J|A|C|S ARTICLES

Published on Web 09/24/2009

Primary Photoinduced Protein Response in Bacteriorhodopsin and Sensory Rhodopsin II

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Abstract: Essential for the biological function of the light-driven proton pump, bacteriorhodopsin (BR), and the light sensor, sensory rhodopsin II (SRII), is the coupling of the activated retinal chromophore to the hosting protein moiety. In order to explore the dynamics of this process we have performed ultrafast transient mid-infrared spectroscopy on isotopically labeled BR and SRII samples. These include SRII in D_2O buffer, BR in $H_2{}^{18}O$ medium, SRII with ^{15}N-labeled protein, and BR with $^{13}C_{14}{}^{13}C_{15}$-labeled retinal chromophore. Via observed shifts of infrared difference bands after photoexcitation and their kinetics we provide evidence for nonchromophore bands in the amide I and the amide II region of BR and SRII. A band around 1550 cm^{-1} is very likely due to an amide II vibration. In the amide I region, contributions of modes involving exchangeable protons and modes not involving exchangeable protons can be discerned. Observed bands in the amide I region of BR are not due to bending vibrations of protein-bound water molecules. The observed protein bands appear in the amide I region within the system response of ca. 0.3 ps and in the amide II region within 3 ps, and decay partially in both regions on a slower time scale of 9−18 ps. Similar observations have been presented earlier for BR5.12, containing a nonisomerizable chromophore (R. Gross et al. **J. Phys. Chem. B** 2009, *113*, 7851−7860). Thus, the results suggest a common mechanism for ultrafast protein response in the artificial and the native system besides isomerization, which could be induced by initial chromophore polarization.

1. Introduction

Retinal binding proteins as bacteriorhodopsin (BR),[1] halorhodopsin (HR),[2,3] sensory rhodopsin II (SRII)[4] and proteorhodopsin (PR)[5] execute different light driven biological functions, i.e. trans-membrane ion pumps or light sensors. Despite these differences, the respective function in each system is based on a photoinduced all-*trans* to 13-*cis* isomerization reaction of the retinal chromophore, covalently bound to a lysine residue via a protonated Schiff base. The static (e.g., absorption maximum λ_{max}) as well as the dynamic properties of each system are governed by the specific interaction between the chromophore and the chromophore binding pocket. Chromophore dynamics during the primary photoreaction have been investigated in numerous studies by means of time-resolved transient absorption in the UV/vis and mid-infrared regime as well as by time-resolved Raman techniques. For a recent review see ref 6 and work cited therein. After excitation, the isomerized and vibrationally relaxed chromophore in its electronic ground state (K-state) is formed on a time scale of a few picoseconds. A crucial process during this time is the coupling of the excited and reacting chromophore to the protein moiety, which then, thermally driven, carries out the biological function on longer time scales.

Protein structural alterations during the photocycles of BR, HR, and SRII have been studied e.g. by means of nanosecond to millisecond time-resolved Fourier transform infrared (FTIR) spectroscopy. For example, absorbance changes in the amide I and amide II region of BR,[7−9] HR[10] and SRII[11] have been

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 10.1021/ja904218n CCC: $40.75

associated with functionally relevant protein backbone distortion. Concerning the initial step of the photocycles, i.e. the trans−cis isomerization of the chromophore, structural differences between the ground state and the cryogenically trapped K-like product state have been deduced from static FTIR studies[9,11−15] and X-ray diffraction on protein crystals.[16,17] Thus, albeit on the basis of quasi-stationary experiments, these studies already suggest the existence of various processes pertaining to the protein moiety that could be induced very quickly by the photoreaction of the chromophore. However, only little is known about the temporal evolution of the protein response and questions raise as: Is it possible to observe such protein alterations at ambient temperature? How fast do they occur? Can they be assigned on a molecular level?

Femtosecond time-resolved IR spectroscopy is an approach to monitor protein as well as chromophore related dynamic processes (e.g., conformational changes) at ambient temperature in real time equally efficient. The transient IR absorption spectra obtained earlier for the study of the primary reactions of BR,[18] HR[19] and SRII[20] have been used so far predominantly to extract contributions due to the reacting retinal chromophore in order to elucidate the respective reaction scheme. However, in addition the spectra comprise the contributions from those IR active vibrational modes of the protein or protein bound water molecules that are perturbed by the photoreaction. In fact, in the amide I and amide II region of the transient IR spectra of BR, HR and SRII, indications for a protein response on the time scale of the primary photoreaction have been found already. Similar observations have been reported for PR in the amide II region[21,22] and the amide I region.[23]

In the present work, using various isotopically labeled protein samples of BR and SRII (Table 1), we have compiled evidence for a number of protein alterations in the amide I and amide II region in BR and SRII on the time scale of the photoreaction. The protein samples include isotopically labeled buffer, ^{15}N isotopically labeled protein as well as $^{13}C_{14}{}^{13}C_{15}$ isotopically labeled retinal chromophore. In combination with the kinetic analysis of the transient absorbance data, the observed frequency shifts allow in principle to distinguish unambiguously between infrared bands of the retinal chromophore and the protein moiety including protein bound water molecules. In particular, we provide evidence for ultrafast protein response in BR and SRII within 3 ps or faster as observed in the amide I and amide II region. The associated absorbance differences evolve further with 9−18 ps, i.e. significantly slower than the primary chromophore dynamics. Contributions of water molecules (H_2O bending mode) are not explicitly observed but cannot be excluded. The results demonstrate the complexity of the processes taking place in the protein moiety concomitantly with chromophore excitation and isomerization.

Table 1. Investigated Systems

labeled site	system	abbreviation
no isotopic labeling	SRII-wild-type (wt)	SRII
	BR-wild-type (wt)	BR
buffer	SRII-D_2O	SRII-D_2O
	BR-$H_2{}^{18}O$	BR-$H_2{}^{18}O$
apoprotein	SRII-^{15}N-protein[a]	SRII-^{15}N
chromophore	BR-$^{13}C_{14}{}^{13}C_{15}$-retinal	BR-^{13}Cret

[a] ^{15}N labeling of the apoprotein includes the nitrogen of the Schiff base linkage between chromophore and protein.

2. Materials and Methods

2.1. Preparation of SRII, BR, and Isotopically Labeled Samples. SRII. Heterologous synthesis of SRII and purification of *Escherichia coli* membranes. The pET27bmod construct[24] was transformed into BLR(DE3)pLysS cells (Merck Biosciences). The transformed bacterial cells were grown at 37 °C in TB. At an OD_{600} of 0.6−0.7 initiation of T7 RNA polymerase expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (1 mM). At the same time 10 μM all-*trans*-retinal was added. The cells were harvested 4 h postinduction by centrifugation (4500 g, 10 min, 4 °C), resuspended in buffer medium (1 mM EDTA, 10% glycerol, 10 mM Tris, pH 8.0) and immediately frozen. To induce autolysis by the endogenous lysozyme, frozen cells were thawed at 37 °C and treated with a pinch of DNase and RNase. To avoid proteolytic activity, phenylmethanesulphonylfluoride (PMSF) (1 mM) was added. Cell lysis was supported by sonfication. In the first centrifugation step (20000 g, 15 min, 4 °C), cell debris were removed and membranes were collected from the supernatant by centrifugation (110000 g, 40 min, 4 °C).

Purification of SRII by IMAC. Membranes (see above) were resuspended in buffer medium A (300 mM NaCl, 10 mM imidazole, 50 mM Na_2HPO_4, pH 8) containing 2% DDM (Glycon Biomedicals; www.glycon.de) and stirred for 16 h at 4 °C to allow solubilization of intrinsic proteins. After centrifugation of the solubilized membranes (110000 g, 30 min, 4 °C), the supernatant was incubated with Ni-NTA agarose (Qiagen; http://www.quiagen.com) for 2 h. The Ni-NTA suspension was transferred onto a chromatography column, washed with 10 volumes of the Ni-NTA agarose bed volume with buffer medium B (300 mM NaCl, 10 mM imidazole, 50 mM Na_2HPO_4, pH 8, 0.5% DDM) and afterward, with 10 column volumes of buffer medium C (300 mM NaCl, 60 mM imidazole, 50 mM Na_2HPO_4, pH 8, 0.5% DDM). Subsequently, recombinant SRII was eluted with an appropriate amount of buffer medium D (300 mM NaCl, 500 mM imidazole, 50 mM Na_2HPO_4, pH 8, 0.05% DDM). To remove the imidazole the eluate was diluted 1:15 with buffer medium E (10 mM Tris, pH 8, 0.05% DDM) and transferred onto a chromatography column containing diethyl amino ethane (DEAE) and washed

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with 10 volumes of the DEAE bed volume with buffer medium F (20 mM NaCl, 10 mM Tris, pH 8, 0.1% DDM). The imidazole free recombinant SRII was eluted with buffer medium G (500 mM NaCl, 10 mM Tris, pH 8, 0.1% DDM).

SRII-^{15}N. Fully uniformly ^{15}N labeled SRII was expressed in *E. coli* grown on synthetic minimal medium M9,[25] which contained 0.1% ^{15}N NH_4Cl and purified as described.[24,26]

For reconstitution into polar lipids (preparation as described in[27]) SRII and lipids were mixed in a 1:28 molar ratio together with detergent-adsorbing Biobead (Boehringer Mannheim). After filtration of Biobead, the reconstituted proteins were pelleted by centrifugation.

BR. Suspensions of bacteriorhodopsin were prepared according to published procedures.[28]

SRII-D_2O and BR-$H_2{}^{18}O$. H_2O/D_2O and $H_2O/H_2{}^{18}O$ exchange was performed by diluting a pellet of SRII in buffer based on D_2O and BR in a medium based on $H_2{}^{18}O$. This procedure was performed three times for D_2O and twice for $H_2{}^{18}O$. Before preparing protein films (see below) BR was kept in contact with bulk $H_2{}^{18}O$ medium for five days.

BR-^{13}Cret. The $^{13}C_{14}{}^{13}C_{15}$-labeled retinal was synthesized as previously described.[29] ^{13}C-labeled BR was prepared by incubation of apomembrane of BR (20 mM Phosphate buffer, pH 7) with 1.5 equivalents of the labeled retinal (dissolved in 1% ethanol of the total suspension volume) at 25 °C for two hours.

For preparation of protein films suspensions of the described proteins in 15 mM KCl, 1 mM Tris, pH 7 were used, only the films BR- $H_2{}^{18}O$ were based on a suspension of BR in 15 mM KCl.

All experiments were carried out on protein films which contain just sufficient water to ensure full protein stability and function and to avoid unnecessary absorption losses by water. The sample absorbance was set to about 1 OD at the respective absorption maximum ($\lambda_{max,BR} = 570$ nm, $\lambda_{max,SRII} = 500$ nm). Sample integrity was ensured first by controlling the static UV/vis and FTIR spectra before and after the experiments and second by flash-photolysis measurements of the photocycle duration. During the fs-pump−probe experiments the sample was rotated and moved laterally to the exciting and probing laser beams in order to provide fresh sample conditions at each laser shot. All experiments were performed at room temperature.

2.2. Transient Absorption Spectroscopy. A commercial Titanium-Sapphire laser system (CPA 2001, Clark-MXR Inc., Dexter, Michigan) with a repetition rate of 635 Hz was used as a source for generating ultrashort pump and probe pulses by means of nonlinear optics as described earlier.[19]

The pump pulses were generated by means of a home-built noncollinear parametric amplifier (NOPA), tunable between 470−765 nm and a focal width of about 200 μm and a pulse energy of 0.2 μJ at the sample position. Samples of BR and SRII were excited at the respective absorption maximum of $\lambda_{max,BR} = 570$ nm and $\lambda_{max,SRII} = 500$ nm. The probe pulses in the mid-IR were obtained from a two-stage optical parametric amplifier (OPA) followed by difference frequency generation. The transmitted IR pulses with a spectral width of 100 cm^{-1} were dispersed in a polychromator and the spectrally resolved intensity was detected by a 32-channel-MCT-array (Infrared Systems). The system response (250−330 fs full width at half maximum) and time zero were determined by pump−probe experiments on a thin silicon sample.

During the measurements every second pump pulse was chopped and the pump-induced absorption differences $\Delta A(t,\lambda_{pr})$ were measured as a function of the delay time t between pump and probe pulse at probe wavelength λ_{pr} and were evaluated on a single-shot basis. Negative absorption changes indicate the disappearance of IR absorption and thus depopulation of e.g. electronic ground-state vibrations (bleach bands). Positive absorbance changes display the absorption of newly populated states. The detected absorbance changes were quantitatively analyzed by a global multiexponential fit (eq 1) for delay times >150−300 fs using

$$\Delta A(t,\lambda_{pr}) = A_0(\lambda_{pr}) + \sum_{i=1}^{N} A_i(\lambda_{pr}) \times e^{-t/\tau_i} \qquad (1)$$

where $A_0(\lambda_{pr})$ is the pump-induced difference absorption spectrum after long delay times (60−80 ps in our experiments) and $A_i(\lambda_{pr})$ are the decay-associated amplitude spectra (DAS) of the corresponding time constants τ_i. Note that a global fit as an average of absorbance transients at many different wavelengths is prone to ignore respectively suppress such additional kinetic components that appear in a spectrally narrow range only. Therefore, in addition to the global fit, the data were analyzed by a multiexponential fit of single absorbance transients.

3. Results

In order to identify earliest nonchromophore contributions in the transient IR spectra, various isotopically labeled retinal proteins were investigated. A list of the studied systems is given in Table 1. The choice of samples and covered wavenumber regions was determined by respective sample availability and degree of homogeneity of sample films.

SRII and BR are the respective native retinal proteins. In SRII-D_2O, where the buffer is based on D_2O, exposed exchangeable protons undergo H/D-exchange. This includes protein bound water molecules and exchangeable protons of the apoprotein as well as the protonated chromophore Schiff base. Therefore a spectral shift is expected for the chromophore C═NH stretching vibration (ca. 20 cm^{-1})[11] and for the H_2O bending vibration from ~1660 cm^{-1} to the region around 1200 cm^{-1} [30]. Isotopic effects are also expected for the amide I (ca. 10 cm^{-1} or less)[31] and amide II (ca. 90 cm^{-1})[32] bands, mixtures of NH bending with C═O and C−N stretching vibrations of the peptide units, respectively. In BR-$H_2{}^{18}O$ the protein is suspended in a medium based on $H_2{}^{18}O$, with a down-shift (7 cm^{-1}) of its bending vibration.[33,34] Only those protein bound H_2O molecules with access to the surrounding buffer are expected to exchange with $H_2{}^{18}O$. ^{15}N labeling of the apoprotein in SRII (SRII-^{15}N) causes a spectral shift (ca. 15 cm^{-1}) of the

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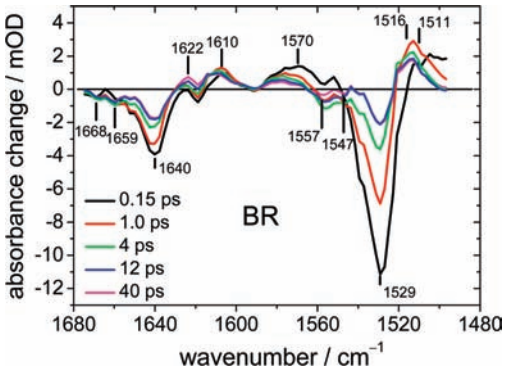

Figure 1. Difference spectra of BR at selected delay times after excitation.

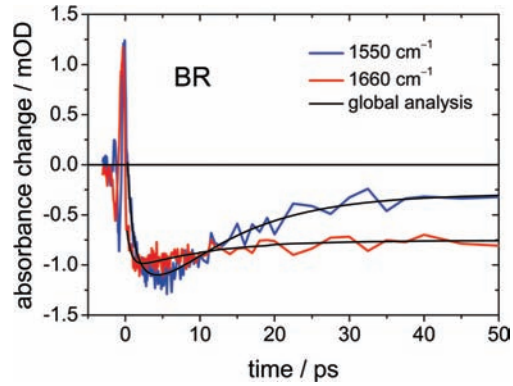

Figure 2. Absorption transients of BR in the amide I and amide II region.

amide II mode.[35−37] Moreover, all vibrations of nitrogen-containing groups are affected, especially the chromophore C═NH stretching vibration (13 cm^{-1}).[38] However, in the case of isotopic labeling of the apoprotein no influence on the chromophore vibrations except for the Schiff base is expected.

^{13}C labeling of C-atoms 14 and 15 of the retinal chromophore in BR-^{13}Cret influences the spectral positions of chromophore bands, for example C═C, C−C, and C═NH stretching vibrations[39] (see below), but not protein bands.

The following parts summarize the results of the femtosecond time-resolved IR measurements on all the listed systems, especially the observed isotopic band shifts. First, the results concerning the chromophore dynamics in BR and SRII are described. They confirm the mainly biphasic reaction dynamics during the all-*trans* to 13-*cis* isomerization as reported earlier.[18,20] Moreover, in both systems absorbance difference bands in the amide I and amide II region are observed associated with a unique time constant of ∼11 ps, indicating a nonchromophore origin. Then, the results on the labeled systems are presented with focus on evidence of nonchromophore bands in the amide I and amide II region.

3.1. Chromophore Dynamics in BR and SRII. 3.1.1. BR. Transient IR absorption difference spectra of BR are depicted in Figure 1. The bleach bands of the C═C (1529 cm^{-1}) and C═NH stretching vibration (1640 cm^{-1}) of the all-*trans*-retinal chromophore[39] are the dominant features. They decay partially within a few picoseconds when the ground state BR$_{570}$ (subscript indicates λ_{max} of the specific state) becomes repopulated in accordance with the isomerization quantum yield $\Phi < 1$ (0.64 for BR[40−42]). The positive (product) signals around 1516, 1610, and 1622 cm^{-1} correspond to the K-state.[43] The instantaneous positive absorption difference at 1570 cm^{-1} decays in parallel with the excited electronic state (see below) and is thus assigned to S_1-vibrational modes of the chromophore.

Absorbance changes observed around 1550 (1545−1560 cm^{-1}) and above 1650 cm^{-1} (1655−1670 cm^{-1}) are suggested to be nonchromophore bands for the following reasons. Above 1640 cm^{-1}, the position of the C═NH stretch, no further chromophore modes are expected[39,44] (not taking into account C−H and N−H stretching vibrations). With respect to the band around 1550 cm^{-1}, earlier studies have proposed the existence of a fast protein response in BR,[45] HR,[19] PR[21] and SRII.[20] Similar bands in shape and spectral position have been observed earlier in several IR experiments concerning the primary events in the photocycle in retinal proteins.[43,46,47] The kinetic behavior of the absorbance transients of BR around 1550 and 1660 cm^{-1} is quite similar (Figure 2). Here, biexponential single transient fits reveal an appearance within less than 2 ps and a partial decay within about 11 ps, i.e. significantly slower than chromophore-associated dynamics (see below). However, above 1650 cm^{-1} negative absorption differences are observed at 150 fs (Figure 1).

A global analysis of the BR data according to eq 1 yields three time constants: τ_1-BR = 0.6 ± 0.1 ps, τ_2-BR = 3.2 ± 0.1 ps and τ_3-BR = 11.1 ± 0.6 ps. The corresponding DAS are shown in Figure 3. They are consistent with a branching reaction from the excited electronic state to the isomerized electronic ground state J and to the initial all-*trans* state with 0.6 ps, followed by vibrational cooling and torsional relaxation with about 3 ps in both paths (K formation and BR$_{570}$ recovery).[18,48−50] Compare Supporting Information (Figure SI 1) for the BR reaction scheme. In the ethylenic stretch region, the negative amplitude of the A_1-BR around 1511 cm^{-1} indicates the fast formation of the J state (early band at 1511 cm^{-1} in Figure 1), which decays to K (1516 cm^{-1})[51] with τ_2-BR (positive amplitude at 1511 cm^{-1} of A_2-BR). The wavenumber of the red-shifted

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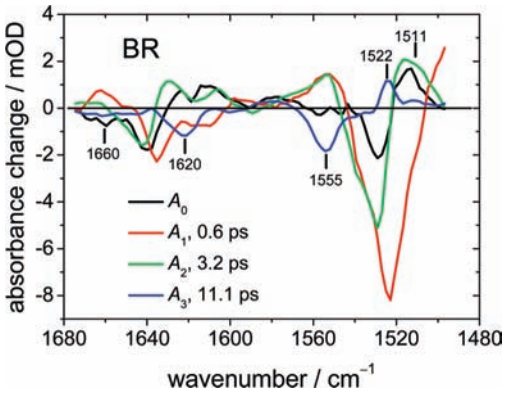

Figure 3. Decay-associated spectra ($A_i = A_i$-BR) of the amide I and amide II region of BR obtained by a triexponential global analysis.

ethylenic stretch in J and K follows the linear correlation[52,53] with the respective electronic absorption maximum. The negative amplitudes of A_1-BR and A_2-BR above 1520 cm^{-1} display the fast formation of hot BR_{570} and its (blue-shifted) vibrational ground state, respectively. The DAS in the region of the C═NH stretching vibration around 1640 cm^{-1} exhibit analogous spectral features and are interpreted accordingly. Thus, these results fully confirm earlier reports on the chromophore isomerization dynamics that included the vibrational fingerprint region around 1160−1210 cm^{-1} with marker bands for the chromophore configuration.[18,45]

Moreover, in extension to earlier studies,[18] an additional, "slow" DAS with τ_3-BR = 11 ps is clearly observed. Its amplitude spectrum exhibits a positive peak at 1522 cm^{-1} and negative peaks at 1555, 1620 and around 1660 cm^{-1} (small). The minima at 1555 and 1660 cm^{-1} correspond well in their spectral position with the potentially nonchromophore bands described above. Consequently, the spectral and temporal characteristics of A_3-BR with significant contributions in the amide I and amide II region suggest a process associated primarily with the protein moiety and not with the chromophore, the dynamics of which are determined by the shorter τ_1-BR and τ_2-BR.

3.1.2. SRII. The primary chromophore reaction dynamics of SRII have been found so far[20,54] to be quite similar to that of BR. Despite one alteration concerning a further decay channel of the excited electronic state (cf. Supporting Information, Figure SI 2) the extended study presented here reproduces the main features of the reaction scheme. The IR difference spectra of SRII at various delay times are depicted in Figure 4. The main differences with respect to BR concern the spectral position but not the kinetics of the dominant chromophore vibrational bands. In accordance with the blue-shifted absorption maximum of SRII at 500 nm, the bleach of the ground state ethylenic stretch is found at 1548 cm^{-1}.[11,55] The corresponding Schiff base C═NH stretching vibration is assigned to the bleach band at 1652 cm^{-1}.[11,55] However, it should be noted that this negative absorbance feature is about twice as broad as the corresponding

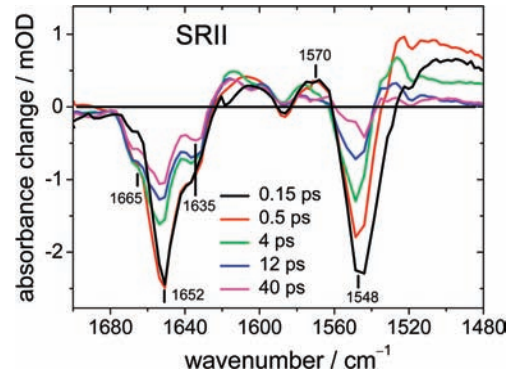

Figure 4. Difference spectra of SRII at selected delay times after excitation.

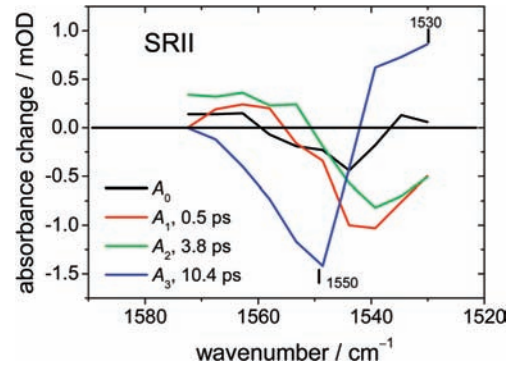

Figure 5. Decay-associated spectra ($A_i = A_{i,II}$-SRII) of the amide II region of SRII obtained by a triexponential global analysis.

band in BR and exhibits shoulders around 1665 and 1635 cm^{-1}, indicating strongly overlapping bands. Both bands, the ethylenic and the C═NH stretch, recover partially in accordance with the isomerization quantum yield $\Phi < 1$ (0.5 for SRII[56]). On the respective low-energy side product bands appear within 0.5 ps, displaying the formation of the 13-*cis* chromophore (cf. Figures SI 3 and 4, Supporting Information for spectra and kinetics of the fingerprint region which reveal the isomerization with ca. 0.5 ps.). The positive band around 1570 cm^{-1} appears within the system-response time and is assigned to excited state vibrational absorption.

As in BR, the amide I (above 1652 cm^{-1}) and amide II region exhibit bands with kinetics that are distinct from those of the chromophore. Analogous to the arguments given for BR, these bands are suggested to reflect dynamics of the protein moiety. Examples are found at 1558 and around 1665 cm^{-1}. A biexponential single transient fit yields a risetime of the bleach bands of about 1 ps and a partial decay with 11 ps (cf. Supporting Information, Figure SI 5). In particular the shoulder in the absorption difference spectra above the C═NH bleach is observed at 150 fs (Figure 4).

The global analysis of the amide II region between 1530 and 1570 cm^{-1} reveals three time constants: $\tau_{1,II}$-SRII = 0.5 ± 0.1 ps, $\tau_{2,II}$-SRII = 3.8 ± 0.8 ps and $\tau_{3,II}$-SRII = 10.4 ± 1.0 ps (subscript: index of time constant, amide region). The corresponding DAS are shown in Figure 5. Note that in the amide II region (between 1530 and 1570 cm^{-1}) the experimental spectral resolution for this sample was reduced by a factor of 2. This has to be taken into account when comparing the results on

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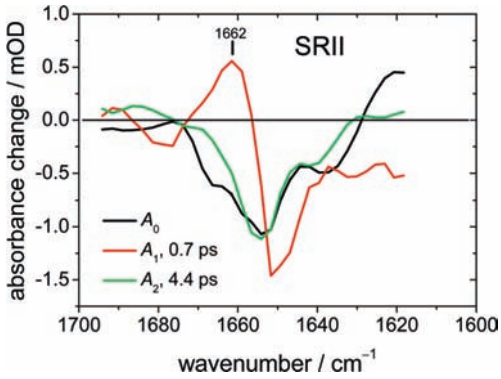

Figure 6. Decay-associated spectra ($A_i = A_{i,\mathrm{I}}$-SRII) of the amide I region of SRII obtained by a biexponential global analysis.

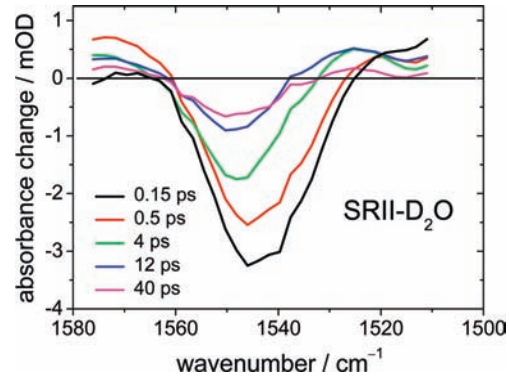

Figure 7. Difference spectra of SRII in D_2O buffer in the amide II region at selected delay times after excitation.

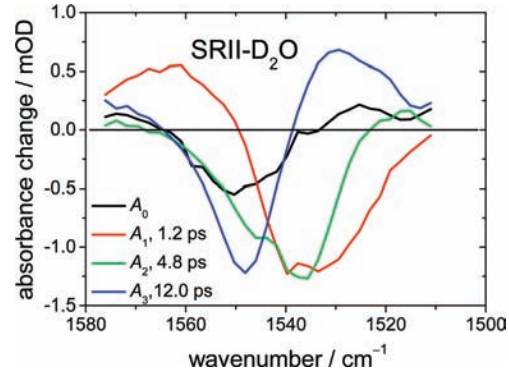

Figure 8. Decay-associated spectra ($A_i = A_{i,\mathrm{II}}$-SRII-D_2O) of the amide II region of SRII in D_2O buffer obtained by a triexponential global analysis.

this sample with those on modified SRII systems. $\tau_{1,\mathrm{II}}$-SRII and $\tau_{2,\mathrm{II}}$-SRII confirm the results in the fingerprint region with configurational marker bands (cf. Supporting Information, Figure SI 3), of earlier IR-experiments[20] and of transient absorption experiments in the visible.[54] They are consistent with a reaction scheme as described for BR. Spectral differences between the presented DAS of BR and SRII in this region are related to a stronger spectral overlap of the ethylenic stretch bands of SRII_{500} and SRII-K as compared to BR (Figure 1). This observation is in keeping with the small red-shift of electronic absorption of K_{510} of SRII[57] relative to SRII_{500} and leads to smaller IR difference signals at long delay times (in the K-state). It further implies a stronger spectral overlap of $A_{1,\mathrm{II}}$-SRII and $A_{2,\mathrm{II}}$-SRII.

Global analysis of the amide I region between 1615 and 1695 cm^{-1} does not reveal three but two DAS with time constants $\tau_{1,\mathrm{I}}$-SRII $= 0.7 \pm 0.1$ ps and $\tau_{2,\mathrm{I}}$-SRII $= 4.4 \pm 0.3$ ps (Figure 6), as well in keeping with the results described above. The interpretation of the DAS is not as straightforward as in the case of BR, possibly due to the stronger spectral overlap of the C=NH stretch and nonchromophore bands. However, the negative amplitudes of $A_{1,\mathrm{I}}$-SRII and $A_{2,\mathrm{I}}$-SRII around 1652 cm^{-1} clearly show the biexponential (partial) recovery of SRII_{500}.

As in BR, a third, slow time constant is necessary to fit the data. In the amide II region the global fit reveals $\tau_{3,\mathrm{II}}$-SRII $=10.4 \pm 1.0$ ps. $A_{3,\mathrm{II}}$-SRII exhibits a positive and a broad negative band around 1530 and around 1550 cm^{-1}, respectively. In the amide I region a third DAS could not be extracted from the data, however around 1665 cm^{-1} the bleach is observed at 150 fs and further increases with about 1 ps and decays partially with 11 ps (see above) in contrast to the neighboring C=NH stretch. The delayed rise of the bleach around 1665 cm^{-1} is in accordance with the positive amplitude of $A_{1,\mathrm{I}}$-SRII at 1662 cm^{-1}. Thus, the results on SRII in the amide region suggest protein related processes (as in BR), that are kinetically distinct from the chromophore dynamics.

3.2. Isotopically labeled systems. 3.2.1. SRII in D_2O-buffer. Amide II. The transient spectra and the corresponding DAS of SRII-D_2O are depicted in Figure 7 and Figure 8, respectively. Given the lower spectral resolution in Figure 5 (see above), spectral position and time ordering of the DAS are very similar to those of SRII in H_2O (Figure 4 and Figure 5). Thus, chromophore kinetics (here monitored mainly via its ethylenic stretch) as well as the slow kinetics of the bands in the amide II region ($A_{3,\mathrm{II}}$-SRII-D_2O) are basically unaffected by H/D-exchange. Especially the absorbance transients around 1550 cm^{-1} (cf. Supporting Information, Figures SI 5 and 6) exhibit the same kinetics for rise and partial decay as in H_2O. For the chromophore ethylenic stretch no spectral shift is expected.[11,39] Possible spectral shifts of nonchromophore bands include amino acid residues with exchangeable protons and amide II bands. FTIR experiments on BR have shown[32] that about 20−30% of the peptide groups undergo H/D exchange in BR, as monitored by a partial amide II band-shift from about 1550 to 1460 cm^{-1}. Thus, assuming similar conditions for H/D exchange in SRII, the observation that $A_{3,\mathrm{II}}$-SRII-D_2O is basically identical to $A_{3,\mathrm{II}}$-SRII suggests the existence of peptide groups (or amino acid residues) that are perturbed by the photoreaction and do not undergo H/D exchange. However, the existence of perturbed peptide groups that do undergo H/D exchange cannot be excluded (the region around 1460 cm^{-1} was not covered).

Amide I. Possible spectral effects due to H/D exchange in this region include the shift of the chromophore C=NH stretching vibration,[39] the shift of the water bending vibration from 1660 cm^{-1} to ca. 1200 cm^{-1}, unspecific shifts of amino acid residues with exchangeable protons and a down-shift (ca. 10 cm^{-1} or less) of exposed amide I vibrations.[31]

The difference spectra in this spectral region (Figure 9) exhibit pronounced changes with respect to those in H_2O buffer (Figure 4). The chromophore C=ND stretching vibration shifts from the congested region around 1652 cm^{-1} (in H_2O) to 1631 cm^{-1} as expected.[11,39] The band exhibits an apparent spectral width of ca. 14 cm^{-1}, in accordance with the C=NH stretch in BR. In the region above 1640 cm^{-1} this shift reveals at least two bleach bands at 1659 and 1668 cm^{-1} already at 150 fs, as

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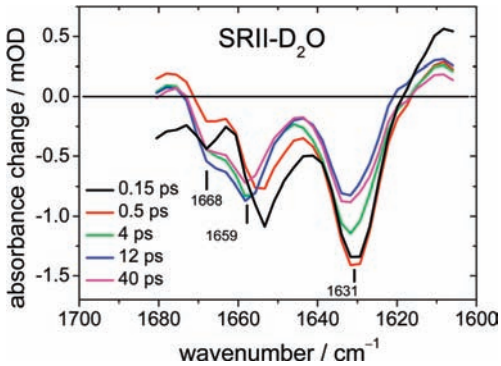

Figure 9. Difference spectra of SRII in D_2O buffer in the amide I region at selected delay times after excitation.

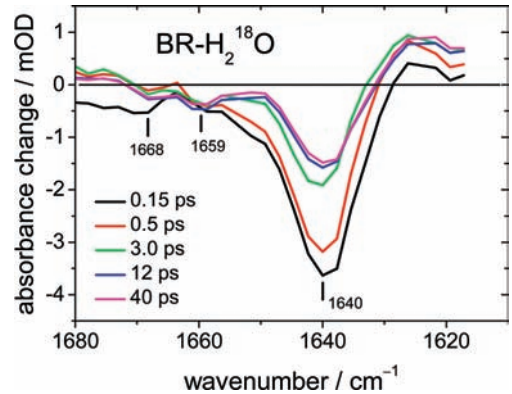

Figure 10. Difference spectra of BR in $H_2{}^{18}O$ in the amide I region at selected delay times after excitation.

detected similarly in BR. Further, the shoulder at 1635 cm^{-1} in the H_2O sample (Figure 4) is not discernible in the D_2O sample.

As in SRII the global analysis of this region yields only two time constants $\tau_{1,I}$-SRII-D_2O = 0.3 ± 0.1 ps and $\tau_{2,I}$-SRII-D_2O = 2.4 ± 0.2 ps (Figure SI 7, Supporting Information). The corresponding DAS mainly describe the chromophore dynamics as monitored by the C=ND stretch. In addition, yet not without contradictions, they also represent the dynamics of the nonchromophore bands above 1631 cm^{-1}. However, Figure 9 shows that all bleach signals above the C=ND stretch at 1631 cm^{-1} which must be attributed to protein bands, rise within 0.5 ps or faster and persist on the time scale of the experiment. In contrast to SRII, the bleach signal between 1660 and 1670 cm^{-1} does not show a partial decay but remains constant (Figure SI 6, Supporting Information), indicating an H/D exchangeable group.

The results on SRII-D_2O clearly confirm the existence of a complex fast nonchromophore response, as displayed by the bands above the chromophore C=ND stretch at 1631 cm^{-1}. The H/D exchange causes the band at 1653 cm^{-1} (C=NH in H_2O) as well as the slow (ca. 11 ps) component between 1660 and 1670 cm^{-1} to disappear. At this point, the described H/D effects are noted, but their molecular origin cannot be assigned unequivocally. However, the bands observed at 1659 and 1668 cm^{-1} cannot be due to water molecules (exposed to H/D exchange). Further, the fact that the slow (ca. 11 ps) component is not observed although negative bands remain around 1660 and 1670 cm^{-1} suggests the existence of two types of vibrational oscillators that are affected by the primary reaction: One which is H/D exchangeable while the other is not.

3.2.2. BR-$H_2{}^{18}O$. In this system exclusively exchangeable protein bound water molecules are affected. Upon $^{16}O/^{18}O$ exchange the bending vibration of water is expected to shift down 7 cm^{-1}.[33,34] Thus, only the amide I region was investigated. Much care was taken to enable maximal $H_2O/H_2{}^{18}O$ exchange (cf. Materials and Methods). Similar preparations in earlier FTIR experiments[12,58] have shown that in fact water molecules that are affected by the primary reaction should be exchanged by our procedure. This does not guarantee that all affected water molecules are exchanged. Note that the quality (homogeneity of the film) of this sample was not as good as that of BR-H_2O.

Amide I. The transient spectra (Figure 10) are virtually identical with those of BR. Especially the bands at 1640 cm^{-1} (chromophore C=NH stretch) and the nonchromophore bands at 1659 and 1668 cm^{-1} are detected as in BR. The latter show analogous kinetics as in the native system and are also observed at 150 fs. A band-shift in the order of 7 cm^{-1} is not distinguishable. The global analysis in this region (Figure SI 8, Supporting Information) reveals only the two time constants, τ_1-BR-$H_2{}^{18}O$ = 0.8 ± 0.1 ps, τ_2-BR-$H_2{}^{18}O$ = 2.9 ± 0.3 ps, related to the chromophore dynamics. The kinetics detected basically reproduce the kinetics observed in BR. However, the third slow time constant is discernible neither in the DAS nor in the kinetics of single absorbance transients, possibly due to the limited signal/noise.

The results (including a global analysis, Supporting Information, SI 8) clearly indicate that the bands around 1659 and 1668 cm^{-1} represent protein- and not water-associated modes. The equivalent observation was made for SRII-D_2O. This is surprising since it has been shown earlier that in BR and in SRII the pentagonal water cluster next to the chromophore Schiff base undergoes structural changes during transition to the K state.[12,13] The results presented here cannot exclude an $H_2{}^{18}O$ effect. However, if it exists, it is too small to be identified by this experiment (cf. Supporation Information, Figure SI 6).

3.2.3. SRII-^{15}N. ^{15}N-labeling of the protein including the ε-^{15}N of lysine 205 is expected to shift the amide II band, the peptide vibration with mainly C−N stretch plus N−H bend contributions.[59] In photosystem (PS) I and II a ^{15}N induced shift of 15 cm^{-1} has been found for the amide II mode.[35−37] The amide I mode, mainly peptide C=O stretch with minor N−H bend contribution, shifts only 1−3 cm^{-1} in PSI,II.[35−37] Shifts of chromophore bands concern mainly the C=NH stretching vibration. Resonance Raman experiments on [ε-^{15}N]lysine216 labeled BR found a downshift of this band of 13 cm^{-1} but no shift of the chromophore ethylenic stretch.[38]

Amide II. The transient absorbance difference spectra in Figure 11 show at early delay times the initial bleach band of the ethylenic stretch at 1548 cm^{-1} as expected. However, the spectrum at 40 ps exhibits significant differences with respect to the 40 ps spectrum in SRII (Figure 4). They include the pronounced negative band at 1536 cm^{-1} and the positive band at 1555 cm^{-1} in SRII-^{15}N as opposed to the negative shoulder around 1555 cm^{-1} and no shoulder around 1536 cm^{-1} in SRII. The transients around 1536 cm^{-1} occur instantaneously, increase slightly within ca. 4 ps and decay partially within 8−18 ps according to biexponential single transient fits. Thus, their kinetic behavior is similar to that of the transients around 1555 cm^{-1} in SRII.

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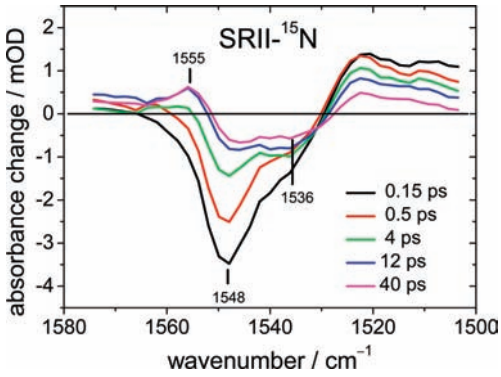

Figure 11. Difference spectra of SRII-^{15}N in the amide II region at selected delay times after excitation.

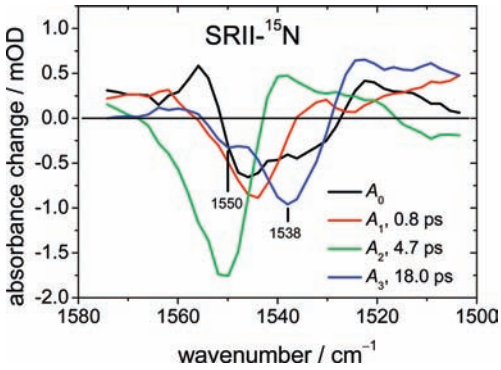

Figure 12. Decay-associated spectra ($A_i = A_{i,II}$-SRII-^{15}N) of the amide II region of SRII-^{15}N obtained by a triexponential global analysis.

A global analysis yields three time constants, $\tau_{1,II}$-SRII-^{15}N $= 0.8 \pm 0.1$ ps, $\tau_{2,II}$-SRII-^{15}N $= 4.7 \pm 0.5$ ps and $\tau_{3,II}$-SRII-^{15}N $= 18 \pm 3.4$ ps (Figure 12), which are slightly longer compared to those of SRII but still of similar magnitude. Most important in the context of the assignment of protein bands is the slow component, $A_{3,II}$-SRII-^{15}N. It exhibits a general red-shift compared to $A_{3,II}$-SRII (Figure 5). In particular, the major negative peak shifts from ca. 1550 cm^{-1} in SRII to 1538 cm^{-1} in SRII-^{15}N, i.e. a comparable position as the shoulder in the transient spectra in SRII-^{15}N (Figure 11). This shift is in full accordance with the expected isotope shift of an amide II band (see above). Besides this observation, a shoulder of $A_{3,II}$-SRII-^{15}N remains at 1550 cm^{-1}, the region of the ground state C═C stretch. Since this (slow) contribution can hardly be associated with a chromophore band, it is here attributed to either an amide II band or a band of an amino acid side chain shifted to that spectral position upon ^{15}N-labeling. Another possibility is that of a protein band which does not shift but becomes apparent by the isotopic down-shift of a superimposed amide II band. The positive part of $A_{3,II}$-SRII-^{15}N (below 1540 cm^{-1}) has also changed compared to $A_{3,II}$-SRII. It is less red-shifted as expected for the amide II isotopic shift of 15 cm^{-1}. This indicates a complex superposition of protein bands (amide II or amino acid side chains) with different isotopic shifts.

$A_{1,II}$-SRII-^{15}N and $A_{2,II}$-SRII-^{15}N in Figure 12 and the respective DAS in Figure 8 and 5 show some mismatch although the chromophore dynamics are not expected to change upon ^{15}N-labeling. In part this can be due to the lower spectral resolution in Figure 5 (see above). In addition, it cannot be excluded that $A_{1,II}$-SRII-^{15}N and $A_{2,II}$-SRII-^{15}N comprise contributions that describe the formation of protein bands besides the contributions of chromophore related processes. Consequently, the observed

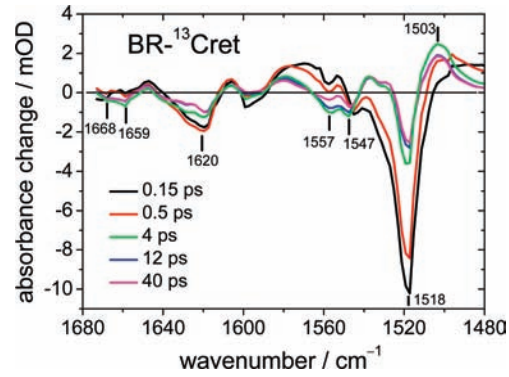

Figure 13. Difference spectra of BR-^{13}Cret at selected delay times after excitation.

mismatch could indicate the ^{15}N-induced shift of these contributions.

In conclusion, the observed shift of 12 cm^{-1} in the slowest DAS by ^{15}N labeling clearly proves the assignment of the bands at 1550 and 1536 cm^{-1} in SRII and SRII-^{15}N, respectively, to protein bands and strongly support their assignment to amide II bands. The changed shape of $A_{3,II}$-SRII-^{15}N with respect to $A_{3,II}$-SRII further suggests that they are associated with protein bands with varying degree of ^{15}N isotopic shift.

Amide I. Experiments on ^{15}N labeled SRII in this region primarily reveal the essential shift of the chromophore C═NH stretch (see above) from 1652 cm^{-1} in SRII to 1635 cm^{-1} in SRII-^{15}N. No significant shifts of amide I bands are expected and observed (data not shown).

3.2.4. BR-^{13}Cret. These experiments are complementary to those on the labeled (apo-)protein and should corroborate the identification of protein related bands in the transient spectra. Only chromophore vibrational bands are expected to shift. In particular, as shown by resonance Raman spectroscopy and normal-mode analysis of BR and BR-^{13}Cret,[39] the chromophore ethylenic stretch shifts from 1527 to 1517 cm^{-1} (observed) and from 1528 to 1520 cm^{-1} (calculated), respectively. Accordingly, the C═NH stretch shifts from 1640 to 1622 cm^{-1} (observed) and from 1639 to 1616 cm^{-1} (calculated), respectively.

Amide II. The survey of the IR difference spectra in the amide II region (Figure 13) and comparison with the unlabeled BR (Figure 1) reveals great similarities. The major difference is the down-shift of ca. 11 cm^{-1} of those features that are related to the chromophore ethylenic stretch, i.e. the strong bleach at 1518 cm^{-1} (1529 cm^{-1} in BR) and the corresponding product band at 1503 cm^{-1} (1516 cm^{-1} in BR). Both shifts are in agreement with the predicted one. In contrast, the region between 1545 and 1580 cm^{-1} remains unaltered with respect to BR. This concerns in particular the negative bands at 1547 and 1557 cm^{-1} and their (slow) kinetics in single absorbance transient fits (data not shown). Normal mode analysis of BR and BR-^{13}Cret has shown[39] that additional chromophore C═C stretches (besides the ethylenic stretch) in this region undergo isotopic shifts of about 6−9 cm^{-1}. Since no spectral shifts with respect to BR are observed between 1540 and 1580 cm^{-1}, the bands at 1547 and 1557 cm^{-1} are assigned to protein vibrations.

This view is supported by the decay-associated spectra (Figure 14). Three time constants are obtained, τ_1-BR-^{13}Cret $= 0.7 \pm 0.1$ ps, τ_2-BR-^{13}Cret $= 2.6 \pm 0.1$ ps and τ_3-BR-^{13}Cret $= 9.2 \pm 0.7$ ps, which agree well with the results from BR (Figure 3). Besides a spectral downshift of ca. 11 cm^{-1} the spectral signature of A_1-BR-^{13}Cret and A_2-BR-^{13}Cret in the region 1490−1580

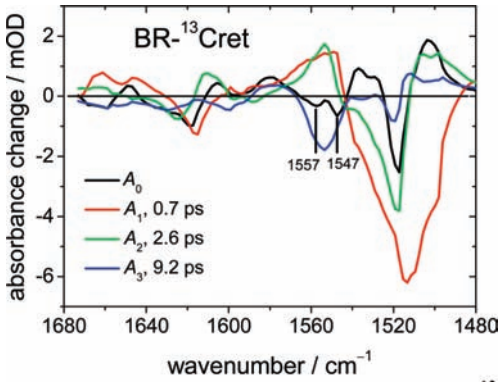

Figure 14. Decay-associated spectra ($A_i = A_i$-BR-^{13}Cret) of the amide I and amide II region of BR-^{13}Cret obtained by a triexponential global analysis.

cm^{-1} remains the same, in accordance with the respective difference spectra. In the spectral region 1540−1580 cm^{-1} (the slow) A_3-BR-^{13}Cret is found unaltered with respect to BR, strongly suggesting its assignment to protein related processes. The observation that neither A_1-BR-^{13}Cret, A_2-BR-^{13}Cret nor A_3-BR-^{13}Cret exhibit the spectral feature of A_0-BR-^{13}Cret with its double band at 1547 and 1557 cm^{-1} implies that the double band appears with the experimental system response.

A surprising result is associated with the changed and spectrally shifted feature of A_3-BR-^{13}Cret between 1510 and 1525 cm^{-1} (Figure 14) compared to BR (Figure 3). Assuming that (the slow) A_3-BR-^{13}Cret exclusively represents protein dynamics, it should not change upon chromophore isotopic labeling. The effect of isotopic labeling could be accounted for by assuming that the chromophore is coupled to slower dynamics in contrast to the previous assumption.

Amide I. The line of arguments for the amide I region is very similar to that for the amide II region. In BR-^{13}Cret the chromophore C=NH stretching mode shifts as expected from 1640 to 1620 cm^{-1} (Figure 1 and Figure 13) and the doublet at 1659 and 1668 cm^{-1} remains at the same spectral positions as in BR. It is still apparent at long delay times and the corresponding transients exhibit the same features as in the wild-type (data not shown). Accordingly, the decay-associated spectra (Figure 14) show a great resemblance to those of the unlabeled BR besides the 20 cm^{-1} shift of A_1-BR-^{13}Cret and A_2-BR-^{13}Cret. Thus, since all chromophore modes of all-*trans*- and 13-*cis*-retinal between 1500 and 1640 cm^{-1} are downshifted by the $^{13}C_{14}{}^{13}C_{15}$-labeling of the chromophore[44,60] and since no chromophore modes are expected above 1640 cm^{-1}, the small bands at 1659 and 1668 cm^{-1} must represent nonchromophore modes.

In summary, on the basis of spectral and kinetic observations, the results on the ^{13}C-isotopically labeled chromophore in BR-^{13}Cret clearly corroborate the assignment of the bands at 1659 at 1668 cm^{-1} in the amide I region and the bands at 1547 and 1557 cm^{-1} in the amide II region to protein vibrational modes.

4. Discussion

On the basis of the observed isotope shifts and the kinetics in the transient IR spectra, the results are summarized as follows:

(i) In the amide region of BR and BR-^{13}Cret negative bands at 1547, 1557, 1659, and 1668 cm^{-1} are assigned to nonchromophore vibrational modes. The observed bands in the amide I region are not caused by protein bound water molecules although the latter are expected to contribute to the IR difference signals. In SRII strong evidence is provided for a (negative) protein band at 1550 cm^{-1}. The spectral shift of this band in the isotopically labeled SRII-^{15}N is indicative for an amide II mode. Further, in SRII a band at 1635 cm^{-1} is assigned to a protein vibrational mode which is suggested to undergo H/D exchange in D_2O buffer. In SRII-D_2O bands at 1659 and 1668 cm^{-1} are attributed to either amide I vibrations or modes of amino acid side chains.

(ii) The temporal evolution of these protein bands is characterized by a fast apparent rise and a slower partial decay. The determination of the risetime in the amide II region is affected by spectrally overlapping contributions of the electronic ground state bleach and the excited electronic state absorption as part of the respective A_1 (decaying with τ_1) and by contributions of A_2 (decaying with τ_2). Thus, it cannot be excluded that the bands appear within the system-response time of ca. 300 fs. However, they clearly appear not later than the primary reaction of the chromophore. In contrast, in the amide I region the absorption difference spectra undoubtedly show that protein bands already appear within the system response, i.e. before chromophore isomerization. The partial decay observed in the amide II and amide I region around 1550 and 1660 cm^{-1} is characterized by τ_3, in the range of 9−18 ps. This slow component is easily identified by the global fit in the amide II region of BR and SRII whereas in the amide I region this is only the case for the BR systems. However, in BR and SRII it is identified by fits of single absorbance transients in both regions. It must be noted that after the partial decay the bands persist on the time scale of the experiment (40 ps) with no indications for a further decay.

The results clearly show the existence of ultrafast protein-associated processes in BR and SRII that are triggered by the photoreaction of the chromophore. In the amide I region they become apparent on the time scale of the system response. In the amide II region they appear latest with chromophore isomerization and evolve further on the 9−18 ps time scale (τ_3).

IR difference bands similar to those reported here have been observed earlier in static low-temperature FTIR experiments concerning the K-state in BR and SRII. Diagnostically conclusive in this respect is the amide I region which is known to be quite sensitive to the secondary protein structure and is more clear than the amide II region since it comprises less chromophore modes than the latter. The low-temperature FTIR spectra[9,11,15,43] of the K-state in BR, SRII and SRII-D_2O exhibit specific differences in amplitudes and spectral position as compared to the time-resolved spectra at long delay times (K) presented in this work. This is attributed to the different accessibility of the various degrees of freedom at different temperatures. However, it is obvious that protein alterations do take place on the time scale of the primary reaction at ambient temperature.

More indications for protein alterations in the K-state have been observed earlier by low-temperature FTIR studies on BR[12,14,61] and SRII[13] with focus on the stretching vibration of protein bound water molecules. For BR, changes of O−D stretching vibrations of three water molecules that belong to a pentagonal cluster in the Schiff base region have been reported.

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This suggests a considerable perturbation of the hydrogen bond network during chromophore isomerization. Similar features of water bands were observed in SRII indicating analogous structural changes of internal water molecules in both systems. This view is supported by X-ray studies of the K-state of BR[17,62] and SRII[16,63] demonstrating a rearrangement of specific amino acid residues and the water cluster in vicinity of the retinal Schiff base.

Considering the nature of the photoinduced retinal isomerization in BR and SRII, several mechanisms for the coupling between the chromophore and its environment can be envisaged. Electronic excitation of the chromophore leads to a charge transfer along the polyene chain, which could almost instantaneously polarize and alter the charge distribution in the chromophore binding pocket, including charged amino acid residues and the hydrogen bond network. The following electronic ground state recovery and the trans−cis isomerization of the covalently linked chromophore with its charged Schiff base nitrogen then further perturb the chromophore environment sterically and again by charge displacement. Each of these processes, i.e. polarization, perturbation of H-bonds and conformational alterations or combinations of them can cause changes in the IR spectra. Whereas retinal isomerization itself is more than plausible to cause the appearance of the protein bands on the time scale of 0.5 ps, our observation of even earlier changes in the IR spectra (amide I) within the system response points to a likewise rapid process that precedes isomerization. In fact, in recent analogous experiments on BR5.12[45] (BR reconstituted with a nonisomerizing, structurally locked retinal chromophore) we have found protein alterations very similar in kinetics and spectral appearance to those observed for BR. In BR5.12 negative IR difference bands around 1550 cm^{-1} and at 1660 and 1669 cm^{-1} were found to appear within a few picoseconds or even instantaneously and thus much faster than the S_1 decay time of this locked system (18 ps[64]). They further show a temporal evolution on a longer time scale and persist for much longer (130 ps in that experiment) than the excited state lifetime. Since in BR5.12 the chromophore is photochemically inactive and returns to the electronic ground state with a S_1 lifetime of 18 ps, it was concluded that the observed light induced protein alterations cannot be caused by isomerization. Instead they have been associated with the excitation induced change of electronic dipole moment $|\Delta\mu|$ and with the altered chemical reactivity that was found on longer time scales in photoexcited nonisomerizable artificial (BR-) pigments.[65] In consequence, the findings in BR5.12 and their similarities with native BR strongly suggest, that the observed ultrafast protein alterations in BR and SRII are at least partly caused by intramolecular charge transfer.

Excitation induced polarization of the chromophore and of the surrounding protein moiety in native retinal proteins has been widely discussed in the literature as a process that might act together with the isomerization in a cooparative manner.[66−68] Magnitude and impact of this ultrafast "field-jump" on the BR protein moiety and its temporal evolution have been studied in several ways. The dipolar characteristics of the chromophore in BR have been characterized e.g. by second harmonic generation in purple membrane films[69] and for $|\Delta\mu|$ values between 13 D[70] and 34 D[71] have been reported. Theoretical considerations have linked a fast twisting motion of the electronically excited chromophore prior to isomerization to an increase of the charge translocation.[72] The involvement of retinal binding site tryptophanes in increasing charge translocation following light absorption was proposed based on second harmonic generation measurements of artificial pigments.[73] Following the absorption dynamics of the nearby tryptophan residue Trp86 after retinal excitation,[74] the rapid Stark-shift of the Trp86 absorption band on a sub-50 fs time scale was traced back to the underlying retinal charge translocation. The Stark-shift then reaches a long-lived plateau on the time scale of the isomerization process (500 fs), indicating a changed charge distribution as compared with the BR ground state. A different type of experiment has dealt with the sudden polarization in BR by detection of light-induced coherent terahertz radiation with femtosecond time resolution.[75] The principal component of the terahertz emission could be well described by excited-state intramolecular electron transfer within the retinal chromophore. An additional slower process (3−4 ps) was associated with the redistribution of an H-bond near the chromophore. Similarly, resonant optical rectification to the infrared has been used to quantify the sudden light-induced polarization in BR.[71] Further, molecular dynamics simulations[76] and photon echo experiments[77] have suggested a fast dielectric response in BR within 50−100 fs without larger scale conformational changes and additional diffusive components.

Although an assignment of the observed ultrafast protein IR bands to specific amino acid residues or specific peptide units is not possible at the moment on grounds of the observed isotope shifts, the results of this work give strong arguments for the existence of polarization induced protein alterations in BR and SRII. Further studies are required to reveal how they might possibly be linked to chromophore isomerization, i.e. to what extent they are part of the multidimensional reaction coordinate describing the primary reaction. A slower component of the response in the native systems could be represented by the third time constant τ_3 of ca. 9−18 ps. Usually, the primary reaction dynamics in BR and SRII have not been associated with such

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a slow time constant. Reports on slower components in the chromophore dynamics of BR concern minor amplitudes in excited state fluorescence decay[78−80] and resonance Raman measurements.[50] In contrast, the results presented here very clearly exhibit this slow component and indicate an additional process in the relaxation dynamics of the perturbed protein-chromophore complex, which is not easily noticed in electronic spectroscopy.

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Acknowledgment. R.G., M.W., C.S., and R.D. acknowledge support by Deutsche Forschungsgemeinschaft (DFG), OTLAP, and RIMB Forschungsschwerpunkte Rheinland-Pfalz. H.E.N. and R.D. acknowledge grants from Stiftung Rheinland-Pfalz für Innovation. R.G. was supported by DFG Graduiertenkolleg 792. M.S. acknowledges support from the US-Israel Binational Science Foundation and from Kimmelman center for Biomolecular Structure and Assembly. We thank J. Tittor for technical assistance and S. Ruhman and I. Radu for stimulating discussion.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

JA904218N