single replacements at position 82 in br (like R82A and R82Q) are of particular interest, because Arg-82 has been proposed to be involved in multiple types of interactions, as part of the counterion of the Schiff base proton (electrostatic interaction), in interactions with the primary and terminal proton release groups (electrostatic and structural-conformational interactions), and during folding (structural-electrostatic interaction). Though the second site revertant R82A/G231C displays in many respects wild type properties (pK_a of Asp-85, rate of light-dark adaptation, kinetics of photocycle and of proton release and uptake, and kinetic isotope effects), small but distinct differences exist: (i) the pK_a of Asp-85 is down shifted by 0.5 pH units compared with wt, (ii) the Hill coefficient is 0.9 in R82A/G231C but 1.6 in wt, (iii) the pK_a of the terminal release group (XH) was determined both in the unphotolyzed protein and from the pH dependence of the M-rise to be about 0.5 pH units lower than in wt, and (iv) the amplitude of the M-rise and of the proton signal are about 80% of the wt amplitude.

The second site revertant R822A/G231C displays in many respects wild type properties (pK_a of Asp-85, rate of light-dark adaptation, kinetics of photocycle and of proton release and uptake, and kinetic isotope effects), small but distinct differences exist: (i) the pK_a of Asp-85 is down shifted by 0.5 pH units compared with wt, (ii) the Hill coefficient is 0.9 in R82A/G231C but 1.6 in wt, (iii) the pK_a of the terminal release group (XH) was determined both in the unphotolyzed protein and from the pH dependence of the M-rise to be about 0.5 pH units lower than in wt, and (iv) the amplitude of the M-rise and of the proton signal are about 80% of the wt amplitude.

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In wild type, Asp-212 (helix G) presumably is directly hydrogen-bonded to Tyr-185 (helix F), Tyr-57 (helix B) and via water molecules (44) to Arg-82 and Asp-85 (helix C) forming a three-dimensional hydrogen-bonding network (9, 10, 23). Asp-85 and Asp-212, together with Arg-82 form the complex counterion to the protonated Schiff base in the retinal binding pocket (45). Note, that the neutral alanine residue in the mutant R82A is not capable of participating in a hydrogen-bonded network. We found that the most striking similarity between the mutants R82A, G231C, and R82A/G231C is the loss of cooperativity in the purple to blue transition (n = 1.6 in wild type to n = 0.8–1.1 in the mutants indicated). In addition, the pK_a values of both Asp-85 and the terminal release group XH in the unphotolyzed protein R82A/G231C are down shifted by about 0.5 pH units compared with wt. These results indicate a changed electrostatic environment of Asp-85 in the complex counterion and an altered interaction of ionizable residues in the proton release channel. This different electrostatic environment in the retinal binding pocket of R82A/G231C compensates for the removed positive charge of the arginine residue. A compensation of this positive charge might be realized by a removal of the negative charge of Asp-212 from the vicinity of Asp-85. This could occur
by a structural change or by protonation. Conformational changes on the cytoplasmic surface close to helix G are suggested from the altered labeling stoichiometry with 5-iodoacetamidofluorescein observed between G231C and R82A/G231C. Asp-212 resides in the same helix (helix G) as Gly-231. A conformational change in helix G introduced by the mutation G231C can lead to a different interaction of the Asp-212 side chain with the neighboring residues in R82A/G231C in such a way that the side chain of Asp-212 no longer participates in the complex counterion. Recent time-resolved electrical measurements (19) provide evidence that the movement of the positively charged arginine 82 side chain makes a substantial contribution to the amplitude of the electrical signal in wt. This amplitude is clearly reduced in the mutants G231C and R82A/G231C. 2 This result can be interpreted with the absence of the movement of Arg-82 already in the single mutant G231C. Note that the loss of cooperativity in the purple to blue transition was also observed in the single mutant G231C. Therefore, it might be that in the mutant protein G231C the arginine 82 side chain is already pointing outward to the extracellular surface and does not contribute to the proton release process. A replacement of Arg-82 with alanine is then not expected to alter substantially the properties of G231C. Furthermore, the results of the H/D isotope effect on the kinetics of the M-rise, on the other hand, indicate a reestablishing of a wt-like hydrogen-bonded network facilitating proton pumping (e.g. Asp-96, Asp-212, Asp-85, Tyr-185, and Glu-204). However, whether double mutants other than R82A/G231C are able to rescue R82A and which residues are involved in the revertancy mechanism is subject to further extensive mutagenesis studies.

In conclusion, we have shown that changes at the cytoplasmic surface can alter structural and functional interactions, which occur in the extracellular part of the protein and vice versa, probably because of a rearrangement of H-bonded networks mediated by conformational changes. We demonstrated with experiments on the mutants R82A, G231C, and R82A/G231C that allosteric interactions occur between the extracellular and cytoplasmic surfaces of bacteriorhodopsin. Such long range propagation of electrostatic and conformational changes is of potential importance for signal transduction in heptahelical G-protein-coupled receptors, where binding of ligands occurs mostly on the extracellular side resulting in the active form of the receptor and where binding and activation of the G-protein takes place at the cytoplasmic surface.

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Evidence for Long Range Allosteric Interactions between the Extracellular and Cytoplasmic Parts of Bacteriorhodopsin from the Mutant R82A and Its Second Site Revertant R82A/G231C

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