Time-resolved Detection of Transient Movement of Helix F in Spin-labelled Pharaonis Sensory Rhodopsin II

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Sensory rhodopsin II (also called phoborhodopsin) from the archaeal Natronobacterium pharaonis (pSRII) functions as a repellent phototaxis receptor. The excitation of the receptor by light triggers the activation of a transducer molecule (pHtrII) which has close resemblance to the cytoplasmic domain of bacterial chemotaxis receptors. In order to elucidate the first step of the signal transduction chain, the accessibility as well as static and transient mobility of cytoplasmic residues in helices F and G were analysed by electron paramagnetic resonance spectroscopy. The results indicate an outward tilting of helix F during the early steps of the photocycle which is sustained until the reformation of the initial ground state. Co-expression of pSRII with a truncated fragment of pHtrII affects the accessibility and/or the mobility of certain spin-labelled residues on helices F and G. The results suggest that these sites are located within the binding surface of the photoreceptor with its transducer.

Keywords: phoborhodopsin; bacterial signal transduction; phototaxis; site-directed spin labelling; EPR spectroscopy

Introduction

The response of Archaea and Bacteria towards external stimuli is mediated by receptor-regulated phosphorylation pathways. In the case of archaeal phototaxis, the receptors, sensory rhodopsin I and II (SRI, SRII), induce an intermolecular, probably transmembrane conformational change in their cognate transducer molecules (HtrI, HtrII) (Zhang et al., 1999), thereby modulating the auto-phosphorylation rate of a bound cytoplasmic histidine kinase. SRI and SRII belong to the family of visual pigment-like seven-helical membrane proteins with the two ion pumps bacteriorhodopsin (BR) and halorhodopsin as additional members. Recent progress in the structural analysis of BR and its main photo-intermediate M have revealed a movement of helix F towards the external lipid phase (Luecke et al., 1999a). This observation might also be of relevance for the mechanism of the signal transfer from the SR receptors to their corresponding Htr.

Direct structural information on the sensory rhodopsins is not yet available. SRII from Natronobacterium pharaonis (pSRII) undergoes considerable alterations in the amide bond region when excited by light (Engelhard et al., 1996), which could be interpreted as conformational changes of the protein. In another set of experiments it could be shown that the illumination of SRI facilitates the oxidative intermolecular cross-linking of the cysteine HtrI mutant I64C (Zhang & Spudich, 1998). These experiments clearly demonstrate that HtrI exists as a dimer. Photo-activated SRI triggers conformational changes within the two HtrI molecules which influences the proximity or mobility of the two cysteine residues in position 64. The
interaction of receptors with their cognate transducers has been assessed by phototaxis and photocycle measurements of chimeric HtrI (Zhang et al., 1999). The deletion of most of the C-terminal domain of HtrI demonstrated that an N-terminal fragment of about 150 amino acid residues is sufficient to alter the photochemical properties of SRI in a similar way to the SRI/HtrI complex (Krah et al., 1994; Perazzona et al., 1996). These experiments indicated that the specificity of the SRI/HtrI interaction is confined to the two transmembrane helices and a hydrophilic stretch of 90 residues subsequent to the cytoplasmic end of TM-2.

In order to obtain more information about the structure and conformational dynamics of pSRII with and without its transducer pHtrII, site-directed spin labelling and electron paramagnetic resonance (EPR) spectroscopy is employed (for a review on site-directed spin labelling of proteins see Hubbell et al. (1998)). Taking recently obtained structural and EPR data from BR (Essen et al., 1998; Luecke et al., 1998; Pebay-Peyroula et al., 1997; Pfeiffer et al., 1999; Rink et al., 2000) into account, residues from the first and last turn of helix F (K157, S158, L159, and Y160) and helix G (F210, I211, A212, and L213), respectively (Figure 1), were mutated to cysteine and subsequently chemically modified with the methanethiosulphonate nitroxide spin label to yield the side-chain R1. To study the interaction of pSRII with its transducer, selected mutants were co-expressed with a fragment of pHtrII. The EPR spectra of these constructs enabled the elucidation of the receptor transducer interaction.

Results

The mobility of nitroxide side-chains

The shape of the electron paramagnetic resonance spectra reflects the re-orientational motion of the nitroxide side-chain, which depends on the degree of interaction of the spin label with nearby groups. Strong interaction between the nitroxide and neighbouring residues of the protein results in a powder-like spectral pattern with a resolved hyperfine line in the high field region of the spectrum. This is obvious for the spectra of S158R1, L159R1, Y160R1, I211R1, A212R1 and L213R1 (Figure 2, left panel, continuous lines). The spectra are similar if not identical to those of the samples solubilized in dodecyl-maltoside (DM) (Figure 2, left panel, dotted lines), which suggests that the interactions restricting the motions are intramolecular.

The spectrum of K157R1 exhibits two distinct extremes in the low and high field regions. This may be due to an anisotropic motion of the side-chain in a certain ordering potential reflecting two conformations of the nitroxide side-chain with different steric restrictions. The mobile component may reflect an orientation towards the exterior of the protein, whereas the immobilized component faces the protein interior. This observation would be evidence for the spin label side-chain being located close to a helix-helix contact interface. Similar spectral shapes were found for the nitroxide side-chains in bacteriorhodopsin at positions 156 and 168, which are positioned within the boundary of helices E and F (Pfeiffer et al., 1999).

The spectrum of F210R1 displays considerable line broadening which disappears upon solubilization in DM. Low temperature measurements (data not shown) of F210R1 reconstituted into lipids revealed dipolar as well as Heisenberg exchange interactions, indicating direct van der Waals contact of nitroxides (Miick & Millhauser, 1992). Hence, we conclude that pSRII-F210R1 forms at least dimers with the G helices in vicinity.

The accessibility of the nitroxide side-chains for paramagnetic quenchers

The motional analysis of the nitroxides was supplemented by measuring their accessibility for freely diffusing paramagnetic probe molecules (Altenbach et al., 1990). The collision frequency between oxygen and a spin label is low in the tightly packed interior of a protein but high in the lipid bilayer. On the other hand, collisions with water-soluble chromium oxalate (CrOx) are likely
if the spin label faces the aqueous phase. As a relative measure for the collision frequency the electron longitudinal relaxation time is determined by the method of continuous wave power saturation (Altenbach et al., 1989).

The data are presented in Figure 3 in terms of the dimension-less parameter $\Pi_1$, which is proportional to the collision frequency of the nitroxide with either oxygen or CrOx. The two-dimensional surface defined by $\Pi_1$ and $\Pi_{CrOx}$ is divided into three areas, each one being characteristic for a side-chain exposed towards one of the three environments, water, lipid bilayer, or protein interior (Farahbakhsh et al., 1992; Pfeiffer et al., 1999). The highest collision frequency with CrOx is revealed for the side-chains attached to positions 157 and 158. These residues must be oriented towards the aqueous phase. Low values for both, $\Pi_{CrOx}$ and $\Pi_{oxygen}$ were found for side-chains at positions 159, 160, 211 and 213, showing that these residues have to face the protein interior. Positions 212 and 210 reveal accessibilities for oxygen typical for side-chains located at the interface between protein and lipid bilayer. The unexpectedly high accessibility of A212R1 towards oxygen might be attributed to its proximity to both the protein surface and a hydrophobic environment, as one would expect for the cytoplasmic channel. After solubilization, the value of $\Pi_{oxygen}$ dramatically increases for F210R1, whereas the corresponding value for A212R1 remains unchanged. This observation further supports our conclusion derived from the analysis of the nitroxide mobility and spin-spin interaction, namely that the nitroxide at position 210 interacts with a neighbouring pSR1Ii molecule in the native lipid bilayer. Similar dependencies on the solubilizing environment were found from an opto-acoustic analysis of pSR1I, which may also be interpreted as an effect of specific protein-protein interaction in the lipid bilayer (Losi et al., 1999). This latter observation indicates that the dimerization is not due to the spin label but due to a general property of pSR1I in lipid environment. By analogy to the orientations of corresponding amino acid side-chains of BR, a structural model of the cytoplasmic extensions of helices F and G is shown in Figure 4, which accounts for the above results.

Changes of the nitroxide mobility upon light excitation and optical spectroscopy

In order to analyse structural changes of pSR1I after photo-excitation, EPR difference spectra (excited-dark state) were monitored directly during a magnetic field scan (see Materials and Methods and Steinhoff et al., 1994). Considerable amplitude changes are observed in the difference spectra of L159R1 and I211R1 and in decreasing order also for S158R1 and L213R1 (Figure 2, right panel). Transient EPR signals could also be detected for K157R1 at the indicated B-field values (see below), although changes are hardly visible in the difference spectrum due to the lower signal-to-noise ratio. In all cases the spectral patterns are in line with an increased nitroxide mobility during the photocycle. In the dark state of pSR1I the nitroxides of S158R1, L159R1 and I211R1 are in contact with the F-G inter-helical surface (see Figure 4). Assuming that during the photocycle helices F and G would be displaced relative to each other, the mobility of these spin label side-chains should be altered, which is indeed observed. The detected transient mobilization of these spin label side-chains including that observed for position 157 renders an outward movement of helix F likely. No mobility changes are observed for the nitroxide of F210R1, in agreement with its orientation towards...
the lipid phase. At least a small movement of helix G is suggested by the difference spectrum detected for L213R1. It is interesting to note that the photocycle analysis of Y160R1 displays decreased turnover rate with a transient accumulation of the O intermediate. Similar results were obtained with the spin-labelled BR mutants F171R1 and T170R1 (Rink et al., 2000). A reason for this finding could be, that the relatively larger side-chain R1 hinders helix F from returning to its original location due to sterical restrictions.

To gain more detailed information about the kinetics of the observed mobility changes the time courses of the EPR signal after photo excitation were compared with those of the optical transients (Figure 5). With the exception of Y160R1, the photocycles of the spin labelled pSRII mutants were indistinguishable from that of wild-type pSRII (Chizhov et al., 1998). For all samples the rise time of the EPR signal is faster than the time resolution of the experimental set up (1 ms). Apparently, the corresponding conformational change is either faster or coincides with the formation of M (deprotonated retinal Schiff' base). The decay occurs in the time range of the recovery of the pSRII initial state (Figure 5(a) and (b); 500 nm traces) or of the reprotonation of the Schiff base (Figure 5(a) and (b); 390 nm traces). To distinguish between these two possibilities the experiments were repeated in the presence of azide, which predominantly accelerates the rate of the Schiff base reprotonation (Figure 5(c); 390 nm and 500 nm traces). It is obvious that the EPR signal follows the decay of the O-state (late photocycle intermediate) and not the reformation of the protonated Schiff base (Figure 5(c), 550 nm trace). Therefore the recovery of the initial conformation might be correlated with the re-isomerization of retinal to the all-trans conformation occurring during the O-decay as verified by FTIR-spectroscopy (M. Hein, F. Siebert & M.E., unpublished results). However, it should be noted that other authors concluded from FTIR and retinal extraction experiments that the O-intermediate of the SRII photocycle is characterized by an all-trans configuration of the retinal chromophore (Bergo et al., 2000; Imamoto et al., 1992).

Co-expression of pSRII with the truncated transducer

To determine the interaction of pSRII with its transducer pHtrII both membrane proteins were co-expressed in Escherichia coli. The construct, consisting of the open reading frame of pSRII and the entire transducer, led to serious protein aggrega-
tion, which could hardly be purified (data not shown). Therefore, a shortened transducer was designed. Proceeding on the assumption that in HtrI and pHtrII analogous domains are involved in binding the corresponding receptors a N-terminal sequence of 159 amino acid residues was selected (Krah et al., 1994; Perazzona et al., 1996).

If the movement of helix F is connected to the physiological response, the outward directed residues S158R1 and F210R1 should be within or close to the interface of the transducer binding site. Therefore the constructs pSRIIS158C/t-HtrHis; pSRIIL159C/t-HtrHis; and pSRIIF210C/t-HtrHis were prepared either to monitor the transducer binding (S158, F210) or the movement of helix F (L159).

To allow for in vivo assembly of pSRII with t-HtrHis a plasmid was constructed with both coding sequences under the control of two independent T7-promoters. In Coomassie stained gels of total cell lysates after induction with 1 mM IPTG, a new band with an apparent molecular mass of about 18 kDa is observed which can be assigned to the truncated transducer (t-HtrHis).

During the purification via Ni-NTA chromatography the presence of pSRII is obvious from its colour with an absorption spectrum identical in shape ($\lambda_{\text{max}} = 498$ nm) to that of wt-pSRII. In addition to t-HtrHis, pSRII-mutants become clearly visible on SDS-PAGE at an apparent molecular mass of about 22 kDa. Since the His-tag has been located at the C terminus of t-Htr the co-purification of receptor and transducer clearly indicates that both proteins do form a complex that withstands the applied conditions. The identity of the t-HtrHis was confirmed by N-terminal sequencing as well as Western-blot analysis using an antibody (rabbit) against the cytoplasmic portion of t-Htr (data not shown). The complexes were spin labelled as described above and finally purified to >90% purity.

**Effect of transducer binding on the mobility and accessibility of the nitroxide side-chains**

Figure 6 represents the EPR spectra of the two mutants pSRII-S158R1 and pSRII-F210R1 in the
presence and absence of t-Htr. The binding of t-Htr to pSRII slightly decreases the mobility of the spin label side-chain of pSRII-S158R1 (Figure 6(a)). The accessibility of this site for CrOx is dramatically decreased (Figure 6(a), insert), indicating that it is blocked by the bound transducer. On the other hand, the values for Πox remain almost unaltered at a low level which is in line with a position of the spin-label side-chain at the protein/water boundary.

The dimerization of pSRII is hampered in reconstituted pSRII-F210R1/t-HtrHis complex as indicated by the loss of dipolar line broadening. On the other hand, the oxygen accessibility of the nitroxide at position 210 remained at the same reduced level (Figure 6(c)). These results confirm that the shortened transducer binds to pSRII in close vicinity of helices F and G. The low Πox level present even in the solubilized sample (Figure 6(b)) suggests that the interaction of pSRII with t-HtrHis is not interrupted by the detergent.

**Effect of transducer binding on the light-induced changes**

In the reconstituted pSRII the nitroxide of S158R1 shows a transient mobilization on light excitation (see Figure 2, right lane). In Figure 7(a) the corresponding difference spectrum is compared with the maximal amplitudes of the EPR-transients of pSRII-S158R1/t-HtrHis complex measured at three distinct B-field strengths. It is obvious that the spectral changes are oppositely directed at the respective magnetic field values. Therefore, the spin label of the transducer-bound pSRII S158R1 encounters a transient immobilization during the photocycle.

The kinetic of this process is illustrated in Figure 7(b). As can be depicted from the traces of the absorbency changes measured at 390, 500, and 550 nm in the complex, the photocycle seems to be retarded and the O-amplitude is reduced. For a detailed analysis, the data were fitted to a model in which the apparent rate constants were assigned to the irreversible steps of a unidirectional sequence of intermediates (see Müller & Plesser, 1991; Chizhov et al., 1996). However, the global fit analysis revealed almost no changes of the rate constants as shown in Table 1. The assumed kinetic model allows the calculation of absolute spectra of kinetic states, which in the case of the mutant S158R1 are quite similar to those of the wild-type (Chizhov et al., 1998). The states P6 and P7 (Figure 8), belonging to the decay times τ6 and τ7, represent fast quasi-equilibria between at least two archetypal chromophore states (M and O). While in P6 the equilibrium is dominated by M, it is shifted in P7 almost completely in favour of O. In case of transducer binding both quasi-equilibria in P6 and P7 are dominated by the M-state. In addition to the presence of the O-state, considerable absorption observed at 500 nm indicates the contribution of the N-state. These results can explain the apparent slower decay of the M-state which originates from the shift of the M ⇔ O partitioning in the quasi-equilibria in favour of M. It is important to note, that the variance of the photocycle is merely observed for S158R1/t-Htr but not for wild-type pSRII/t-Htr or L159R1/t-Htr.

As expected from the kinetic analysis made above, the EPR signal of S158R1/t-Htr shows a
retarded decay but seemingly does not coincide with optical traces at indicative wavelengths. The calculated time constant $\tau_x \approx 1.5$ seconds for the EPR-signal decay might display the recovery of the pSRII-ground state, as it has the same order of magnitude as the corresponding time constant $\tau_y$. It is interesting that $\tau_y$ could not be fixed for S158R1/t-Htr in the global fit analysis of the optical data, probably due to a small optical amplitude.

In case of the mutant L159R1/t-Htr the kinetic properties of the EPR-transients are independent from the binding of the transducer fragment. Therefore the transient mobilization of the spin label at position 159 is fully maintained. This clearly points to the unimpaired outward directed movement of helix F on binding t-HtrHis.

**Discussion**

The results described above allow the orientation of the nitroxide side-chains bound to pSRII to be assessed in relation to their micro-environment. The immobilized spin label side-chains L159R1 and Y160R1 are orientated towards the interior of the protein. The dense atomic packing in this region prevents oxygen and CrOx reaching the nitroxide (see Figure 3). In contrast, the accessibility data of the spin label side-chains at positions K157 and S158 are evidence for their orientation towards the aqueous phase. However, the second spectral component found for K157R1 reflects a high degree of immobilization pointing to a conformation of the side-chain with considerable intra and/or intermolecular interactions. Taking the atomic data of BR (Essen *et al*., 1998; Luecke *et al*., 1999b) into account and assuming structural homologies, an interaction of K157R1 with helix E seems possible.

The spectra and accessibility data of the spin label side-chains I211R1, A212R1 and L213R1 in helix G reflect proximity to other nearby protein residues. By analogy to atomic coordinates of the corresponding amino acid residues in BR (I222, L223 and L224), the pSRII residues could also be orientated towards helices F, C and A. The accessibility data for F210R1 provide evidence that the spin label is oriented towards the lipid phase, whereas K157R1 and S158R1 face the aqueous phase. These results also reflect the different immersion depths of helices G and F into the lipid bilayer. Figure 4 presents a scheme of the relative orientation of helices F and G. Comparing these data with those obtained for BR, it becomes evident that helices F and G not only are similarly oriented to each other and to the other parts of the protein but have also the same boundary separating cytoplasmic residues from those immersed in the membrane.

Apparently, the structural alterations of helix F are strikingly similar for BR and pSRII. An outward movement of the cytoplasmic end of the helix has been originally proposed on the ground of electron diffraction studies of BR (Subramaniam *et al*., 1993). In addition, recent X-ray structural analysis of BR (Luecke *et al*., 1999a) has indicated that structural changes in the cytoplasmic part of helix G occur during the formation of M. Similar
results were obtained by Rink et al. (2000), who observed transient immobilization of residues in the interface of helices F and G. In contrast, the analogous residues in pSRII experience a mobilization during the photocycle. If one takes the results of L213R1 into account, helix G undergoes only minor changes which might be directed towards helix F. The differences between pSRII and BR might be explained by the structural diversities in this region as will be described below.

The π-bulge found in BR at position Ala215 enables a hydrogen bond connection Ala215O-W501-Trp182Ne between helices F and G. The side-chain of Trp182 is located above the kink-region of the helix F and experiences a tilt upon retinal isomerisation. Thereby it is supposed to trigger conformational changes in helix F as it might function as a lever arm. An extensive database sequence alignment of various rhodopsins showed that Ala215(BR) is strongly conserved with the sole exception of the three known SRII-proteins bearing the more bulky threonine and serine at this position (see Figure 1). This might hinder the formation of the π-bulge in helix G. Therefore the helical movements of F and G may be disconnected, which accounts for the results described here.

In a recently published review, Spudich (1998) proposed a model of a unified molecular mechanism for the archaebacterial ion-pumps and photoreceptors. The key step in the mechanism is the disruption of the salt bridge between helices C and G which triggers the outward tilting of helix F. In the case of pSRII this salt bridge exists between the protonated Schiff base and the negatively charged carboxyl group of Asp75. On light excitation the Schiff base proton is transferred to Asp75 (Bergo et al., 2000; Engelhard et al., 1996), thereby neutralizing both functional groups. Only in the last steps of the photocycle is the salt bridge reformed. The EPR data presented in this work constitute the first experimental evidence for the movement of the cytoplasmic side of helix F in pSRII, thus supporting the proposal of Spudich.

The movement of helix F may provide the trigger for the activation of the transducer as EPR experiments with the receptor-transducer complexes suggest. Although only an N-terminal fragment of pHtrII was used for the studies, it is nevertheless representative for the native complex. This assumption is justified, because the expression of pSRII-F86D together with t-HtrHis in oocytes inhibits the light-activated vectorial proton transfer as it has been observed for the full length transducer (G. Schmies, M.E., G. Nagel & E. Bamberg, unpublished results). Therefore, t-HtrHis serves as an adequate model system to elucidate the signal transfer from receptor to transducer.

The co-expression of the pSRII mutants S158R1 (helix F) and F210R1 (helix G) with t-HtrHis alters the accessibilities of the outwardly oriented spin label side-chains. These data suggest that helices F and G are close to or within the binding interface. The disintegration of the dimeric F210R1 mutant on transducer binding also supports the interaction of helices F and G with t-Htr. It is not clear which

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**Table 1. Rate constants of pSRII analogues and pSRII/tHtrII complexes**

<table>
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<tr>
<th>Protein</th>
<th>τ₁ (μs)</th>
<th>τ₂ (μs)</th>
<th>τ₃ (μs)</th>
<th>τ₄ (μs)</th>
<th>τ₅ (ms)</th>
<th>τ₆ (ms)</th>
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<td>18</td>
<td>70</td>
<td>1800</td>
<td>120</td>
<td>250</td>
<td>380</td>
<td>1400</td>
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<tr>
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<td>30</td>
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<td>70</td>
<td>240</td>
<td>500</td>
<td>900</td>
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<td>7</td>
<td>25</td>
<td>2000</td>
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<td>190</td>
<td>390</td>
<td>1100</td>
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<tr>
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<td>38</td>
<td>550</td>
<td>71</td>
<td>170</td>
<td>440</td>
<td>*</td>
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<tr>
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<td>830</td>
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<td>230</td>
<td>340</td>
<td>600</td>
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<tr>
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<td>10</td>
<td>41</td>
<td>920</td>
<td>62</td>
<td>240</td>
<td>390</td>
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Rate constants calculated by the global fit analysis of optical transients measured between 360 and 660 nm for pSRIIHs and the two mutants S158R1 and L159R1 with and without the co-purified tHtrHis. *For τ₈ of pSRIIS158R1/tHtrHis see the text.

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**Figure 8.** Absolute spectra (— —) of kinetic states P6 and P7 derived from the difference spectra of these intermediates calculated from global fit procedure on the full wavelength scan of S158R1His and S158R1/tHtrHis. For reasons of comparison the ground spectrum is shown in each panel (continuous line).
of the two or whether both transmembrane helices of t-Htr are involved in binding to the receptor. Furthermore, the side-chain of S158R1 becomes immobilized during the photocycle, indicating that this site approaches transiently the transducer. This contact has an impact on the energetic barriers between chromophore states M, O and N, thereby changing their contribution to quasi-equilibria in favour of the M-state. Discrepancies between EPR and vis-absorption changes could be due to the specificity of monitors. Whereas the spin label senses local structural changes, absorbance changes detect alterations in the chromophore environment.

Taking recent proposals on the mechanism of chemotactic signal transfer into account (e.g. Ottemann et al., 1999) the C-terminal transmembrane helix (HTM-2) could be implicated. The invariance of light-induced changes of the nitroxide mobility of L159R1 to binding of the transducer implies that the outward movement of helix F is not sterically hindered in the receptor-transducer complex. This is in line with the finding that the photocycle kinetics of this mutant and the wild-type do not change when bound to t-HtrHis. On the other hand, a modulation of the photocycle of the SrII by its transducer HtrII has recently been described by Sasaki & Spudich (1998). Whether this discrepancy is due to the different receptors used has to be clarified.

In summary, the data presented in this work indicate an outward tilting of helix F which is correlated with the early steps of the photocycle and which is sustained in time over at least three orders of magnitude until the O-intermediate decays back to the ground state. Co-expression with an N-terminal part of pHtrII provides first experimental evidence that the movement of helix F could be the trigger for a conformational change of the transducer transmembrane helices as a result of direct physical contact with the effector molecule.

Material and Methods

DNA manipulation

Bacterial strains

As a host for DNA manipulations E. coli XL1 was used. Gene expression was carried out in E. coli BL21 (DE3).

pSRII-cysteine mutants

Starting with the plasmid pET27bmod-pSRIIIHis (Hohenfeld et al., 1999) cysteine-encoding mutations were introduced via a two-step PCR procedure according to the overlap extension method described by Ho et al. (1989). The final PCR products were ligated into pET27bmod using the NcoI and HindIII restriction sites. Plasmid DNA of positive clones of E. coli XL1 transformants was sequenced (ABI-Prism BigDey terminator kit) to verify the introduced mutations.

Constructs for co-expression of pSRII with the transducer fragment

The DNA sequence coding for the N-terminal fragment (1-159), called t-Htr in this study, was amplified using as a template the sub-cloned KpnI-SacI portion of the pHtrII genomic sequence (Seidel et al., 1995). With the primer 5'-CGCGTCAATAAACCCCATGGGCCCTG-3' and the reverse primer 5'-CCGCTCTGAATTCCGTGTCTGATCTC-3' restriction sites are introduced to facilitate the sub-cloning into pET27bmod (Klostermeier et al., 1998) with an additional His-tag. Due to this cloning strategy Ser2 is changed to Ala resulting in the following N and C-terminal sequences: MALNV t-HtrHis. Finally, the 5'-BglII/3' HindIII fragment of the resulting plasmid was excised which contains the t-HtrHis coding region as well as the upstream T7 promoter region.

For the co-expression construct the DNA of pSRIII and of its mutants (F210C, L159C, S158C) were amplified by PCR as described (Hohenfeld et al., 1999) but with a modification of the reverse primer 5'-AACAGGGATCCCTTAGTCGGCCACC-3' now maintaining the original stop codon and introducing a downstream Hin-dIII fragment of the resulting plasmid was excised and ligated with the 5'-BglII/3' HindIII and ligated with the 5'-BglII/3' HindIII fragment (T7prom + t-HtrHis) described above.

Protein expression and spin labelling

pSRII/His mutants as well as the complex pSRIII/ t-HtrHis were expressed in E.coli BL21(DE3) as described by Shimono et al. (1997) and purified as outlined by Hohenfeld et al. (1999) except that the buffer 300 mM NaCl, 25 mM sodium phosphate (pH 8) was used in all steps. For cell disruption and membrane isolation 2 mM EDTA was added. The spin label (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulphonate (MTSSL; TRC, Toronto) was covalently attached to the cysteine residues of the solubilized pSRII mutants by the method described by Pfeiffer et al. (1999). The label was mixed at a concentration of 1 mM with a freshly purified protein sample (300 mM NaCl, 25 mM sodium phosphate (pH 8 0), 1% DM). Excess label was removed by Ni-NTA (Qiagen, Hilden) chromatography. The yield was about 90% as determined by EPR and absorption spectroscopy. Finally, pSRII-mutants and the purified complex were reconstituted into purple membrane lipids as described by Losi et al. (1999) and buffer for all samples was adjusted to 150 mM NaCl, 10 mM Tris (pH 8).

EPR measurements

EPR cw-setup

EPR experiments were performed using a home-made EPR spectrometer equipped with a Bruker dielectric resonator. Spectra were recorded at 293 K with the microwave power set to 1.2 mW and B-field modulation amplitude adjusted to 1.5 G. The whole setup was controlled by a personal computer, which also performed 12 bit analogue-to-digital data acquisition at up to 20 kHz sample rate.
Power saturation

For power saturation experiments in the presence of molecular oxygen or chromium CrOx the samples were loaded into gas-permeable TPX capillaries (Jagmar Ltd., Kraków, Poland). The samples were deoxygenated or oxygenated by passing nitrogen or oxygen around the sample capillary. Saturation curves were determined from the peak-peak amplitudes of the centre line measured at seven different incident microwave power levels in the range from 0.5 to 80 mW. The saturation behaviour of the samples was parametrized by the quantity $P_{1/2}$ which is defined as the power level of the incident radiation at which the amplitude of the saturated line is half of the amplitude in the absence of saturation. Values for this parameter were calculated from a fitting procedure by the method of Altenbach et al. (1994). The $\Delta P_{1/2}$ values calculated from the difference in $P_{1/2}$ values in the presence and absence of the relaxing agent were divided by the peak-peak centre line width and normalized by the same quantity of a DPH standard sample to obtain the dimensionless accessibility parameter $\Pi$.

Time-resolved EPR and flash photolysis

Light pulses generated by a xenon flash lamp (bulb supplied by ILC Technologie) at approximately 80 J electrical pulse energy and 30 µs pulse duration were used for photo-excitation. Difference spectra between the photo-activated and the initial states were recorded during a single B-field scan by subtraction of EPR signals which were averaged within two different time intervals of equal length but different delays after the flash. The delays were set to 0 and one second, respectively, the length of both intervals was adjusted to one second. According to this detection scheme the EPR signal averaged during the first time interval consists of components corresponding to the photocycle intermediates. The EPR signal present after the recovery of the ground state is sampled during the second interval. The kinetics of the EPR signal changes were measured at fixed B-field values, where the difference spectra represent local extremes. The time courses of these transients were found to be independent from the selected B-field values. Between 256 and $10^{3}$ EPR transients (3G modulation) were sampled at a rate of 0.125 s$^{-1}$ with the integration time set to 1 or 10 ms. Optical measurements were performed with a laser flash photolysis setup similar to that described by Chizhov et al. (1996). For complete analysis of pSRII-mutants photo reaction, traces were recorded in the spectral range of 360-660 nm with steps of 10 nm at 25(±1)°C. 25 transients were averaged for each wavelength. Data treatment and evaluation was the same as described by Chizhov et al. (1998).

Acknowledgements

The financial support of the Deutsche Forschungsgemeinschaft (SFB 394, En 87 10/39) is gratefully acknowledged. We thank R.P. Seidel for providing the genomic clones of pSRII and pHtrII.

References


Edited by W. Baumeister

(Received 18 April 2000; received in revised form 30 June 2000; accepted 4 July 2000)