Properties of bacteriorhodopsin derivatives constructed by insertion of an exogenous epitope into extra-membrane loops

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Bacteriorhodopsin (BR) is folded into a bundle of seven α -helices which is embedded in the cellular membrane of *Halobacterium salinarum*; these helices are connected by short extra-membrane loops, three on the cytoplasmic side and three on the outside. Oligonucleotide-directed insertion or replacement mutagenesis was used to integrate the C-terminal sequence (13 amino acids long) of Sendai virus L-protein individually into each of the six helix-connecting loops. The altered gene products were obtained by expression of the mutant genes in either *Escherichia coli* or *Schizosaccharomyces pombe* and were used to reconstitute BR in proteoliposomes. In four cases (altered loops B/C, C/D, D/E or E/F), the mutant BRs were found to be fully functional as judged by light-driven proton pumping and photocycle kinetics. Within the four functional BR variants, recognition of the viral epitope by a monoclonal antibody is restricted to modified loops B/C and E/F. Immunogold staining of *S.pombe* cells producing either of the two latter BR variants shows that the protein is distributed among various cellular membranes but is not present in mitochondrial membranes. Sequence alteration of loop A/B or F/G resulted in loss of function, most plausibly due to a folding defect of the respective proteins. These results on the one hand document differences in structural importance of the various BR extra-membrane loops and on the other hand open the door to the construction of multifunctional membrane proteins via loop replacement mutagenesis of BR.

Key words: bacteriorhodopsin/immunotag/membrane protein/protein engineering/*Schizosaccharomyces pombe*

Introduction

Bacteriorhodopsin (BR) is the light-driven proton pump of *Halobacterium salinarum* (formerly called *Halobacterium halobium*). The protein is embedded in the lipid bilayer of purple membrane as a bundle of seven helices, connected by extra-membrane loops (Figure 1). In its mature form,

BR consists of 248 amino acids plus a retinal moiety attached by a Schiff base linkage to the ϵ -amino group of Lys216 (Oesterhelt and Stoeckenius, 1971; for reviews see Stoeckenius and Bogomolni, 1982; Khorana, 1988; Tittor, 1991). A detailed structural model of the helix bundle has been derived from electron diffraction of two-dimensionally crystalline BR, whereas the extra-membrane loops were disordered (Henderson *et al.*, 1990). Knowledge of this structure, together with a large body of biophysical, biochemical and genetic data, provided the basis for formulating a mechanism of light-driven proton translocation (for reviews see Mathies *et al.*, 1991; Oesterhelt *et al.*, 1992; Khorana, 1993). Other halobacterial retinal proteins (halorhodopsin and sensory rhodopsin) have the same topology as and show extensive sequence similarities with BR within the seven trans-membrane helices, yet they have extra-membrane loops of varying length (Oesterhelt *et al.*, 1992).

The protein architecture sketched above already suggests the possibility of using BR as a structural scaffold in the construction of biological membranes with new and pre-defined properties by replacing the extra-membrane parts of BR with exogenous polypeptide modules of known function. In such a scheme, the conspicuous purple colour of chimeric BRs could be used as a convenient macroscopic indicator of correct overall folding and membrane insertion. Beyond this, the promotion of light-driven proton translocation could be integrated into the construction of multifunctional membrane proteins. This idea gained support

Fig. 1. Topological model of BR. The amino acid sequence encoded by the synthetic gene (*bos*) contains glutamic acid at the N-terminus and lacks the C-terminal aspartic acid which is encoded in the natural gene (*bop*) but post-translationally removed in *Halobacterium salinarum*. A–G are α -helical trans-membrane segments according to the structural model of Henderson *et al.* (1990). A retinal moiety is attached to amino acid residue 216 (lysine, designated K in the one-letter code).

Fig. 2. Insertion of an epitope derived from the Sendai virus L-protein into the helix-connecting loops of BR. The left half of the figure lists the amino acid sequences of each of the six extra-membrane loops according to Figure 1. The right half illustrates the corresponding loop sequences after insertion of the viral epitope (13 amino acids in length, shaded sequence). All constructs were obtained by oligonucleotide-directed mutagenesis of the synthetic gene, *bos*. Insertion of the epitope has the following consequences for length (number of amino acid residues) and net charge of the loops (before/after insertion); A/B: 5/14, -1/-4; B/C: 16/24, -1/-4; C/D: 6/18, -2/-4; D/E: 7/21, +2/-2; E/F: 9/15, 0/-5; F/G: 7/20, 0/-4.

from a number of observations. In at least some specific cases, stretches of amino acid sequence could be deleted from extra-membrane loops without impairing BR function (Gilles-Gonzales *et al.*, 1991) and BR could even be reconstituted from purified polypeptide subfragments, lipids and retinal (Huang *et al.*, 1981; Liao *et al.*, 1983; Popot *et al.*, 1987; Kahn and Engelman, 1992). Here we describe the systematic alteration of extra-membrane loops of BR by insertion of an epitope (13 amino acids in length) of Sendai virus L-protein. As a result, we identify four loops that can be sequence-modified without concomitant loss of BR function and can now serve as target sites for more complex constructions.

Results

Construction of variant *bos* genes

In a previous paper (Pompejus *et al.*, 1993), we described the construction of a synthetic gene (*bos*) of bacterio-opsin (BO, apo-protein of BR), its expression in *Escherichia coli* and renaturation of the gene product to give functional BR. Our present study deals with the production and functional characterization of a set of six variant BRs, in which the extra-membrane loops have been modified individually by insertion of a tridecapeptide sequence corresponding to the C-terminus of Sendai virus L-protein. This epitope is highly hydrophilic and carries four negative charges (see Figure 2);

Fig. 3. Analysis of purified BO derivatives by immunoblotting. Variant BOs were produced in *E. coli* by overexpression of the corresponding genes and purified as described by Pompejus *et al.* (1993). **Upper panel:** protein blot stained with monoclonal antibody BR2, directed against a C-terminal epitope of BO. **Lower panel:** corresponding protein blot stained with monoclonal antibody VII-E-7, directed against the Sendai virus epitope illustrated in Figure 2. A/B: BO with Sendai epitope inserted between helix A and helix B. Corresponding nomenclature applies to B/C, C/D, D/E, E/F and F/G (for structural details refer to Figure 2). w.t.: Wild-type BO; M: gel position of marker proteins of indicated relative molecular mass ($\times 1000$).

these properties are compatible with its being exposed on the membrane surface. It is recognized with high selectivity by monoclonal antibody VII-E-7 (Einberger *et al.*, 1990) and

Fig. 4. Colour phenotypes of *S.pombe* clones transformed by various BO genes. Cells were grown in liquid culture, harvested by centrifugation and converted to protoplasts. (–), cells transformed with parent vector pEVP11; *bop*, cells expressing natural *bop* gene of vector pEVPbop (Hildebrandt *et al.*, 1989); *bos*, cells expressing synthetic *bos* gene of plasmid pEVPbos; A/B to F/G, cells carrying the same vector but coding for BOs with Sendai epitope insertions in the loops indicated (for details and nomenclature refer to Figure 3).

has been used before as an immunotag for gene expression studies (Döring *et al.*, 1991).

The structural model schematically outlined in Figure 1 provided the conceptual framework for planning the epitope insertion mutants of BR summarized in Figure 2. Since loops C/D, D/E and F/G are relatively short, insertion of the Sendai epitope at these sites was chosen rather than replacement of the respective original amino acid sequences. Duplication of Ser132 within loop D/E, the position of which is less well defined than that of the other loops (Fimmel *et al.*, 1989; Engelman *et al.*, 1980; Henderson *et al.*, 1990), was taken as an additional precaution to keep distance to the helices on either side of the epitope. For the construction of BRSEN-B/C and BRSEN-E/F, several residues of the corresponding loops were replaced with the Sendai epitope. This decision was based on results of Gilles-Gonzales *et al.* (1991), who showed that deletions within loops B/C or E/F did not impair BR function, as judged by UV/VIS absorption spectroscopy and light-driven proton translocation. Finally, replacement of residues Val34–Pro38 by the epitope led to BRSEN-A/B. The increase in loop size caused by epitope insertion ranged from six additional residues in BRSEN-E/F to 14 in BRSEN-D/E. For experimental details refer to Materials and methods.

Expression of variant *bos* genes in *E.coli*

For bulk production of variant BOs in *E.coli*, the corresponding variant *bos* genes were inserted into expression vector pLZPWB1, in analogy to our previous study (Pompejus *et al.*, 1993). With all constructs tested, ~100–150 mg β -Gal454/BO fusion protein per litre *E.coli* culture accumulated as water-insoluble inclusion bodies. Site-specific proteolysis by IgA protease in the heterogeneous phase liberated variant BOs, which could be purified to apparent homogeneity by extraction into organic solvent with similar efficiency and yield (data not shown) as described for wild-type BO (Pompejus *et al.*, 1993).

Purified BO variants were investigated by immunoblotting for binding of two different monoclonal antibodies, BR2, directed against a C-terminal epitope of BO (Figure 3, upper

panel), and VII-E-7, specific for the Sendai epitope (lower panel). The latter gave a strong signal with BOSEN-A/B, BOSEN-B/C, BOSEN-E/F and BOSEN-F/G but bound only weakly to BOSEN-C/D and not at all to BOSEN-D/E or to a control sample of wild-type BO.

Most preparations show an additional more rapidly moving band which is visible only in Western blots and not with Coomassie stain. This band, tentatively attributed to partial BO proteolysis, appears in all samples analysed with the monoclonal antibody directed against the C-terminal sequence of BR. The lack of reaction of this more rapidly moving material with the anti-Sendai monoclonal antibody in the case of BOSEN-A/B localizes the presumptive cleavage site to the area in between the respective epitope insertion points of BRSEN-A/B and BRSEN-B/C. This interpretation is supported by the observed relative molecular mass of the more rapidly moving material.

Production of variant bacteriorhodopsins in *Schizosaccharomyces pombe*

For expression of the *bos* gene and derivatives in *S.pombe* all DNA constructs were cloned in expression vector pEVP11 (Russell and Nurse, 1986; for details refer to Materials and methods). It had been shown earlier that transformation of the fission yeast by recombinant pEVP11 containing the natural BO gene (*bop*) under control of the alcohol dehydrogenase (*adh*) promoter leads to the production of functional BR if retinal is added to the culture medium (Hildebrandt *et al.*, 1989, 1991).

In analogy to these results, we found that expression of *bos* and variants in the fission yeast also leads to the synthesis of functional BR. Purple pellets of fission yeast cells could be harvested from strains carrying the *bos*, *bos*SEN-B/C, *bos*SEN-C/D, *bos*SEN-D/E or *bos*SEN-E/F genes (Figure 4). Expression of *bos* and the four variants is at least as strong as that of *bop*, even though the codon usage of the *bos* gene is very different from that of *bop* (Pompejus *et al.*, 1993). This result further emphasizes the versatility of *S.pombe* for the production of exogenous proteins. In contrast, transformation of *S.pombe* with pEVP11

Fig. 5. *In situ* visualization of Sendai epitope in *S. pombe* cells by immunofluorescence microscopy. Cells were grown in liquid culture and fixed as described in Materials and methods. **Upper panel:** fields of cells stained with monoclonal anti-Sendai antibody and fluorescein-coupled goat anti-mouse IgG. **Lower panel:** same fields but viewed under interference contrast. The scale bar in field E/F represents 10 μ m. For nomenclature refer to Figure 3.

derivatives carrying *bosSEN-A/B* or *bosSEN-F/G* leads to a colourless phenotype (Figure 4).

Full-length transcription of genes *bosSENA/B* and *bosSENF/G* was demonstrated as described under Materials and methods; however, no corresponding gene product could be detected in Western blot experiments (data not shown). The most plausible explanation for this is that the proteins are proteolytically degraded either because they have a structural defect that renders them incompetent in proper folding or because the functional conformation providing protection against proteolysis is inaccessible because *in vivo* the proteins cannot be channelled into the membrane insertion pathway. In order to distinguish between these two possibilities, *BOSEN-A/B* and *BOSEN-F/G* were produced in the denatured state in *E. coli*, purified and subjected to the reconstitution procedure published earlier (Pompejus *et al.*, 1993). In contrast to wild-type BO and *BOSEN-D/E* produced in parallel by the same procedure, *BOSEN-A/B* and *BOSEN-F/G* could not be reconstituted to functional BR derivatives (data not shown). We interpret these findings as support of the notion that BR folding is sensitive to the structure of loops A/B and F/G.

***In situ* immunochemical visualization in *S. pombe* cells of bacteriorhodopsins carrying the Sendai epitope**

The four *S. pombe* transformants producing BRs with an integrated Sendai epitope were tested for presentation of the epitope *in situ* by indirect immunofluorescence microscopy (for details see Materials and methods). Strong fluorescence was observed with clones producing *BRSEN-B/C* or *BRSEN-E/F*, whereas cells producing *BRSEN-C/D* or *BRSEN-D/E* did not accept the fluorescent label (Figure 5).

The intracellular localization of the variant BRs in *S. pombe* was determined by *in situ* immunogold staining. BR derivative *BRSEN-E/F* is distributed fairly evenly between various cellular membranes such as plasma membrane, endoplasmic reticulum and vesicles; mitochondrial membranes are not stained (Figure 6). Construct *BRSEN-B/C* shows an essentially identical distribution pattern but the extent of labelling is less pronounced. Neither cells producing wild-type BR nor those producing *BRSEN-C/D*

or *BRSEN-D/E* are labelled by the anti-Sendai antibody (data not shown). In this respect, the results of the two *in situ* immunostaining experiments agree not only with one another but also with the immunoblotting data of material produced in *E. coli* (see Figure 3). Since mitochondrial membranes are apparently inaccessible to the variant BRs, they seem to enter membraneous compartments of the cell in a controlled manner—via the endoplasmic reticulum, followed by vesicle transport—rather than through non-specific association. In the absence of a leader peptide, this seems surprising at first. Conceivably, however, one or several of the seven trans-membrane segments of BR could act as an internal, non-cleavable topogenic sequence as has been found for bovine opsin (Audiger *et al.*, 1987).

Enrichment of variant bacteriorhodopsins from extracts of *S. pombe*

Wild-type BR and the four variants produced by *S. pombe* were enriched from total membrane fractions as outlined in Figure 7. BR and derivatives obtained by this procedure, while not homogeneous, were of sufficient quality for functional analysis by biochemical and biophysical methods.

Functional characterization of variant BRs *in vitro*

Functional characterization of variants *BRSEN-B/C*, *BRSEN-C/D*, *BRSEN-D/E* and *BRSEN-E/F* *in vitro* included UV/VIS absorption spectroscopy, flash photolysis difference spectroscopy and light-induced proton translocation across synthetic membranes. As judged by these criteria, all four variants were indistinguishable from one another and from wild-type BR. Below, details are described for wild-type BR and *BRSEN-B/C* only. The observation of a photobleachable absorption maximum at or near 550 nm for light-adapted species in 1% octyl glucoside (Figure 8: panels A and B for wild-type BR, panels E and F for *BRSEN-B/C*) is in good agreement with previous observations (Dencher and Heyn, 1978; Shkrob *et al.*, 1978). Furthermore, all samples exhibited a typical light-dependent blue shift of the absorption maxima (Table I). Photocycle kinetics of BR and variants were monitored by flash photolysis spectroscopy (Uhl *et al.*, 1985; Tittor *et al.*,

Fig. 6. Intracellular localization of BRSEN-E/F in transformed *S.pombe* cells. Ultrathin cell sections were stained with anti-Sendai antibody and goat anti-mouse antibody coupled to ultra-small gold particles; the gold particles were visualized by silver enhancement. ER, endoplasmic reticulum; M, mitochondrion; V, vesicle. Scale bar represents 500 nm.

1987). Spectral changes in the millisecond range were virtually identical for wild-type BR (Figure 8, panels C and D), for BRSEN-B/C (panels G and H) and for the other variants (data not shown). In summary, presence of the Sendai epitope in loops B/C, C/D, D/E or E/F has no significant influence on the UV/VIS spectral properties of BR, including photocycle kinetics.

The four BR variants, incorporated into liposomes, showed light-dependent acidification of the bulk water. The effect is verified as trans-membrane proton translocation by its breakdown upon addition of the protonophore FCCP (Figure 9). Thus, wild-type and variant BRs are found embedded in the liposomes preferentially in the outside-out orientation. This is surprising in view of the fact that purified BR incorporated into liposomes normally displays inside-out orientation (Racker and Stoekenius, 1974; Rigaud *et al.*,

Fig. 7. Flow diagram of BR production and enrichment. *S.pombe* cells are converted to protoplasts by enzymatic digestion and homogenized mechanically. The membrane fraction containing the bulk of BR is collected by centrifugation (9600 g, 15 min). BR or respective derivatives are solubilized with Triton X-100 and enriched by adsorption/desorption to Q-Sepharose with concomitant exchange of Triton X-100 for octyl glucoside. For experimental details refer to Materials and methods.

1983). Whole cell envelopes of *H.salinarium*, on the other hand, do show outside-out orientation of BR (Lanyi and MacDonald, 1977). Happe *et al.* (1977) showed that the preferential orientation is not firmly determined, but dependent on (for example) the pH of the bulk medium and the nature of lipids used for reconstitution.

Specific initial rates of proton pumping were determined under the assumption of identical molar extinction coefficients for wild-type BR and all the variants tested. Limited purity of the proteins used for integration into liposomes and the less than perfect reproducibility of the overall procedure leave rather wide margins of error (Table I, third row). Within these margins, however, again no significant differences could be observed. Possible detection of small differences in pumping rates has to await the preparation of homogeneous BR variants.

Discussion

We have used the C-terminal tridecapeptide of Sendai virus L-protein as a tool to probe the BR molecule for candidate sites that tolerate insertion of exogenous and functional polypeptide modules. The long-range goal of the project is grafting a variety of functional domains on to biological membranes in a structurally controlled manner. Results obtained from our present, orienting study (summarized in Figure 10) are encouraging albeit not entirely straightforward.

Fig. 8. Spectroscopic characterization of wild-type BR and BRSEN-B/C. Protein samples were prepared according to the procedure outlined in Figure 7. (A–D) Wild-type BR; (E–H) BRSEN-B/C. (A and E) Absorption spectra of light-adapted samples in 1% OG, before (solid line) and after photobleaching (broken line). (B and F) Corresponding difference spectra (absorbances corrected for values observed with the photobleached samples). (C and G) Flash photolysis spectra taken at three time points after the flash. (D and H) Time course after flash of absorption at three wavelengths.

Fig. 9. Light-driven proton translocation by BR and BRSEN-B/C in soybean lipid liposomes. Proteoliposomes containing BR and BRSEN-B/C were obtained by the detergent dilution method as described (Pompejus *et al.*, 1993). Arrows indicate time points of switching light on or off or, respectively, the addition of protonophore (FCCP, 3 μ M final concentration). The measurements were calibrated by adding a known amount of protons. For nomenclature refer to Figure 3.

Table 1. Summary of spectroscopic and proton translocation properties

	Dark adapted (nm)	Light adapted (nm)	Specific activity (H ⁺ /BR/s)
w.t.	543 $\pm$ 3	549 $\pm$ 2	0.37 $\pm$ 0.30
B/C	543 $\pm$ 2	549 $\pm$ 3	0.19 $\pm$ 0.11
C/D	538 $\pm$ 2	544 $\pm$ 3	0.30 $\pm$ 0.10
D/E	542 $\pm$ 3	548 $\pm$ 2	0.28 $\pm$ 0.15
E/F	538 $\pm$ 2	544 $\pm$ 4	0.12 $\pm$ 0.01

Three independent preparations of each species were separately investigated (collection of raw data as illustrated in Figures 8 and 9). Wavelength values in nm refer to absorption maxima in 1% OG. Dark adapted samples were kept in darkness for 24 h before spectra were recorded. Specific activities listed refer to initial pumping rates. For nomenclature see legend to Figure 3.

Four out of six constructed variant *bos* genes are expressed in transformed *S.pombe* cells and direct the accumulation of BR loop variants that are practically indistinguishable from the wild-type protein with respect to UV/VIS absorption spectra, photocycle kinetics and light-driven proton pumping, even though some of the alterations introduced into the loops by the presence of the Sendai epitope are quite drastic (length, net charge etc., see Figure 2). As discussed above, the lack of protein accumulation in the case of *bos*SEN-A/B and *bos*SEN-F/G is most likely due to a folding defect of the corresponding gene products. It is not clear at present, whether loops A/B and F/G are generally inaccessible to biomembrane design by loop replacement or whether the particular epitope used here inserted in this specific manner is causing the defect. Since BR can be reconstituted from individual polypeptide segments A, B and C–G (Kahn and Engelman, 1992) it is clear that the integrity of loop A/B is not a prerequisite for proper folding and membrane insertion; our finding rather seems to suggest that there are at least some ways of connecting helices A and B that are incompatible with the acquisition of a functional BR structure. It should be kept in mind, for instance, that BOSEN-A/B carries a particularly pronounced sequence alteration, in which (according to the most recent structural models of BR) the replacing epitope even extends into the

Fig. 10. Summary of results obtained with BR variants produced in *S.pombe*. A filled circle under 'chromophore' indicates that the respective transformed clone is purple. n.t.: not tested, due to impaired BR production.

beginning of helix B. More work is needed to delineate these constraints more precisely.

Insertion of the Sendai epitope (amino acid sequence: DGLSGDIEPYDSS) into extra-membrane loops of BR potentially introduces a second function into the molecule, i.e. binding of the respective monoclonal antibody. As elaborated above, four loop variants were fully functional with respect to all BR properties tested. Only two of these, however (BRSEN-B/C and BRSEN-E/F), are able to bind the monoclonal antibody. There are at least three possible explanations for the lack of binding by BRSEN-C/D and BRSEN-D/E; (i) most simply, this could be due to hitherto unappreciated constraints of amino acid sequence flanking the tridecapeptide epitope; (ii) the surrounding in which the epitope is embedded could force upon it a conformation incompatible with antibody binding; (iii) the epitope could be buried inaccessibly under neighbouring structure. The first explanation is favoured by the observation that all immunochemical results collected so far are completely parallel in three different kinds of experiments: Western blots, *in situ* immunofluorescence of fixed cells and immunogold staining of ultrathin sections. The more interesting possibilities, (ii) and (iii), however, are not ruled out yet but would require preservation of at least some elements of similar secondary structure under widely different experimental conditions.

Taken together, our results demonstrate that structural integrity of loops B/C, C/D, D/E and E/F is not a prerequisite of BR function and that the construction of multifunctional membrane proteins on the basis of BR as a structural scaffold is a feasible proposition. Loops B/C, C/D, D/E and E/F are now clearly identified as prime candidates for future constructions of more complex loop replacements. These findings extend in a systematic manner individual observations made previously with BR that was either assembled from subfragments or carried deletions in loop sequences (see Introduction). This tolerance to modifications of extra-membrane parts of the protein is not limited to BR. *E.coli* Lac permease, for example, remained active after individual loops were modified by the insertion of oligohistidine tracts (McKenna *et al.*, 1992); furthermore, transport-competent Lac permease can be assembled *in vivo* from separately produced subfragments (Wrubel *et al.*, 1990; Bibi and Kaback, 1992). Antigens have been presented on the *E.coli* cell surface by insertion into putative

extramembrane loops of the outer membrane porins LamB or PhoE (Agterberg *et al.*, 1987; Charbit *et al.*, 1986, 1987, 1991; Martineau *et al.*, 1991). For LamB it was shown that neither folding nor function were impaired by these insertions (Charbit *et al.*, 1986, 1991). These observations support the assumption that structural pliability of surface loops is a widespread phenomenon with membrane proteins. Thus, biomembrane design as suggested here could possibly be based on a variety of membrane proteins other than BR, though for reasons elaborated above, we consider BR as a particularly attractive case.

Conceivably, the same or other epitopes could be employed in at least two additional lines of investigations. Numerous photo- and chemoreceptors are of the seven transmembrane α -helices type and many of them are known to interact with transducer proteins (Dohlman *et al.*, 1991). Epitope insertion into loops exposed on the cytoplasmic side may preserve signal uptake but impair interaction with the G-protein, thus defining the substructures on the receptor side that are relevant for signal relay. In addition, epitopes of sufficient affinity to a given antibody could be used as anchoring sites for a variety of antibody conjugates and thus facilitate rather flexible design of functionalized membranes without the need of covalent insertion of a functional module.

Materials and methods

Enzymes and chemicals

DNase I, RNase A, T4 polynucleotide kinase and T4 DNA ligase were purchased from Boehringer Mannheim; Taq DNA polymerase was from Boehringer Mannheim and Cetus. T7 DNA polymerase type II was from New England Biolabs, Sequenase™ was from United States Biochemicals and M-MLV reverse transcriptase was from Gibco/BRL. Restriction enzymes were from Boehringer Mannheim, New England Biolabs and Gibco/BRL. 'Lysing enzymes' from *Trichoderma harzianum* and 'lyticase' were from Sigma. *Neisseria* type II IgA protease was a gift from J. Pohlner (Max-Planck-Institut für Biologie, Tübingen). Soybean lipids were obtained from Fluka, SDS from Bio-Rad, and ampicillin, dNTPs, octyl glucoside (OG) and CHAPS from Boehringer Mannheim. Tween 20, Triton X-100, poly-L-lysine and all-trans retinal were from Sigma. Q-Sepharose Fastflow was purchased from Pharmacia. All other chemicals were of analytical grade and supplied by Merck or Sigma.

Antibodies

The monoclonal antibody (VII-E-7) against a 13 residue C-terminal epitope of Sendai virus L-protein (DGLSLDIEPYDSS, Einberger *et al.*, 1990) was a gift from H. Einberger and H.P. Hofschneider (Max-Planck Institut für Biochemie, Martinsried bei München). The monoclonal antibody BR2 (May, 1990), directed against the C-terminal part of BR was a gift from K. May and D. Oesterhelt (Max-Planck Institut für Biochemie, Martinsried bei München). The alkaline phosphatase- and fluorescein-conjugated goat anti-mouse IgGs were purchased from Sigma. Goat anti-mouse antibodies bound to ultra-small gold particles were from Aurion (Wageningen).

Strains

E. coli strain HB101 (Boyer and Roland-Dussoix, 1969) was used to propagate and amplify *E. coli/S. pombe* shuttle vectors. With the fission yeast, *bos* gene expression was achieved with *S. pombe* *h⁻ leu1-32* (gift from P. Nurse).

Microbiological procedures

E. coli procedures were as described by Pompejus *et al.* (1993). Media used for *S. pombe* were YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) and minimal medium according to Moreno *et al.* (1991). Minimal medium was supplemented with leucine (150 μ g/ml) when required. *S. pombe* transformations were performed essentially as described by Moreno *et al.* (1991). Growth of fission yeast was monitored by measuring the optical density of the cultures at 600 nm.

DNA procedures

Standard DNA procedures, such as plasmid isolation, ligation, restriction analysis of plasmids and isolation of DNA fragments were as described

by Sambrook *et al.* (1989). DNA sequence analysis of clones containing mutant *bos* genes was performed using Sequenase™ as described (Sanger *et al.*, 1977; Ansorge *et al.*, 1987).

Oligonucleotide synthesis, PCR and directed mutagenesis

Synthetic 2'-deoxyoligonucleotides used for directed mutagenesis and PCR were prepared using the Applied Biosystems 380B synthesizer as described by Beaucage and Caruthers (1981) and purified by reversed phase HPLC (Fritz *et al.*, 1978). SEN-A/B (68mer): 5'-CAAGGGTATGGGAGATG-GTTCATTAGGTGATATTGAACCATACGATTCATCAGCTAAGA-AATTCTATG; SEN-B/C (82mer): 5'-CGCGAAGCCAGTAGATAG-GATTCTGTGATGAATCGTATGGTTCAATATCACCTAAGCTTCC-ATCCATCGTTCAGACCGTAACC; SEN-C/D (80mer): 5'-GCAGAGC-TAGATAGTACCCTGTGATGAATCGTATGGTTCGATATCACCT-AATGAACCATCAGCCTGAACAAGTAGAGC; SEN-E/F (83mer): 5'-GCTTGAAAGTGCTGGCGACCTCAGGTGATGAATCGTATGG-TTCGATATCACCAATGCTTCCATCGCTTTAGAAGTTGAATCC; SEN-F/G (83mer): 5'-GGCAGAGTTTCAATGTTCAAACCCACTGA-TGAATCGTATGGTTCGATTACACCTAAGCTTCCATCCACAATA-CCGGCTCCCTCGG. The mutant gene *bos*SEN-A/B was constructed by the gapped duplex DNA method using SEN-A/B as the mutagenic primer (Friedrich, 1990; Stanssens *et al.*, 1989). For the construction of *bos*SEN-B/C, *bos*SEN-C/D, *bos*SEN-E/F and *bos*SEN-F/G the corresponding oligonucleotides listed above were phosphorylated using polynucleotide kinase and used for mutagenesis by the method of Kunkel *et al.* (1987) with pMa5IgAbos (Pompejus *et al.*, 1993) as template. *bos*SEN-D/E was constructed by PCR mutagenesis (Higuchi *et al.*, 1988) using Taq DNA polymerase and LACIGABOS (Pompejus *et al.*, 1993) and SEN-D/E as the mutagenic primers (45 s denaturation at 94°C, 60 s annealing at 37°C and 90 s elongation at 72°C), followed by insertion of the resulting amplified DNA into the *bos* gene as a fragment bordered by an *Eco*RI and a *Cl*aI restriction site. All mutations were verified by DNA sequence analysis.

Protein gel electrophoresis and Western blotting

Proteins were separated by SDS-PAGE (Laemmli, 1970), electrophoretically transferred to nitrocellulose and stained immunochemically as previously described (Pompejus *et al.*, 1993).

Production and purification of chimeric BOs in *E. coli*

Chimeric *bos* genes were cloned as *Eco*RI-XbaI fragments from the pMa5IgAbos derivatives into the *E. coli* expression vector pLZPWB1 (a gift from W. Wetekamp, Hoechst AG). Production, protease cleavage with IgA protease and purification of chimeric BOs was done essentially as described for wild-type BO (Pompejus *et al.*, 1993).

Production of chimeric bacteriorhodopsins in *S. pombe*

The *bos* gene and derivatives *bos*SEN-B/C, *bos*SEN-C/D, *bos*SEN-D/E, *bos*SEN-E/F and *bos*SEN-F/G were inserted between the unique *Bam*HI and *Hind*III recognition sites of pEVP11 (Russell and Nurse, 1986). Gene sequences correspond to the previously published *bos* gene (Pompejus *et al.*, 1993) but codon 1 (GAA, Glu) is preceded by the sequence 5'-AGATCT-ACATATG. In pEVP*bos*SEN-A/B this sequence is 5'-AGATCTCATG. These adaptor sequences provide for a translation start codon and also for a *Bgl*II recognition site and hence for ligation compatibility of the corresponding DNA fragment with the *Bam*HI site of the vector. The *Hind*III recognition site bordering the *bos* gene on the promoter-distal end is from the pLZ1*bos* vector (Pompejus *et al.*, 1993). Expression of the various *bos* genes is under control of the constitutive promoter of the *S. pombe* *adh* gene. For each of the seven constructs, transformants were selected on solid minimal medium and 10 individual clones each were analysed for production of BR. 2 ml minimal medium were inoculated and cells were grown at 30°C. After 30 h of growth, 0.2 ml of each culture were transferred to 20 ml minimal medium and 10 μ l all-trans retinal (1 mg/ml ethanolic solution) was added in mid-logarithmic growth phase (after 12, 18 and 24 h). *S. pombe* cultures producing BR were identified by their purple cell colour after centrifugation (5 min at 4000 g).

Analysis of transcription of chimeric *bos* genes in *S. pombe*

Yeast cells were harvested in mid-logarithmic growth phase and RNA was isolated essentially as described by Schmitt *et al.* (1990). Specific RNA was detected applying PCR after reverse transcription of total RNA (Gaudette and Crain, 1991) using 100 units of M-MLV reverse transcriptase and 6 μ g of total *S. pombe* RNA. The primer for reverse transcription was 5'-GTCTGACGTCGCCGCTGCGCCGTGCC. The same primer was used for PCR, along with LACIGABOS (Pompejus *et al.*, 1993) as the second primer. PCR conditions for 25 cycles were 45 s denaturation at 94°C, 1 min hybridization at 55°C and 1 min elongation at 72°C with extension for 1 s per cycle.

Protein extracts of *S. pombe*

Total *S. pombe* protein extracts for immunoblotting were produced as described by Moreno *et al.* (1991).

Isolation of wild-type and chimeric BRs from *S. pombe* membranes

S. pombe cells producing wild-type or chimeric BRs were grown in 1 litre of minimal medium supplemented with all-*trans* retinal (12, 18 and 24 h after inoculation, 1 mg each) and harvested in mid-logarithmic growth phase (centrifugation 15 min, 4000 g, 4°C). Cells were washed twice with 10 ml 1.2 M sorbitol, 40 mM disodium hydrogen phosphate, 54 mM citric acid, 40 mM EDTA and 0.2% (v/v) β -mercaptoethanol, adjusted with sodium hydroxide to pH 5.6 and kept for 30 min at 30°C in 10 ml 1.2 M sorbitol, 40 mM disodium hydrogen phosphate, 54 mM citric acid, 0.2% (v/v) β -mercaptoethanol, pH 5.6, containing 10 mg each 'lysing enzymes' and 'lyticase'. Protoplasts were homogenized by 20 strokes with a Dounce homogenizer. Soluble proteins were removed by centrifugation (10 min, 9600 g, 4°C) and the BR-containing membrane fraction was solubilized with 10 ml 1% (v/v) Triton X-100. Insoluble contaminants were removed by an additional centrifugation step (30 min, 13 000 g, 4°C).

Enrichment of BR and detergent exchange via adsorption/desorption on ion-exchange columns

Detergent exchange with concomitant enrichment of BR was achieved by adsorption/desorption to ion exchange resin as described previously (Scherler *et al.*, 1991; Pompejus *et al.*, 1993). 10 ml Triton X-100 solubilized membrane fraction was applied to a 2 ml Q-Sepharose column equilibrated with 1% Triton X-100, 10 mM potassium phosphate, pH 5.6. The resin was washed with 5 bed volumes of 1% (v/v) Triton X-100, followed by 5 bed volumes of 10 mM potassium phosphate, pH 5.6, 1% (w/v) OG. Wild-type and chimeric BRs were eluted with 2 ml 2 M NaCl, 1% OG, 1 mM potassium phosphate/phosphoric acid, pH 5.6. Chromophore concentrations were determined by absorption spectroscopy to be in the range 3–6 mM assuming that $\epsilon_{550} = 51\,000\text{ M}^{-1}\text{ cm}^{-1}$ (Rehorek and Heyn, 1979) for the entire series of samples.

Absorption spectroscopy and photocycle kinetics

UV/VIS absorption spectra of BRs were measured with a Uvikon 930 spectrophotometer (Kontron). The absorbance at 650 nm was used to define the zero absorbance baseline. Dark-adapted spectra were recorded with samples stored in the dark for 24 h. Light adaptation of BRs was performed by illumination with a 150 W illuminator (1 min, 10 cm distance, Schott OG 515 yellow filter). For photobleaching of the chromophore, the treatment was extended to 10 min. Fast difference spectra of BR and variants were measured as described earlier (Uhl *et al.*, 1985; Tittor *et al.*, 1987).

Preparation of proteoliposomes and measurement of light-driven proton translocation

4 mg asolectin were suspended in 200 μ l boiling water and sonicated under argon until clarity. To 136 μ l of vesicles preformed in this way, 50 μ l BR or variants (see above) and 14 μ l 0.5 M OG (final concentration 1%) were added and the mixture was kept on ice for 5 min. Proteoliposomes were formed by diluting this suspension in argon-purged 2 M NaCl. Typically, 75 μ l of the suspension were injected into 1 ml salt solution and kept on ice for at least 1 h (Hackett *et al.*, 1987). Light-driven proton pumping activities were measured as described by Pompejus *et al.* (1993) except that data were collected and processed with the help of the computer program Dalog (R.Merkel, this laboratory). In control experiments, the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to a final concentration of 3 μ M.

Immunofluorescence microscopy

Cells were fixed in liquid culture by adding formaldehyde to a final concentration of 3.7% (w/v), followed by addition of 0.1 vol 1 M potassium phosphate buffer pH 6.5. After 30 min incubation at 30°C, cells were collected by centrifugation and resuspended in 3.7% (w/v) formaldehyde, 0.1 M potassium phosphate buffer pH 6.5. After two washing steps with 0.1 M potassium phosphate buffer pH 6.5, cells were incubated for 30 min at 30°C in 10 ml 1.2 M sorbitol, 40 mM disodium hydrogen phosphate, 54 mM citric acid, 0.2% (v/v) β -mercaptoethanol, pH 5.6 containing 10 mg each 'lysing enzymes' and 'lyticase'. Residual enzyme was removed by washing with 10 mM Tris-HCl pH 7.6, 1.2 M sorbitol. *S. pombe* cells were attached to poly-L-lysine-coated coverslips and fixed with methanol and acetone as described by Harlow and Lane (1988) and Pringle *et al.* (1991). Fixed cells were incubated for 2 h with VII-E-7 culture supernatant, after which fluorescein-conjugated goat anti-mouse antibody was added (1:1000 dilution in PBS/Tween, 1 h incubation). Cells were mounted in 1% (w/v) *p*-phenylenediamine (Johnson and Nogueira Araujo, 1981) and

viewed with a Zeiss Axiophot microscope equipped with a 100 $\times$ objective and photographed with TMAX-400 film (Kodak).

Electron microscopy

Yeast cells were fixed in suspension with 2% (w/v) formaldehyde and 0.02% (v/v) glutaraldehyde in PBS, pH 7.2 for 1 h. Formaldehyde was freshly prepared by depolymerizing paraformaldehyde. After fixation, the cells were embedded in 10% gelatin and cubes of $\sim 1\text{ mm}^3$ were cut and infiltrated with 2.3 M sucrose. Cryosections of $\sim 100\text{ nm}$ thickness were made with a cryo-ultramicrotome (Ultracut/FC4 Reichert-Jung, Vienna, Austria) at about -120°C according to Tokuyasu (1973). The cryosections were collected on Formvar- and carbon-coated nickel grids. Immunolabelling was done as described previously (Humbel and Biegelmann, 1992). In short, the grids were incubated overnight with the supernatant of hybridoma cell cultures producing the VII-E-7 monoclonal antibody (Einberger *et al.*, 1990). Primary antibodies were detected with goat anti-mouse antibodies bound to ultra-small gold particles (Aurion, Wageningen, The Netherlands). In order to visualize the ultra-small gold particles they were enlarged by silver deposition as described previously (Stierhof *et al.*, 1990). The cryosections were embedded in methylcellulose containing 0.6% uranyl acetate (Tokuyasu, 1978).

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