# Bacteriorhodopsin Can Function without a Covalent Linkage between Retinal and Protein

Ulrike Schweiger, Jörg Tittor,* and Dieter Oesterhelt

*Max-Planck-Institute for Biochemistry, D-82152 Martinsried, FRG*

*Received August 16, 1993; Revised Manuscript Received November 1, 1993*®

ABSTRACT: Light energy is transferred from retinal to the protein in bacteriorhodopsin after absorption of a photon resulting in changes of protein conformation. To examine whether the covalent bond, formed by the carbonyl group of retinal and the ε-amino group of lysine 216, is essential for this process, a mutant with lysine 216 replaced by alanine was expressed in *Halobacterium salinarium* L33 ($BO^-$, $retinal^+$). Reconstitution of the chromoprotein with varying retinylidene-*n*-alkylamines was possible in isolated membranes as well as in whole cells. When the protein in membranes with retinylidene Schiff bases of *n*-alkylamines of different lengths was reconstituted, the most stable chromoprotein was formed with retinylideneethylamine. The absorbance maximum was at 475 nm in alkaline solution and 620 nm in acidic solution. At neutral pH values both species equilibrate with a third one absorbing maximally at 568 nm. Reconstitution of whole cells with retinylideneethylamine led to a specific proton pump activity of 30 mol of protons per mol of BR per minute. This value indicates a lower limit of transport; no light saturation could be reached in these measurements in contrast to wild-type BR where transport activities of 162 mol of protons per mol of BR per minute under identical conditions can be achieved. Action spectra from flash photolysis experiments revealed that only the 568-nm form led to a M-intermediate with a half-time of decay of 17 ms. In summary, it could be shown that the covalent linkage between retinal and the protein is basically not required for the function of bacteriorhodopsin as a light-driven proton pump.

The retinal protein bacteriorhodopsin functions as a light-driven proton pump in the plasma membrane of halobacteria and can be isolated as a crystalline array called the purple membrane (PM).[1] The chromoprotein consists of 248 amino acids with retinal covalently linked to the ε-amino group of lysine 216 via a Schiff base (SB) (Mullen et al., 1981; Lemke & Oesterhelt, 1981; Katre et al., 1981; Bagley et al., 1981). The retinal can be removed from the native protein with hydroxylamine to give the apoprotein BR and retinaloxime. By subsequent addition of retinal to the apomembrane, the chromophore is reconstituted. This reaction has been used to study the specificity of the retinal–protein interaction in great detail (Schreckenbach & Oesterhelt 1977; Schreckenbach et al., 1977, 1978; Towner et al., 1981). As a summary the following three step model for reconstitution was developed. The species have been designated by the value of their maximal absorbance:

$$\text{retinal} + \text{BO} \xrightarrow{a} 400\text{ nm} \xrightarrow{b} 430/460\text{ nm} \xrightarrow{c} 568\text{ nm}$$

In reaction a, the retinal is noncovalently fixed in the binding pocket, and planarization of the cyclohexene ring with the polyene chain occurs (Schreckenbach et al., 1978). This reaction is mediated by a still unidentified basic group with a p*K* of 3.8. Reaction b is not understood in molecular terms; however, it is known that the 430/460 chromophore is not reduced with borohydride, indicating that the carbonyl group of the retinal is no longer accessible as it was in the 400 nm chromophore. Furthermore, the reaction is blocked for the 9-*cis* isomer. Reaction c is the rate-limiting step in the overall process and is characterized by the formation of the SB (Gärtner et al., 1983) resulting in a protonated SB with a p*K* >13 (Kalisky et al., 1977). This reaction is exclusive for *all-trans*- and 13-*cis*-retinal. A second basic group with a p*K* of 4.6 was required for this step and was suggested to be the lysine 216 itself (Schreckenbach et al., 1978).

The large "opsin" shift (Nakanishi et al., 1980) of the absorbance maximum in reaction c from 460 to 568 nm was explained in part by the increased distance of the protonated SB and a negatively charged counterion in the protein. The counterion was suggested to be Asp212 or Asp85 (Mogi et al., 1988; de Groot et al., 1990; Subramaniam et al., 1992).

An important question regarding the maintenance of the covalent linkage of the retinal during the catalytic cycle is of concern. In the longest living intermediate of the BR photocycle, the M-state, the retinal remains linked to the protein via the SB (Lewis et al., 1974). The photocycle is completed within 5 ms under physiological conditions, and this velocity is attributed to the energy stored in the protein involving a twist of the lysine side chain. Experimental evidence from FTIR measurements suggest that also a twist around the C=N bond exists (Gat et al., 1992). BR and the closely related chloride pump halorhodopsin (HR) are the only known cases in nature where retinal thermally reisomerizes in the millisecond time range, and in both cases the SB is not hydrolyzed during the photocycle. The question therefore arises whether these two phenomena correlate.

An additional interesting point which addresses the energy coupling of the retinal–protein interaction in the isomerization reactions and the transfer of the energy to the protein to drive the back-reaction can also be raised. If the energy is

* Author to whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* December 15, 1993.

[1] Abbreviations: RSB, retinylidene-*n*-alkylamine as an endogenous Schiff base; SB, Schiff base as a functional group in chemical terms; BO, bacterioopsin; BR, bacteriorhodopsin; HR, halorhodopsin; PM, purple membrane; K216A, bacteriorhodopsin with lysine 216 replaced by alanine; FTIR, Fourier transform infrared spectroscopy; CCCP, 3-(carbonyl cyanide 3-chlorophenylhydrazone).

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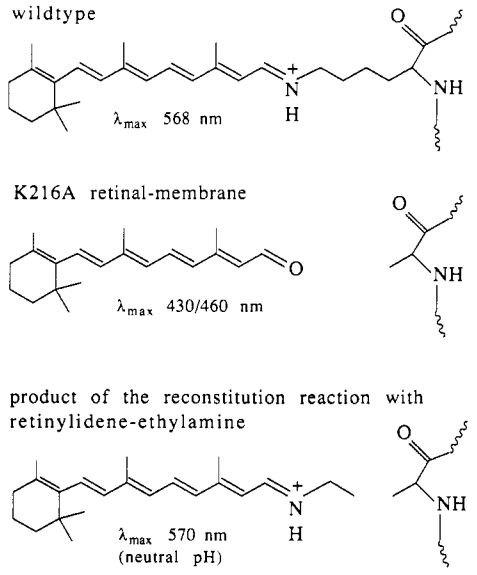


FIGURE 1: Retinal chromophores in the wild-type and mutant K216A before and after addition of retinylideneethylamine.

transferred other than via the covalent linkage, the SB would not be a prerequisite for function.

Essentially there are two models for the retinal isomerization sequence in BR, the C-T model and the 13,14-di-*cis* model (Fodor et al., 1988; Zhou et al., 1993). For a thorough discussion of both proposals, see Zhou et al. (1993). If the retinal isomerizes around the 13,14 double bond alone (as discussed in the C-T model) a larger compensative twist in the lysine side chain must therefore occur.

To address the question concerning the necessity of the covalent bond between retinal and lysine 216 in bacteriorhodopsin, the lysine residue was changed to alanine and the protein was homologously expressed. Due to this alteration, no retinal could be covalently bound in the binding pocket. Retinylidene-*n*-alkylamines (RSB)[2] were added and replaced the retinal. In this way a situation similar to the wild-type was created, containing a SB but no covalent linkage between retinal and the protein (Figure 1). The mutation of lysine to alanine was chosen because alanine was a smaller residue than lysine, and, after reconstitution with the RSB, the polarity inside the binding pocket was not changed dramatically. The resulting chromoproteins were examined for function and characterized spectroscopically. Similar experiments have been reported for rhodopsin, and it was shown that the covalent linkage was not required for activation of the visual cascade (Zhukovsky et al., 1992).

## EXPERIMENTAL PROCEDURES

All chemicals used were of analytical grade.

*Mutagenesis and Expression.* Site-directed mutagenesis of the *bop*-gene was performed according to Ferrando et al. (1993). The mutant construction in *Escherichia coli* was confirmed by nucleotide sequence analysis (DNA-Sequencer 374A, Applied Biosystems).

*Halobacterium salinarium* L33 (Wagner et al., 1981) was used for transformation (Cline et al., 1991). Expression of wild-type and mutated bacterioopsin was as previously described (Ni et al., 1990; Ferrando et al., 1993). Transformed *Halobacterium* cells could be distinguished from untransformed ones by the use of a monoclonal antibody binding specifically to the C-terminus of bacterioopsin according to Oesterhelt and Krippahl (1983). For a confirmation of the mutation, the total DNA from the transformants was isolated (Vogelsang et al., 1983), and the *bop* gene was amplified by PCR and sequenced. The expression level of the mutated chromoprotein was determined by western blot analysis (Oesterhelt & Krippahl, 1983) and was found to be 5 mg = 180 nmol of BR per liter of cell culture. This is 20% of the expression level of bacteriorhodopsin in L33 transformed with the *bop*-wild-type plasmid (Ferrando et al., 1993).

*Synthesis of Schiff Bases.* Preparation of the retinal Schiff bases (RSB) was according to Ball et al. (1949). The amines were checked for purity by determination of the refractive index and were used without further purification. The *all-trans*-retinal was dissolved in 2-propanol, and the concentration was measured spectroscopically, using a molar extinction coefficient of 48 900 at 368 nm in *n*-hexane (Groenendijk et al., 1980). The Schiff bases were stored at 4 °C in alkaline solution in the presence of an excess of the amine to prevent hydrolysis (Morton & Pitt, 1955).

*Membrane Fractionation.* BR containing membrane were isolated as described by Oesterhelt and Stoeckenius (1974) for PM. The resulting membrane fraction is called the retinal membrane. The protein to chromophore ratio of absorbance at 280 and 460 nm was 2.65:1.

Apomembranes were prepared from retinal membranes by reaction with hydroxylamine as described (Oesterhelt, 1982) but without illumination. Retinaloxime was removed by the use of bovine serum albumin (Katre et al., 1981) in the presence of 0.1 M sodium chloride.

*Retinal Extraction and Analysis.* Retinal was extracted and analyzed by high-performance liquid chromatography according to Fischer et al. (1981). Illumination conditions employed a 455-nm cut-off filter (24 mW/cm$^2$, 30 min) and a 450-nm cut-on filter (15 min, 0.15 mW/cm$^2$) for blue light illumination.

*Preparation of RSB Membranes.* Apomembrane protein (18 nmol) (determined by western blot analysis) or retinal membrane was mixed with an equimolar amount of the RSB in a total volume of 1 mL which contained 100 mM sodium borate buffer pH 9.5 at 20 °C in the dark to yield the RSB membrane.

*Determination of $pK_a$ Values.* The $pK_a$ values were determined using two different methods for adjustment of pH value. To prevent aggregation during acid–base titration, the retinal membranes were embedded in a polyacrylamide gel in 10 mM sodium phosphate buffer as described (Fischer & Oesterhelt, 1979). The acid–base titration was performed at 20 °C with 0.1 N sulfuric acid.

A second method to adjust the pH value was used in p*K* determinations of the RSB membranes. Aliquots of 10 mM hydrochloric acid were added to the membrane suspensions in 10 mM sodium borate and 10 mM sodium chloride. The spectra were recorded 30 s after addition of the acid. To prevent degradation, and hence to improve the stability of the protein, the titration was performed at 7 °C.

*Spectral Measurements.* Absorption spectra during acid–base titration were recorded on an Aminco DW2000 spectrophotometer (American Instrument Co., Silver Spring, MD) at a slit width of 3.0 nm. For recording spectra during reconstitution reactions, the same apparatus was used as for

[2] Throughout this report we denote reconstitution as the reaction of retinylidene-*n*-alkylamines or retinal with apoprotein resulting in the occurrence of a red-shifted absorbance maximum compared to that of free retinal or retinylidene-*n*-alkylamine in solution. This indicates an interaction between the protein and the prosthetic group and therefore the reconstitution of the chromophore.

examination of the photochemistry (see below). The data were processed and fitted with the computer program Sigma Plot.

Flash photolysis experiments were carried out in a multichannel flash photolysis apparatus. The reconstituted chromoprotein of the RSB membrane was excited with a laser flash of 18 ns (excimer-dye laser-system EMG100, FL 3002, Lambda Physics, Göttingen, FRG). The time resolution was 130 μs (Uhl et al., 1985).

For the action spectrum, the wavelength of the laser flashes was varied from 460 to 620 nm in steps of 5 nm. The maximal absorbance changes at 390 nm after the laser flash were related to the number of photons of the exciting laser flash according to Plancks formula and normalized. Due to its instability, the chromoprotein was freshly reconstituted for each set of experiment.

*Reconstitution of the Chromoprotein in Whole Cells.* After centrifugation, the cells were resuspended in basal salt to an optical density at 578 nm of 4 (Oesterhelt, 1982). The Schiff base solution (RSB) was neutralized prior to use to pH 9.5–10 with concentrated hydrochloric acid. Retinylideneethylamine (130 nmol/mL) was added to the cell suspension, and the suspension was maintained in the dark. To remove the excess of RSB, the cells were centrifuged and washed with basal salt adjusted to pH 8.75.

*Determination of BR Activity in Whole Cells.* The measurement of proton pump activity was performed according to Oesterhelt (1982) at 20 °C, pH 8.0. Copper sulfate solution (1%, $d$ = 1 cm) and a cut-off filter (570 nm) were used to select actinic light from the light source (200 W Hg–Xe lamp 901 B0011; Engelhard Hanovia Inc., Newark, NJ). The light intensity on the sample was 2 W/cm$^2$, which was not sufficient for light saturating conditions. For determination of the specific BR activity, the protein concentration was measured by western blot analysis. A quantitative reconstitution reaction was assumed. It was confirmed by a control experiment that it is possible to measure bacteriorhodopsin activity in L33, although it contained HR. In a suspension of untransformed L33 cells, the HR activity was quenched completely in the presence of 0.25 mM tetraphenylphosphonium bromide as used in the BR assay.

## RESULTS

*Characterization of Retinal Membrane from K216A.*[3] Isolation of a membrane fraction following the standard protocol for purple membrane (Oesterhelt & Stoeckenius, 1974) yielded two bands on a sucrose gradient, one with a buoyant density of PM and a second membrane fraction with a density of 1.13 g/cm$^3$. The PM fraction was further examined and was found to contain two main absorbance maxima at 430 and 460 nm and shoulders at shorter wavelengths (Figure 2, spectrum 1). This absorbance pattern resembled an intermediate occurring during the reconstitution of wild-type apomembrane and *all-trans*-retinal. A third peak, which was smaller, had an absorbance maximum at 630 nm. There was a strong accordance of this spectrum with that reported by Alex et al. (1992) who dissolved *all-trans*-retinal in chloroform or *n*-hexane in the presence of excess trifluoroacetic acid. The putative significance of the similarity of the spectra is discussed below.

[3] Retinal membrane is the membrane fraction of bacteriorhodopsin K216A isolated from *Halobacterium salinarium* L33 under PM conditions. RSB membranes contain mutated bacterioopsin K216A reconstituted with the denoted retinylidene-*n*-alkylamines.

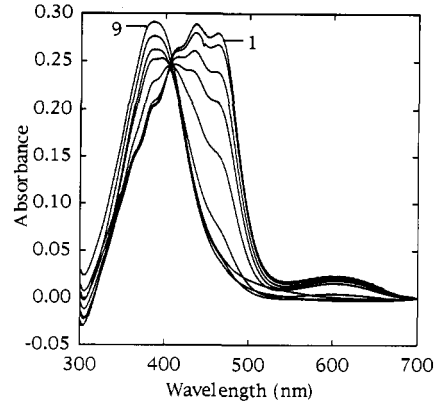


FIGURE 2: pH dependence of the absorbance maximum of the retinal membrane. Spectra were recorded in 10 mM sodium chloride and 10 mM sodium phosphate from pH 5.6 to 2.0 after adjustment of pH with 10 mM hydrochloric acid. From 1 to 9: pH 5.6, 5.00, 4.7, 4.3, 4.1, 3.7 3.5, 3.0, 2.0. The p$K$-value was determined as 3.9 (plot not shown).

Titration of the retinal membrane to lower pH values resulted in a loss of the absorbance at 430/460 nm and the appearance of a species absorbing maximally at 387 nm (Figure 2, spectrum 9). The p$K$ for this transition was at 3.9 and was identical to the one found for a functional group involved in ring-chain planarization in the wild type (Schreckenbach et al., 1978). Interestingly, the absorbance maximum at 630 nm vanished with the same p$K$ as the one at 430/460 nm. Moreover, the described spectroscopic behavior was strikingly identical to that of wild-type apomembrane mixed with 11-*cis*-retinal when considering the $\lambda_{max}$ values, the fine structure, and the p$K$ values of the transition (Schreckenbach et al., 1977).

Removal of the endogeneous retinal from the membranes was performed by reaction with hydroxylamine and extensive washing yielding apomembranes. When these membranes were mixed with *all-trans*-retinal at room temperature, a time constant of 5 s for the formation of the 430/460 nm chromophore was found, identical to the one for the 430/460 nm chromophore reconstitution reaction of wild-type apomembrane with *all-trans*-retinal. The similarity of the double absorbance maximum 430/460, the p$K_a$ of the transition to 387 nm, and the kinetics of its appearance between wild-type and mutant K216A justifies the assignment of this species in the mutant to a planarized retinal in the binding pocket of the apoprotein.

Illumination of the membranes with continuous white light (10 mW/cm$^2$) resulted in bleaching of the chromophore. The appearance of an absorbance around 380 nm in the difference spectrum indicated an abolishment of the ring-chain planarization upon photoisomerization.

Indeed, 82% of the retinal extracted from the dark-adapted membrane fraction was *all-trans*. Illumination (455 nm cut-off filter, 24 mW/cm$^2$, 30 min) resulted in the following retinal isomers: 21% 13-*cis*, 17% 11-*cis*, 27% 9-*cis*, and 36% *all-trans*. The occurrence of 27% 9-*cis*-retinal species and the absorbance maximum around 380 nm are responsible for the bleaching of the chromoprotein as described above. This was due to the inability of the 9-*cis* isomer to lead to a planarized ring chain configuration (Schreckenbach & Oesterhelt, 1977). Subsequent illumination with blue light (450 nm cut-on filter, 0.15 mW/cm$^2$, 15 min) increased the amount of *all-trans*-retinal (to 62%) whereas the amount of 11-*cis* and 9-*cis*-retinal decreased to 4 % and 6%, respectively. The amount of 13-*cis*-retinal was less affected by blue light (28%).

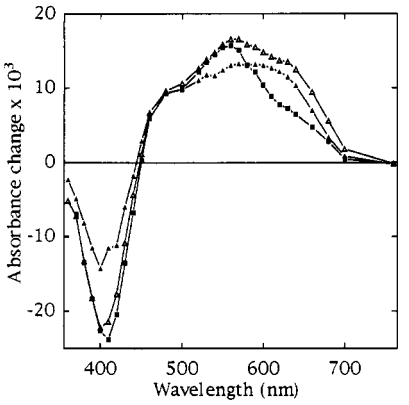


FIGURE 3: Chromophores formed after mixing of apomembranes with methyl-RSB (closed squares), ethyl-RSB (open triangles), and *n*-propyl-RSB (closed triangles). Equal molar amounts of the RSBs and apomembrane were used in each case. The spectra shown were recorded 4 min after addition of the respective RSB.

Table 1: Time Constants for the Reconstitution of the Chromoprotein with Various Schiff Bases and Apomembrane

| Schiff base | time constants |
|---|---|
| retinylidenemethylamine | 31 s |
| retinylideneethylamine | 44 s |
| retinylidene-*n*-propylamine | 79 s |

*Reconstitution of Retinylidene Schiff Base Membranes.* Retinal membranes and apomembranes were used in a reconstitution reaction with retinylidene-*n*-alkylamines. RSBs made from retinal and *n*-alkylamines of different length (methyl, ethyl, *n*-propyl) were mixed with the membranes in a molar ratio of 1:1. The formation of a chromophore with an absorbance maximum shifted bathochromic, compared to the RSB in solution, was observed in each case. Figure 3 shows the difference spectra of the reconstitution reaction of apomembranes with the respective RSB at pH 9.2. The chromophores formed with methyl-RSB and ethyl-RSB had difference maxima at 475 nm in alkaline and at 620 nm in acidic solution. In the neutral pH range both species were present together with an additional one at 568 nm. The chromophore employing the *n*-propyl-RSB had the same difference maxima at alkaline and acidic pH values, but not at neutral pH values. Time constants of 44 s for the monoexponential increase of absorbance at 620 nm were determined for the reaction of apomembrane with ethyl-RSB and 167 s at room temperature for the reaction with the retinal membrane. The time constants of the reconstitution reaction with RSBs increased with increasing length (Table I). The reconstituted chromoproteins were stable for at least 3 h if kept in complete darkness.

*Properties of the Chromoprotein Reconstituted from Apomembrane and Ethyl-RSB.* All further experiments were carried out with the chromoprotein containing ethyl-RSB because it spectroscopically mostly resembled the wild-type and comes close to the number of carbon atoms in the binding site. The difference of one methylene group is compensated by the two hydrogens of the ethyl and the alanine moieties.

*(a) pH Dependence of Chromoprotein Absorbance.* The acid–base titration of the chromoprotein was complicated by the following restrictions: (1) Free RSB changes its absorbance maximum from 365 to 400 nm upon titration with 0.1 N hydrochloric acid (p*K* 9). (2) The free RSB hydrolyzes at neutral pH values and is stable only at alkaline or acidic pH values (Morton & Pitt, 1955). (3) Reconstituted chromoprotein is stable for less than 2 h at room temperature. To slow down degradation, measurements were performed at 7 °C.

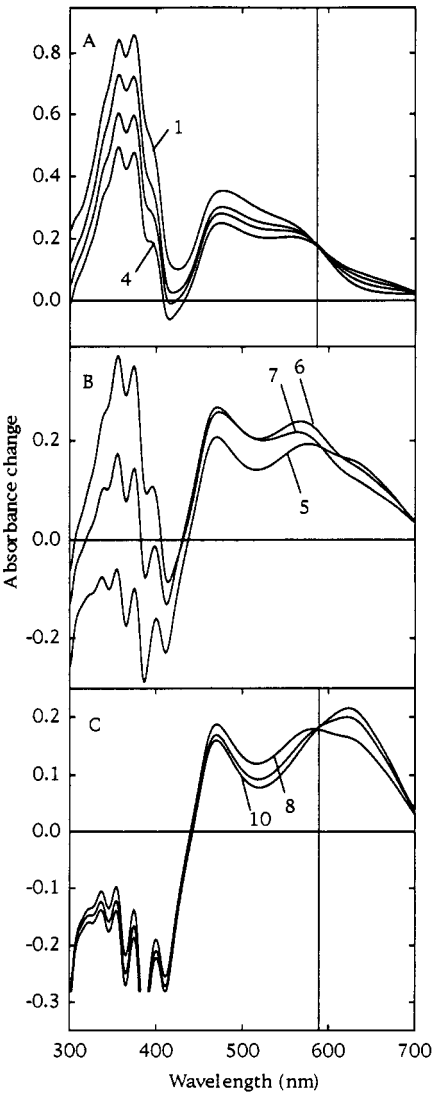


FIGURE 4: pH dependence of chromophore absorption in ethyl-RSB. Spectra from the apomembrane (10–15 nmol) mixed with 10 nmol of retinylideneethlylamine were recorded between pH 3.95 and 11.6 (7 °C, 10 mM sodium chloride and 10 mM sodium phosphate, titration with 10 mM hydrochloric acid). Control experiments of retinylideneethylamine without apomembranes showed excessive absorbance changes between 300 and 450 nm. (A) Spectra at pH 11.6 (1), 10.9 (2), 10.3 (3), and 9.5 (4) showing the transition of a chromophore absorbing maximally at 475 nm to a chromophore absorbing at 620 nm with an isosbestic point at 592 nm. (B) Spectra at pH 9.2 (5), 7.9 (6), and 6.1 (7) showing the chromophore absorbing maximally at 568 nm. (C) Spectra at pH 5.6 (8), 5.0 (9), and 3.95 (10) with an isosbestic point at 597 nm.

At alkaline pH values the absorbance maximum of the chromoprotein was at 475 nm (alkaline form) and changed to 620 nm at acidic pH (acidic form). Only in the range of 11.60–9.50 and 5.61–3.95 (Figure 4 A,C) this transition showed isosbestic points at 592 and 597 nm, respectively. In the neutral pH range, a third species absorbing maximally at 568 nm (neutral form) interfered with the transition of the alkaline and acidic form (Figure 4B). Absorbance changes were corrected for the above described pH dependence of the free RSB and analyzed at 620, 568, and 475 nm from pH 4 to 11 (Figure 5). p*K* values for the described transitions were found at 8.8 (from alkaline to the neutral form) and 6.6 (from neutral to the acidic form). Due to the complexity of the reactions occurring concomitantly during the pH titration, we estimate the accuracy of the p*K* values as ±0.3 units.

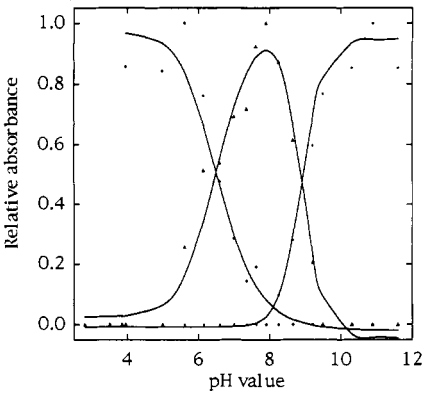


FIGURE 5: Quantitation of the spectral transitions shown in Figure 4A–C. The relative absorption changes at 475 nm (closed squares), 568 nm (closed triangles), and 620 nm (closed rhombs) are plotted versus pH.

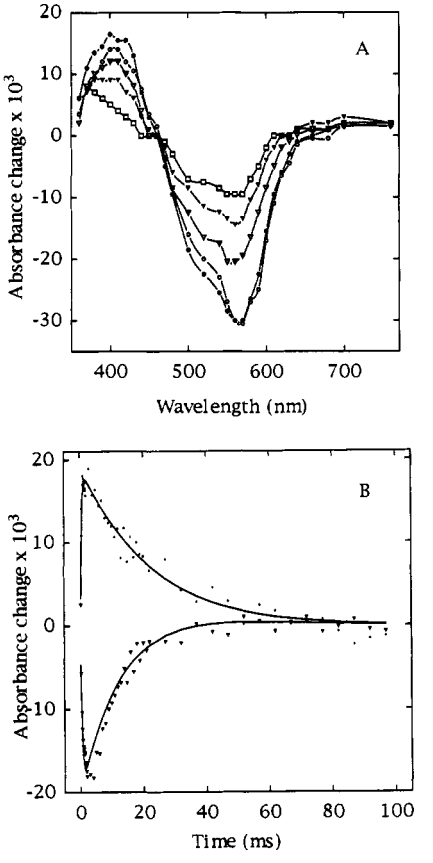


FIGURE 6: Flash photolysis of ethyl-RSB membrane in 0.1 M Tris, pH 8.0. Photobleaching by the measuring light was corrected for by substraction, and the resulting spectra at distinct times after excitation are shown as (A) 0.65 ms, closed circles; 1.3 ms, open circles; 7 ms, open triangles; 12 ms, closed triangles, 22 ms open squares. (B) Kinetics of the absorbance at 390 nm (closed circles) and 570 nm (closed triangles). The solid line represents the simultaneously fitted curves with three exponential functions. The parameters are given in Table 2.

*(b) Flash Photolysis.* Flash induced difference spectra taken at different times (0.65, 1.3, 7, 12, and 22 ms) after flash excitation of ethyl-RSB membranes at pH 8.0 are shown in Figure 6 A. An intermediate absorbing maximally at 410 nm clearly occurred and decayed. The kinetics recorded at 390 and 570 nm are shown in Figure 6B and were fitted simultaneously by a sum of three exponential functions. The time constants and the amplitudes at the respective wavelengths are given in Table 2. The action spectrum in Figure 7 shows that the M-intermediate was exclusively formed upon excitation of the 568-nm species. Excitation of the 475-nm chromophore by measuring light lead to the occurrence of a species which absorbs maximally at 610 nm in the difference spectra (data not shown). This decayed slowly (in the minute time range), and therefore the photoreaction of the 475-nm chromophore is the dominating one upon steady-state illumination with light >500 nm. Consequently, this was the main photoreaction observed and had to be accounted for to yield the one shown in Figure 6A. The photoreaction of the 620-nm chromophore led to a species absorbing in the difference spectrum maximally at 500 nm with an amplitude that reflects 20% of the decreased 620 nm absorbance.

Table 2: Result of Fitting the Laser-Induced Absorbance Changes Occurring during the Photoreaction of the 568-nm Chromophore Reconstituted from Apomembrane and Retinylideneethylamine with Three Time Constants[a]

| | time constants | | |
|---|---|---|---|
| | $\tau_1$ | $\tau_2$ | $\tau_3$ |
| | 0.4 ms | 15 ms | 19 ms |
| | relative amplitudes | | |
| | $i$ = 1, 390 nm | | $i$ = 2, 570 nm |
| $b_i$ | 0.32 | | 0.59 |
| $c_i$ | 0.68 | | 0.41 |

[a] The following equation was used: $f_i = a_i \exp(-t/\tau_1) + b_i \exp(-t/\tau_2) + c_i \exp(-t/\tau_3)$

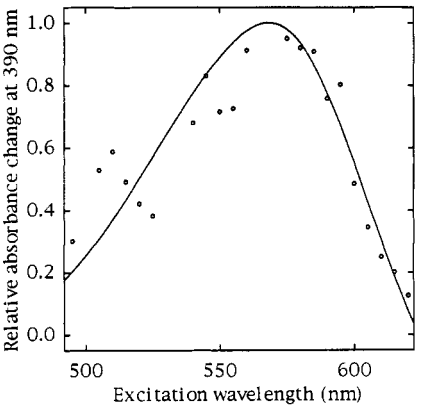


FIGURE 7: Action spectrum of maximal absorbance change at 390 nm after laser flash. The apomembrane of K216A reconstituted with retinylideneethylamine was excited by a laser flash at wavelengths in the range from 470 to 620 nm. The absorbance changes and the energy of the laser were measured, and the energy of excitation according to $E = hc/\lambda$ and the absorption change per photon were calculated and normalized. The solid line represents a standard purple membrane absorbance spectrum for comparison.

*Measurement of Proton Pump Activity in Cell Suspensions.* For measurement of the proton translocating activity of the chromoprotein retinylideneethylamine was added to cell suspensions. To prevent lysis of the cells, the pH value of the RSB has been adjusted to 9.5–10 before the addition. Light-induced pH changes in the medium are shown in Figure 8. Upon illumination (570 nm cut-off filter, 2 W/cm$^2$) a specific proton pump activity of 30 nmol of protons per nmol of BR per minute was measured. Illumination of the same cell suspension a second time produced about half of this value. Subsequent illuminations resulted only in a minor further decrease of activity. The total amount of protons pumped in the photostationary state showed a similar behavior as the initial rate. No pH changes could be measured upon illumination before addition of retinylideneethylamine.

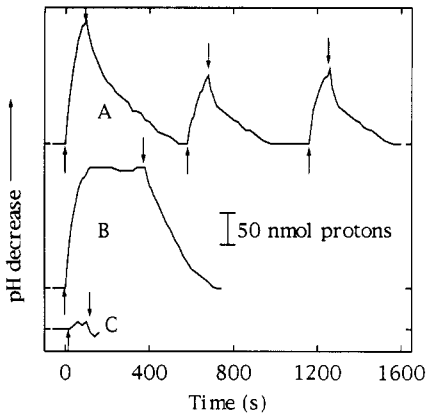


FIGURE 8: Light-induced acidification of a suspension of *H. salinarium* L33 K216A cells after adding retinylideneethylamine as described under Experimental Procedures. (A) Illumination was repeated for three times, and every light period (100 s) was followed by 480 s in the dark. (B) The cells were illuminated for 380 s. (C) After addition of CCCP to a final concentration of 0.05 mM, no proton translocation activity was detected.

## DISCUSSION

*Characterization of the Retinal Membrane.* Upon introduction of mutation K216A in bacteriorhodopsin, a fraction with the same buoyant density as the purple membrane could be isolated. The mutated protein with retinal noncovalently bound forms a crystalline lattice as in wild-type BR, and the buoyant density gives an additional hint that the structural organization of the protein is not drastically altered.

The retinal membrane had the same properties as an intermediate occurring during reconstitution of wild-type apomembrane with *all-trans*-retinal with respect to three points: (a) a double absorbance maximum at 430/460 nm, (b) the same $pK_a$ value for the transition to the 387-nm form, and (c) the same time constant for the increase of absorbance at 430/460 nm upon addition of retinal or RSB to the wild-type or mutant apomembrane, respectively. In the intermediate formed during reconstitution, the retinal molecule is proposed to be planar (Schreckenbach et al., 1978) but not yet covalently bound to the protein (Gärtner et al., 1983). In addition, the fine structure of the absorbance spectrum of the retinal membrane resembles the product of reconstitution of wild-type apomembrane and 11-*cis*-retinal (Schreckenbach et al., 1977). In wild-type, no 568-nm chromophore is formed upon addition of 11-*cis*-retinal because of steric hindrance. The origin of the absorbance shoulders at 412, 384, 363, and 344 nm is only known for that at 412 nm. It is the Soret band of a cytochrome occurring as impurity in the membrane preparation. Both the wild-type apomembrane with 11-*cis*-retinal and the mutant retinal membrane the chromophores contain free retinal of different geometry, but this geometry apparently does not influence the spectral behavior.

The spectrum of the retinal membrane had a striking similarity with a spectrum of *all-trans*-retinal in chloroform or *n*-hexane in the presence of a 2000- or 3000-fold excess of trifluoroacetic acid (Alex et al., 1992). Thus, the three main peaks at 430, 460, and 630 nm of the chromoprotein can be interpreted in the terms of hydrogen bonding and a protonated retinal interacting with anionic groups of the protein. Aspartic acids 212 and or 85 would be suitable candidates. The absorption maximum at 630 nm has not been observed during reconstitution of wild-type BR.

*Significance of the $pK_a$ values of the RBS Membranes in Comparison to Wild-Type.* The chromophore in wildtype and mutant can be discussed in terms of a proton donor/acceptor complex (Fischer & Oesterhelt, 1979). The $\pi$-electron system of retinal is affected by two protonatable groups leading to shifts of the absorbance maximum between 605 nm (at acidic pH values) and 460 nm (at alkaline pH values). In K216A RSB membranes these values are 620 and 475 nm, respectively. As the proton donor, the Schiff base was assigned (Druckmann et al., 1982). Asp85 could be identified as a proton acceptor upon acidification (Metz et al., 1992). We have no indication that the proton acceptor has changed in the mutant K216A. Moreover, removal of the carboxylic group by site-directed mutagenesis led to similar absorbance shifts of the chromophore as did its protonation (Mogi et al., 1988; Lanyi et al., 1992; Subramaniam et al., 1992; Turner et al., 1993).

In the mutant BR $pK_a$ values of 6.6 (for the transition 620 to 568 nm) and 8.8 (568 to 475 nm) were found. A mixture of chromophores therefore exists under physiological conditions and narrows the range of existence of the active 570-nm chromophore. The respective $pK_a$ values for the wild-type are 3.2 (605–568 nm) and $13.3 \pm 0.3$ (568–460 nm) in aqueous suspension of purple membranes (Druckmann et al., 1982) stabilizing the active chromophore species over the entire physiological pH range. These shifts were also reported for all mutations in the vicinity of the protonated SB. For example, changes of Asp85, Arg82, and Asp212 (Lanyi et al., 1992; Subramaniam et al., 1992; Turner et al., 1993) led to chromophores where the transitions of different chromophore species merged toward neutral pH values. Obviously the electrostatic interactions defining the extreme p*K* values found in the wild-type are disturbed in quite different mutations.

*Photocycle Measurements.* The main observations made were (a) an M intermediate did occur, (b) exclusively the 570-nm species produced M, (c) M-decay was still the rate-limiting step in the photocycle, and (d) M decayed in 17 ms to the initial state. The recovery of the ground state was a factor of about 1.5 slower than the reaction in wild-type under identical conditions (data not shown). This result further implied that the thermal reisomerization (the transition from N to O in wild-type) was not slowed because in this case it would have been detected instead of the M decay as the rate-limiting reaction.

Species absorbing maximally around 610 nm are found in wild-type BR at low pH values or in some mutated BR molecules at physiological pH values. In all these cases the negative charge of the carboxylic group is neutralized and no formation of M can be observed (Metz et al., 1992; Lanyi et al., 1992; Fahmy et al., 1993). The missing formation of M in the photoreaction of the 610-nm species of K216A mutant is interpreted in the same way.

Less is known for the photoreaction of the 500-nm species. In the basal salt ether system such a species produces as a photoproduct a M intermediate at high pH values, but no proton was released to the medium (Fischer & Oesterhelt, 1979). In K216A at pH 9.5 only a red-shifted intermediate occurred upon excitation with a 480-nm flash with a difference maximum at 640 nm. This reaction was not reversible within 1 s.

*The Proton Transport Activity.* The prerequisite for reconstitution of the mutant protein in whole cells with the retinylidene Schiff bases is the substitution of endogenous retinal in the binding site. This reaction occurred spontaneously. Thus, it was not necessary to express the mutant BR in a retinal deficient strain or to inhibit retinal formation by addition of nicotine during growth of cells. Both conditions would have led to lower expression levels of BR.

*H. salinarium* L33 is a strain which lacks BR but contains the chloride pump HR. By addition of tetraphenylphosphonium bromide, passive proton fluxes due to HR activity could be quenched (Oesterhelt, 1982), so that acidification of the medium upon illumination in the case of transformed cells is solely due to the action of genetically introduced bacteriorhodopsin. Thus, L33 cells were an appropriate strain for these measurements.

The proton transport activity of the reconstituted chromoprotein in whole cells yielded an initial rate of 30 mol of protons pumped per minute and mol of BR at pH 8. Taking into account that three chromophores are present at this pH value but only one is active, the proton transport activity of K216A is comparable to L33 cells transformed with the wild-type bacterioopsin gene. The fact that the activity was reduced in repetitive illuminations may be due to the parallel photoreactions of the 475- and 620-nm chromophores leading to translocation inactive species at 610 and 500 nm. This may have led to a decrease of the 568-nm chromophore concentration, and consequently transport activity would be reduced.

In summary we conclude that the covalent linkage to the protein is neither required for the red shift of the absorbance maximum of the chromophore nor for the thermal 13-*cis* to *all-trans* isomerization reaction. Although we did not investigate the thermodynamic efficiency of the proton pump and the quantum yield of the primary reaction, we can state that the thermal reisomerization was not drastically altered as deduced from the kinetics of the M-decay and the concomitant recovery of the initial state. The observed transport rates of protons which resembled that of wild-type clearly indicated that the covalent linkage was also not required for the energy transfer from the chromophore to protein. These data strongly support the interpretations from the molecular dynamic calculations indicating the interactions in BR to be mainly electrostatic (Zhou et al., 1993).

Thus the question remains about the real function of the covalent linkage. Leaving aside the unknown thermodynamic potential of the mutated protein and the unknown quantum yield of the primary reaction at least three advantages of the use for the covalent linkage became apparent (1) higher stability, (2) the microenvironment of the protonated Schiff base can select the active 570-nm chromophore over a wide pH range, and (3) the amino group of a lysine side chain is more readily available in nature than methyl- or ethylamine.

## ACKNOWLEDGMENT

We thank U. Haupts, M. Rüdiger, and E. Ferrando-May for continuous interest and discussions, especially in difficult phases of the work, and Drs. W. Havelka and N. A. Dencher for critical reading of the manuscript.

## REFERENCES

Alex, S., Le Thanh, H., & Vocell, D. (1992) *Can. J. Chem. 70*, 880–887.

Bagley, H., Huang, K., Radhakmishman, R., Ross, A., Takagaki, Y., & Khorana, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2225–2229.

Ball, S., Collins, F. D., Dalvi, P. D., & Morton, R. A. (1949) *Biochem. J. 45*, 304–307.

Cline, S. W., Lam, W. L., Charlebois, R. L., Schalckwyk, L. C., & Doolittle, W. F. (1989) *Can. J. Microbiol. 35*, 148–152.

de Groot, H., Smith, S., Courtin, J., van den Berg, E., Winkel, C., Lugtenburg, J., Griffin, R., & Herzfeld, J. (1990) *Biochemistry 29*, 6873–6883.

Druckmann, S., Ottolenghi, M., Pande, A., Pande, J., & Callender, R. H. (1982) *Biochemistry 21*, 4953–4959.

Fahmy, K., Weidlich, O., Engelhard, M., Sigrist, H., & Siebert, F. (1993) *Biochemistry 32*, 5862–5869.

Ferrando, E., Schweiger, U., & Oesterhelt, D. (1993) *Gene 125*, 41–47.

Fischer, U. C., & Oesterhelt, D. (1979) *Biophys. J. 28*, 211–230.

Fischer, U. C., Towner, P., & Oesterhelt D. (1981) *Photochem. Photobiol. 33*, 529–537.

Fodor, S. P. A., Ames, J. B., Gebhard, R., Vandenberg, E. M. M., Stoeckenius, W., Lugtenburg, J., & Mathies, R. A. (1988) *Biochemistry 27*, 7079–7101.

Gärtner, W., Towner, P., Hopf, H., & Oesterhelt, D. (1983) *Biochemistry 22*, 2637–2644.

Gat, Y., Grossjean, M., Pinevsky, I., Takei, H., Rothman, Z., Sigrist, H., Lewis, A., & Sheves, M. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2434–2438.

Groenendijk, G. W. T., Jansen, P. A. A., Bontig, S. L., & Daemen, F. J. M. (1980) *Methods. Enzymol. 67*, 203–220.

Kalisky, O., Goldschmidt, C., & Ottolenghi, M. (1977) *Biophys. J. 19*, 185–189.

Katre, N. V., Wolber, P. K., Stoeckenius, W., & Stroud, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4068–4072.

Lanyi, J. K., Tittor, J., Varo, G., Krippahl, G., & Oesterhelt, D. (1992) *Biochim. Biophys. Acta 1099*, 102–110.

Lemke, H. D., & Oesterhelt, D. (1981) *FEBS Lett. 128*, 255–260.

Lewis, A., & Spoonhower, J. (1974) *Proc. Natl. Acad. Sci. U.S.A., 71*, 4462–4466.

Metz, G., Siebert, F., & Engelhard, M. (1992) *FEBS Lett. 303*, 237–241.

Mogi, T., Stern, L. J., Marti, T., Chao, B. H., & Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4148–4152.

Morton, R. A., & Pitt, G. A. J. (1955) *Biochem. J. 59*, 128–134.

Mullen, E., Johnsin, A. H., & Akhtar, M. (1981) *FEBS Lett. 130*, 187–193.

Nakanishi, K., Balogh-Nair, V., Arnaboldi, M., Tsujimoto, K., & Honig, B. (1980) *J. Am. Chem. Soc. 102*, 7945–7947.

Ni, B., Chang, M., Duschl, A., Lanyi, J., & Needleman, R. (1990) *Gene 90*, 169–172.

Oesterhelt, D. (1982) *Methods. Enzymol. 88*, 10–17.

Oesterhelt, D., & Stoeckenius, W. (1974) *Methods.Enzymol. 31*, 667–686.

Oesterhelt, D., & Krippahl, G. (1983) *Ann. Microbiol. (Paris) 134B*, 137–150.

Schreckenbach, T., & Oesterhelt, D. (1977) *Fed. Proc. 36*, 1810–1814.

Schreckenbach, T., Walckhoff, B., & Oesterhelt, D. (1977) *Eur. J. Biochem. 76*, 499–511.

Schreckenbach, T., Walckhoff, B., & Oesterhelt, D. (1978) *Biochemistry 17*, 5353–5359.

Subramaniam, S., Greenhalgh, D. A., & Khorana, H. G. (1992) *J. Biol. Chem. 267*, 25730–25733.

Towner, P., Gärtner, W., & Oesterhelt, D. (1981) *Eur. J. Biochem. 117*, 353–359.

Turner, G. J., Miercke, L. J. W., Thorgeirsson, T. E., Kliger, D. S., Betlach, M. C., & Stroud, R. M. (1993) *Biochemistry 32*, 1332–1337.

Uhl, R., Meyer, B., & Desel, H. (1985) *J. Biochem. Biophys. Meth. 10*, 35–48.

Vogelsang, H., Oertel, W., & Oesterhelt, D. (1983) *Meth. Enzym. 97*, 226–241.

Wagner, G., Oesterhelt, D., Krippahl, G., & Lanyi, J. K. (1981) *FEBS Lett. 131*, 341–435.

Zhou, F., Windemuth, A., & Schulten, K. (1993) *Biochemistry 32*, 2291–2306.

Zhukovsky, A., Robinson, P. R., & Oprian, D. D. (1991) *Science 251*, 558–560.