Chromophore of Sensory Rhodopsin II from *Halobacterium halobium*

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ABSTRACT: The photoreceptor sensory rhodopsin II (sR-II) was enriched 120-fold from cell membranes of *Halobacterium halobium*. The final preparation yields sR-II with a specific content of 3 nmol of sR-II/mg of protein. The spectroscopic measurements were performed on the enriched photoreceptor solubilized in digitonin. In the absolute absorption spectrum of the partially purified receptor, the main peak in the visible range corresponded to sR-II with a maximum at 488 nm. Cytochromes contributed to the spectrum only in a minor band at 415 nm. The extinction coefficient of sR-II was estimated from difference spectra during bleaching with hydroxylamine to be 48 000 M^{-1} cm^{-1}. The reduced chromophore displayed a pronounced fine structure which is due to the coplanarity of the retinyl residue. The isomeric composition of the chromophore from the enriched photoreceptor was determined in retinal extracts in HPLC. The dark-adapted sR-II contains 80% *all-trans*- and 20% 13-*cis*-retinal. After illumination, the ratio changed to 1:1, indicating a trans–cis isomerization during the photocycle of sR-II.

Under conditions of low oxygen tension and lack of substrates, *Halobacterium halobium* can utilize photophosphorylation (Danon & Stoeckenius, 1974; Oesterhelt & Krippahl, 1983). This photosynthetic-like process is caused by bacteriorhodopsin (bR),[1] a light-driven proton pump. A second ion pump, halorhodopsin, takes part in the net salt uptake by the cells during growth [for a review, see Oesterhelt and Tittor (1989)]. Not only the ion translocation but also the phototaxis of the bacteria is mediated by the same family of intrinsic membrane proteins. A characteristic property of these proteins is a retinylidene chromophore bound to a bundle of seven helices via a protonated Schiff base [for reviews, see Spudich and Bogomolni (1988) and Oesterhelt et al. (1992)].

Two retinal pigments are responsible for the phototactic behavior, and their particular properties enable the bacteria to avoid damaging light from shorter wavelengths and to be attracted by light above 520 nm, conditions optimal for the functioning of the two energy converters.

The photoreceptor which was discovered first and has been described extensively is sensory rhodopsin I (sR-I). It is responsible for the positive phototactic reaction of the bacteria toward green to orange light. Additionally, via a two-photon process, a negative reaction to near-UV light is observed (Bogomolni & Spudich, 1982; Hazemoto et al., 1983). The second sensory rhodopsin sR-II, also named phoborhodopsin or P480, is involved in the photophobic answer to blue light (Takahashi et al., 1985; Wolff et al., 1986; Marwan & Oesterhelt, 1987).

sR-I was recently isolated and sequenced (Schegk & Oesterhelt, 1988; Blanck et al., 1989), but little is known about the molecular and structural properties of sR-II and its chromophore. This is due not only to its low concentration in the cellular membrane but also to its sensitivity and instability to external conditions (Scharf et al., 1992a). Therefore, major investigations are concerned only with its photoreaction (Tomioka et al., 1986; Shichida et al., 1988; Imamoto et al., 1991) and its physiology (Marwan et al., 1990; Yan et al., 1991).

sR-I and sR-II are distinguished physiologically by their expression. While the biosynthesis of sR-I, like the two ion pumps, will be induced under anaerobic conditions, sR-II is produced constitutively by the cell. This observation suggests that sR-II has appeared quite early in the evolution of retinal pigments, thus protecting the respiring and fermenting cell from exposure to harmful irradiation. Furthermore, the simple wavelength regulation of the chromophore with a maximum at 490 nm might indicate that sR-II arose first, while sR-I and the ion pumps were developed later during evolution acquiring the ability to adsorb light with longer wavelengths (Takahashi et al., 1990).

The few existing data on the molecular properties of sR-II consist of the molecular weight, which is in the same range as that of the other retinal pigments. The absorption was determined by difference spectroscopy (Scherrer et al., 1987) and has its maximum at 490 nm with a shoulder at 460 nm. This fine structure has been explained by a special interaction of the retinal with the opsin (Takahashi et al., 1990). The isomeric composition of the retinal has not yet been determined. However, data gained from the sR-II like pigment (psR-II) from *Natronobacterium pharaonis* (Imamoto et al., 1992) suggest an all-trans configuration also for sR-II. On light excitation, sR-II undergoes a photocycle which is similar to that of bR. Like in bR, the main intermediate is a species with a hypsochromically shifted absorption maximum. This observation was explained by a deprotonated Schiff base, but in the absence of further data the formation of a free retinal cannot be excluded. For a better understanding of the function of this photophobic receptor, more molecular data are required. In the present study the partial purification of sR-II and its functional and molecular properties are described.

MATERIALS AND METHODS

Materials. Digitonin was purchased from Aldrich (Heidenheim, FRG). All chromatography media were obtained from Pharmacia (Freiburg, FRG) except hydroxyapatite Bio-Gel HT from Bio-Rad (Munich, FRG). Activated manganese-(IV) oxide was a product of Alfa Products (Karlsruhe, FRG). ^3H- and ^{14}C-labeled compounds were obtained from Amer-

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[1] Abbreviations: bR, bacteriorhodopsin; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; hR, halorhodopsin, PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; sR, sensory rhodopsin.

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sham Buchler (Braunschweig, FRG). All other chemicals used were of analytical grade.

Strain and Cell Culture. *H. halobium* mutant D1 (bR$^-$, hR$^-$, sR-I$^-$, sR-II$^+$, obtained from E. K. Wolff) was grown aerobically on peptone medium according to Oesterhelt and Stoeckenius (1974). After 60 h, cells with an optical density at 800 nm of 1.6 (ca. 1.5×10^9 cells/mL) were harvested and washed with basal salt (medium without peptone) buffered with 10 mM HEPES, pH 7.0.

Buffers and Solutions. All buffers containing digitonin were heated prior use for 5 min at 100 °C.

Buffer A: 3 M NaCl, 0.3 % (w/v) digitonin, 10 mM HEPES, pH 6.5. *Buffer B*: 3 M NaCl, 20% saturated ammonium sulfate (at 60 °C), 0.3% digitonin, 10 mM HEPES, pH 6.5. *Buffer C*: 3 M NaCl, 100 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.3% digitonin, 10 mM HEPES, pH 6.5. *Buffer D*: 3 M NaCl, 400 mM NaH_2PO_4, 2 mM $MgCl_2$, 0.3% digitonin, 10 mM HEPES, pH 6.5. *Detergent buffer*: 4% digitonin, 3 M NaCl, 10 mM HEPES, pH 5.5.

Purification of sR-II. All steps were carried out at room temperature.

Preparation of Cell Membranes. Washed *H. halobium* cells from 20 L of cell culture were resuspended in 400 mL of basal salt buffer to which 20 mg of DNase was added. The cells were lysed by a triple freeze–thaw procedure. Membrane fractions were centrifuged for 60 min at 360000*g* and washed three times with 400 mL of 3 M NaCl, 10 mM HEPES, pH 7.0.

Solubilization of Cell Membranes. The cell membrane was suspended in 3 M NaCl, 10 mM HEPES, pH 5.5, to a protein concentration of about 40 mg/mL, mixed with a 1.5-fold volume of the detergent buffer, and stirred for 14 h in the dark. The final protein/detergent ratio was 1:1.5 at a protein concentration of about 15 mg/mL. After centrifugation for 60 min at 360000*g*, the supernatant containing the solubilized sR-II was collected.

Butyl-Sepharose Chromatography. The supernatant was adjusted to conditions of buffer B and applied to a butyl-Sepharose column (5.0 × 14 cm) which had been equilibrated with buffer B. After washing with two bed volumes of buffer B, sR-II was eluted by a linear gradient from buffer B to buffer A in 12 h at a flow rate of 40 mL/h. Fractions were collected and assayed for photolytic activity (Scharf et al., 1992a) and protein content. The sR-II peak fractions were combined.

Hydroxyapatite Chromatography. The combined fractions of the butyl-Sepharose chromatography were concentrated by centrifugation in Centriprep 10 and then adjusted to a protein concentration of 2 mg/mL and to the conditions of buffer C. The solution was applied to a hydroxyapatite column (2.6 × 2 cm) which has been equilibrated with buffer C. After washing with five column volumes buffer C, sR-II was eluted in a linear gradient from buffer C to buffer D in 8 h at a flow rate of 20 mL/h. The fractions were assayed for photolytic activity and protein content, and the sR-II peak fractions were combined.

Gel Filtration. Fractions from the hydroxyapatite chromatography were concentrated in Centricon C10 tubes to a protein concentration of 3.5 mg/mL and were subjected to 0.5-mL portions to FPLC gel filtration (Superdex 200, HR16/60). The column was equilibrated and developed in buffer A at 0.5 mL/min. Peak fractions were manually collected and assayed spectrophotometrically for sR-II and cytochrome content.

Preparation of the [^{3}H]Retinal. [11,12(*n*)-$^3H_2$]retinol was freed from butylated hydroxytoluene (added to prevent oxidation) by HPLC (Beckman Instruments) using a reverse-phase column (μBondapak, C18) and hexane/diethyl ether (7:3) as eluant. Retinol was oxidized to retinal by a 30-fold excess of MnO_2 in dichloromethane and 0.2% pyridine. The suspension was shaken under argon for 30 min in the dark until the reaction was completed. The aldehyde was finally purified by HPLC.

Extraction of Retinal. Retinal from partially purified sR-II was extracted, and the isomers were separated on HPLC according to Scherrer et al. (1989) using two reverse-phase C18 columns (C18, μBondapak) in series with 8% diethyl ether in hexane as solvent.

Bleaching and Regeneration of sR-II. Partially purified sR-II was bleached with 10 mM hydroxylamine in buffer A, pH 8.0, for 30 min at room temperature and irradiated with yellow light (filter GG 470; Schott, Mainz, FRG) from a 200-W halogen lamp. Hydroxylamine was removed by gel filtration on a Sephadex G-15 column in buffer A.

After concentration in Centriprep CP10 tubes the G-15 eluate was incubated for 2 h with *all-trans*-[^{3}H]retinal in ethanol (the final concentration of ethanol did not exceed 1%). Nonlabeled *all-trans*-retinal in an equimolar ratio was added to regenerate all opsins.

SDS–PAGE and Fluorography. Reduction of the Schiff base was carried out according to Scherrer et al. (1987) with slight modifications. One milliliter of the regenerated sample was mixed with 20 mg of solid cyanoborohydride and 15 μL of 2 M sodium acetate buffer, pH 4.5. The reaction mixture was illuminated with yellow light for 4 h.

Gel electrophoresis was performed essentially using the procedure of Laemmli (1970) in a 2-mm flat gel with 10–17.5% acrylamide in a linear gradient for the separation gel. Samples were desalted by Centricon centrifugation. For fluorography [^{14}C]methylated proteins and ^{3}H-labeled bacteriorhodopsin [performed according to Peters et al. (1976)] were used as molecular weight markers. The gel was soaked for 30 min in an autoradiography enhancer (Amplify, Amersham), dried, and exposed to a Kodak X-Omat AR film at −70 °C.

Protein Determination. The protein content was determined after total hydrolysis of the samples in 6 N HCl with 0.1% phenol at 110 °C for 24 h with a Biotronik LC 7000 amino acid analyzer.

Spectroscopy. The visible spectra were recorded on a Perkin Elmer Lambda 9 double-beam spectrophotometer. The spectrophotometric assay for sR-II used during purification was carried out as described in Scharf et al. (1992a).

RESULTS

The assay for the activity of sR-II used during purification was based on its photochemical activity by measuring the return to the ground state after photoexcitation at 480 nm. The kinetics of the recovery of the original state depend on external conditions, e.g., pH, salt concentration, and nature of the salt and detergent. Since these parameters had to be varied during the purification procedure, absolute values for the receptor content were only available from absorption difference spectroscopy between native and hydroxylamine bleached samples.

Purification. The solubilization of native membranes under nondenaturating conditions for sR-II presented a special problem due to the instability of sR-II. Among the detergent classes tested (Table I) the following examples inactivate the

Table I: Influence of Selected Detergents on the Stability of sR-II

	class I, desactivation	class II, 20–30% active	class III, 40–75% active
detergents	LDAO Zwittergent 312 NP-40 Tween 20 MEGA-9 octyl glucoside	cholate taurocholate glycocholate CHAPS CHAPSO	dodecyl maltoside digitonin

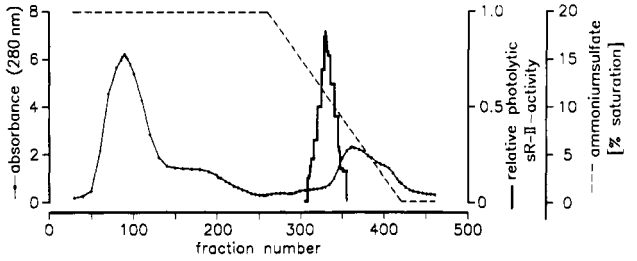

FIGURE 1: Elution profile of the butyl-Sepharose column chromatography. For experimental details, see Materials and Methods.

receptor already at very low concentrations: lauryldimethylamine oxide (LDAO), Zwittergent 312, Tween-20, nonanoyl *N*-methylglucamide (MEGA-9), and octyl glucoside. Trihydroxy bile acids like cholate, taurocholate, and glycocholate solubilized the membrane with a remaining photolytic activity of sR-II of about 12–25%, while the synthetic detergents of the CHAPS series could preserve 30–35% of the photolytic activity of the receptor during solubilization. A solubilization of the membranes with a residual sR-II activity of 40% could be achieved with dodecyl maltoside, but chromatographic experiments of this solubilized fraction resulted in varying activities between different experiments.

The best results could be obtained with digitonin which solubilized about 75% of photolytic active sR-II and stabilized it during the purification procedure. The solubilized material was chromatographed over butyl-Sepharose. Binding of sR-II to the gel matrix could be achieved by addition of ammonium sulfate in 20% saturation (at 60 °C), whereas most of the impurities were immediately washed out (Figure 1). The sR-II could be eluted in a linear to zero decreasing gradient of ammonium sulfate. The peak fraction of sR-II appeared at an ammonium sulfate saturation of about 12%. sR-II was 20-fold enriched in this chromatographic step. Other alkyl groups as ligands instead of the butyl group like octyl or phenyl ligands were not used, because sR-II adsorbed so strongly to the matrix that it could not be eluted in an active state.

Following this purification step, chromatography on hydroxyapatite was applied which required particular starting conditions. The best results were obtained by choosing a buffer composition (100 mM P_i, 2 mM $MgCl_2$) which selectively binds the main part of the sR-II fraction while most of the other proteins were not held back. The elution of the adsorbed proteins was carried out in a linear to 400 mM increasing phosphate gradient and the sR-II elution started at 200 mM P_i (Figure 2). The yield in this chromatographic step was low, only 58%. The adsorption to and the desorption from the matrix probably led to inactivations. The enrichment factor in this step was about 4, and 85% impurities could be separated.

In the final step, size-exclusion chromatography on Superdex 200 was performed resulting in an additional separation of sR-II from cytochromes which interfere in later spectroscopic studies. The purification factor in this step was only 1.4, but it led to a final preparation of sR-II 122-fold enriched over

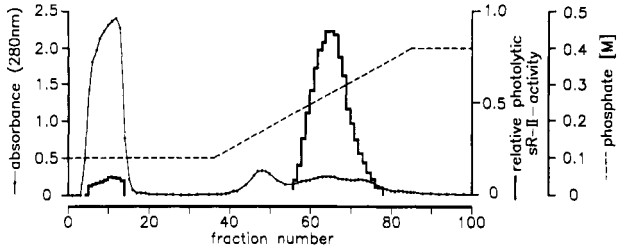

FIGURE 2: Elution profile of the hydroxyapatite column chromatography. For experimental details, see Materials and Methods.

the membrane fraction, with a 26% yield and a specific sR-II content of 3.3 nmol.

An overview of the purification of sR-II is presented in Table II. The corresponding SDS–PAGE of the final fraction is shown in Figure 3a. For the molecular identification of retinyl proteins, radioactively labeled retinal was used for reconstitution, followed by a reduction of the Schiff base between chromophore and protein to a nonhydrolyzable bond. Proteins prepared by this procedure could be identified after gel electrophoresis by fluorography (Figure 3b). The spectroscopic product of the reduction process is demonstrated in Figure 6 and will be discussed in a later section. Only one retinyl protein with an apparent molecular weight of 24 000 was found in the sR-II-enriched preparation (Figure 3b, lane 1). The Coomassie stain of the SDS gel shows a single band in the corresponding region in the final fraction of sR-II (Figure 3a, lane 2), which could be assigned unambiguously to the single band in the fluorogram.

Photocycle. To examine the function of the protein, the photocycle kinetics were measured at different wavelengths ranging from 360 nm to 500 nm and analyzed using the global fit method (Müller et al., 1991). The amplitude spectra and time constants were compared with the corresponding data from the membrane-bound receptor (Scharf et al., 1992a). No differences were found in the apparent absorption maxima of the intermediates. However, the turnover of the photocycle proved to be slower. The time constants of the first-order reactions are listed in Table III. Especially, the formation of the UV intermediate (characterized by τ_1 and τ_2) is slowed down with factors of 7 and 4.6, respectively. The decay of sR-II_{360} and thus the formation of the long-living intermediate sR-II_{540} (τ_3) was nearly unaffected. The regeneration of the ground state from sR-II_{540} (τ_4) was prolonged with a factor of 3.6 to 1.2 s.

Spectral Properties. Figure 4 shows the absolute spectrum of enriched sR-II. Maximum absorption occurs at 488 nm, with a shoulder at 460 nm according to the membrane-bound state of sR-II. The absorption maximum around 415 nm is caused by the Soret band of the cytochromes. The pigment contributes to the extinction at 490 nm in membrane preparations only 2.5% (Scharf et al., 1992a). The absorption at 415 nm mainly due to cytochromes was 145 times larger than the sR-II absorption at 490 nm. During the purification procedure, most of the cytochromes could be separated, leading to an absolute spectrum where the cytochrome absorption in the Soret region is seen only as a shoulder of the sR-II absorption spectrum.

The extinction coefficient of sR-II was calculated from the absorbance difference spectrum of the native minus the hydroxylamine-bleached sample (Figure 5). The spectral properties of the solubilized receptor with an absorption maximum of 488 nm and the typical fine structure resemble that of the receptor in native membranes (Takahashi et al., 1990; Scharf et al., 1992a). The absorbance decrease with

Table II: Purification of Sensory Rhodopsin II[a]

stage of purification	protein (mg)	sR-II content (nmol)	sR-II specific content (nmol/mg of protein)	purification	yield
membrane fraction	2005	72	0.036	0.75	133
solubilized fraction	1830	50	0.027	1	100
butyl-Sepharose fraction	73	38	0.52	19	76
hydroxyapatite fraction	10.5	22	2.1	78	44
gel filtration eluate	3.9	13	3.3	122	26

ulture (strain D1). Protein content was determined by amino acid analysis after scopy between native and hydroxylamine bleached fraction.

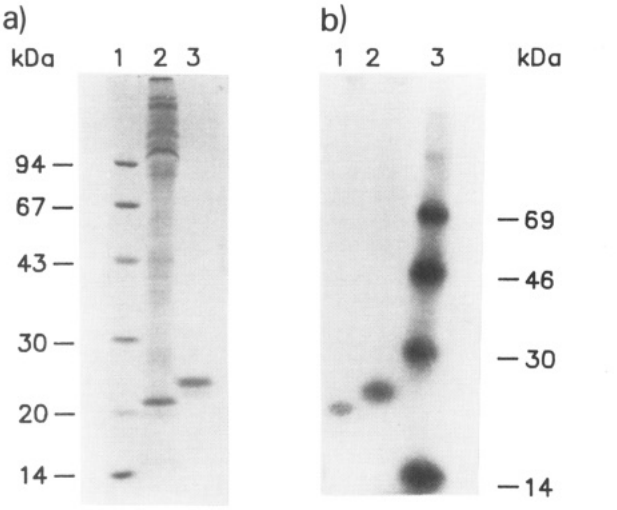

FIGURE 3: (a) Coomassie stain of the purification fractions after SDS–PAGE: lane 1, molecular weight marker; lane 2, gel filtration eluate; lane 3, bacteriorhodopsin. (b) Autofluorogram of [^{3}H]retinal-labeled proteins after SDS–PAGE: lane 1, enriched sR-II; lane 2, bacteriorhodopsin; lane 3, molecular weight marker.

Table III: Time Constants of the Photocycle of sR-II in Native Membrane Preparation and in the Partially Purified, Solubilized State

half-life times	sR-II in membranes[a] (ms)	sR-II solubilized (ms)
τ_1	0.16	1.13
τ_2	1.8	8.36
τ_3	119	153
τ_4	333	1207

[a] Data from Scharf et al. (1992a).

a minimum at 360 nm is mainly due to the formation of retinal oximes. The corresponding extinction coefficient at 360 nm was estimated from bleaching bR under similar conditions. Such an approximation seems justified because the interfering β-band of bR has a negligible contribution at 360 nm. A second problem, the possible syn and anti forms of the retinal oximes, can be neglected because they have comparable extinction coefficients (Imamoto et al., 1992). Taking these precautions into account, the differential extinction coefficient for retinal oxime at 360 nm can be estimated to be 33 600 M^{-1} cm^{-1}. This value provides an ϵ_{488} = 48 000 M^{-1} cm^{-1} for sR-II which is comparable to the extinction coefficient of sR-I (54 000 M^{-1} cm^{-1}; Spudich & Bogomolni, 1984) and *Chlamydomonas* rhodopsin (50 000 M^{-1} cm^{-1}; Beckmann & Hegemann, 1991).

The reaction of sR-II with sodium cyanoborohydride results in the reduction of the Schiff base linkage between retinal and protein to a C–N single bond. Illumination of the sample with yellow light accelerated the reduction. The spectral changes occurring after illuminating for 4 h are shown in Figure 6. The disappearance of the absorption in the blue light region is accompanied by the formation of an absorption band in the near-UV region with three main peaks at 338, 356, and 374 nm. A three-peaked spectrum is generally expected for a molecule containing 5 double bonds with maximal π–π orbital interaction (Reppe, 1970) affording a planarization of the unsaturated hydrocarbon chain. Possible causes of such fine-structured absorption bands could be, e.g., a retinal derivative with all double bonds shifted toward the ring by one position (a retroretinyl). Such an *all-trans*-retroretinyl chromophore was found in the photoconverted form of reduced bR (Peters et al., 1976). Another possibility, the interaction of the retinyl moiety with amino acid side chains, could also lead to coplanarity of the cyclohexene ring and the side chains and thus to a pronounced fine structure. To distinguish between the two alternatives, the retinyl–protein interaction was destroyed by the addition of 10% ethanol. After this treatment, the fine structure and the red shift of the absorption band in reduced sR-II is lost and turned into a single absorption band with a maximum at 324 nm. The same behavior was described for dark-reduced bR (Schreckenbach et al., 1977). The shoulder at about 415 nm in the difference spectrum is due to the γ-band of the remaining cytochromes which are also sensitive to cyanoborohydride.

Configuration of Retinal. The isomer composition of the chromophore in retinal-containing photoreceptors could be obtained by chromatography of retinal extracts. An analysis of the sR-II chromophore from cell membrane extracts by HPLC is not possible due to the low receptor content as well as the interference by other extractable organic compounds. Thus, extraction was carried out from solubilized and enriched sR-II. As far as HPLC analysis shows, retinal is the only substance in the extract which absorbs at 365 nm (Figure 7). The extraction technique of Scherrer et al. (1989) was applied, which has shown that thermal isomerization at 0 °C is negligible. This was confirmed by treating different isomers by the same procedure. In the extracts from dark-adapted sR-II, 20% 13-cis and 80% all-trans isomer were determined (Figure 7a).

After the sample was illuminated with actinic light, the ratio changes to an equimolar portion of both isomers (Figure 7b). Other cis isomers besides the 13-cis form could not be detected. The changes due to illumination occur very fast. An 80-ms light flash filtered through a 480-nm interference filter shifted the isomer ratio to the light adapted form. To guarantee that the changes of the isomer ratio under illumination are an effect of the cycling photoreceptor, an illuminated sample was allowed to adapt in the dark for 6 h at room temperature. A comparison of the isomer ratio in this sample with the light-adapted species reveals that this time was sufficient to generate the dark-adapted form. The results of the different illumination experiments are listed in Table IV. The isomeric distribution in dark-adapted sR-II is similar to that found in dark-adapted sR-I (Tsuda et al.,

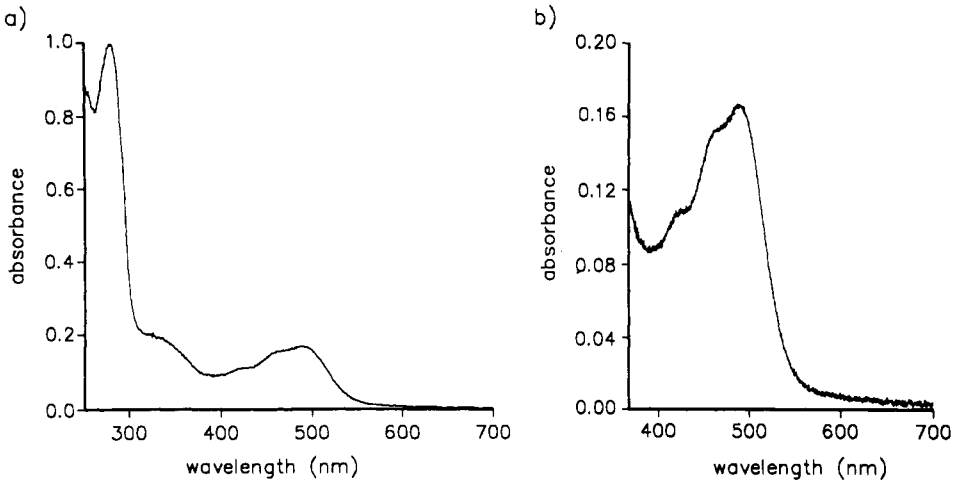

FIGURE 4: Absolute absorption spectrum of the final sR-II fraction in buffer A.

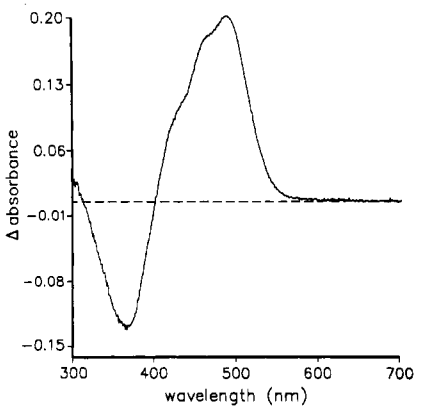

FIGURE 5: Difference spectrum between a native and a hydroxylamine-bleached sR-II sample. Samples were either sR-II in buffer A, pH 8, or sR-II in buffer A, pH 8, bleached in 10 mM hydroxylamine illuminated with yellow light (200 W, GG470) for 30 min.

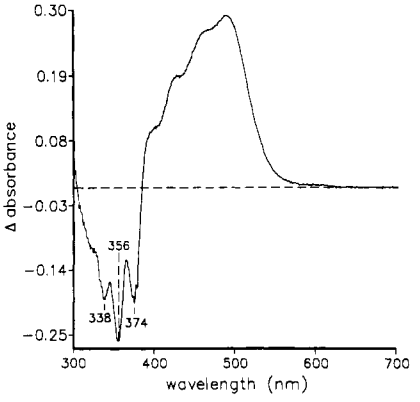

FIGURE 6: Difference spectrum between a native and a sodium cyanoborohydride reduced sR-II fraction. Samples were either native sR-II in buffer A, 30 mM sodium acetate, pH 4.5, or sR-II reduced with cyanoborohydride for 4 h under constant illumination with yellow light (200 W, GG470).

1985), although the 13-cis portion is slightly higher. This is probably an effect of the solubilization, which might shift the ratio of the isomers more toward the cis component, as already observed for solubilized sR-I (Schegk, 1989).

DISCUSSION

With the mutant D1, a strain becomes available, which is not only deficient in the retinal dependent ion transporters bR and hR, but which also lacks sR-I (Stoeckenius et al., 1988). Thus, a purification and the spectroscopic analysis of sR-II

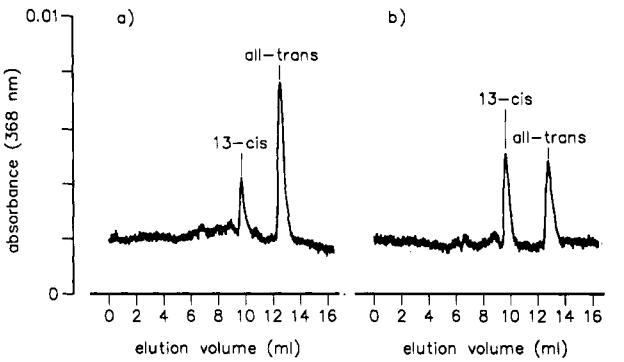

FIGURE 7: Chromatogram of the HPLC separation of retinal isomers present in the sR-II fraction: (a) dark-adapted sample; (b) illuminated sample. HPLC conditions: 2 C18 μBondapak columns in series; solvent, 8% diethyl ether in hexane; flow rate, 1 mL/min.

Table IV: Retinal Isomeric Composition under Different Illumination Conditions

illumination conditions, λ = 480 nm	all-trans	13-cis
dark	78%	22%
80 ms	54%	46%
10 s	49%	51%
1 min	47%	53%
30 min	47%	3%
30 min, 6 h in the dark	76%	24%

become possible without the presence and conceivable possible interference of the other retinal proteins.

The isolation of sR-II had so far been hindered because of several reasons. First, the very low receptor content (about 400 copies per cell; Otomo et al., 1989) of only one-tenth as compared to the concentration of sR-I demands large amounts of cell suspension as starting material. In the present study, 20 L of cell culture was used. The corresponding membrane fraction contained only about 40 pmol of sR-II/mg of protein (Scharf et al., 1992a).

A second problem arises from the high sensitivity of the receptor toward most of the available detergents. This makes a Tween washing of the membrane as the first and simple purification step successfully used in the case of hR and sR-I impossible (Steiner & Oesterhelt, 1983; Schegk & Oesterhelt, 1988). Of the many detergents evaluated (Table I), only digitonin, also used as a classical substance for the solubilization of rhodopsin (Hubbard, 1953), solubilized and stabilized sR-II. However, digitonin is only soluble in aqueous solutions after boiling and subsequently cooling the detergent to room

temperature. Because the solution remains still slightly turbid, the buffers used for purification are not very well defined. Unfortunately, the high aggregation number of digitonin (Smith & Pickels, 1940) does not support purification steps based on size differences like gel filtration.

Finally, the membrane-bound receptor is only stable in solutions with concentrations >2.0 M NaCl (Scharf et al., 1992a). Thus, it was essential to keep the protein under high-salt buffer conditions not only during purification like in the case of hR (Steiner & Oesterhelt, 1983) and sR-I (Schegk & Oesterhelt, 1988) but even during the preparation of the membrane. The necessity of high-salt buffer conditions also reduces the choice of available chromatography media, e.g., ionic-exchange chromatography.

From the remaining possible purification methods, hydrophobic interaction chromatography on butyl-Sepharose, hydroxyapatite chromatography, and size-exclusion chromatography were applied. Unlike hR and sR-I, sR-II could be adsorbed at and desorbed from solid supports without becoming inactivated. Furthermore, binding of the pigment to hydroxyapatite could also be achieved at concentrations at 100 mM phosphate in the present of 2 mM $MgCl_2$. For acidic proteins, elution at low phosphate concentrations is expected. Therefore, sR-II has a less acidic surface as, e.g., halorhodopsin from *N. pharaonis* which could be eluted at concentrations of 5 mM phosphate (Duschl et al., 1990). Employing these purification procedures, the final preparation of sR-II was 122-fold enriched over that of the starting membrane preparation with 26% yield and a specific sR-II content of 3.3 nmol of sR-II/mg of protein.

The kinetic scheme of the photocycle of solubilized and enriched sR-II is comparable to the membrane-bound state of the pigment. This indicates that under these conditions the system is still intact. The lipid environment in the membrane-bound state of the receptor seems to have little influence on the basic steps of the photoreaction. Although the shape of the digitonin micelle is different from the dimension of the lipid membrane of the halobacterial cell, the detergent-embedded photoreceptor maintains its native structure. The interaction between membrane lipids and the receptor protein is therefore not crucial for the primary process, namely, the photoreception.

The pigment after solubilization shows the same absorption spectrum as in the membrane-bound state. The absorption maximum and the shoulder could be observed in membrane suspensions as well as in the isolated form. It was assumed that the appearance of the fine structure originates from the fixation of the β-ionone ring and its side chain (Takahashi et al., 1990). The presence of the shoulder in the solubilized sR-II emphasises that the structure of the protein remains nearly unaffected after removal of the natural lipid environment, an observation which was already suggested from the unperturbed photocycle (see Table III).

The retinyl chromophore remains planar even after reduction of the double bond of the Schiff base. This can be demonstrated in analyzing the fine structure of the absorption spectrum of the reduced sample. In principle, the three sharp maxima can arise from a retroretinyl or a planarized chromophore. The former structural element can be proven on denaturing the protein by, e.g., ethanol (Hegemann, 1983). Under these conditions, the stringent environment of the retinal moiety is broken and the chromophore can relax to a twisted configuration. Bacteriorhodopsin displays a similar behavior (Schreckenbach et al., 1977). The reduction of bR under illumination produces a reaction product with a three-peaked absorption band (λ_{max} = 360 nm) similar to the reduced sR-II. Schreckenbach et al. (1977) concluded from this observation and other arguments that the long-lived M intermediate possesses a planarized conformation of the retinyl moiety which is not disrupted if the retinyl remains covalently attached to the opsin. Comparing these data with those of sR-II, one can state that the coplanarity of the retinyl chromophore is found not only in the ground state (contrary to bR!) but most probably also in the long-lived intermediate sR-II_{360} which is accessible to the reduction agent.

The extraction experiments have shown that in the dark retinal adopts almost 80% the all-trans and 20% the 13-cis configuration (Table IV). On light excitation, the cis content is increased, but not to much more than 50%. This observation contrasts data gained with bacteriorhodopsin where in the dark–light adaptation process only the trans configuration is obtained. The reaction is accompanied by a bathochromic shift of the absorption maximum. In the case of sR-II, an effect on the absorption maximum was not observed. This could have two explanations. First, the light–dark adaptation process is so fast that it cannot be observed with the techniques available (time resolution $\cong$ 100 ms). Or second, the cis portion is caused by the slow photocycle so that intermediates are also contributing to the trans/cis proportion. Since it is unlikely that the light–dark adaptation process is as fast as the photocycle turnover, it seems plausible to assume the second statement to be true. Interestingly, extraction experiments with sR-I have revealed that on light excitation the content of 13-*cis*-retinal is also increased (Tsuda et al., 1985). The authors conclude from this experiment the isomerization of retinal in sR-I_{370} to be in the 13-cis configuration. Furthermore, the sR-II-like pigment from *N. pharaonis* psR-II contains also *all-trans*-retinal which is isomerized during the photocycle to the 13-cis configuration (Imamoto et al., 1992). After light excitation, apparently, all three receptors sR-I, psR-II, and sR-II undergo a trans $\Rightarrow$ cis $\Rightarrow$ trans isomerization cycle of the retinal chromophore.

The isomerization of the retinal chromophore around the 13,14 bond to yield a 13-cis configuration is followed by the deprotonation of the Schiff base. However, a hydrolysis of the Schiff base linkage is also conceivable. Furthermore, it seems likely that as it was observed in psR-II a carboxyl group is protonated (Scharf et al., 1992b) concomitantly with the formation of psR-II_{390}. The physiological active configuration is the transition between sR-II_{360} and sR-II_{540} (Yan et al., 1991) indicating at least three necessary requirements for the function of this photoreceptor: isomerization around the 13,-14 double bond, deprotonation of the Schiff base, and protonation of a carboxyl group.

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Registry No. 13-*cis*-Retinal, 472-86-6; *all-trans*-retinal, 116-31-4.