Spin Labeling of *Natronomonas pharaonis* Halorhodopsin: Probing the Cysteine Residues Environment

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Halorhodopsin from *Natronomonas pharaonis* (pHR) is a light-driven chloride pump that transports a chloride anion across the plasma membrane following light absorption by a retinal chromophore which initiates a photocycle. Analysis of the amino acid sequence of pHR reveals three cysteine residues (Cys160, Cys184, and Cys186) in helices D and E. Here we have labeled the cysteine residues with nitroxide spin labels and studied using electron paramagnetic resonance (EPR) spectroscopy their mobility, accessibility to various reagents, and the distance between the labels. It was revealed by following the d_1/d parameter that the distance between the spin labels is ca. 13−15 Å. The EPR spectrum suggests that one label has a restricted mobility while the other two are more mobile. Only one label is accessible to hydrophilic paramagnetic broadening reagents leading to the conclusion that this label is exposed to the water phase. All three labels are reduced by ascorbic acid and reoxidized by molecular oxygen. The rate of the oxidation is accelerated following retinal irradiation indicating that the protein experiences conformation alterations in the vicinity of the labels during the pigment photocycle. It is suggested that Cys186 is exposed to the bulk medium while Cys184, located close to the retinal ionone ring, exhibits an immobilized EPR signal and is characterized by a hydrophobic environment.

Introduction

Halorhodopsin (HR) is a light-driven chloride ion pump in the cytoplasmic membranes of halobacteria. Since its discovery in the archea *Halobacterium salinarum*,[1] various types of HRs have been reported,[2−4] but the most extensively studied are those of *Halobacterium salinarum* (sHR) and *Natronomonas pharaonis* (pHR).[5] Like sHR, pHR also transports chloride into the cell.[6] The light is absorbed by an all-trans retinal chromophore that is covalently bound to a protein (opsin) via a protonated Schiff base (SB) linkage.

Light absorption by the retinal chromophore induces retinal isomerization from the all-trans to 13-cis isomer and initiates a photocycle in which the chloride anion is transferred between several binding sites, resulting in a net translocation of one chloride from the extracellular membrane to the cytoplasmic side.[7,8] The photocycle of sHR and pHR was studied by several methods. The intermediates of the photocycle and their absorption spectra were determined by time-resolved absorption spectroscopy.[9,10] In addition the photocycle was studied by infrared spectroscopy[11−13] as well as by resonance Raman spectroscopy.[14−16] The details of the photocycle are still under debate, but it is generally agreed that the photocycle comprises (besides the ultrafast primary events) K, L, N, O, HR′, and HR intermediates. The O intermediate is accumulated in the pHR photocycle but not in sHR.[17] In pHR the chloride anion is released between the N and the O intermediates, and the uptake process takes place either between the O and the HR′ intermediates [7,18,19] or in a transition between two spectrally silent O intermediates.[8]

The amino acid sequence of HR was determined[20] and has revealed considerable similarity to bacteriorhodopsin (bR). In keeping with the lack of proton pumping activity of HR, sequence alterations were found in crucial residues. HR is lacking the equivalent of Asp85, which serves as the SB proton acceptor in the bR photocycle. This residue is replaced in HR by threonine, which is part of the chloride binding site. In bR Asp96 is the proton donor to the Schiff base in the second half of the photocycle and is substituted by alanine in HR.

The chloride is bound in the vicinity of the SB as detected by resonance Raman spectroscopy.[21,22] Recently, a high-resolution structure of sHR indicated that the chloride anion is part of the SB counterion and replaces Asp85 of bR.[23]

Unlike bacteriorhodopsin that does not have cysteine residues, sHR contains two cysteines (Cys145 and Cys169) in helices D and E, while three cysteine residues (Cys160, Cys184, and Cys186) were detected in pHR in helices D and E. The crystal structure of sHR[23] reveals that Cys145 is located relatively close to the polyene chain of the retinal (6 Å between SH and C9). Cys169 is part of the retinal binding site located close to the retinal β-ionone ring (3.94 Å between SH and C4). The functional role of the cysteine residues is not clear, however, it was shown that a disulfide bond is not formed. Free SH groups could be titrated in sHR, and the protein was cleaved by SH-specific reagents into two fragments.[24] Support for a functional role of the cysteine residues in HR was obtained from studies with $HgCl_2$, which indicated an effect on light-dependent chloride transport.[25]

Electron paramagnetic resonance (EPR) spectroscopy is a powerful tool for studying the structure and conformational dynamics of proteins.[26,27] The method is based on the substitution of cysteine residues with spin-labeled side chains.[28] The EPR spectrum yields information on the nitroxide side chain

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mobility,[29] solvent accessibility,[30] polarity of the immediate environment of the nitroxide, as well as the distance between spin labels if they are in close proximity.[31−33] In the present studies, we have used EPR spectroscopy to study pHR by labeling its three cysteine residues with nitroxide side chains. The studies aimed at obtaining structural information on pHR and comparing its tertiary structure to the known structure of sHR. In addition, we have studied the accessibility of the nitroxide side chains to the bulk water, distances between the labels, and light-induced conformational alterations in the vicinity of the nitroxide side chains. We have revealed that only one nitroxide is exposed to the water bulk phase while the other two are located in a relatively hydrophobic environment. The distances between the spin labels detected in this study support the similarity of the pHR and sHR tertiary structures.

Experimental Section

Sample Preparation. Solubilized pHR (0.05% *N*-dodecyl-β-D-maltoside (DM) detergent) was expressed in *Escherichia coli* and isolated according to a previously published method.[34] Briefly, Transformed *E. coli* BL21(DE3) cells were grown at 37 °C in 2TY medium supplemented with 50 μg/mL kanamycin. At an OD_{578} of 0.6−0.7, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 10 μM all-trans retinal were added. After an induction period of 2.5 h the cells were harvested, washed, and resuspended in a Tris-HCl buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 8.0) and finally broken up in a microfluidizer. Membranes were sedimented at 100 000*g* for 1 h at 4 °C and solubilized in buffer S (1.5% DM, 300 mM NaCl, 50 mM MES, 5 mM imidazole, pH 6.0) for 16 h at 8 °C. After centrifugation of the solubilized membranes (100 000*g*, 1 h, 4 °C), the supernatant was incubated with Ni−NTA agarose for 1 h at 8 °C. The Ni−NTA resin was filled into a chromatography column and washed extensively with buffer W (0.15% DM, 300 mM NaCl, 50 mM MES, pH 6.0) with an increasing imidazole concentration (≤50 mM) to remove unspecifically bound proteins. Subsequently, the histidine tagged proteins were eluted in buffer E (0.05% DM, 300 mM NaCl, 50 mM Tris-HCl, 150 mM imidazole, pH 7.5).

The spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl)-methanethiosulfonate (MTSSL; Toronto Research Chemicals, Canada) was dissolved in dimethyl sulfoxide and was covalently attached to the cysteine residues of pHR protein to yield the spin-labeled side chain. A 30 μL solution of 50 mM MTSSL in dimethyl sulfoxide was diluted with a 1 mL suspension of 15 μM pHR in 50mM Tris buffer (pH 7.5), 0.3M NaCl, and 0.05% DM. The suspensions were stirred at room temperature for 14 h. The noncovalently bound spin label was removed by overnight dialysis. EPR spectra of the spin-labeled pHR were measured to estimate the extent of labeling and to ensure removal of nonbound spin label. The spin label 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Sigma) in dimethyl sulfoxide was covalently attached to the cysteine residues of the pHR protein to yield the spin label side chain as described for the MTSSL reagent. (A 100-fold excess of the radical was used.)

For the free radical control experiments, we have used the stable nitroxyl radical 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO−OH) at concentrations of 1−8 μM. The concentration of the covalently bound pHR protein was determined by double integration of the EPR spectra, using TEMPO−OH for calibration.

Reduction of the Spin-Labeled Pigment with Ascorbic Acid. The spin label pHR−TEMPO was reduced by the addition of 0.5mM L-(+)-ascorbic acid (Merck) at pH 7.5 to a solution of the pigment suspension. The reduction reaction was monitored by the disappearance of EPR signals at 3480G and 3490G for the bound immobilized species and for the bound mobilized species, respectively. Experiments were carried out in the dark or under illumination with a halogen lamp with an output of 150 W equipped with a heat-absorbing filter and a 550 nm glass cutoff filter. Illumination was kept steady for the whole experiment.

Reoxidation of the Spin-Labeled Pigment with Molecular Oxygen. After complete reduction of the spin-labeled pigment, 3 μL ascorbate oxidase (100 units suspended in 400 μL of H_2O) was added to a 60 μL solution of the reduced pigment suspension. The reoxidation reaction was monitored by the time-dependent increase in the peak intensity of the EPR spectra central component (3510G). Experiments were carried out in the dark or under illumination with a halogen lamp with an output of 150 W equipped with a heat-absorbing filter and a 550 nm glass cutoff filter.

EPR Measurements. All measurements were performed on an ELEXSYS 500 spectrometer (Bruker), using a flat cell (volume 60 μL) at 23 °C. The low-temperature EPR experiments were carried out in 50 mM Tris buffer (pH 7.5), 0.3 M NaCl, 0.05% DM/glycerol (50/50) solutions using a capillary. The measurements were carried out using a temperature control unit (Euroterm, ER 4131VT, Bruker) with an accuracy of ±1 K.

Results and Discussion

Spin Labeling of pHR. Amino acid analysis of the pHR protein[20] indicated that the protein contains three cysteine residues, Cys160, Cys184, and Cys186. An attempt was made to spin label the three SH groups in pHR. The shape of the EPR spectra measured at room temperature reveals the degree of nitroxide radical mobility, which depends on the protein structure in the vicinity of the spin label binding site. pHR was spin-labeled by MTSSL via a thiol−disulfide exchange reaction. The EPR signal was composed of a relatively immobilized EPR signal along with a more mobile signal (still less mobile than unbound free radical) and probably some unbound free radicals (Figure 1A).

To support binding through a disulfide bond the labeled protein was reacted with 1,4-dithiothreitol (DTT) and 2-mercaptoethanol. Both reagents induced disappearance of the immobilized components along with simultaneous appearance of a free radical triplet (data not shown). This observation indicated a disulfide bond between the spin label and the protein. The pHR−MTSSL complex was not stable, and following incubation overnight at 4 °C a fraction of the bound label probably hydrolyzed to yield a free radical (Figure 1B) that was removed by the dialysis process. The EPR spectrum of the dialyzed protein indicated that the ratio between the relatively mobilized and immobilized EPR signals was altered such that the fraction of the immobilized signal was increased (Figure 1C). It is plausible to suggest that following incubation the mobilized labeled radical hydrolyzed faster than the immobilized one. Additional reaction with the reagent (MTSSL) following the decomposition and dialysis processes led to recovery of the original EPR spectrum. This observation indicates the formation of a free thiol following label disappearance and supports the hydrolysis process rather than a possible reaction with a free neighboring protein thiol that will prevent further reaction with MTSSL. However, a possible reaction of the label with a free neighboring thiol cannot be completely excluded if the formation of the disulfide bond is reversible following addition of more

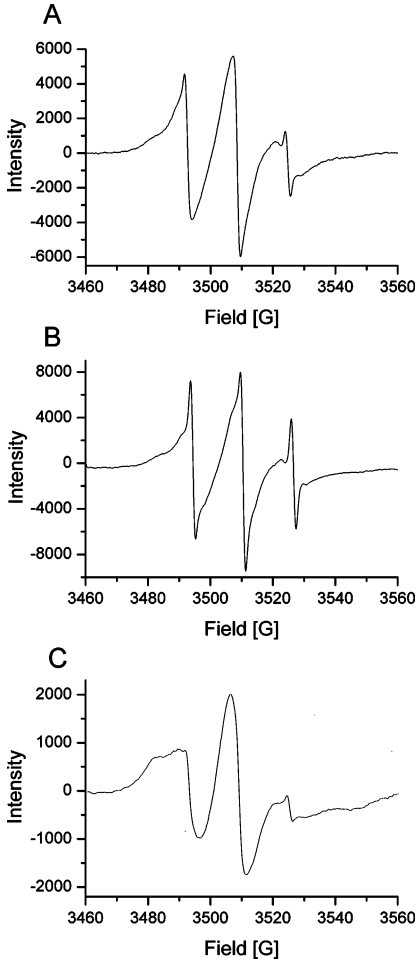
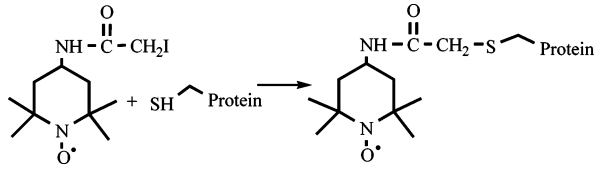


Figure 1. EPR spectra of spin-labeled pHR with MTSSL. (A) The spectrum was taken in 50 mM Tris buffer pH 7, 0.3 M NaCl, 0.05% DM. (B) After overnight at 4 °C. (C) Following dialysis and removal of the free label.

SCHEME 1



MTSSL reagent. Note that hydrolysis of thiol labeling with sulfhydryl reagents such as Ellman's reagent and 4,4′-dithiodipyridine was previously demonstrated.[35]

Since labeling with MTSSL was not sufficiently stable, we have spin-labeled pHR (in 50 mM buffer Tris pH 7, 0.3 M NaCl, 0.05% DM) with 4(2-iodoacetamido)−TEMPO, which yields a stable carbon−sulfur bond (Scheme 1). The EPR spectrum is shown in Figure 2A. Similarly to the reaction with MTSSL, two EPR signals were observed corresponding to relatively mobilized and immobilized labels. The typical degree of modification was 0.4 ± 0.1. The labeling process did not affect the absorption maximum of pHR, which was identical (578 nm) to that of the native protein.

Several methods for tracking distances (**r**) between spin labels have been proposed for continuous wave EPR.[33] Basically, in one approach, the spectral line shape is simulated by convoluting the spectrum of an isolated spin label (e.g., with no dipole−dipole contribution) with the distribution function of spin labels in the sample.[36−39] The second approach relies on an empirically found spectral parameter, the ratio of the peak heights d_1/d,

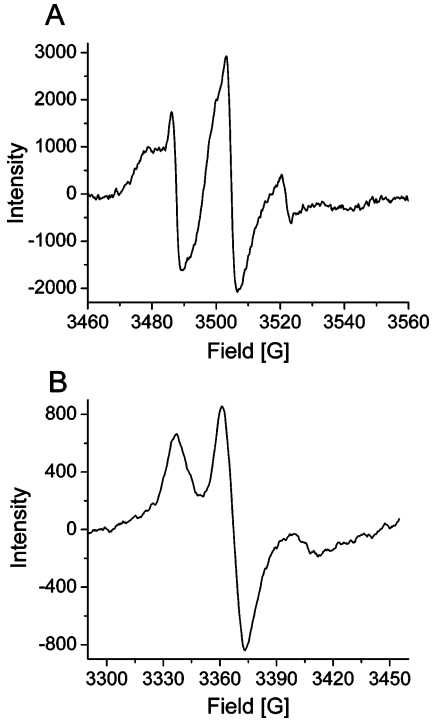


Figure 2. (A) EPR spectrum of spin-labeled pHR with 4(2-iodoacetamido)−TEMPO. The sample was dissolved in 50 mM Tris buffer pH 7, 0.3 M NaCl, 0.05% DM. (B) EPR spectrum of pHR−TEMPO at 140 K in buffer/glycerol (50/50) mixture.

TABLE 1: EPR Spectra d_1/d Ratio

	temperature	d_1/d
TEMPO−OH (free radical)	140 K	0.38
bR−163C-TEMPO	140 K	0.35
pHR−TEMPO	200 K	0.48
	140 K	0.48

which is sensitive to the distance between interacting spins.[31a] d_1 is the sum of amplitudes of two extreme peaks (low and high field), and d is the amplitude of the central peak of the nitroxide EPR signal. To a good approximation, the d_1/d ratio is inversely proportional to the distance between interacting spins in rigid molecules. The validity of this empirical equation has been confirmed for a broad range of spin-labeled systems.[40−42] The d_1/d parameter is therefore a convenient measure of the dipole−dipole broadening effect. In this study, we chose this parameter to characterize the strength of dipole−dipole interactions. Using the previously measured calibration curves[31a] and the d_1/d values obtained by us (Table 1), we estimated the distance **r** between the spin labels. In the absence of interaction between the radicals, a value of $d_1/d < 0.4$ is expected.[31a] To further support it, we have measured the d_1/d parameter in bR−163C-TEMPO pigment, which has only one nitroxide label and obtained a value of 0.35. In contrast, for pHR−TEMPO a d_1/d value of 0.48 was detected (Table 1). The latter value leads to a distance estimation of 13−15 Å between the nitroxide radicals. (The dependence of the parameter d_1/d on the distance between the paramagnetic centers (**r**) was studied for a chaotic (random) distribution of the centers[31a] and for a fixed distance between them (the rigid biradical model). In the latter case the following empirical formula was proposed:[31b] $\mathbf{r} = 9.3 + 0.77/\Delta$, where $\Delta = (d_1/d) - (d_1/d)_o$. In the present studied case (three potential modification sites), the application of the formula is in principle incorrect. However, for qualitative evaluation, three different dislocations of spin-labeled SH groups in the protein (A, B, and C) can be suggested: (1) The radicals are in the vertex of

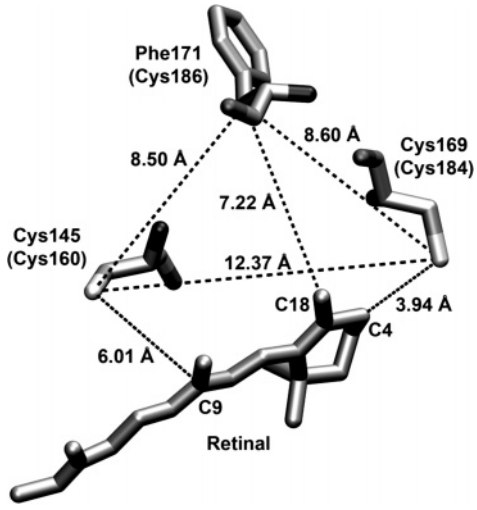


Figure 3. Structure of the retinal region in sHR. The picture is based on the structure of PDB code 1E12. Only three amino acids, Cys145, Cys169, and Phe171, with distances from the retinal moiety and from one another, are shown. The residues shown in parentheses correspond to residues in pHR.

an equilateral triangle AB = AC = CB. (2) The three labels are located on one straight line AB = BC > AC. (3) The radicals are in the vertex of an isosceles triangle, where AB = BC < AC. On the basis of a strong dependence of the dipole−dipole interaction on the distance between the radicals, one can readily obtain for the three cases 11.2 Å < **r** < 15.3 Å, which is in a good agreement with the estimate of the *r* value (see above) for the chaotic radicals distribution. This analysis also indicates that even a mixture of disubtituted and trisubstituted cysteines will not affect significantly the suggested distance.) We note that protein−protein interactions as a source for dipole broadening can be excluded since the results obtained were not affected by dilution of the sample. The protein modification degree of the studied samples was 0.4 ± 0.1. Less than 100% occupancy of SH groups by spin labels will lead to overestimation of distances. The possible error in distance calculation as a function of fractional occupancy of paramagnetic centers was previously carried out.[43] These calculations indicate that 50% occupancy will result in an error of <15% in the detected distance. Therefore, this error will not affect our main conclusions.

At present, the crystal structure of pHR was not reported. Since the primary structures of sHR and pHR are highly homologous (66%),[44] it is conceivable that their tertiary structures would be quite similar. Cys160 and Cys184 in pHR correspond to Cys145 and Cys169, respectively, in sHR, while Cys186 in pHR replaces Phe171. The crystal structure of sHR reveals that Cys145 is located in the same plane and relatively close to the polyene chain of the retinal (6 Å between SH and C_9). Cys169 is part of the retinal binding site located close to the retinal β-ionone ring (3.94 Å between SH and C_4). Phe171 is located far from the retinal, being more exposed to the bulk (Figure 3). Distances measured between the three residues in sHR reveal distances of about 8.5−12 Å between all the three residues.

Approximate distances of 13−15 Å measured with pHR−TEMPO fit the distances between the corresponding residues in the crystal structure of sHR and may confirm conservation in the tertiary structures of the two proteins.

Accessibility to Ferric Cyanide ($K_3Fe(CN)_6$) and Chromium Oxalate (CrOx). Exposure of the spin labels to the aqueous phase can be evaluated by probing the interaction of the label with water-soluble paramagnetic probes. The interaction is reflected in spin (Heisenberg) exchange, which results from a direct collision of a paramagnetic species with the nitroxide radical. This event is experimentally detected by a line width broadening and a decrease of signal peak intensity.[45−47] $K_3Fe(CN)_6$ and CrOx are excellent choices for water-soluble paramagnetic spin exchange reagents that can affect the nitroxide EPR signal in the case where they can freely diffuse from the bulk water to the labelled nitroxide. The EPR spectrum broadening effect of such paramagnetic probes depends on the product of its translocational diffusion and its local concentration as well as accessibility of the nitroxide radical covalently bound to the protein.

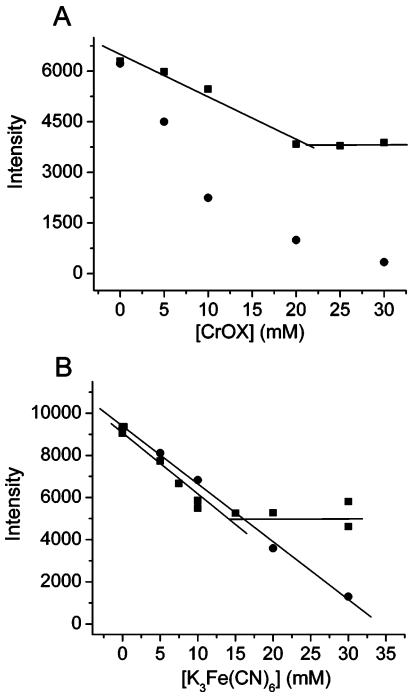


Figure 4. (A) Effect of CrOx concentration on the pHR−TEMPO EPR signal at 3490 G (■) and the free TEMPO−OH radical in solution (●). (B) Effect of $K_3Fe(CN)_6$ concentration on the EPR signal at 3490 G of pHR−TEMPO (■) and the free TEMPO−OH radical (●).

Different concentrations of CrOx were added to the labeled protein, and the broadening of the EPR spectrum was monitored by the decrease in signal intensity (Figure 4A). While a decrease of about 40% of the relatively mobilized signal (at 20 mM CrOx) could be detected, no effect on the immobilized signal was monitored (Figures 5A and 5B). Interestingly, addition of higher concentrations of CrOx did not induce further decrease in the mobilized signal. The labeled protein−CrOx interactions were monitored in the dark as well as with steady-state illumination, but no light effect was detected. To shed further light on the protein−CrOx interaction the spin exchange interaction was monitored between CrOx and free TEMPO−OH radical in solution containing the same buffer used for the labeled pHR studies. In contrast to the labeled pHR, a complete broadening and signal disappearance of the free TEMPO−OH occurred at ca. 30 mM CrOx (Figure 4A).

We have conducted similar experiments with $K_3Fe(CN)_6$. At 10 mM $K_3Fe(CN)_6$ a decrease of about 45% of the mobilized EPR signal peak intensity was detected due to signal broadening. Increasing the $K_3Fe(CN)_6$ concentration did not yield further decrease in the EPR peaks (Figure 4B) in keeping with the results obtained for CrOx. This observation was in contrast to free TEMPO−OH radical in buffer solution in which complete signal quenching was observed following addition of ca. 30 mM

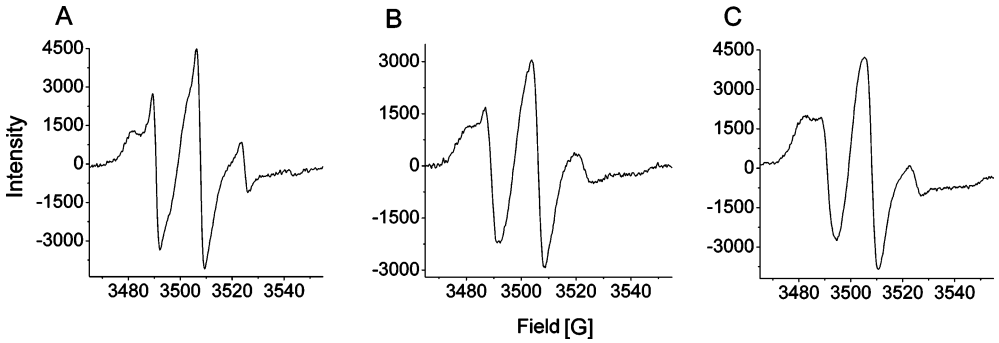


Figure 5. EPR spectra of (A) pHR−TEMPO, (B) pHR−TEMPO in the presence of 30 mM CrOx, and (C) pHR−TEMPO in the presence of 30 mM $K_3Fe(CN)_6$.

$K_3Fe(CN)_6$ (Figure 4B). The immobilized signal was not affected by the addition of $K_3Fe(CN)_6$ (Figure 5C).

As mentioned above pHR contains three cysteine residues. Two are located in helix E and one in helix D. The EPR spectrum clearly indicates that one label covalently bound to the protein is immobilized. We suggest that the second relatively mobilized peak corresponds to the other two labels. The immobilized label does not interact with water-soluble reagents (such as CrOx and $K_3Fe(CN)_6$), indicating that the label is not exposed to the bulk and it is not accessible to these reagents. Therefore, we can conclude that the label is located in a hydrophobic environment. Furthermore, retinal irradiation does not open a new channel for the water-soluble reagents. The observation that only part of the mobilized signal is quenched by the reagent is probably associated with the signal comprising two mobilized labels of which only one is accessible to the water phase. This suggestion is valid even if the cysteine residues are not uniformly labeled and the sample consists of a protein mixture. Thus, out of the three labeled cysteine residues, two are located in a hydrophobic environment and one is exposed to the water phase either directly or through an exposed channel. It is tempting to suggest that labeled Cys186, which corresponds to Phe171 in sHR, is the one exposed to the water phase, probably through a water-exposed channel. This suggestion relies on the X-ray structure of sHR in which Phe171 (corresponds to Cys186 of pHR) is closer to the outside medium than the two cysteines embedded closer to the retinal chromophore. It implies that the label of Cys186 is relatively mobilized.

Reduction and Reoxidation of the Spin-Labeled Pigment. Nitroxide spin labels can be easily reduced by ascorbic acid[48] to give hydroxylamine, which is a diamagnetic moiety. The pHR labeled pigment was reduced by ascorbic acid monitored by a decrease in EPR signal intensity (Figures 6, 7A and 7B). Incubation for ca. 2 h with ascorbic acid led to a total disappearance of the EPR signals. After this process only ascorbic acid peaks could be detected in the EPR spectrum (Figure 8B). The bound mobilized nitroxide radical reacted 7 times faster than the bound immobilized one ($k = 0.00419\ s^{-1}$ and $k = 0.00059\ s^{-1}$, respectively). The rate of the bound mobilized signal reduction was similar to that of free TEMPO−OH radical in solution for similar radical and reagent concentrations. The reduction reaction rate was followed in the dark and under illumination with a $\lambda > 550$ nm cutoff filter, absorbed exclusively by the retinal chromophore. However, irradiation did not have any effect on the reaction rate (Figure 6).

After complete reduction of the spin labels the remaining ascorbic acid was oxidized by addition of ascorbate oxidase, thereby allowing for reoxidation of the spin labels. The ascorbic acid EPR signal immediately disappeared followed by a recovery of the labeled protein EPR spectrum due to a spontaneous oxidation with O_2 (Figures 7C and 7D). The oxidation reaction of the labeled protein was monitored by an intensity increase of the EPR spectrum central component (at 3510 G) (Figure 7). The reaction rate was followed in the dark as well as under illumination ($\lambda > 550$ nm cutoff filter). It is evident that the reaction was accelerated by light ~3 times relative to the dark (Figure 8) ($k = 0.00121\ s^{-1}$ at steady-state illumination and $k = 0.000415\ s^{-1}$ in the dark). The recovery yield following addition of the enzyme was about 40%. The use of H_2O_2 led to the recovery of an additional 30% of the radical signal to a total of 70%.

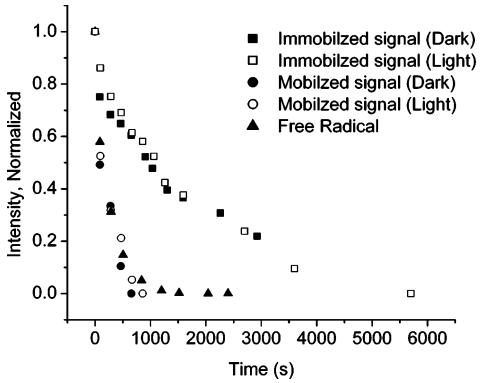


Figure 6. Decrease of pHR−TEMPO EPR signal due to reaction with 0.5 mM ascorbic acid. The reduction reaction was followed in both dark and light and monitored at 3480G for the bound immobilized species and at 3490G for the bound mobilized species. The free radical TEMPO−OH concentration was 8×10^{-6} M.

In contrast to the effect of water-soluble broadening reagents described above, the reaction with ascorbic acid was completed with all three labeled radicals. It is plausible that ascorbic acid, which is more hydrophobic than CrOx or $K_3Fe(CN)_6$, is capable of penetrating to more hydrophobic regions in which the labels are located.[49] The difference between the reduction reaction rates of the relatively mobilized and the immobilized EPR signals may indicate that the immobilized nitroxide is located in a more hydrophobic environment since its reduction rate is slower. Therefore, the immobilized EPR signal may be identified with the labeling of Cys184 located closer to the retinal ionone ring. The ionone ring is forced by the protein environment to adopt a defined conformation, and it is surrounded by hydrophobic protein residues. Thus, it is plausible that Cys184 is located in a hydrophobic and rigid environment. Interestingly, the rate of the reoxidation process is affected by light, indicating that after light absorption the protein experiences conformational alterations that affect all three labels. The light used ($\lambda > 550$ nm) is absorbed solely by the retinal chromophore and not by the reduced spin label, and therefore excited-state involvement of the spin label can be excluded. We note that previously[50] similar

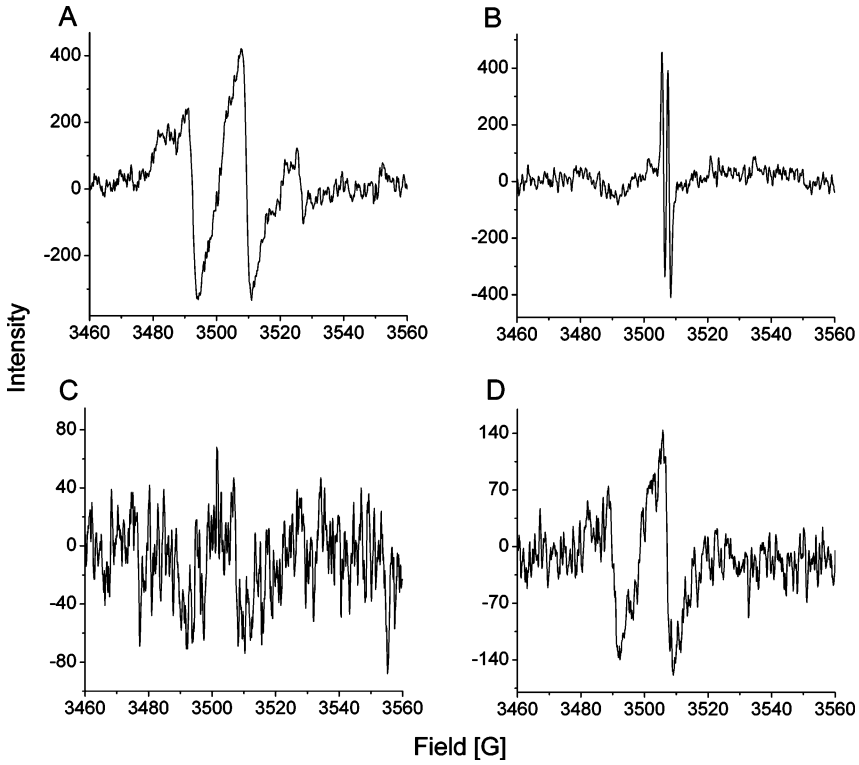


Figure 7. EPR apectra of pHR−TEMPO reduction by ascorbic acid and the reoxidation following the addition of ascorbate oxidase. (A) pHR−TEMPO. (B) After incubation with 0.5 mM ascorbic acid for 1.5 h. (C) Subsequent addition of ascorbate oxidase. (D) Following incubation with ascorbate oxidase for 3 h.

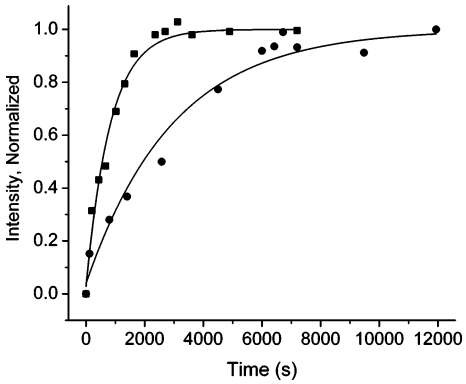


Figure 8. Recovery of the nitroxide radical upon addition of ascorbate oxidase to an incubated solution of pHR−TEMPO with ascorbic acid for 2 h. Progress of the reoxidation reaction under dark (●) and light (■) conditions measured by EPR signal intensity monitored at the central component of the spectra (3510 G).

reoxidation light acceleration was detected in an artificial pigment of bacteriorhodopsin spin-labeled at the A103C mutant located in the cytoplasmic side. This region is known to experience conformation alteration during bacteriorhodopsin photocycle.[51−53]

Conclusions

It was previously shown that in bacteriorhodopsin helix F undergoes an outward movement during the late M photochemically induced intermediate and a subsequent approach of helix G toward the proton channel.[54−56] These changes may be crucial for the pigment function and proton pumping activity. In the present studies we have detected light-induced conformational alterations in pHR in the vicinity of the three protein cysteine residues located in helixes E (Cys184 and 186) and D (Cys160). These conformational alterations are reflected in rate modification of the nitroxide side chain reoxidation reaction. The photocycle of pHR is lacking the M intermediate, and thus the conformational alterations should be associated with other photocycle intermediates. The identification of these intermediates should be the subject of future studies. Out of the three pHR cysteine residues only one is exposed to the bulk water phase and collides with hydrophilic reagents. Comparison of pHR and the known sHR structure suggests that Cys186 of pHR is exposed to the bulk water while the other two (Cys184 and Cys160) are embedded in a more hydrophobic environment in the retinal ring and polyene vicinity. In addition, it can be concluded that the environment of Cys186 is not very hydrophilic since its substituted nitroxide still reacts with ascorbic acid, which is more hydrophobic than CrOx or ferric cyanide, and moreover with oxygen, which is relatively hydrophobic. The relative mobilized EPR signal is quenched due to reaction with ascorbic acid faster than the immobilized signal, which may suggest that the cysteine residue corresponds to the immobilized signal located in a more hydrophobic environment. One of the mobilized signals corresponds to Cys186. Since the protein enforces ring−chain s-trans planar conformation it is plausible that the environment in the ring vicinity is quite rigid. Therefore, it is tempting to speculate that the immobilized EPR signal corresponds to Cys184, located very close to the retinal ring, rather than to Cys160. The distance of 13−15 Å between the nitroxide radicals revealed by our studies is in keeping with the distance between the cysteine residues of sHR as detected by the X-ray structure. This finding strongly supports the assumption that the tertiary structure of pHR is similar to that of sHR.

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