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Chemical reconstitution of a chloride pump inactivated by a single point mutation

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The arginine residue R108 plays an essential role in the transport mechanism of the light-driven anion pump halorhodopsin (HR) as demonstrated by complete inactivation of chloride transport in mutant HR-R108Q. In the presence of substrate anions, guanidinium ions bind to the mutant protein with affinities in the mM range, thereby restoring transport activity and photochemical properties of wild type. One guanidinium ion and one anion are bound per molecule of HR-R108Q. For HR wild type, HR-R108Q–guanidinium and HR-R108K, differences in transport activity and anion selectivity are found which may be explained by effects of anion solvation. The agreement between light-induced FTIR difference spectra of HR wild type and HR-R108Q–guanidinium demonstrates that no structural changes occur in the reconstituted mutant and that the photoreactions of wild type and reconstituted mutant are identical. Furthermore, an IR absorbance band of the guanidino group of R108 can be identified at 1695/1688 cm^{-1}. In HR-R108Q, a guanidinium ion binding close to the mutated residue is proposed to mimick the role of the R108 side chain as the anion uptake site. Thus the wild type reaction mechanism is reconstituted.

Key words: chloride transport/Fourier transform infra-red/guanidinium/halorhodopsin/specific mutagenesis

Introduction

Halorhodopsin (HR) is one of four photoactive retinal proteins found in the cytoplasmic membrane of *Halobacterium salinarium*. Halorhodopsin and bacteriorhodopsin (BR) act as light-driven electrogenic ion pumps coupling light absorption with vectorial ion translocation. HR transports chloride ions into the cell and BR protons to the outside (recent reviews: Lanyi, 1990, 1993; Oesterhelt *et al.*, 1992). Sensory rhodopsins I and II are photoreceptors which mediate the phototactic behaviour of the cell (recent reviews: Spudich and Bogomolni, 1992; Oesterhelt and Marwan, 1993). The two ion pumps BR and HR share common structural and functional features: the structures are indistinguishable at a resolution of 7 Å, suggesting similar three-dimensional structures consisting of seven transmembrane helices (Havelka *et al.*, 1993, 1995). In both proteins the retinal chromophore is bound via a protonated Schiff base to a lysine residue in the seventh helix and interacts with the protein in a similar way. Both chromophores undergo a thermoreversible all-*trans* to 13-*cis* isomerization after photoexcitation (Braiman and Mathies, 1980; Diller *et al.*, 1987; Fodor *et al.*, 1987). Functional similarities are reflected by the capacity of HR to act as a two-photon-driven proton pump (Bamberg *et al.*, 1993) and the indication that BR at low pH may translocate chloride ions (Der *et al.*, 1989). Characteristic differences between HR and BR, on the other hand, can be correlated with their differing functions (Oesterhelt and Tittor, 1989; Oesterhelt *et al.*, 1992). At physiological pH only HR exhibits characteristic anion-dependent effects, which have been detected by various spectroscopic methods (Lanyi, 1990; Braiman *et al.*, 1994). During the photochemical cycle of the proteins the Schiff base deprotonates in BR but remains protonated in HR. The two photocycle intermediates of HR with lifetimes in the ms range, HR520 and HR640, equilibrate in an anion-dependent manner (Oesterhelt *et al.*, 1985; Tittor *et al.*, 1987). The aspartic acids D85 and D96 which play a crucial role in the proton transport mechanism of BR (Mogi *et al.*, 1988; Butt *et al.*, 1989; Gerwert *et al.*, 1989) are replaced in HR by threonine and alanine, respectively.

Anion effects on chromophore absorption and on the $C{=}NH^+$ vibrational frequency of the Schiff base were explained by assuming two anion binding sites in HR (Schobert *et al.*, 1986; Pande *et al.*, 1989; Lanyi, 1990). At least one of these sites, termed site II, seems to be functionally important for anion translocation. It binds specifically the anions which are transported by HR and its occupation in the dark results in the preferential formation of HR520 after photoexcitation, which correlates with transport activity. When there is no anion at this site only HR640 is observed upon photoexcitation (Schobert *et al.*, 1983; Lanyi *et al.*, 1990). The anion binding sites have been assigned hypothetically to two positively charged arginines in the HR primary sequence which are expected to be located within the membrane-spanning region (Blanck and Oesterhelt, 1987). Site II was thought to be mainly constituted by R108 and to be responsible for anion uptake from the extracellular side, whereas site I was thought to be involved in anion release and to be represented by R200 (Lanyi, 1990; Oesterhelt *et al.*, 1992). Another model suggested the existence of a single binding site and two different conformational states of the protein which are adopted in an anion-dependent manner (Walter and Braiman, 1994). In analogy to the counterion complex of the protonated Schiff base assumed for BR (de Groot *et al.*, 1989), a model for the anion uptake site in HR was proposed by Ames *et al.* (1992) consisting of R108 and the protonated Schiff base as the positively charged part and D238 and the bound anion as the negative charges. This model was supported by detection of hydrogen

Table I. Specific anion transport activities of wild type and mutant HR

Anion/strain	Wild type	R108Q	R108Q+ guanidinium	R108K
Chloride	280	0	66	8
Bromide	300	8	122	24
Nitrate	125	0	20	3

The values given in ions per min and HR molecule were determined from light-induced pH changes of cell suspensions in basal salt solution with the indicated anion. Experimental error ± 10%.

bonding between the protonated Schiff base and the anion (Walter and Braiman, 1994). Similar charge distributions around the Schiff base in HR and BR are supported by two observations: anions affect the chromophore absorption in BR mutants lacking D85, which in HR is replaced by T (Marti *et al.*, 1991). Analogous anion-induced absorption shifts are observed in the blue forms of BR at low pH (Fischer and Oesterhelt, 1979), where D85 is protonated (Metz *et al.*, 1992), and of HR from *Natronobacterium pharaonis* in the absence of anions (Scharf and Engelhard, 1994).

All of the proposed models for anion binding and anion translocation mechanism of HR include as an essential part the arginine residue 108, which is conserved in all known HR sequences (Soppa *et al.*, 1993). Nevertheless, no direct experimental evidence has so far been presented for the importance of this amino acid side chain. Here, we report on investigations of two specific R108 mutants of HR: R108K conserving the positive charge, and R108Q with a neutral residue at this position. The results presented demonstrate that R108 is indeed a crucial residue for the function of HR and provide insight into the mechanism of anion binding.

Results

Transport activity of mutant HRs

Cells overexpressing wild type or mutant HR (Heymann *et al.*, 1993) were suspended in basal salt solution in the presence of 50 μM of the protonophor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). For wild type HR, light-induced alkalinization is observed under these conditions due to passive CCCP-mediated proton influx in response to electrogenic chloride transport (Oesterhelt, 1982). The replacement of arginine 108 by lysine resulted in a largely reduced transport activity compared with the wild type, while introduction of a neutral glutamine residue inactivates chloride transport completely (Table I). The small signal detected for the mutant HR-R108Q (Figure 1A, a) could be attributed to a light-induced artefact also seen in samples without cells. Addition of 0.1 M guanidinium chloride to a HR-R108Q cell suspension caused light-induced alkalinization (Figure 1A, b) and thus indicated reconstitution of the chloride transport activity for the HR-R108Q mutant. When TPP^+ was added to a concentration of 0.5 mM, the signal disappeared again (Figure 1A, c) because the passive proton influx was outweighed by TPP^+ influx. No effect of guanidinium could be observed for wild type HR, for the mutant R108K, or for the BR- and HR-negative recipient strain HN5 (data not shown). The reactivation of the inactive

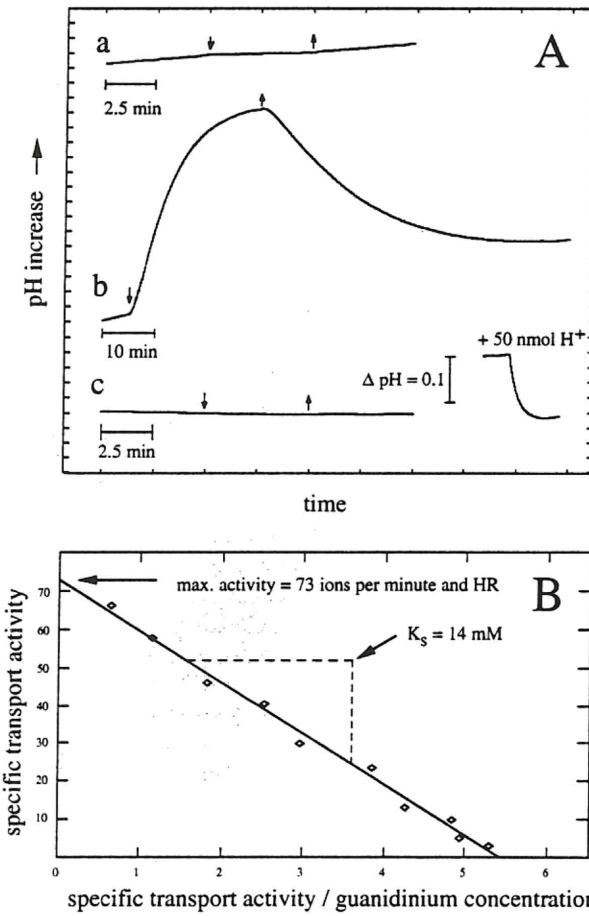

Fig. 1. (**A**) Light-induced pH changes in cell suspensions containing HR-R108Q. Cells were suspended in basal salt solution with chloride as the anion. The onset of illumination with yellow light is indicated by a downward arrow, the end of illumination by an upward arrow. The initial pH value of each trace is 6.90. (**a**) Illumination of cells overexpressing HR-R108Q; (**b**) signal after addition of 0.1 M guanidinium chloride to the same sample; (**c**) signal after addition of 0.5 mM TPP^+ to the sample in (b); calibration with HCl. (**B**) Guanidinium titration of the chloride transport activity of HR-R108Q. The light-induced initial pH change in a cell suspension as in (A) is titrated by guanidinium addition; the specific chloride transport activity is plotted according to Eadie–Hofstee.

HR-R108Q mutant will be called the 'guanidinium effect' in the following text. Addition of 0.1 M urea or tetramethylammonium chloride to the cell suspension did not cause reconstitution of activity in HR-R108Q, whereas subsequent addition of guanidinium did activate the pump. The dependence of reconstituted transport activity on the guanidinium concentration exhibited hyperbolic saturation behaviour and was analysed according to Eadie–Hofstee (Figure 1B). This revealed a maximal specific activity of 73 ± 8 chloride ions per min and HR molecule, and an affinity constant for guanidinium of K_S = 14 ± 3 mM.

As for wild type HR, bromide and nitrate are also substrates for the mutant HR-R108K and HR-R108Q–guanidinium (Table I). Bromide is transported by HR-R108Q at low rates even without guanidinium, while chloride and nitrate are transported only in the presence of this cofactor. The discrimination between different anions becomes more pronounced in the HR mutants: the ratio of activities for nitrate:chloride:bromide

Table II. Chromophore absorption maxima of HR-R108Q (HR wild type)

Guanidinium	Sulfate	Chloride	Bromide	Nitrate
0	578 (576)	579 (578)	575 (582)	578 (568)
100 mM	578 (576)	568 (578)	567 (583)	566 (568)

Membranes were suspended in basal salt solutions with the indicated anions. Accuracy ± 2 nm.

is ~1:3:6 for R108Q–guanidinium, 1:3:8 for R108K and 1:2.2:2.4 for wild type.

Absorbance shifts

All measurements on membrane suspensions including flash photolysis experiments were done consistently in the same salt solutions of high ionic strength also used in the transport experiments with cell suspensions, except that 20 mM Tris–H_2SO_4 buffer was added and the pH adjusted to 7.0. Table II shows λ_{max} values of membrane-bound HR-R108Q and HR wild type in the presence of different ions. Guanidinium has nearly no influence on the chromophore absorption of wild type (Table II, c.f. values in brackets in rows 1 and 2). Guanidinium also does not affect HR-R108Q in sulfate solution (column 1), but in the presence of transported ions it induces blueshifts of 8–12 nm (columns 2–4). Without guanidinium, on the other hand, different anions hardly influence the absorption maximum of HR-R108Q (row 1), while HR wild type exhibits a blueshift upon nitrate binding (Schobert *et al.*, 1986). HR-R108Q is anion sensitive only in the presence of guanidinium; the addition of a substrate anion induces blueshifts of ~10 nm (row 2). A blueshift can result from electrostatic changes near the retinal binding site, if a negative charge or partial charge of a dipole approaches the protonated Schiff base (Blatz *et al.*, 1972; Grossjean and Tavan, 1988). Anion-induced blueshifts have been found for the blue membrane of BR at low pH (Fischer and Oesterhelt, 1979) and for the anion-free blue form of HR from *N.pharaonis* (Scharf and Engelhard, 1994). In our case the blueshift is caused by simultaneous binding of guanidinium and a substrate anion, therefore it can be concluded that the anion binds closer to the Schiff base than does the guanidinium cation. The difference of anion effects on wild type (Table II, values in brackets) or mutant with guanidinium (row 2) may be explained by different interactions between the chromophore and either anion plus arginine or anion plus guanidinium.

Both the blueshift of HR-R108Q and its transport activity need the simultaneous presence of guanidinium and the substrate anion. To examine this correlation further, the concentration dependencies were determined. The effect of guanidinium binding on the absorption maximum at saturating chloride concentration was analysed according to Eadie–Hofstee (Figure 2A, upper graph). The first two deviating data points, marked by stars, are not included in the linear regression. The binding constant K_S = 11 ± 3 mM agrees within experimental error with the binding constant measured for the transport activity of the cells (Figure 1B). The same correspondence of affinities determined from the blueshift and the transport activity experiments was found with bromide or nitrate as anions, with values of 6 and 20 mM, respectively (Table III).

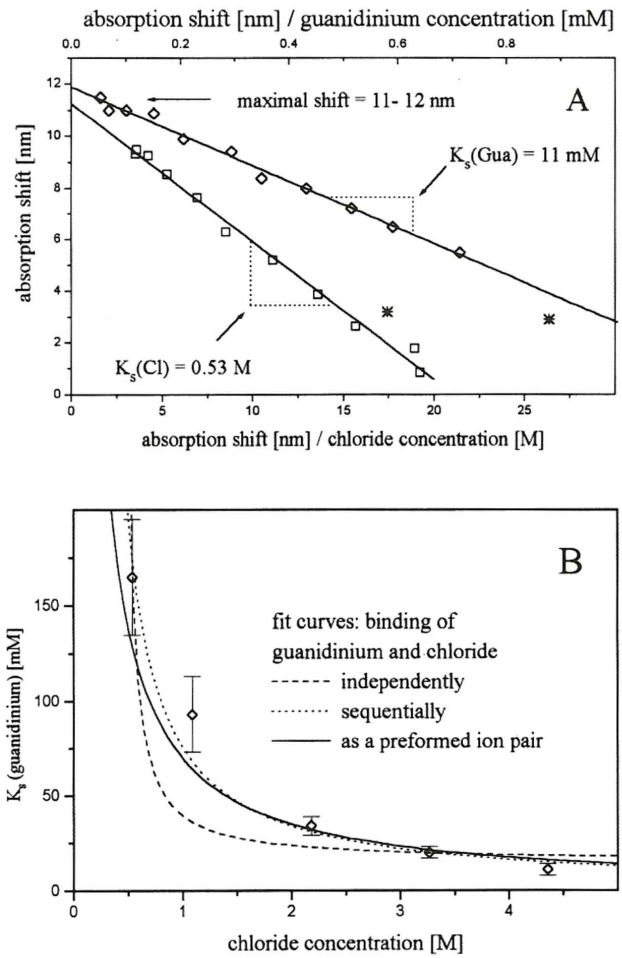

Fig. 2. (**A**) Titration of the absorption maximum of membrane-bound HR-R108Q. Membranes were suspended in basal salt solution containing chloride (upper graph) or sulfate as anion and 0.2 M guanidinium (lower graph).The shift of the chromophore absorption maximum upon guanidinium (upper graph) or chloride addition (lower graph) is plotted according to Eadie–Hofstee. (**B**) Chloride dependence of the guanidinium binding constant. The apparent binding constant is determined as in (A) for samples at various chloride concentrations. The ionic strength of the basal salt solution is maintained by sulfate.

The chloride dependence of the chromophore absorption maximum was determined by adding chloride to a sample in sulfate solution at a guanidinium concentration of 0.2 M. Eadie–Hofstee analysis revealed a chloride binding constant of 0.53 ± 0.05 M (Figure 2A, lower graph). The affinity for bromide is higher, while it is lower for nitrate (Table III). The corresponding anion titration of transport activity was difficult due to the low stability of cells suspended in sulfate solution. For bromide, however, the half-maximal effect could be observed at ~0.3–0.5 M, which was in agreement with the bromide-induced absorption shift. Table III summarizes the results obtained by the different titration experiments. Guanidinium ions induced transport activity and absorption shifts and in the case of bromide also formation of the HR520 intermediate (see below), with the same affinity (column 1). The nature of the anion modulates the affinity for guanidinium identically for all three effects. The anion affinities determined for transport and absorption shift are the same for

Table III. Titration experiments on HR-R108Q

	Apparent binding constants	
	Guanidinium ions (mM)[a]	Anions (M)[b]
Transport activity	in Cl^-: 14 ± 3 in Br^-: 6 ± 1 in NO_3^-: 20 ± 4	nd Br^-: 0.3–0.5 n.d.
Absorbance shift	in Cl^-: 11 ± 3 in Br^-: 6 ± 1 in NO_3^-: 21 ± 4	Cl^-: 0.53 ± 0.05 Br^-: 0.30 ± 0.02 NO_3^-: 1.3 ± 0.1
Flash-induced HR520 formation	in Br^-: 8 ± 2	n.d.

The binding constants were obtained by Eadie–Hofstee analysis of saturation curves, as shown in Figures 1B, 2A and 3A.
[a] Guanidinium sulfate was added to cell or membrane suspensions in basal salt solution with the indicated anion.
[b] The indicated anion was added to cell or membrane suspensions in basal salt solution containing sulfate with 0.2 M guanidinium. n.d., not determined.

bromide (column 2), but were not determined for the other anions.

Figure 2B shows the chloride dependence of the guanidinium binding constant, determined from the absorption shift, as performed in Figure 2A. The observed increase of K_S with decreasing chloride concentration is predicted by models which assume sequential binding of guanidinium and chloride (dotted line) or binding of the two ions as a preformed ion pair (solid line). Independent binding of the ions (dashed line), however, is clearly excluded.

The specificity for guanidinium was examined by use of substituted guanidinium salts. 1-Methyl guanidinium and 1-ethyl guanidinium ions induced a maximal blue-shift of 9 nm with binding constants of 17 or 33 mM, respectively. No influence on the absorption spectrum was found for 1,1-dimethyl guanidinium ions, arginine, urea, tetramethyl ammonium or ammonium chloride up to a concentration of 0.4 M.

Flash photolysis

Flash photolysis experiments were performed on membrane-bound HR-R108Q suspended in basal salt solutions with different anions. Figure 3A shows a series of difference spectra for a bromide sample starting with the spectrum at 260 µs up to the spectrum at 327 ms after the excitation flash. Only the main features of the complex pattern of these difference spectra will be considered. A red-shifted intermediate relative to the initial state is transiently detectable, similar to the truncated photocycle of HR wild type in the absence of transportable anions (Lanyi and Vodyanoy, 1986; Tittor *et al.*, 1987). After addition of guanidinium at a concentration of 0.2 M, a blue-shifted intermediate is detected (Figure 3B) which corresponds to the HR520 intermediate of the wild type photocycle in the presence of chloride or bromide. Therefore the guanidinium-induced change in the photochemical behaviour of HR-R108Q agrees with the change of the wild type photocycle upon binding of anions to site II (Lanyi and Vodyanoy, 1986; Tittor *et al.*, 1987). Guanidinium titration of the HR520 formation measured

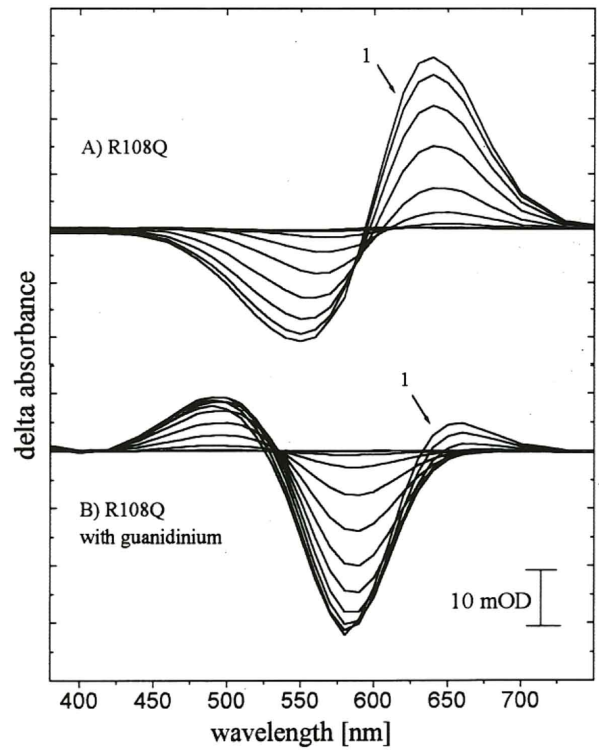

Fig. 3. Flash-induced difference spectra of membrane-bound HR-R108Q in basal salt solution containing bromide as anion. The first spectrum is 260 µs after the flash, the following spectra 0.52, 1.04, 2.08, 4.34, 8.34, 31, 62, 122, 247 and 327 ms after the flash. (**A**) without guanidinium; (**B**) with 0.2 M guanidinium.

as maximal absorption change at 490 nm revealed an apparent guanidinium affinity of 8 ± 2 mM, in agreement with the guanidinium affinities measured for the induced transport activity and the absorption shift (Table III). With chloride instead of bromide, the same effect was observed, but compared to bromide only ~30% of the transient positive absorption change at 490 nm was found (data not shown) in qualitative agreement with the lower transport activity for chloride (Table I). No guanidinium-induced change was observed in the case of sulfate, where only the red-shifted photoproduct was observed (data not shown).

FTIR difference spectra

FTIR difference spectra between the HR520 intermediate stabilized under photostationary conditions at 253 K and the initial state HR578 are shown in Figure 4A for HR wild type and in Figure 4B for HR-R108Q in the presence of guanidinium. The largest signal seen in Figure 4A, the band at 1523 cm^{-1}, corresponds to ~2% of the absolute IR absorption of the protein in this spectral region. Figure 4C shows the difference spectrum for HR-R108Q without guanidinium measured under the same conditions. All samples contained sodium bromide at saturating concentration. The difference spectrum of the wild type protein in membrane-bound form (Figure 4A) agrees very nicely with the spectrum of lipid-reconstituted samples published by Braiman *et al.* (1994).

Addition of guanidinium to a wild type sample did not change the difference spectrum (data not shown). The spectra of HR-R108Q with (Figure 4B) and without

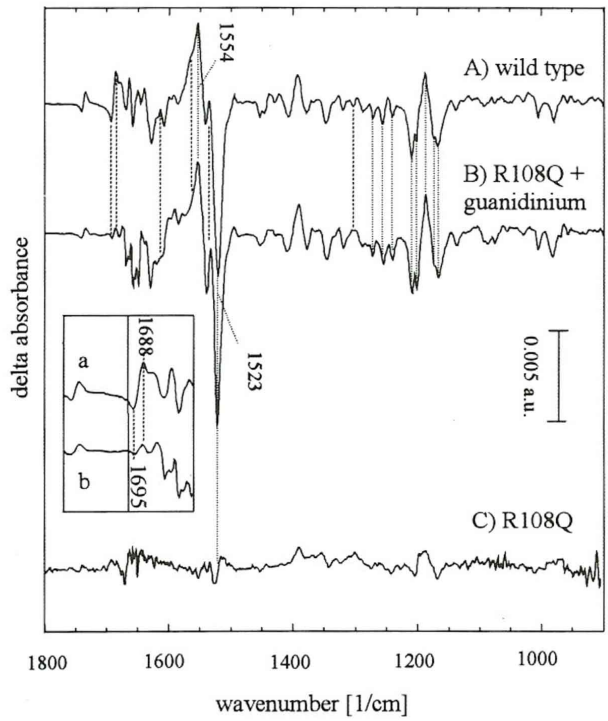

Fig. 4. (**A**) Low temperature light-induced FTIR difference spectrum of HR wild type at saturating bromide concentration. The spectrum was taken at 253 K as difference between the sample in light and dark; negative bands correspond to the initial state of HR, positive bands to the intermediate HR520. Resolution is 2 cm^{-1}. (**B**) Same for HR-R108Q in the presence of guanidinium; (**C**) same for HR-R108Q without guanidinium. Inset: spectral region 1650–1750 cm^{-1} (**a**) HR wildtype, enlarged from A (**b**) HR-R108Q−guanidinium, enlarged from B.

guanidinium (Figure 4C), however, exhibit clear deviations due to the guanidinium-induced change of photochemical behaviour also observed in the visible spectral range (Figure 3). Comparing the spectra of wild type (Figure 4A) and the reconstituted mutant (Figure 4B), a striking similarity is observed. In particular, the main chromophore bands of the wild type spectrum are well reproduced for the mutant as indicated by the dotted lines: (i) the difference band of the C=C stretching vibration which shifts from 1523 to 1554 cm^{-1} corresponds to the shift in the visible range from HR578 to HR520 (Aton *et al.*, 1977); (ii) the band pattern between 1150 and 1280 cm^{-1} caused mainly by C−C stretching, C−C−C and C−C−H bending vibrations indicates that the same photoisomerization from all-*trans* to 13-*cis* takes place in the reconstituted mutant as in the wild type and that the interactions of retinal with the wild type or mutant protein are almost identical. Perturbations of protein or chromophore structure induced by the mutation can be excluded, or at least, they are reversed by guanidinium addition. The agreement between the two FTIR difference spectra in Figure 4A and B proves that HR-R108Q−guanidinium undergoes the same light-induced intramolecular reaction as wild type HR.

Besides the general agreement between the spectra of wild type and reconstituted mutant some specific differences are found, which are indicated by the dashed lines in Figure 4A and B. Deviations around 1650 cm^{-1} are not considered, due to the low signal-to-noise ratio of the mutant spectrum in this region caused by the large background absorbance of amide I and H_2O bands. Effects of the mutation are observed at ~1690, 1620, 1540 and 1305 cm^{-1}. Here only the 1690 cm^{-1} band is taken into consideration as a more detailed discussion of the specific differences will be presented in a forthcoming publication. The inset in Figure 4 shows spectra of wild type (a, enlarged from Figure 4A) and mutant (b, enlarged from Figure 4B) in the region between 1750 and 1650 cm^{-1}. The wild type difference band at 1695/1688 cm^{-1} is reduced to relatively low intensity in the spectrum of the reconstituted mutant. Therefore this difference band can be assigned to a frequency shift of the R108 guanidino absorbance in the HR578 to HR520 reaction, demonstrating that the reaction of this residue can be monitored by its IR signals in FTIR difference spectra of HR. The remaining difference band in the spectrum of the reconstituted mutant may be caused by a frequency shift of the absorbance of the externally added guanidinium.

Discussion

A change of the residue R108 in HR by site-directed mutagenesis resulted in a drastic reduction of the chloride transport activity to a few percent of the wild type level for R108K, and virtually to zero for R108Q (Table I). This demonstrates the central role of R108 for the function of HR. Since major structural changes induced by the mutation are excluded by the results of FTIR experiments, the inactivation is a specific effect of the removal of the guanidino group of R108, supporting models which involve R108 as an important residue for the interaction with the transported anion (Oesterhelt and Tittor, 1989; Lanyi, 1990; Ames *et al.*, 1992; Oesterhelt *et al.*, 1992).

The inactive mutant R108Q can be reactivated by guanidinium ions (Figure 1). Depending on the anion added, 20–40% of the specific activity of wild type HR can be reconstituted (Table I). The most obvious interpretation is that the externally added guanidinium ion is able to replace the guanidino group of arginine 108 by binding close to residue 108 and mimicking the arginine side chain of the wild type protein. This idea is supported by the fact that no guanidinium effect is observed for the mutant R108K or the wild type. The lysine and arginine side chains may prevent guanidinium binding either spatially and/or electrostatically, while the shorter neutral glutamine residue may allow it (c.f. side chains drawn in Figure 5). The concentration dependence of the guanidinium effect for R108Q (Figure 1B) shows that binding of one guanidinium ion per HR molecule is sufficient for reactivation, as no deviations from linearity are seen in the Eadie−Hofstee plot. Due to its low chemical reactivity, the guanidinium ion presumably is interacting only electrostatically with the protein environment, acting as a non-covalently bound cofactor. Indeed, the guanidinium-induced spectroscopic effects on HR-R108Q could be reversed by washing the membrane suspension with guanidinium-free buffer (data not shown).

Using suspensions of HR-R108Q isolated in a membrane-bound form, an influence of guanidinium on the chromophore absorption maximum was found (Figure 2, Table II). A blueshift of ~10 nm is observed only if

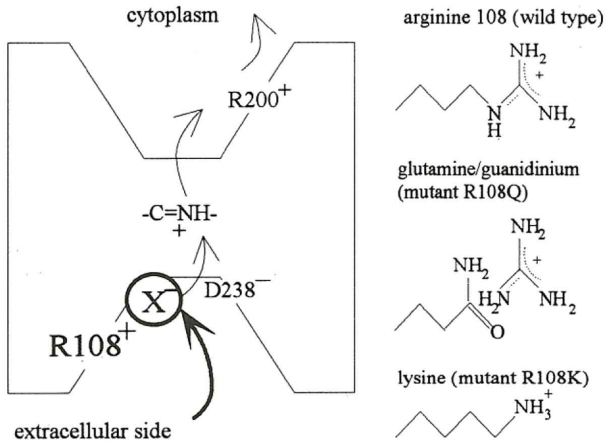

Fig. 5. Scheme of anion binding and translocation in HR. The anion uptake residue R108 investigated in this work is accentuated. On the right, the three 108 residues are drawn for which anion transport activity was found. The involvement of R200 has not been proven so far.

guanidinium as well as one of the anions transported by HR are present. The blueshift and transport activity in the mutant HR-R108Q occur only in the presence of guanidinium and a transportable anion, clearly indicating that the blue-shifted species is also the active one. Indeed, titrations of transport activity and absorbance shift revealed corresponding binding constants for bromide as well as for guanidinium in chloride, bromide or nitrate solution (Table III). The difference between the guanidinium binding constants with different substrate anions demonstrates an influence of the anion on the guanidinium binding, indicating that guanidinium and anion bind cooperatively to the protein as an ion pair. This is supported by the stoichiometry of one guanidinium and one chloride per molecule of HR-R108Q which is needed to induce the absorption shift, as concluded from the linearity of the Eadie–Hofstee plots (Figures 2A and 3A). Data on the chloride dependence of the guanidinium affinity (Figure 3B) do not allow one to discriminate between sequential binding or binding of a preformed guanidinium chloride ion pair, but do exclude independent binding of the two ions. This is as expected for a neutral binding site in which the first bound ion creates an electrostatic attraction for the second ion.

The binding site is highly specific for guanidinium ions. A single methyl or ethyl substituent at the guanidinium ion significantly reduces the affinity, as shown by titration of the absorption maximum. The 1,1-dimethyl derivative does not bind, and no effects were observed for arginine, ammonium or tetramethyl ammonium ions or urea. This strong steric effect indicates binding of the cofactor inside the protein and supports the idea that guanidinium replaces the arginine side group at residue 108, as suggested in Figure 5. The guanidinium specificity is more pronounced than the specificity found for the reconstitution effect of BR-D96N induced by azide and other anions (Tittor *et al.*, 1989). Azide was shown to act by a specific effect on an internal hydrogen bonding network rather than by replacing the proton binding site D96 (le Coutre *et al.*, 1995).

The correlation between reconstituted translocation activity and anion-induced absorption shift demonstrates that binding of the substrate to the protein is the crucial reaction which is prevented by the replacement of R with Q, and which can be reconstituted by addition of guanidinium. For HR wild type, the site involved in substrate binding was termed site II (Schobert *et al.*, 1986). Its assignment to R108 has already been discussed as a hypothesis (Lanyi, 1990; Oesterhelt *et al.*, 1992), but no experimental evidence has been available so far. The results presented here prove the participation of R108 at this binding site. According to the location of R108 between the Schiff base and the extracellular surface in the proposed structural model of HR (Havelka *et al.*, 1995), the R108 binding site is responsible for the uptake of substrate anions from the extracellular side. Other residues also may contribute as in the 'active site' model proposed by Ames *et al.* (1992), including the protonated Schiff base as positive, and D238 and the bound anion as negative charges. The investigation of the R108 mutants, however, demonstrates that in such a complex the arginine is of main importance.

On analysing the effects of various anions an increased selectivity for bromide over chloride is found in the mutants (Table I) which is reflected by the binding constants observed for halide-induced absorption shifts of HR-R108Q–guanidinium (Table III). The selectivity change may be explained by effects of anion solvation. Upon binding of halide ions to HR wild type at least part of the hydration shell of the anion is replaced by protein residues which 'solvate' the bound anion by formation of a weakly hydrated ion pair, as has been demonstrated by FTIR experiments (Braiman *et al.*, 1994). Due to its lower hydration energy and lower hydration number, dehydration of bromide is facilitated compared to chloride. This could be the reason for the increased bromide selectivity of the mutants which have manipulated anion binding sites, while the binding site in HR wild type can be considered to be optimized for chloride binding.

The guanidino group of arginine is known for its good capability of hydrogen bonding and ion pairing in a number of proteins (Hannon and Anslyn, 1993). Interestingly, arginine residues important for anion permeation have also been found in mammalian anion channels, in particular for the cystic fibrosis transmembrane conductance regulator (Sheppard *et al.*, 1993) and the inhibitory glycine receptor (Shiang *et al.*, 1993). Therefore, the results on the crucial importance of an arginine in halorhodopsin and on its binding mechanism by dehydrating the anion may reflect more general principles of anion–protein interactions.

The correlation between the appearance of the HR520 photoproduct and transport activity found for HR wild type also holds for the mutant HR-R108Q, since guanidinium induces transport and HR520 formation with the same affinities (Table III and Figure 4A and B). This finding confirms the idea of HR520 being a key intermediate in the anion transport mechanism (Tittor *et al.*, 1987; Zimanyi and Lanyi, 1989; Oesterhelt *et al.*, 1992). The HR520 intermediate was investigated in more detail by static FTIR difference spectroscopy. The difference spectra for HR wild type (Figure 4A) and for the mutant HR-R108Q with guanidinium (Figure 4B) agree very well, while the spectrum of the mutant without guanidinium (Figure 4C) clearly deviates, thus demonstrating the significance of

the similarity between the spectra 4A and 4B. In particular, the pattern of chromophore bands in the spectra of wild type and reconstituted mutant is nearly identical. Therefore photoproducts and initial states are very similar; guanidinium reactivates anion transport by reconstituting the wild type mechanism for HR-R108Q. This supports the interpretation that guanidinium binds at the residue Q108 overtaking the role of the guanidino side group, and that binding of guanidinium plus anion to HR-R108Q is equivalent to the binding of an anion to HR wild type. The picture of replacing R108 by Q108 and guanidinium, as shown in Figure 5, suggests that guanidinium remains at its place during the photochemical reaction cycle of the protein associated with anion transport. However, at present it cannot be excluded that the guanidinium ion is shuttling between Q108 and the extracellular surface, thereby transporting the anion to the binding site inside the protein.

A specific difference between wild type and mutant spectra is found around 1690 cm^{-1}, where the band pattern seen in the wild type spectrum is much less intense in the mutant spectrum. For the signal still present in the mutant spectrum there are two possible explanations: it could be caused (i) by the externally added guanidinium undergoing the same reaction or environmental changes in the mutant as the guanidino group of R108 does in the wild type, or (ii) by other groups besides R108 which contribute to the band structure in the wild type and remain unchanged in the mutant. Experiments with isotopically labelled guanidinium should allow a more precise band assignment and identification of further signals from the 'active site'. Braiman *et al.* (1994) attributed the 1695/1688 cm^{-1} band to a C–N vibration of an arginine due to its position and its anion sensitivity. It was interpreted to be caused by disruption of the ion-pairing interaction between a halide ion and arginine during the light-induced HR578 → HR520 reaction. The arginine which binds the anion in the HR578 state is now identified as R108. From this starting location the anion is transmitted, after photoexcitation, to a location where it is presumably interacting mainly with the protonated Schiff base (Oesterhelt *et al.*, 1986; Ames *et al.*, 1992; Walter and Braiman, 1994), leading to formation of the HR520 intermediate. Finally the anion is passed on to the cytoplasm. Until now, no further interactions with protein residues within this anion release pathway have been detected. However, involvement of an anion binding site termed site I and participation of another arginine residue, R200, have been proposed (Lanyi *et al.*, 1990; Oesterhelt *et al.*, 1992). Figure 5 summarizes the results on the uptake binding site and shows the proposed steps of anion translocation. Further studies will be addressed to the elucidation of the complete anion pathway through the protein to achieve a more detailed understanding of the anion translocation mechanism of halorhodopsin.

Materials and methods

Bacterial strains

Specific mutants of HR were overexpressed in the BR and HR negative strain HN5 of *Halobacterium salinarium* (K.Rumpel and D.Oesterhelt, in preparation) using the bop-hop-cartridge described by Heymann *et al.* (1993). Details of mutagenesis and expression will be published elsewhere. The expression level of mutant HR was determined from the chromophore absorption in the total membrane fraction, using the HR wild type extinction coefficient of $\varepsilon = 50\,000$ l/(mol×cm) (Steiner and Oesterhelt, 1983). Mutant HR was expressed similar to wild type HR at a level of ~50–60% compared to BR in the strain S9 (Heymann *et al.*, 1993).

Anion translocation experiments

Anion translocation experiments were performed according to Oesterhelt (1982). The cells were suspended in basal salt solutions without sodium citrate, containing either chloride salts as in the standard protocol (4.28 M NaCl, 30 mM KCl and 80 mM $MgSO_4$) or with NaCl and KCl substituted by the corresponding bromide, nitrate or sulfate salts. Eight ml of the cell suspension in a 10-ml cuvette thermostated at 25°C were adjusted to pH 6.90–6.95. Illumination with yellow light (OG 515 filter, Schott) yielded an irradiance of ~100 mW/cm^2 at the place of the sample. The initial rates of pH change were measured and calibrated by adding known amounts of HCl. Quantitation of HR in the sample was performed as described above. A 1 M stock solution of guanidinium sulfate in basal salt was used for titration of HR-R108Q activity.

UV–visible spectroscopy

HR containing membrane fractions were prepared as described by Oesterhelt and Stoeckenius (1974) for the purple membrane (PM), with only minor changes (Heymann *et al.*, 1993). For spectroscopic experiments, a membrane fraction with an apparent buyoant density of ~1.15–1.16 g/cm^3 was prepared which exhibits an absorption ratio between 280 nm and the chromophore peak of ~2.0. The membranes were suspended in the basal salt solutions also used for the translocation experiments, but buffered to pH 7.0 with 20 mM Tris–H_2SO_4. UV–visible spectra were recorded with an Aminco DW2A spectrophotometer connected to a personal computer. Flash-induced difference spectra were recorded with a home-made flash photolysis apparatus with a time resolution of 130 μs (Uhl *et al.*, 1985).

FTIR spectroscopy

For the FTIR measurements, 100 μl of a membrane suspension adjusted to pH 6.5, containing 150–200 μg HR, 100 mM NaBr and, in the case of R108Q, 5 mM guanidinium sulfate, were dried, rehydrated and placed in the FTIR spectrometer (Bruker IFS 88, equipped with MCT detector) as described by Souvignier and Gerwert (1992). The sample was light-adapted for 10 min [80 W halogen lamp, OG 530 filter (Schott)] and cooled to 253 K. Static difference spectra with 2 cm^{-1} resolution were achieved by recording first a reference spectrum of 128 single scans, then a spectrum of the photostationary state, while the sample was illuminated as above, followed again by a dark spectrum. This cycle was repeated 30–50 times for averaging. The difference of the second dark spectrum and the reference spectrum served as a control for complete relaxation of the sample, for baseline stability and for the signal-to-noise ratio.

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