Specific arginine and threonine residues control anion binding and transport in the light-driven chloride pump halorhodopsin

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The light-driven chloride pump halorhodopsin (HR), a halobacterial retinal protein, was studied by comparing wild type with specific mutants. Changes of conserved arginine and threonine residues in the transmembrane regions could be classified in two categories: in the extracellular half of the molecule, mutations influence anion uptake and binding. R108 mutations abolish all anion effects previously attributed to two distinct binding sites and change the characteristic photochemistry. Neutral residues at position 108 completely inactivate the pump. T111 increases the affinity of this anion binding site without being essentially important. In the photochemical cycles of the mutants T111V and Q105E, a red-shifted absorbing intermediate is enriched indicating retarded anion uptake. On the cytoplasmic side, mutations do not change anion binding properties of the unphotolyzed protein, but slow down anion release thereby reducing the chloride transport activity and the photocycling rate. The lowest activity is found for T203V, while R200 mutations have weaker effects. Thus, in the symmetrically arranged pairs R108/T111 and T203/R200, threonine and arginine play different roles, reflecting high affinity anion uptake by the former and effective anion release catalyzed by the latter residues. A model for the anion transport mechanism in HR is suggested comprising the specific functions of channel-lining residues.

Keywords: channel-lining residues/chloride transport/halorhodopsin/ion pump/mutagenesis

Introduction

The retinal protein halorhodopsin (HR) is a light-driven chloride pump in the plasma membrane of the halophilic archaeon *Halobacterium salinarum*, which populates habitats with NaCl concentrations of more than 4 M. During cell growth HR helps to maintain the osmotic balance between cytoplasm and extracellular medium by transporting chloride ions into the cell against the membrane potential. HR shows structural and functional similarities to its well-studied proton pumping counterpart bacteriorhodopsin (BR). BR has become a model system for studying membrane protein structure, transmembrane proton transport, bioenergetics, protein-photophysics and -photochemistry (reviewed by Mathies *et al.*, 1991; Oesterhelt *et al.*, 1992; Lanyi, 1993). The analysis of HR and its transport mechanism, on the other hand, can elucidate specific characteristics of anion translocation,

and comparison with BR may reveal general principles of ion specificity and transport. A general concept for ion translocation by halobacterial retinal proteins (Haupts *et al.*, 1997) rationalizes results on BR and its mutants (Tittor *et al.*, 1994), HR (Bamberg *et al.*, 1993) and sensory rhodopsin I, which is also capable of light-driven proton transport (Bogomolni *et al.*, 1994; Haupts *et al.*, 1996). However, compared with the detailed knowledge about the proton transport mechanism in BR, in particular as obtained by characterizing specific mutants, much less information is available on the molecular properties of HR (reviewed by Lanyi, 1990; Oesterhelt, 1995).

Electron cryo-microscopy proved nearly identical structures for HR and BR at a resolution of 7 Å (Havelka *et al.*, 1995). Seven transmembrane helices form a central pore, which is divided into an extracellularly (EC) and a cytoplasmically (CP) oriented half-channel by the chromophore all-*trans* retinal which is covalently bound via a protonated Schiff base to a lysine residue in helix G. The structural conservation between the two proteins is particularly high in the retinal binding pocket formed by 21 residues in the BR structural model (Henderson *et al.*, 1990), 14 of which are conserved in HR. In both proteins, light induces an all-*trans* to 13-*cis* photoisomerization of the chromophore, followed by a series of thermal relaxation steps leading back to the initial state in the millisecond time regime; this reaction cycle is called the photocycle. Functional similarities between the two pumps are reflected by the facts that (i) BR can be converted into a chloride pump by the mutation D85T (Sasaki *et al.*, 1995), (ii) HR as well as specific BR mutants can act as two-photon-driven inward proton pumps (Bamberg *et al.*, 1993; Tittor *et al.*, 1994), and (iii) HR from *Natronobacterium pharaonis* can act as an one-photon-driven outward proton pump in the presence of azide (Váró *et al.*, 1996).

The differing functions of HR and BR, on the other hand, are reflected by characteristic molecular properties. In HR, the Schiff base remains protonated during the photochemical cycle, while it is transiently deprotonated in BR. HR exhibits characteristic anion dependent effects in the initial state as well as in its photochemical cycle (reviewed by Lanyi, 1990; Oesterhelt, 1995), some of which are similarly found for the anion-transporting BR mutant D85T (Brown *et al.*, 1996), but not for wild type BR. These effects have been explained by assuming two anion binding sites in HR (Schobert *et al.*, 1986). In analogy to the essential proton binding sites in the two half-channels of BR, D85 and D96 (Braiman *et al.*, 1988; Butt *et al.*, 1989), the anion binding sites in HR have been assigned hypothetically to two arginine residues, R108 in the EC and R200 in the CP half-channel (Lanyi *et al.*, 1990; Oesterhelt *et al.*, 1992). These arginines are the only positively charged groups in the transmembrane region of HR beside the Schiff base. After establishing

homologous HR overexpression (Heymann *et al.*, 1993), the function of individual residues could be clarified by analyzing specific HR mutants. Experiments on the mutants R108K and R108Q proved this arginine to be indeed essential for anion binding (Rüdiger *et al.*, 1995). On both the EC and CP side there are threonines about one helix turn closer to the Schiff base, T111 next to R108 and T203 next to R200. These four residues forming two arginine/threonine pairs in symmetric positions with respect to the Schiff base are conserved among all known halorhodopsins (with R200 being replaced by K in some HR variants) (Mukohata, 1994; Oesterhelt, 1995). Here we investigate the anion translocation pathway in HR by mutational analysis focusing on these conserved arginine/threonine pairs.

Results

Overexpression of halorhodopsin mutants

Specifically mutated halo-opsin (*hop*) genes were constructed and homologously overexpressed according to Heymann *et al.* (1993) with some modifications. Compared with the *Escherichia coli*-*H.salinarum* shuttle vector pJH-F formerly used for *hop* expression (Heymann *et al.*, 1993), the new expression vectors pMR3 and pMR4 are smaller, and they are suicide vectors without a halobacterial replication origin, therefore integrating into the genome of transformed *H.salinarum* cells. Maintenance of the vector as a plasmid is not required for overexpression since integration also occurs for bacterio-opsin (*bop*) or *hop* expression plasmids containing halobacterial replication origins (Ferrando *et al.*, 1993; Heymann *et al.*, 1993). Indeed, no difference in the HR expression levels was found using either pJH-F, pMR3 or pMR4. The transformation efficiency, however, was clearly higher for the small vectors pMR3 or pMR4. About 40–50% expression of HR wild type corresponding to 120 000–150 000 copies per cell could be achieved compared with the BR content in the strain S9. The same was found for most of the HR mutants, although R108I and R108H expression was lower by a factor of ~5.

Anion transport activity

The effect of specific amino acid replacements on HR activity was examined by an anion transport assay (Oesterhelt, 1982). Illumination of HR expressing cells suspended in basal salt solution supplemented with the protonophore CCCP causes passive proton influx in response to electrogenic anion transport into the cells. This can be measured in the suspension as light induced alkalization. The signal was extrapolated to saturating light intensity. Calibration of the pH change by adding known amounts of HCl and spectroscopic determination of the HR content in an aliquot of the suspension resulted in values for specific anion transport activity.

Figure 1A shows the anion transport activities of mutants in the EC half of the molecule. Replacement of R108 by a neutral residue (Q or I) completely inactivates the pump. The mutants R108K and R108H exhibit only 2 or 8% of the wild type activity. Since the pK_a value of the histidine side group is 6.5 in solution, the R108H activity was determined at pH values between 7.5 and 5.5. The small activity increase at lower pH, however, was

Fig. 1. Specific anion transport activity of wild type HR and specific mutants for chloride and nitrate ions. (A) Mutants in the extracellular, (B) in the cytoplasmic half of the molecule.

also observed for wild type HR (not shown), preventing a conclusion about the influence of charge at residue 108. The mutations Q105E and T111V, which are located close to R108 about one helix turn towards the EC surface or towards the Schiff base, respectively, result in a 25% reduction of chloride transport activity. The anion selectivity is slightly reduced for T111V, but increased for R108K: the ratio of chloride to nitrate transport activity is ~2:1 in wild type, 3:2 in T111V and 3:1 in R108K. The inactive mutant R108Q can be partially reconstituted by adding guanidinium ions (Rüdiger *et al.*, 1995). The activity of chloride transport recovers to 25% of the wild type value, that of nitrate transport to 10% (Figure 1A) and that of bromide transport to 40% (Rüdiger *et al.*, 1995). This effect is specific for R108Q, since no guanidinium effect was found on the activity of wild type HR or any other mutants reported here (including R200A and T203V). The strong effect of R108 mutations and the specific reconstitution by guanidinium demonstrate the essential role of the guanidino group at position 108.

Mutations of R200 in the CP half of the molecule have weaker effects than R108 mutations (Figure 1B). Only R200A causes an activity decrease to ~35% (chloride) or 45% (nitrate) compared with wild type. An R to A mutation, however, changes chemical properties drastically, possibly also causing structural changes. Therefore this mutant does not allow conclusions on a specific

function of R200. The more conservative mutation R to Q, also changing the positively charged to a neutral side chain, inhibits transport by preventing anion binding at the EC side (R108Q), but has no influence at the CP side (R200Q), indicating that R200 plays a less specific role than R108. Surprisingly, R200H shows higher activity than wild type. Of all CP mutations investigated, T203V causes the maximal reduction of activity. Chloride or bromide transport are strongly inhibited in T203V, while nitrate transport is almost unaffected. This anion dependency suggests a specific role of T203 for anion translocation in the CP channel.

Anion dependent chromophore properties

Membrane fractions suspended in 4.4 M substrate anion or 1.4 M sulfate solutions were used for UV-visible spectroscopy and flash photolysis. Chloride, bromide and nitrate are bound and transported by wild type HR, while sulfate is neither specifically bound nor transported thereby serving as a control for effects of ionic strength (Steiner *et al.*, 1984). In wild type HR, the bound anion determines the chromophore absorption maximum, in particular nitrate induces a blue shift compared with the halide bound form (Figure 2A; Steiner *et al.*, 1984; Schobert *et al.*, 1986). In contrast, for R108 mutants the absorption maximum is anion independent within the experimental accuracy (± 3 nm) (Figure 2A). Hence, mutation of R108 affects a site essential for anion binding as well as for transport. Furthermore, the side chain at residue 108 influences the chromophore, since R108Q absorbs at 578 nm, the other mutants at ~ 565 nm. As already observed for transport activity, guanidinium has no effect on wild type HR or any other mutants. For R108Q alone, it restores anion sensitivity of the chromophore, which is detected as a hypsochromic shift in the presence of substrate anions (Figure 2B). The glutamine side chain may therefore be required for guanidinium binding and reconstitution of the binding site.

Beside inducing colour changes, anion binding influences the Schiff base pK_a (Steiner *et al.*, 1984; Schobert *et al.*, 1986) and the extinction coefficient of the chromophore absorption in wild type HR (Schobert *et al.*, 1986; Váró *et al.*, 1995b). The latter effect is absent for all R108 mutants, their absorption spectra showing identical ratios between the absorptions at 280 nm and at the chromophore peak for all anions tested. The Schiff base pK_a in wild type HR solubilized in octylglucoside is 7.4 in sulfate solution and is shifted to ~ 9 by the addition of various anions (Schobert *et al.*, 1986). For HR in the membrane bound state, pK_a values of 8.9 in 1.4 M Na_2SO_4 and 9.5 in 4.4 M NaCl solution were found, confirming a chloride-induced upshift. In the mutant R108Q, the pK_a of the Schiff base is anion independent with a value of 12 in both sulfate and chloride solution; only the simultaneous presence of guanidinium and chloride ions induces a shift to 11.3 (not shown). Therefore the chloride effect on R108Q/guanidinium (lowering of pK_a) differs from that observed for wild type HR [pK_a increase (Schobert *et al.*, 1986)], but is comparable to anion effects on the Schiff base pK_a in BR mutants (Tittor *et al.*, 1994). In summary, mutation of the single residue R108 blocked all anion dependent effects which have been previously attributed to anion binding at two distinct sites (Schobert *et al.*,

Fig. 2. Chromophore absorption maxima of wild type HR and specific mutants in the (A) extracellular and (C) cytoplasmic half of the molecule, determined in membrane suspensions in the presence of different anions, pH 7.0. (B) Effect of guanidinium on the chromophore, shown as difference of the chromophore absorption maximum in the presence of 0.2 M guanidinium sulfate and without guanidinium.

1986; Lanyi *et al.*, 1990; Oesterhelt *et al.*, 1992). Determination of the apparent chloride binding constants to wild type HR in the presence of various nitrate concentrations (Figure 3A) revealed a linear dependence. This can be interpreted by assuming a single binding site to which chloride and nitrate are binding competitively with the intrinsic binding constants 0.09 M and 0.6 M, respectively (Figure 3B).

The mutants with slightly reduced activity, Q105E and T111V, show anion sensitive absorption shifts like wild type HR; only the absorption maxima are altered. For both mutants, chloride induces a blue shift of the absorption maximum compared with that in sulfate, thereby allowing determination of the chloride affinity by titration. The Q105E value was identical to wild type (0.09 M), but a lower affinity (1.0 M) was found for T111V (not shown). Hence the Q105E mutation affects the chromophore absorption, but not the anion binding constant. T111, on the other hand, fulfills an accessory role for anion binding by increasing the affinity.

For all CP mutants investigated, the chromophore

Fig. 3. Chloride and nitrate binding to wild type HR. (A) Titration of the chromophore absorption maximum with chloride in the presence of various nitrate concentrations; the maximal absorbance shift was set to 1.0. (B) Apparent chloride binding constants versus nitrate concentration. Linear fit assuming competitive binding of the two anions to one binding site.

absorption maximum is shifted by anions as for wild type HR (Figure 2C). This is in agreement with R108 being the binding site responsible for all anion dependent effects. Therefore the putative assignment of 'binding site I' to R200 (Lanyi *et al.*, 1990; Oesterhelt *et al.*, 1992) is disproved by these results on specific mutants.

Two properties of HR can cause heterogeneity thereby complicating photochemical studies: first, two chromophore configurations, all-*trans* and 13-*cis*, are found in HR also in the light-adapted state (Lanyi, 1986), and second, at low substrate concentration the anion-bound as well as the anion-free form are present (Váró *et al.*, 1995b). To test for effects of isomer distribution, retinal was extracted from the same samples as used in spectroscopic experiments. In all samples studied (wild type HR, R108Q $\pm$ guanidinium, Q105E and T111V in 4.4 M NaCl or 1.4 M Na₂SO₄ solution), an all-*trans* to 13-*cis* ratio of 2:1 ($\pm$ 5%) was found in the light-adapted state. Therefore spectroscopic differences between these samples are caused by the specific mutations and by interactions of HR with various anions rather than by different isomer ratios. While the photoproducts of the 13-*cis* and the anion-free form have to be separated for a complete description of HR photoreactions (Váró *et al.*, 1995b), here we focussed on clear spectroscopic differences between

Fig. 4. Series of flash induced difference spectra for R108 mutants in chloride (A–D), R108Q in sulfate (E), wild type HR in sulfate (F), R108Q in chloride with 0.2 M guanidinium (G), and wild type HR, Q105E and T111V in chloride solution (H–K). The first spectrum showing the largest absorption change is 0.26 ms after the laser flash, the following spectra showing relaxation to the baseline are taken at 0.52, 0.78, 1.04, 1.43, 2.08, 2.84, 4.34, 5.84, 8.34, 11.34, 16.34, 23, 33, 47, 67, 92, 132, 197, 267 and 327 ms.

samples with identical isomer ratios, and we used samples equilibrated with either NaCl at physiological concentration (4.4 M) or Na₂SO₄ of the same ionic strength (1.4 M). Under these conditions, effects of mixtures between chloride-bound and chloride-free state can be avoided (M. Rüdiger and D. Oesterhelt, in preparation).

Transient absorbance changes

Figure 4 shows series of flash-induced difference spectra. For R108 mutants in the presence of 4.4 M chloride, only a red-shifted intermediate is detected as a transient positive absorbance with maximum around 650 nm, similar to wild type HR in sulfate (Figure 4A–D and F). For all R108 mutants, no difference is found between the pattern in chloride and sulfate solution as typically seen for R108Q

Fig. 5. Flash induced absorption changes at 660 nm for wild type HR and specific mutants in the presence of 1.4 M sulfate or 4.4 M chloride. The data were normalized to either the maximal absorption change at 660 nm (R108Q, Q105E, wild type in sulfate, T203V in sulfate) or to the same negative amplitude at 580 nm (wild type in chloride, T203V in chloride).

(Figure 4C and E). Adding guanidinium to R108Q in the presence of chloride restores the formation of a blue-shifted intermediate, similar to the intermediate called HR520 found for wild type HR in chloride (Oesterhelt *et al.*, 1985) (Figure 4G and H). Characterization of the blue-shifted intermediates by FTIR difference spectroscopy proved identical photoreactions in R108Q/guanidinium and wild type (Rüdiger *et al.*, 1995). Therefore the HR520 formation is correlated with transport activity as is the anion sensitivity of the chromophore, and it represents a reaction characteristic for anion transport in HR. The small transient positive absorption at 660 nm in the R108Q/guanidinium spectra may be attributed to partly non-reconstituted protein, since the relaxation kinetics at 660 nm are similar with and without guanidinium, while the relaxation of HR520 is clearly slower (data not shown). Q105E and T111V form a HR520 intermediate in the presence of chloride as expected due to their transport activity, but a red-shifted positive absorption is observed in addition (Figure 4I and K).

Inspection of the time course of transient absorbance changes at 660 nm in sulfate (1.4 M) and chloride (4.4 M) reveals three characteristic patterns (Figure 5). (i) Wild type HR and T203V show a fast decaying red-shifted intermediate in sulfate, while in chloride HR520 is formed, and only a negative signal can be detected at 660 nm due to bleaching and recovery of the initial state. For T203V in chloride the relaxation is much slower than for wild type (see below). (ii) For the R108 mutants with the defective binding site, chloride does not change the 660 nm transient at all, as shown for R108Q. The red-shifted intermediate decays more slowly than found for wild type in sulfate. (iii) For Q105E (and T111V, not shown), a long-lived red-shifted intermediate is observed in chloride, which decays in parallel to HR520, much more slowly than the signal of the chloride-free sample in sulfate. Therefore it is more likely to be an intermediate of the transport cycle than a product of a different initial state. According to their positions at the EC side, these two mutations may possibly influence anion uptake, and the

Fig. 6. (A) Flash induced absorption changes at 490 and 580 nm for the mutants in the cytoplasmic half of the molecule, all in 4.4 M chloride; the data were normalized to the same negative amplitude at 580 nm (averaged for the time points 0.26–0.78 ms after laser flash). Solid lines: multiexponential fits [one rate for the rise at 490, two rates (k_1 and k_2) for the decay at 490 and recovery at 580 nm]. (B) Correlation of decay rates of the HR520 intermediate with chloride transport activity.

occurrence of this red-shifted intermediate may be explained by retarded anion uptake to the EC binding site (see Discussion).

In the presence of chloride, all CP mutants form HR520 upon light absorption as wild type HR does, and no red-shifted intermediate is found. However, the decay rates of HR520 (observed at 490 nm), corresponding to the rates of recovery of the initial state (observed at 580 nm), differ from the wild type values and correlate with the transport activity: R200H exhibits higher activity and a faster HR520 decay than wild type, T203V the smallest activity and the slowest decay rate (Figure 6A). The decay of the 490 nm and the recovery of the 580 nm signal can be fitted simultaneously with a sum of two exponential functions. In Figure 6B, the fitted decay rates are plotted against transport activities of the CP mutants, demonstrating a nearly quantitative correlation between HR520 decay and anion transport. Due to the location of T203 in the CP half of the molecule, it is concluded that during the HR520 relaxation the chloride is passed through the CP channel catalyzed by the T203 side chain.

Discussion

To study the functional role of individual amino acid residues in the light-driven chloride pump HR, specific mutants were investigated in comparison with wild type with respect to anion transport activity and spectroscopic properties. For mutagenesis, conserved arginine and threonine residues in the extracellular (EC) and the cytoplasmic (CP) half of the molecule were selected. In the following, the specific functions of these proposed channel-lining residues for anion uptake from the extracellular side and anion release to the cytoplasm are discussed, and the results are summarized in a model for the anion transport mechanism in HR.

Anion uptake from the extracellular side

Of all mutations investigated here, changes of R108 have the most striking effects. They nearly annihilate ion transport and change spectroscopic properties, demonstrating the essential role of R108. Since this arginine is conserved in all known halobacterial retinal proteins (Mukohata, 1994; Oesterhelt, 1995), it may also have a general function, e.g. for protein folding or stability. This may explain the low expression of R108H and R108I, the mutants with large chemical changes of the 108 side group. The R108Q mutant, however, is overexpressed to the same level as wild type HR, but is inactive, showing that R108 fulfills a specific role for the function of HR as an anion pump. Chloride and nitrate transport activity, anion sensitivity of the chromophore and occurrence of the HR520 intermediate upon light absorption are cancelled by R108 mutations (Figures 1A, 2A and 4A–D). The reconstitution effect of guanidinium on R108Q (Figures 1A, 2B and 4G) shows the correlation between these properties, which are determined by occupation of the EC binding site R108.

Beside absorption shifts, other anion induced effects such as change of the Schiff base pK_a , formation of the HR520 intermediate in the photocycle and change of the extinction coefficient at the chromophore peak, are absent in R108 mutants. These effects were attributed to two anion binding sites in HR (Schobert *et al.*, 1986), which were hypothetically assigned to R108 and R200 (Lanyi *et al.*, 1990; Oesterhelt *et al.*, 1992). Anion binding to 'site I' (assigned to R200) should induce a blue-shifted absorption and an increase of the Schiff base pK_a . R200 mutants, however, show wild type like properties (Figure 2C), while R108 mutations cancel all these anion effects. By titration experiments on wild type HR, mutual competition between chloride and nitrate can be shown (Figure 3), which is most easily described as competition of the two anions for one single binding site. Hence all anion effects listed above are determined by anion binding to R108. Anion-specific differences (e.g. different absorption maxima) are then caused by the detailed interactions between anion and the surrounding protein residues.

The hydroxyl group of threonine 111 also participates in anion binding, since the mutation T111V reduces the chloride affinity and lowers the anion specificity of the pump. It is, however, not as essential as R108, because the mutant is still active. Threonine at this position, which is homologous to the proton acceptor D85 in BR, is conserved in all known HR sequences (Oesterhelt, 1995).

The BR mutant D85T, but not D85N, exhibits chloride transport activity, and therefore this threonine was suggested to be important for anion transport (Sasaki *et al.*, 1995). However, anions do not bind in exactly the same way to HR as to modified BRs, as demonstrated by the role of the conserved arginine, 108 in HR, 82 in BR. While R108 changes in HR destroy the binding site, anion binding to the 'blue membrane' of BR [formed at pH below 3 due to protonation of D85 (Metz *et al.*, 1992)] is found also for R82 mutants (Balashov *et al.*, 1993), and no contributions of R82 could be identified in FTIR difference spectra of the chloride binding reaction to the blue membrane (le Coutre *et al.*, 1995).

Taken together, R108 is essential for anion uptake from the EC side, while T111 is only an additional participant of the binding site. Anion binding to R108 causes all spectroscopic effects formerly attributed to two distinct binding sites.

Anion release to the cytoplasmic side

Changes of R200 at the CP side have less specific effects than R108 mutations. In particular, the glutamine mutation inhibits transport at the EC side (R108Q), but R200Q shows wild type activity. Direct interactions of R200 with the translocated substrate are therefore not assumed. The largest effect at the CP side has the mutation T203V lowering specifically chloride and bromide transport activity, while nitrate transport is nearly unaffected. This strong discrimination between different anions indicates specific interactions of the T203 side group with halides. Furthermore it suggests that selectivity differences between HRs from different halobacterial species, which have been attributed to sequence variations in the interhelical loops (Otomo *et al.*, 1992), may also be caused by residues in the CP channel. Interestingly, a selection procedure for low activity HR mutants also revealed a T203 change (K.Rumpel and D.Oesterhelt, in preparation). R200 and T203 are conserved in BR as R175 and T178. In the BR structural model (Grigorieff *et al.*, 1996) only T178, but not R175 is oriented towards the CP channel, suggesting participation of this threonine also in chloride transport by BR mutants.

The anion sensitivity of the chromophore is not changed by any of the CP mutants (Figure 2C), disproving former assignments of 'binding site I' to R200, but in accordance with the EC binding site being responsible for all anion effects in the initial state. If anions bind identically to wild type and CP mutants in the unphotolyzed state, changes of transient anion–protein interactions during the transport cycle may be expected to cause the activity differences. Indeed, this is observed in the flash induced difference spectra (Figure 6). For wild type and the various mutants, photocycling rate and transport rate are correlated: the slower the HR520 relaxation and concomitantly the recovery of the initial state, the smaller the transport activity (Figure 6B). In the HR520 state the anion is assumed to be located closely to the Schiff base, as in FTIR experiments anion–Schiff base interactions were detected (Walter and Braiman, 1994). In agreement with this assignment, T203 mutation blocks anion conduction through the CP channel during HR520 decay [step (4) in Figure 7, see below] demonstrating that in HR520 the anion has not yet passed by the 203 residue. In wild type,

Fig. 7. Model for anion binding and translocation by HR. The approximate distances between residues, extracellular and cytoplasmic surface are adapted from the positions of the homologous residues in the BR structure (Grigorieff *et al.*, 1996). For explanation see text.

T203 catalyses anion permeation from the Schiff base to the cytoplasm by specific interactions particularly with halides. These interactions could be governed by anion hydration, structure and polarizability, which may explain the different effects of T203 mutation on chloride and nitrate transport. R200 mutations may cause less specific structural changes in the CP channel which also affect anion conduction and thereby HR520 relaxation. Altogether, the observations on CP mutants enable the correlation of a step of the transport cycle, anion release to the cytoplasm, with a step of the spectroscopically monitored photocycle, HR520 relaxation.

The starting point for the choice of residues for mutagenesis has been the observation of conserved arginine/threonine pairs located in symmetric positions with respect to the Schiff base (Oesterhelt, 1995): R108/T111 in the EC and T203/R200 in the CP half of the molecule. On the other hand, the function of residues interacting with anions is different on the two sides: while high affinity binding is necessary at the uptake side (EC), in the CP channel effective catalysis of anion permeation and only low affinity binding allowing release at an anion concentration above 4 M, is required. This functional asymmetry may be reflected by the different roles of R and T residues in the EC or CP channel, respectively. While the guanidino group of R108 is essential for anion uptake and the hydroxyl group of T111 is only an additional participant, for anion release T203 is obviously more important than R200. Summarizing the data on HR mutants, the effects of mutations in the EC channel may be described as 'thermodynamic', since the binding equilibrium is influenced; destruction of the binding site (R108 changes) even results in an inactive molecule. Mutations on the CP side, on the other hand, have a 'kinetic effect', since they reduce the effectivity of the pump by slowing down anion permeation through the CP channel, but do not principally affect its function.

Anion transport mechanism of HR

In a general description of the various ion transport modes found for halobacterial retinal proteins (Haupts *et al.*, 1997), the transport mechanism is represented by a sequence of six principal reaction steps: retinal isomerization to 13-*cis* or all-*trans* (either photochemical, designated as I*, or thermal, I), ion translocation to or from the Schiff base (T) and switch of the Schiff base accessibility towards the EC or CP side (S). Within this framework, the anion transport mechanism of wild type HR, taking into consideration the results on specific mutants, can be described as follows (Figure 7): in the initial state, the substrate anion occupies a binding site constituted by R108 and T111, also interacting with the protonated Schiff base (Walter and Braiman, 1994), and the Schiff base is accessible for ions from the EC side. (1) Light-induced isomerization (I*) is followed by (2) translocation of this anion towards the Schiff base (T), where it is located in the HR520 intermediate state (Walter and Braiman, 1994). As a prerequisite for anion release to the CP side, now (3) a switch of Schiff base accessibility (S) has to occur, which was assigned to a spectroscopically silent HR520I to HR520II step corresponding to the MI to MII transition in BR (Oesterhelt *et al.*, 1986; Oesterhelt, 1995). The molecular basis of this switch event has not yet been identified. It could consist of a small change such as a single-bond rotation in the retinal (Oesterhelt *et al.*, 1986) or involve protein conformational changes (Ames *et al.*, 1992). (4) Chloride transfer from the Schiff base to the cytoplasm (T) is mediated by interaction of the ion with T203. The lack of this hydroxyl group (T203V mutant) increases the barrier for chloride translocation, therefore the anion sticks to the Schiff base and the lifetime of HR520 is prolonged. To reach the initial state again, (5) retinal now has to reisomerize to the all-*trans* configuration (I), (6) the accessibility has to be reverted to the EC side (S), and the EC binding site has to be reoccupied (see below). Interestingly, exactly the same reaction sequence (I*/T/S/T/I/S) also describes proton transport by wild type BR (Haupts *et al.*, 1997), indicating that the principle of ion translocation mechanism is identical in both proteins. The mechanistic difference between BR and HR then explaining different ion specificity and transport vectoriality, is the substitution of proton transfer away from the Schiff base (in BR) by chloride transfer to the Schiff base (in HR) and vice versa. In agreement with this fact, in the BR initial state the Schiff base is 'proton-bound', meaning occupied with the substrate, and transiently deprotonated during the transport cycle, while it is chloride-free in the HR initial state and transiently chloride-bound.

The sequence of six reaction steps does not include reoccupation of the R108 binding site. The time at which the binding site equilibrates with the extracellular medium does not influence the general transport mechanism, which determines the vectoriality of the pump. The time point of anion reuptake, however, may be responsible for the observed absorbance changes. Assuming that the reoccupation step can be influenced by mutations as well as by experimental conditions (anion concentration or different HR preparations), disagreements between the HR photocycle models published so far (Oesterhelt, 1995) could be rationalized, in particular differences regarding

the occurrence of a red-shifted intermediate: if anion uptake to R108 is slower than anion release from the Schiff base to the cytoplasm, the transient absence of anions from the Schiff base vicinity would cause a red-shifted chromophore absorption. Such an intermediate state has been described as HR640 or HR-O (Oesterhelt *et al.*, 1985; Ames *et al.*, 1992). It was observed for solubilized HR (Lanyi and Vodyanoy, 1986; Tittor *et al.*, 1987), HR from *N.pharaonis* (Váró *et al.*, 1995a), the chloride-transporting BR mutant D85T (Brown *et al.*, 1996), and for *H.salinarum* HR mutants possibly retarding anion uptake (Q105E and T111V, Figure 4I and K); further members of this group may be H95A and H95R, which are located in the extracellular loop between helices B and C and also exhibit HR640 enrichment (Otomo, 1996). If, on the other hand, anion uptake is faster than release, no red-shifted intermediate is enriched, as found for wild type *H.salinarum* HR in the membrane-bound form (Váró *et al.*, 1995b). In either case, however, the transport mechanism can be described as outlined above.

In conclusion, our results on specific HR mutants clarify the role of individual amino acids in the anion translocation mechanism. Chloride is taken up from the EC side by binding to R108, and T111 increases the affinity of this binding site. Chloride release to the cytoplasm is catalyzed by the T203 side chain, whereas no specific role is found for R200, which was previously suggested as another anion binding site. Further studies on HR will be addressed to the coupling mechanism of photoisomerization and internal chloride transfer and to the structural details of the accessibility change to achieve a comprehensive picture of the molecular basis for anion transport in this membrane protein.

Materials and methods

Construction, overexpression and isolation of mutants

Site-directed mutagenesis of the *hop* gene was performed by the procedure of Kunkel *et al.* (1987). The *hop* gene was cloned into the plasmid pJH91 (Heymann *et al.*, 1993) which then carries a fusion gene of the *bop* promoter, the *bop* presequence and the mutated *hop* gene. A *Pst*I-*Cl*aI fragment of pJH91 containing this *bop*-*hop* cartridge was cloned into a pBluescript derivative carrying a 2.3 kb subfragment of the mutated gyrase B gene from *Haloferax sp.* which mediates novobiocin resistance to *Halobacterium* transformants (Holmes and Dyall-Smith, 1991; B. Novosad and J. Soppa, unpublished results). The resulting vector pMR3 (9.6 kb) was reduced in size by digestion with *Sma*I and religation giving the vector pMR4 (6.0 kb). This expression vector carries the *bop*-*hop* cartridge (to be isolated as *Sma*I-*Cl*aI or *Nde*I-*Cl*aI fragment) for expression in *H.salinarum*, selection markers for transformed *E.coli* (ampicillin resistance) and *H.salinarum* cells (novobiocin resistance), and the *E.coli* replication origin, but no halobacterial replication origin. Therefore it is a suicide plasmid with respect to *H.salinarum*. Cells of the BR and HR negative *H.salinarum* strain HN5 (K. Rumpel and D. Oesterhelt, in preparation) were transformed according to Cline *et al.* (1989) with minor modifications (Heymann *et al.*, 1993). ~20–50% of the transformants grown on novobiocin plates (0.5–1 µg/ml) showed blue or purple colour (except for the mutants R108H and R108I). Transformants were analyzed by PCR using genomic DNA as template and primers binding to the *bop* promoter and the *hop* C-terminal sequence (Heymann *et al.*, 1993). The mutations were verified by sequencing this PCR product (DNA sequencer 374A, ABI). Cell growth and isolation of HR containing membrane fractions were performed as described for purple membrane isolation (Oesterhelt and Stoekenius, 1974). The HR expression level was determined from the chromophore absorbance in total cell membranes, using the HR wild type extinction coefficient ($\epsilon = 50\,000\text{ M}^{-1}\text{cm}^{-1}$, Steiner and Oesterhelt, 1983). Mutant as well as wild type HR was expressed to ~40–50% compared with BR in the strain S9.

Anion translocation experiments

Anion transport activity was assayed according to Oesterhelt (1982). The cells were suspended in basal salt solutions without citrate, containing either chloride salts as in the standard protocol (4.28 M NaCl, 30 mM KCl and 80 mM MgSO₄) or with NaCl and KCl substituted by the corresponding nitrate salts. 8 ml of the cell suspension in a 10 ml cuvette thermostatted at 25°C were adjusted to pH 6.90–6.95. Yellow light from a 900 W Xenon lamp (OG515 filter, Schott) was attenuated by various neutral glass filters (Schott) yielding an irradiance of 0.01–0.4 W/cm² at the place of the sample. In control experiments, it was shown that the signal depends linearly on cell concentration in the range of OD₆₀₀ = 0.2–0.5, and that a concentration of 100 µM CCCP saturates the signal. Since light intensity was limiting under the experimental conditions, the initial rate of pH change was extrapolated to a maximal rate by varying intensity and linearizing the data according to Eadie-Hofstee. For calibration, known amounts of HCl were added. Quantitation of HR in the sample was performed as described above.

UV-visible spectroscopy

For spectroscopic experiments, membrane fractions with an apparent buoyant density of 1.15–1.16 g/cm³ were prepared which show an absorption ratio between 280 nm and the chromophore peak of ~2.0–2.5. The membranes were suspended in the basal salt solutions also used in the transport assay, but buffered to pH 7.0 (20 mM Tris-H₂SO₄). UV-visible spectra were recorded with an Aminco DW2A spectrometer connected to a personal computer. Absorption maxima were determined as zero points of the first derivative of the chromophore band. Acid-base titrations to determine the Schiff base pK_a were performed by adding aliquots of 1 N NaOH, measuring the relative absorption decrease at the absorption maximum of the protonated chromophore (570–580 nm), correcting for volume changes and fitting a hyperbolic binding function to the data. Retinal extraction and determination of the isomer ratio by HPLC separation was performed according to Scherrer *et al.* (1989). Flash-induced difference spectra were recorded with a home-made flash photolysis apparatus with a time resolution of 130 µs (Uhl *et al.*, 1985).

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References

- Ames, J.B., Raap, J., Lugtenburg, J. and Mathies, R.A. (1992) Resonance Raman study of halorhodopsin photocycle kinetics, chromophore structure, and chloride-pumping mechanism. *Biochemistry*, **31**, 12546–12554.
- Balashov, S.P., Govindjee, R., Kono, M., Imasheva, E., Lukashev, E., Ebrey, T.G., Crouch, R.K., Menick, D.R. and Feng, Y. (1993) Effect of the arginine-82 to alanine mutation in bacteriorhodopsin on dark adaptation, proton release, and the photochemical cycle. *Biochemistry*, **32**, 10331–10343.
- Bamberg, E., Tittor, J. and Oesterhelt, D. (1993) Light-driven proton or chloride pumping by halorhodopsin. *Proc. Natl Acad. Sci. USA*, **90**, 639–643.
- Bogomolni, R.A., Stoekenius, W., Szundi, I., Perozo, E., Olson, K.D. and Spudich, J.L. (1994) Removal of transducer HtrI allows electrogenic proton translocation by sensory rhodopsin I. *Proc. Natl Acad. Sci. USA*, **91**, 10188–10192.
- Braiman, M.S., Mogi, T., Marti, T., Stern, L.J., Khorana, H.G. and Rothschild, K.J. (1988) Vibrational spectroscopy of bacteriorhodopsin mutants: light-driven proton transport involves protonation changes of aspartic acid residues 85, 96, and 212. *Biochemistry*, **27**, 8516–8520.
- Brown, L.S., Needleman, R. and Lanyi, J.K. (1996) Interaction of proton and chloride transfer pathways in recombinant bacteriorhodopsin with chloride transport activity: implications for the chloride translocation mechanism. *Biochemistry*, **35**, 16048–16054.
- Butt, H.J., Fendler, K., Bamberg, E., Tittor, J. and Oesterhelt, D. (1989) Aspartic acids 96 and 85 play a central role in the function of bacteriorhodopsin as a proton pump. *EMBO J.*, **8**, 1657–1663.

- Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C. and Doolittle, W.F. (1989) Transformation methods for halophilic archaeobacteria. *Can. J. Microbiol.*, **35**, 148–152.
- Ferrando, E., Schweiger, U. and Oesterhelt, D. (1993) Homologous bacterio-opsin-encoding gene expression via site-specific vector integration. *Gene*, **125**, 41–57.
- Grigorieff, N., Ceska, T.A., Downing, K.H., Baldwin, J.M. and Henderson, R. (1996) Electron-crystallographic refinement of the structure of bacteriorhodopsin. *J. Mol. Biol.*, **259**, 393–421.
- Haupts, U., Bamberg, E. and Oesterhelt, D. (1996) Different modes of proton translocation by sensory rhodopsin-I. *EMBO J.*, **15**, 1834–1841.
- Haupts, U., Tittor, J., Bamberg, E. and Oesterhelt, D. (1997) General concept for ion translocation by halobacterial retinal proteins: the isomerization/switch/transfer (IST) model. *Biochemistry*, **36**, 2–7.
- Havelka, W.A., Henderson, R. and Oesterhelt, D. (1995) Three dimensional structure of halorhodopsin at 7 Å resolution. *J. Mol. Biol.*, **247**, 726–738.
- Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J. Mol. Biol.*, **213**, 899–929.
- Heymann, J.A.W., Havelka, W.A. and Oesterhelt, D. (1993) Homologous overexpression of a light-driven anion pump in an archaeobacterium. *Mol. Microbiol.*, **7**, 623–630.
- Holmes, M.L. and Dyal-Smith, M.L. (1991) Mutations in DNA gyrase result in novobiocin resistance in halophilic archaeobacteria. *J. Bacteriol.*, **173**, 642–648.
- Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Rapid and efficient site-directed mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.
- Lanyi, J.K. (1986) Photochromism of halorhodopsin. Cis/trans isomerization of the retinal around the 13–14 double bond. *J. Biol. Chem.*, **261**, 14025–14030.
- Lanyi, J.K. (1990) Halorhodopsin, a light-driven electrogenic chloride-transport system. *Physiol. Rev.*, **70**, 319–330.
- Lanyi, J.K. (1993) Proton translocation mechanism and energetics in the light-driven pump bacteriorhodopsin. *Biochim. Biophys. Acta*, **1183**, 241–261.
- Lanyi, J.K. and Vodyanov, V. (1986) Flash spectroscopic studies of the kinetics of the halorhodopsin photocycle. *Biochemistry*, **25**, 1465–1470.
- Lanyi, J.K., Duschl, A., Váró, G. and Zimanyi, L. (1990) Anion binding to the chloride pump, halorhodopsin, and its implications for the transport mechanism. *FEBS Lett.*, **265**, 1–6.
- le Coutre, J., Rüdiger, M., Oesterhelt, D. and Gerwert, K. (1995) FTIR investigation of the blue to acid-purple transition of Bacteriorhodopsin by use of induced halide binding. *J. Mol. Struct.*, **349**, 165–168.
- Mathies, R.A., Steven, W.L., Ames, J.B. and Pollard, W.T. (1991) From femtoseconds to biology: Mechanism of bacteriorhodopsin's light-driven proton pump. *Annu. Rev. Biophys. Chem.*, **20**, 491–518.
- Metz, G., Siebert, F. and Engelhard, M. (1992) Asp85 is the only internal aspartic acid that gets protonated in the M intermediate and the purple-to-blue transition of bacteriorhodopsin. A solid-state carbon-13 CP-MAS NMR investigation. *FEBS Lett.*, **303**, 237–241.
- Mukohata, Y. (1994) Comparative studies on ion pumps of the bacterial rhodopsin family. *Biophys. Chem.*, **50**, 191–201.
- Oesterhelt, D. (1982) Reconstitution of the retinal proteins bacteriorhodopsin and halorhodopsin. *Methods Enzymol.*, **88**, 10–17.
- Oesterhelt, D. (1995) Structure and function of halorhodopsin. *Isr. J. Chem.*, **35**, 475–494.
- Oesterhelt, D. and Stoekenius, W. (1974) Isolation of the cell membrane of Halobacterium halobium and its fractionation into red and purple membrane. *Methods Enzymol.*, **31**, 667–678.
- Oesterhelt, D., Hegemann, P. and Tittor, J. (1985) The photocycle of the chloride pump halorhodopsin. II: Quantum yields and a kinetic model. *EMBO J.*, **4**, 2351–2356.
- Oesterhelt, D., Hegemann, P., Tavan, P. and Schulten, K. (1986) Trans-cis isomerization of retinal and a mechanism for ion translocation in halorhodopsin. *Eur. Biophys. J.*, **14**, 123–129.
- Oesterhelt, D., Tittor, J. and Bamberg, E. (1992) A unifying concept for ion translocation by retinal proteins. *J. Bioenerg. Biomembr.*, **24**, 181–191.
- Otomo, J. (1996) Influence exercised by histidine-95 on chloride transport and the photocycle in halorhodopsin. *Biochemistry*, **35**, 6684–6689.
- Otomo, J., Tomioka, H. and Sasabe, H. (1992) Properties and the primary structure of a new halorhodopsin from halobacterial strain mex. *Biochim. Biophys. Acta*, **1112**, 7–13.
- Rüdiger, M., Haupts, U., Gerwert, K. and Oesterhelt, D. (1995) Chemical reconstitution of a chloride pump inactivated by a single point mutation. *EMBO J.*, **14**, 1599–1606.
- Sasaki, J., Brown, L.S., Chon, Y.-S., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995) Conversion of bacteriorhodopsin into a chloride ion pump. *Science*, **269**, 73–75.
- Scherrer, P., Mathew, M.K., Sperling, W. and Stoekenius, W. (1989) Retinal isomer ratio in dark-adapted purple membrane and bacteriorhodopsin monomers. *Biochemistry*, **28**, 829–834.
- Schober, B., Lanyi, K.L. and Oesterhelt, D. (1986) Effects of anion binding on the deprotonation reactions of halorhodopsin. *J. Biol. Chem.*, **261**, 2690–2696.
- Steiner, M. and Oesterhelt, D. (1983) Isolation and properties of the native chromoprotein halorhodopsin. *EMBO J.*, **2**, 1379–1385.
- Steiner, M., Oesterhelt, D., Arikawa, M. and Lanyi, J.K. (1984) Halide binding by the purified halorhodopsin chromoprotein. I. Effects on the chromophore. *J. Biol. Chem.*, **259**, 2179–2184.
- Tittor, J., Oesterhelt, D., Maurer, R., Desel, H. and Uhl, R. (1987) The photochemical cycle of halorhodopsin: absolute spectra of intermediates obtained by flash photolysis and fast difference spectra measurements. *Biophys. J.*, **52**, 999–1006.
- Tittor, J., Schweiger, U., Oesterhelt, D. and Bamberg, E. (1994) Inversion of proton translocation of bacteriorhodopsin mutants D85N, D85T and D85,96N. *Biophys. J.*, **66**, 1682–1690.
- Uhl, R., Meyer, B. and Desel, H. (1985) A polychromatic flash photolysis apparatus. *J. Biochem. Biophys. Methods*, **10**, 35–48.
- Váró, G., Brown, L.S., Sasaki, J., Kandori, H., Maeda, A., Needleman, R. and Lanyi, J.K. (1995a) Light-driven chloride ion transport by halorhodopsin from *Natronobacterium pharaonis*. I. The photochemical cycle. *Biochemistry*, **34**, 14490–14499.
- Váró, G., Zimanyi, L., Fan, X., Sun, L., Needleman, R. and Lanyi, J.K. (1995b) Photocycle of halorhodopsin from *Halobacterium salinarum*. *Biophys. J.*, **68**, 2062–2072.
- Váró, G., Brown, L.S., Needleman, R. and Lanyi, J.K. (1996) Proton transport by halorhodopsin. *Biochemistry*, **35**, 6604–6611.
- Walter, T.J. and Braiman, M.S. (1994) Anion-protein interactions during halorhodopsin pumping—halide binding at the protonated Schiff-base. *Biochemistry*, **33**, 1724–1733.

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