The orientation of the light-driven chloride pump, halorhodopsin, in the membrane was determined using antibodies directed against a synthetic peptide which represents the C-terminal segment of the protein.

Antibodies against this decapptide did not bind to right-side-out cell vesicles. Partial inversion by sonication or lysis under low salt conditions exposed this COOH-terminal antigenic site. Antibody binding was removed by preincubation with the decapptide.

The COOH terminus of the molecule is therefore located on the cytoplasmic surface of the membrane.

The cell envelope of Halobacterium halobium is composed of two structures distinguishable by electron microscopy, the cytoplasmic membrane and a protein layer. It has been shown that at low salt concentrations the protein layer disintegrates, whereas the cell membrane is stable (provided a minimum concentration of Mg$^{2+}$ ions is present (1)). These conditions result in the production of right-side-out vesicles.

Located in the membrane are two ion-translocating retinal proteins, bacteriorhodopsin which is a light-driven outward directed proton pump and halorhodopsin (for review see Ref. 2), which, upon illumination, transports chloride ions into the cell interior (3, 4).

So far there is little information on the secondary structure of HR. The DNA-derived amino acid sequence of HR has enabled the prediction of a model of the secondary structure (see Fig. 1). In this model of Blanck and Oesterhelt (5) a long stretch of 20 COOH-terminal amino acids is thought to be exposed on the membrane surface.

Antibodies recognizing a peptide representing this COOH terminus combined with vesicles of defined orientation have been applied here to investigate the topology of HR.

EXPERIMENTAL PROCEDURES

Materials and Methods

H. halobium Strains—The strains used were OD-2, an HR-over-producing strain (6) and Flx-15, an HR" mutant (7).

Cell membrane fractions of strains OD-2 and Flx-15 were prepared according to the method described in Ref. 8; vesicles were prepared following the method described in Ref. 1.

Menadione reductase activity to determine vesicle orientation was tested with the assay described in Ref. 9.

HR was isolated according to the method described in Ref. 8 from strain OD-2. Carboxypeptidase-digested HR was prepared as described in Ref. 10.

Peptide Synthesis and Coupling to Keyhole Limpet Hemocyanin—

The peptide (corresponding to amino acid residues 263-272 of HR) was synthesized by a modified Merrifield solid phase strategy using fluorenyl-methoxy-carbonyl-protected amino acids (amino acids and Fmoc-protected Boc Amino Acids) on a semi-automatic Labotec peptide synthesizer SP 640 (11, 12).

The amino acid composition was determined after hydrolysis in 6 M HCl on a Kontron amino acid analyzer; its purity was further controlled by fast atom bombardment mass spectrometry on a Finnigan MAT 312 mass spectrometer.

The peptide was coupled to keyhole limpet hemocyanin according to the method described in Ref. 13.

Production of Antiserum

Rabbits were immunized with 300 µg of carrier-bound peptide at intervals of 4 weeks. Blood was taken 10 days after the last injection.

Enzyme-linked Immunosorbent Assays (ELISAs)

Standard ELISA—100 µl of an antigen solution (5 µg/ml HR, keyhole limpet hemocyanin-coupled peptide, or 20 µg/ml free synthetic peptide) were incubated in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, pH 7.2) overnight at 4°C in a Costar microtiter plate. After washing twice with PBS-Tween (PBS, to which 0.05% Tween 20 had been added) free sites on the plate were blocked using freshly prepared 0.1% bovine serum albumin in PBSM-Tween (PBSM with the addition of 0.05% Tween 20). The plates were again washed twice followed by incubation with goat-anti-rabbit peroxidase conjugate (Nordic immunologicals) diluted 1:1000 in PBS-Tween 1 (1 h, 20°C). After two washing steps, 100 µl of substrate solution was added (48.5 mM citrate, 103 mM Na$_2$HPO$_4$, pH 5.0, 0.4 µg/ml o-phenylenediamine, 0.4 µl/ml H$_2$O$_2$). The reaction was stopped by the addition of 50 µl of 1.5 M sulfuric acid. Then the absorbance (492 nm) was measured in an ELISA reader (SLT-Lab Instruments, Salzburg, Austria).

ELISAs with Vesicles and Membranes of OD-2 and Flx-15—Membrane fractions and vesicles of OD-2 were prepared as described above. The ELISA was carried out as described above with the exception that a slightly modified buffer system was necessary in order to retain vesicle integrity, namely PBSM (100 mM MgCl$_2$, 70 mM NaCl, 1.35 mM KCl, 0.75 mM KH$_2$PO$_4$, and 4 mM Na$_2$HPO$_4$) instead of PBS and PBSM-Tween (PBSM with the addition of 0.05% Tween 20) replacing PBS-Tween. For antigen coating of microtiter wells, either vesicles or disrupted vesicles (which had been sonified for 10 min in a sonification bath (Bransonic 12)) were incubated at a protein concentration of 0.15 mg/ml, whereas membranes were incubated at 0.05 mg/ml in PBSM overnight.

Competition ELISA—Serum (1:1000 in PBSM-Tween) was preincubated (1 h, 20°C) with known amounts of peptide in a bovine serum albumin-blocked microtiter plate and transferred to a microtiter plate coated with sonified vesicles, followed by the standard ELISA techniques.

RESULTS AND DISCUSSION

A peptide representing the 10 carboxyl-terminal amino acids of HR has been synthesized (see Fig. 1).
Orientation of Halorhodopsin in the Membrane

FIG. 1. Secondary structural model of HR as proposed by Blanck and Oesterhelt (5). The protein is thought to traverse the membrane in seven helical rods beginning with its NH₂ terminus in the periplasm. The position of the synthetic peptide is indicated by the dotted area.

FIG. 2. Comparison of the binding of COOH-terminus-specific antibodies onto cell vesicles and membranes from OD-2 and Flx-15 cells. The antibody binding was measured by the ELISA technique at increasing serum dilution. Note that sonication liberates an antigenic site in the vesicles and that vesicles of the HR- strain Flx-15 show no antibody reaction.

On immunoblot analysis antibodies raised against this peptide bound onto purified HR and onto an identical band in extracts of cell membranes from HR+ strain OD-2. Removal of the COOH terminus of HR with carboxypeptidase treatment removed binding. This confirms that the antibody binding is specific for the COOH-terminal part of HR (data not shown).

In order to localize the COOH terminus of HR, antibody binding studies were performed. For this purpose a defined orientation of vesicles was indispensable. With a menadione reductase (9) assay, the orientation of surface layer-depleted cell vesicles was determined to be approximately 90% right-side-out. After sonication this value dropped to about 60%.

In an ELISA antibody binding was compared between HR-containing membranes and surface layer-free cell vesicles. As can be seen in Fig. 2 the anti-peptide antibody bound with high affinity to membranes of strain OD-2. The same antibody showed reduced, but significant, binding (approximately a 5-fold reduction) to sonified vesicles of OD-2 (see Fig. 2). The reduction in antibody binding in vesicles compared to the membrane preparation may be due to a weaker adsorption of vesicles to the microtiter wells and greater tendency to be washed off during the assay.

No detectable binding was observed onto untreated vesicles of OD-2 (see Fig. 2), indicating that a cytoplasmically located antigenic determinant first became accessible after sonication.

In order to exclude the possibility of unspecific binding of antibody onto sonified vesicles or membranes, the experiments were repeated using the HR- mutant H. halobium strain Flx-15. The antiserum failed to bind onto such preparations showing that HR alone was responsible for the antibody recognition (see Fig. 2).

Preincubation of the COOH-terminal-specific antibody with the synthetic peptide removed, in a titratable fashion, the affinity of the antibody for the sonified OD-2 vesicles as shown in Fig. 3.

These data show that the COOH terminus of HR as an antigenic determinant only becomes accessible to antibodies after partial inversion of the right-side-out vesicles by sonication (whereupon the cytoplasmic membrane becomes exposed to the exterior). Thus it can be concluded that the COOH terminus of HR is located at the cytoplasmic side of the membrane.

This result for the orientation of HR in the membrane is in agreement with protease digestion studies that were performed in parallel by Schobert et al. (10).

Blanck and Oesterhelt (5) postulate in their HR model seven transmembrane helices derived from hydrophilicity and acrophilicity predictions and from comparison with bacteriorhodopsin. It is likely that the topology of HR is similar to that of bacteriorhodopsin; both proteins have a cytoplasmically orientated COOH terminus and, by analogy, HR is thought to traverse the membrane in seven helical rods that are interconnected by loop structures. The NH₂ terminus would then be expected to be periplasmically orientated.

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REFERENCES