Ultrafast Spectroscopy of the Protonated Schiff Bases of Free and C_{13}=C_{14} Locked Retinals

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In this study, the ultrafast pump−probe spectroscopy of the all-trans protonated Schiff base of retinal (*trans*-PSB) in solution, is compared to that of two retinal analogues, *trans*-PSB5.12 and *13-cis*-PSB5.13, in which C_{13}=C_{14} torsional motion is inhibited by a rigid five-membered ring structure. The objective is to obtain measures of internal conversion (IC) dynamics in these polyenes. Contrasting the results with those obtained for the same pigments when attached to their opsin protein, serve to appreciate the protein role in catalyzing energy transduction in bacteriorhodopsin. Several major features appear to be common to all three PSBs: (i) A 50−100 fs process due to a primary relaxation out of the Franck−Condon (FC) region, (ii) A subsequent biexponential decay ($t_1 = 1-2$ ps and $t_2 = 4-7$ ps) of the fluorescent state (FS) assumed to be due to IC, and (iii) Spectral modulations in the FS emission. The three are only marginally effected by locking of the C_{13}=C_{14} bond. With respect to features (i) and (iii) the PSB model compounds behave analogously to the related retinal protein bacteriorhodopsin (bR). However, this does not apply to the FS decay. While in bR, the IC takes place with a 0.5 ps decay time, locking of the C_{13}=C_{14} bond in bR markedly increases the FS lifetime to ∼15 ps. These observations demonstrate the crucial role played by the protein in directing the isomerization action to the active double bond and enhancing the rate of IC. They also prove that these coordinates are not exclusive pathways of IC in the isolated PSB of retinal. The mechanism of ground-state repopulation in the PSBs is discussed in light of these results.

Introduction

A retinal isomer, bound to the protein via a protonated Schiff base linkage with a lysine residue, is the chromophore of all retinal proteins (see ref 1 for a series of review articles). In visual rhodopsins (Rh), the retinal moiety is in its 11-cis configuration, whereas in the photosynthetic protein bacteriorhodopsin (bR) the chromopore is *all-trans* or *13-cis*. Light absorption by rhodopsins triggers characteristic photocycles in the msec time range, reflecting structural transformations in the chromophore and in the surrounding protein matrix, which are responsible for the biological activity of the pigments.

At the focus of retinal protein research are the early light-induced ultrafast events, most commonly investigated by fs−ps spectroscopies (for reviews, see refs 3 & 4). A major issue is the characterization of the early dynamics of the chromophore and of the protein following light absorption. Still unresolved is the exact identification of the steps associated with *cis* → *trans* or *trans* → *cis* isomerization of the polyene, which is a basic light-response feature of all retinal proteins.

The importance of nonphotochemical skeletal modes in the primary relaxation after light absorption was pointed out in the case of bR on the basis of resonance Raman excitation profiles nearly two decades ago.[2] Nevertheless, the "prevailing" approach to the early events in retinal proteins assumed that the primary dynamic consequence of light absorption, i.e., departure out of the Franck−Condon (FC) region, is controlled by substantial torsional motion about the "critical" double bond, C_{11}=C_{12} in Rh and C_{13}=C_{14} in bR, and in the closely related chloride-ion pump, halorhodopsin (hR).[3−15] Accordingly, initiation of the isomerization process is a very primary dynamic consequence of light absorption. Recently, this approach has been questioned by spectroscopic evidence, both in Rh[16−18] and bR,[19−23] indicating that the <50 fs motion out of the FC region, and the corresponding evolution of the flourescent FS (I_{470}) state, does not involve substantial torsion about the critical C=C reaction coordinate. On the basis of ab initio quantum chemical calculations in model compounds, [24,25] it was concluded that the initial excited-state dynamics are dominated by in-plane stretching modes. According to these calculations, the actual *cis* → *trans* or *trans* → *cis* motion, toward a surface-crossing conical intersection (CI), is initiated on the excited state surface only after completion of bond stretching (the ∼500fs FS (I_{470}) → J step). Further questions concerning the exact timing of isomerization in bR were recently raised by a CARS study,[26] suggesting that C_{13}=C_{14} isomerization is delayed to after the ∼3 ps decay of the J intermediate.

Highly relevant to the function of retinal proteins is the role of the protein matrix in controlling the selectivity, quantum yield, and time scale of the primary photoreaction. In this respect, a comparison between the photophysical behavior of the pigments and that of the corresponding protonated Schiff bases (PSB) in solution is compelling. The ultrafast spectroscopy of model PSB isomers in solution has been investigated by fluorescence up-conversion,[27,28] as well as by pump−probe[29,30] techniques. In the case of the all-trans isomer (*trans* PSB, see Scheme 1), an initial fluorescence decay component of about 100 fs was detected,[27,30] which is followed by a slower, 3−7 ps, decay of the excited state.[27,29,30] The ∼100 fs, process was attributed to relaxation out of the FC region, possibly along the C_{13}=C_{14} or C_{11}=C_{12} torsional coordinate.[30] A slower time scale for the first isomerization stage was suggested on the basis of

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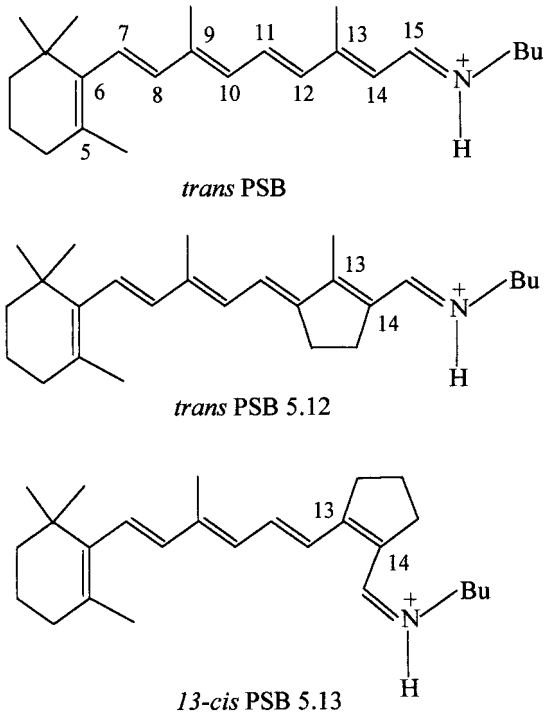

temperature effects on the flourescence decay.[29] Accordingly, the ps decay component is attributed to a ~90°, thermally activated, C═C torsional motion on the S_1 surface.[24,25,29]

In the present work, the involvement of out of plane C═C motion in the primary events in PSB in solution is probed by using the locked retinal derivatives, *trans*-PSB5.12, and *13-cis*-PSB5.13, in which the C_{13}═C_{14} bond is blocked by a five-membered ring (see Scheme 1). The use of this methodology for clarifying the primary events following light absorption has been previously applied by us to bR.[19,20] In variance with previous models, the present data lead to the conclusion that in *trans*- or *13-cis*-retinal PSB, the decay of the FC state is not controlled by substantial torsional motions about the C_{13}═C_{14} bond. Moreover, they raise the possibility that the decay of the fluorescent state as well may be controlled by coordinates other than C═C torsion. Finally, these observations further highlight the crucial role played by the protein in enhancing the rate of IC in bR, and in directing action to isomerization along C_{13}═C_{14}.

Experimental Section

The aldehyde analogues of *trans*-PSB5.12 and *13-cis*-PSB5.13 were prepared according to previously described methods.[19,20] The corresponding protonatd Schiff bases (PSB) were prepared by mixing the aldehyde with excess of *n*-butylamine (10 equiv) in ethanol for 1 h. The ethanol and excess of amine were evaporated and the residue was dissolved in the appropriate solvent and acidified with dilute solution of the desired acid. Complete protonation was monitored by absorption spectroscopy.

The laser system consisted of a homemade multipass amplified Ti:sapphire arrangement. The oscillator producing ~16 fsec pulses was constructed after the design of Asaki et al.,[31] and the amplifier was a modified version of that devised by Bakcus et al.[32] The alterations consisted of reducing the overall length of the amplifier cavity by a factor of 0.75, and introducing coaxial end-pumping and back reflection through the focusing

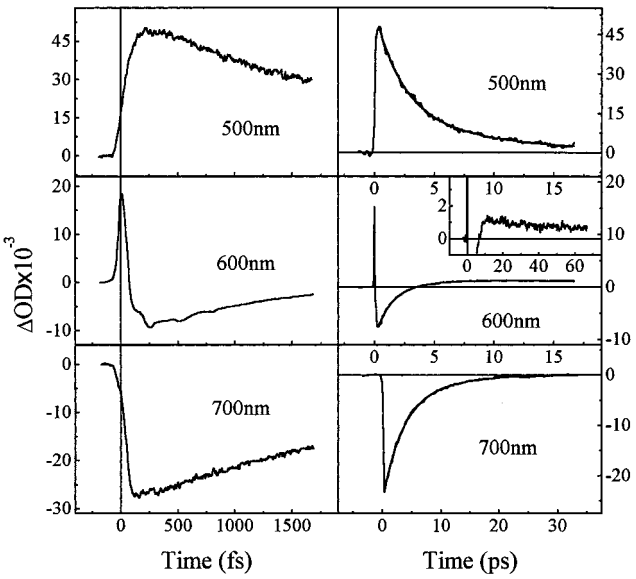

Figure 1. Transient absorption (positive) and emission (negative) scans of *all-trans*-PSB (C_2H_5OH/Cl_3CCOO^-). The slower, ps, scans include nonlinear least-squares fits (see Table 1).

end mirrors. Pumping the amplifier with ~10 mJ at 527 nm, the ultimate output after pulse compression was a 1 kHz train of pulses, 28 fsec in duration, centered at 790 nm (40 nm fwhm), containing ~600 μJ.

Excitation pulses at 395 nm were derived by doubling a small portion of the amplifier output in a 0.1 mm BBO crystal. Probe pulses were generated by interference filtering from a white continuum which was produced by tightly focusing about 1 μJ of the amplifier output in sapphire. The origin of pump/probe delay was determined by conducting Optical Kerr Effect scans in the cell before each differential transmission scan was collected. Dispersion in either arm was precompensated by prism pairs to provide the shortest pump/probe cross correlation in the sample cell. Amplified photodiodes and lock-in detection were used to measure transmission changes as a function of probe delay, using a pump fluence which was reduced to ensure linear power dependence of the data. The samples were peristaltically circulated under a nitrogen atmosphere, through a 100 μm path length cell constructed of stainless steel, and equipped with 150 μm thick quartz windows. Flow rates ensured sample replenishment between laser pulses.

Results

***trans*-PSB.** The ultrafast response of *trans*-PSB in ethanol (with Cl_3COO^- as counterion) is characterized by the prompt formation of a broad absorption between 490 and 650 nm, which is attributed to the initially generated FC state.[30] After ~200 fs, the band is replaced by a stimulated emission, peaking around 670 nm, and by an absorption at ~500 nm. Both features, which decay over several ps, are associated with the fluorescent state (FS) of the molecule.[27,28]

Figure 1 shows traces obtained by us for *trans*-PSB in ethanol at several characteristic wavelengths: 500 nm (FS absorption), 700 nm (FS emission) and 600 nm (intermediate range, FC absorption). The corresponding spectral response between 500 and 700 nm is given in Figure 2a. The data are in very good agreement with those of Hamm et al.,[30] showing that the very early events are dominated by an instantaneous rise of the 600 nm FC absorption, followed by a decay which is completed in ~100 fs. As indicated by the shifted position of the corresponding traces relative to the zero point, evolution of the FS

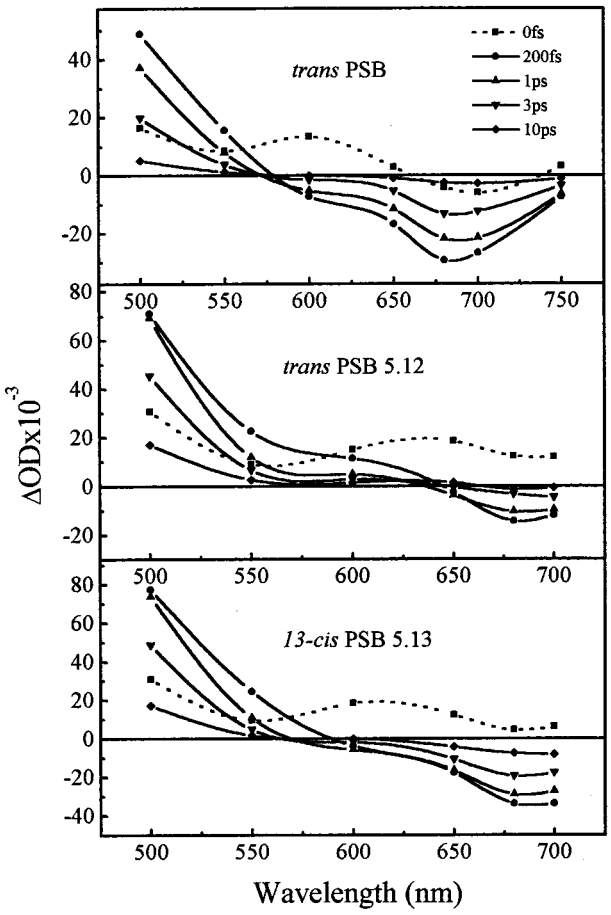

Figure 2. Transient spectral changes following excitation of free and C_{13}═C_{14} locked PSBs.

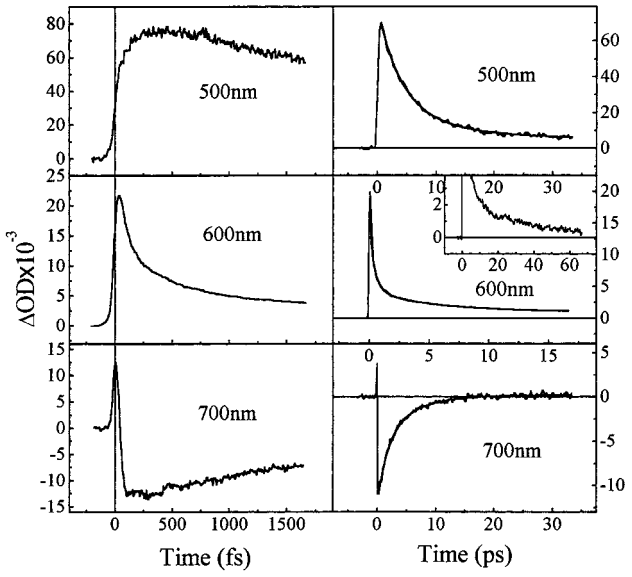

Figure 3. Transient spectral changes observed for *trans*-PSB5.12 (conditions as in Figure 1).

stimulated emission around 700 nm and of the corresponding FS absorption at 500 nm take place in the same (50−100fs) time range. A prominent feature which is evident in Figure 1 is the highly structured nature of the flourescence signal at 600 nm, due to periodic oscillations. An analogous effect has been recently reported by us for bR,[33] but has not been previously detected in PSB. As reported before,[30] these fast dynamic processes are followed by the decay of both emission and

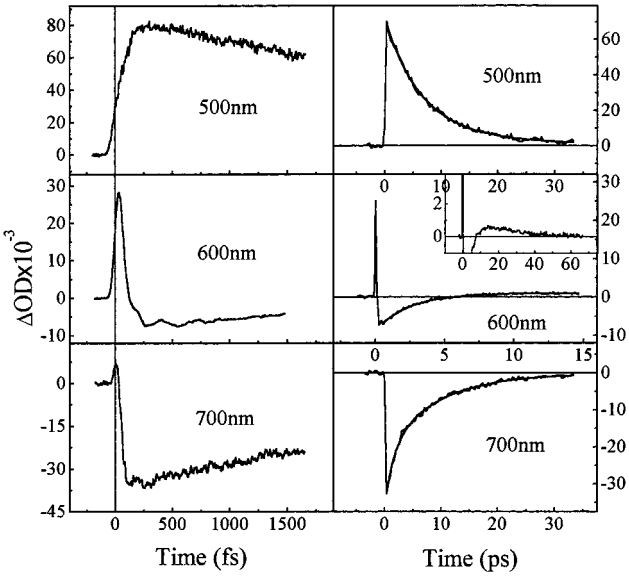

Figure 4. Transient spectral changes observed for 13-*cis*-PSB5.13 (conditions as in Figure 1).

absorption bands in a nonexponential process, with good agreement to two, $t_1 = 1-2$ ps and $t_2 = 4-7$ ps, components. Although these dynamic features are characteristic of the stimulated emission (650−750 nm) and maximum absorption (500 nm) wavelengths, somehow different patterns are observed in the intermediate range, at 550 and 600 nm, where the best fits were obtained with a three exponents analysis. Thus, at 550 nm, we observe a decrease of about a factor of 3 in t_1. An additional feature, at both 550 nm and 600 nm, is the occurrence of a low amplitude, long-lived, absorption decay, with a lifetime of t_3 ∼20 ps.

Table 1 includes data for *trans*-PSB in ethanol, with Cl^- replacing Cl_3CCOO^- as the Schiff base counterion. Also presented are systems in which ethanol is replaced by methylene chloride (with either Cl^- or IO_4^- as counterion). For the extreme, 500 nm absorption and 700 nm emission bands, the major dynamic features in both ethanol and methylene chloride are essentially independent, within ∼30%, of the specific counterion. It is also evident that exchanging ethanol with methylene chloride does not substantially affect t_1 and t_2, as well as the primary 50−100 fs FC → FS relaxation (not shown). It appears, however, that methylene chloride markedly affects t_1, t_2, and t_3 in the intermediate range around 600 nm, also shifting the ∼575 nm isosbestic point (between FS emission and FS absorption) to the red, so that at 600 nm t_1 and t_2 reflect an absorption decay, rather than an emission decay as observed in ethanol (see Figures 1, 2, and Table 1). An additional feature observed in methylene chloride is a substantial increases in the amplitude of the t_3 component at 600 nm, as well as a considerable increase in its lifetime (to ∼100 ps).

***trans*-PSB5.12 and *13-cis*-PSB5.13.** The major photoisomerization pathways of *trans*-PSB in ethanol are ∼20% to the 11-c*is* isomer and ∼6% to *13-cis*,[30,34] whereas *13-cis* PSB isomerizes exclusively to *all-trans*.[35,36,] Thus, if C_{13}═C_{14} torsions play dominant roles in the decay of either or both FC and FS states,[24,25,29,30] one may expect a substantial sensitivity of the early photophysics to the inhibition of such out of plane pathways. We have thus carried out experiments with the two analogues, *trans*-PSB5.12 and *13-cis*-PSB5.13, aiming at comparing their ultrafast photophysical patterns with those of the uninhibited isomers, *trans*-PSB[29,30] and *13-cis*-PSB.[29]

TABLE 1: Curve Fitting Analysis of ps Decay Processes of Various Retinal PSB Systems[a]

system	decay component (ps)	wavelength (nm) 500	550	600	650	680	700	750
trans-PSB C_2H_5OH/Cl_3CCOO^-	t_1	1.5 [+0.6]	0.5 [+0.63][b]	1.1 [−0.48]	1.3 [−0.55]	1.7 [−0.54]	1.5 [−0.52][a]	1.3 [−0.36]
	t_2	5.8 [+0.4]	3.7 [+0.27][b]	2.0 [−0.38]	4.4 [−0.45]	5.5 [−0.46]	6.0[−0.48][b]	5.4 [−0.64]
	t_3		24 [+0.10][b]	∼20 [−0.14]				
trans-PSB C_2H_5OH/Cl^-	t_1		0.6 [+0.64][b]				1.8 [−0.56]	
	t_2		4.1 [+0.27][b]				8.2 [−0.44]	
	t_3		25 [+0.09][b]					
trans-PSB CH_2Cl_2/Cl^-	t_1			0.2 [+0.25]	0.7 [−0.25]		2.4 [−0.24]	
	t_2			11 [+0.20]	5.3 [−0.75]		6.6 [−0.76]	
	t_3			95 [+0.55]				
trans-PSB CH_2Cl_2/IO_4^-	t_1			0.16 [+0.30]	1.4 [−0.48]		2.5 [−0.36]	
	t_2			16 [+0.40]	5.7 [−0.52]		6.7 [−0.64]	
	t_3			128 [+0.30]				
trans-PSB5.12 C_2H_5OH/Cl_3CCOO^-	t_1	2.3 [+0.48]	0.50 [+0.56]	0.5 [+0.60]	2.1 [−1.0]	1.6 [−0.23]	1.5 [−0.60]	
	t_2	7.0 [+0.52]	2.8 [+0.33]	5.0 [+0.24]		7.6 [−0.77]	4.8 [−0.40]	
	t_3		21 [+0.11]	∼20 [+0.16]				
13-cis-PSB 5.13 C_2H_5OH/Cl_3CCOO^-	t_1	2.5 [+0.33]	0.6 [+0.64]		1.6 [−0.57]	0.9 [−0.53]	1.2 [−0.35]	
	t_2	8.8 [+0.67]	2.6 [+0.25]	2.4 [−0.85]	4.3 [−0.43]	4.5 [−0.47]	8.1 [−0.65]	
	t_3		15 [+0.11]	∼20 [−0.15]				

[a] All experiments are at 18 °C. A three exponential fit is given when the two component analysis yielded unsatisfactory fits. Values in parentheses are fractional amplitudes of the corresponding component. Positive signs mark an absorption decay (gain increase), whereas negative signs represent emission decay (gain decrease). [b] Data are unaffected by changing the PSB concentration in the range between 6 OD/cm and 16 OD/cm.

The results of experiments with the two locked PSB molecules, analogous to those described above for *trans*-PSB, are given in Figures 2−4 and Table 1. It is evident that the two analogues are characterized by light-induced patterns which are essentially identical to those of *trans*-PSB with respect to several major features: (a) The prompt appearance of the FC absorption bands around <500 nm and 600−650 nm, which decay over several tens of femtoseconds; (b) parallel to this primary decay, the rise of the ∼500 nm absorption and ∼650 nm stimulated emission bands, attributed to the FS state; (c) the nonexponential decay of the 650−750 nm stimulated emission and 500 nm absorption, which in all cases is fairly represented by two, 1.2−2.5 ps and 5−8 ps, components; (d) the special features in the intermediate 550−600 nm range, namely, the short (∼600 fs) and long (15−20 ps) absorption decay components; and (e) the periodic oscillations giving rise to the structured nature of the signals.

Discussion

FC Decay and FS Evolution. The two C_{13}═C_{14} locked retinals that are the subject of the present work have recently been applied by us for probing the primary events in bacteriorhodopsin (bR).[19,20] In its native forms, bR carries an *all-trans*, or *13-cis* chromophore, which undergoes photoisomerization to *13-cis*, or *all-trans*, respectively. The corresponding artificial pigments, bR5.12 and bR5.13, were found to exhibit a fluorescent state which is spectroscopically similar to that of native bR. Consequently, it was concluded that in bR the primary relaxation out of the FC range is not associated with substantial torsion about the "critical" C_{13}═C_{14} bond. The rate of this relaxation in both native bR and bR5.12 was set as faster than ∼30fs by following the evolution of the absorption and emission bands of the FS.[19,20] No absorption band which may be assigned to the initially generated FC state could be detected. A closer examination of the stimulated fluorescence, with improved time resolution, showed that for native bR the signal at 800 nm arises nearly instantaneously (<30 fs), whereas that at 950 nm is delayed by ∼20 fs.[33] In the case of bR5.12, both signals are delayed with respect to $t = 0$ by ∼20 fs and ∼30 fs, respectively.

Our present data (Figures 1, 3, 4) indicate that analogous patterns are also associated with the free PSB chromophore in solution, with the difference that evolution of the FS absorption and stimulated emission is delayed with respect to $t = 0$ by 50−100 fs. This time scale is longer than that of the related proteins, allowing the detection of a distinct absorption due to the FC state. The presently emerging picture shows that in PSB the primary relaxation out of the FC region exhibits very little dependency on (a) the specific (*all-trans* or *13-cis*) isomer, (b) locking of the C_{13}═C_{14} bond into *all-trans* or *13-cis* configuration, and (c) the nature of the solvent and/or counterion. We are thus led to the conclusion that analogously to bR, and in variance with previous suggestions,[27,30] the primary relaxation in PSB involves intrinsic coordinates of the polyene chromophore, other than torsion about the critical C_{13}═C_{14} or C_{11}═C_{12} bonds. This is in keeping with the quantum mechanical model of Garavelli et al.,[24,25] which interprets the primary motion out of the FC region in terms of in-plane stretching of C═C bonds of the polyene skeleton.

FS Decay. Interpretation of the subsequent decays, in the range between hundreds femtosecond and several picoseconds are best analyzed by considering three main spectral regions: The excited-state absorption on the blue edge around 500 nm, the emission in the red, above ∼650 nm, and the intermediate 550−600 nm region. Our data show that for all three molecules the decay on both red (emission) and blue (absorption) is satisfactorily described in terms of two components, 1.2−2.5 ps and 5−8 ps. This behavior is in very good agreement with the *trans*-PSB work of Hamm et al.[30] (rather than with those of Logunov et al.,[29] and Kandori et al.,[27] in which the emission on the red side was fit to a single, 2.5−4 ps, exponential). Several models, such as biphasic movement on the S_1 surface, a small energy gap between the S_1 minimum and the CI (S_1/S_o funnel point), or two local minima on S_1, have been proposed

to account for the biexponential decay of the FS state.[30] Our present data are incapable of leading to a discrimination between such, or other, general models. However, all of these models for both retinal proteins and model PSB,[24,25,27,29] imply that the decay of the fluorescent state is determined by the rate of a *substantial* (∼90°) twist around a critical (photoisomerizing) C═C bond on the S_1 surface, leading from the FS to the CI funnel. A subsequent non-rate-determining transition from the CI to the S_o surface takes place which is followed by relaxation to the fully isomerized photoproduct. On the basis of a temperature dependence of the fluorescence decay time, a barrier of ∼600 cm^{-1} was ascribed to the FS → CI partial isomerization step.[29]

The question arises concerning the extent to which our present observations are compatible with such interpretation of the FS decay. A problem in this respect is raised by the observation that the (similar) decay patterns observed for the FS of 13-*cis*-PSB and *trans*-PSB, are also maintained in both *trans*-locked and (*13-cis*)- locked chromphores. Thus, if the FS decay is governed by a 90° torsion about the C_{13}═C_{14} or C_{11}═C_{12} bonds, our data would imply that the ∼600 cm^{-1} barrier for such partial isomerization[29] is essentially the same for all processes: (a) trans → *13-cis*, (b) *trans* → *11-cis*, (c) *13-cis* → *trans*, (d) *13-cis* → *13,11-cis*. Computational data on the excited torsional deformation of a model *all-trans*-PSB indicate that motion about all the central double bonds is substantially barrierless, so that locking of one bond (e.g., C_{13}═C_{14}) will not restrain others, such as C_{11}═C_{12}, or C_9═C_{10} yielding analogous FS decay dynamics for processes (a) and (b).[45,46] At present, no such computational data are available for 13-*cis*-PSB, so as to estimate the barriers for processes (c) and (d). However, although it cannot be definitely excluded, it seems unlikely that the same barrier will be maintained for the transition from *13-cis* to *13-cis*/*90° 11-cis*, as required by the *13-cis* PSB5.13 data. In fact, in 13-*cis*-PSB the only isomerization pathway is to all-trans, with no double-cis photoproducts.[35,36] The lack of e.g., a *13-cis*, 90° 11-cis photoproducts may be accounted for by arguing that after decaying to the 90° twisted funnel associated with the *11-trans* → *11-cis* motion, ground-state relaxation will favor a back reaction to the more stable single *13-cis* isomer. Nevertheless, a feasible alternative is that the *13-cis*, 90° *11-cis* configuration is not populated due to a high barrier on the S_1 surface. This would imply that the FS decay is not controlled by a 90° C═C torsion though not excluding small amplitude out of plane, skeletal distortions (including C═C bonds).

In conclusion, the present data point to the possibility that the coordinates which determine the CI between S_1 and S_o may not include C═C isomerization. Accordingly, isomerization will have to take place *after* crossing to S_o. Alternatively, one cannot exclude a mechanism in which the 1.5−2.5 ps and 5−8 ps decays reflect a transition to a minimum on the S_1 ($^1B_u^+$) state whose fluorescence and absorption are both weak or out of the present 500−700 nm observation range. It is possible that this minimum is associated with strong mixing with a second $^1Ag^-$ surface.[42,43] From such a state, substantial torsional motion along a critical C═C coordinate will take place, with no observable changes in emission and absorption. This mechanism may in principle be probed by experiments which will monitor generation of the isomerized photoproduct on a sub-nanosecond time scale. However, such measurements will encounter serious difficulties due to the relatively small spectral difference between the various PSB isomers.[35,36]

We wish to refer here to the ∼0.5 ps (t_1) process and to the $t_3 \cong 20$ ps process, which are both observed in the intermediate 550−600 nm range. Such decay components are respectively faster and slower than the 1.5−2.5 ps and 5−8 ps decay of the FS at the maxima of its absorption and emission bands. A ∼0.3 ps component analogous to our ∼0.5 ps process has been detected for PSB by Hamm et al.[30] and discussed in terms of an initial cooling effect. The weak ∼20 ps decay (∼130 ps in methylene chloride) has not been previously reported, and may represent a residually trapped S_1 population. However, independently of the exact assignments of such components, the fact that they are observed both in the "native" and in the two locked choromphores implies that they cannot be associated with substantial C═C torsions. Thus, they cannot bear on the mechanism of C═C isomerization.

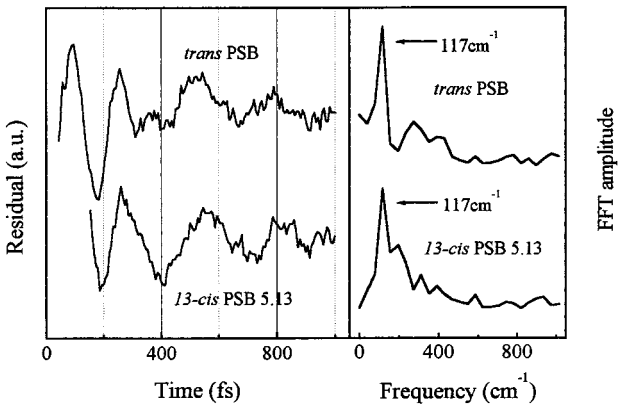

Figure 5. Oscillatory residuals at 600 nm derived by subtracting least-squares fits from the actual data, dividing the result by the fit itself and later multiplying by a constant factor to make the first clear modulation equal in amplitude in the two residuals.

Spectral Modulations. As observed in Figure 5, stimulated emission from native and locked PSBs is superimposed with spectral modulations which are clearly observable during the first picosecond of probe delay. These modulations must be associated with vibrational coherences in the excited state, akin to those reported previously for the native and locked bR's.[33] This portion of the data is particularly significant. First, it reflects motions which are strongly activated by the optical transition, and highlights the coordinates along which large displacements exist between the ground and the reactive excited state. As such, these coordinates are by definition most relevant to the light induced dynamics. Second, direct time domain probing gives us access to the evolution of motions which are not easily characterized by other methods due to their low frequencies. Third, by comparing these modulations to those observed in bR, we may be able to appreciate how the protein influences the primary light induced motions of the prosthetic group in the combined system.

From the preliminary data presented in Figure 5, three main conclusions may already be drawn: (a) FFTs of the spectral modulations contain frequencies close to those observed for bR.[33] (b) As in the protein, the modulations in emission also appear to arise after a short incubation time. (c) Even on a qualitative level, observable differences exist in the structure of the modulations observed in the native and in the *cis* locked PSB. The similarities described in (a) and (b) already suggest that the modulations observed in the protein systems must mainly be associated with nuclear motions triggered by the photon absorption in the retinal moiety itself. The significant differences observed between the vibrational coherences in native PSB, and in bR, involving both their frequency spectrum and phases of appearance with respect to the zero of time, will be quantitatively analyzed in light of the known assignments

of the Raman active modes in both systems. This analysis should clarify the final point in the previous paragraph. It may also shed light on the suggested spectral overlap of near-IR absorption and emission bands from the excited state in bR, which are separately observed in the case of retinal PSB in ethanol.

Relevance to the Primary Events in Retinal Proteins. We finally wish to focus on the primary photophysical features which distinguish the free *all-trans*- and 13-*cis*-PSB chromophores from the related bR pigment, as well as on those which are common to both free and protein-embedded polyenes. Such a comparison bears on the mechanism through which the protein controls the biological activity of the pigment. First, we note that in the case of the PSBs, we observe an early evolution on a time scale of 50−100 fs that must represent departure out of the FC state, which exhibits a distinct absorption spectrum. Although an initial absorption attributable to the FC state is observable in the case of bR5.12 at ∼560 nm (see Figure 7d in ref 33), the FC → FS transition in native bR has not been resolved, most probably due to its occurrence on a time scale which is shorter than our time resolution (20−30 fs). It therefore appears that the bR protein matrix catalyzes the FC → FS process by a factor of at least 3, as compared to the free chromophore in solution. Despite such differences in time scales, it seems that in both PSB and bR the FC → FS relaxation is not markedly affected by locking of the C_{13}═C_{14} bond. This is consistent with the conclusion that the primary relaxation in both systems is not controlled by out of plane C═C motions. As discussed above, a further analogy between the free and protein embedded chromophores are the early spectral modulations which reflect the same intrinsic nuclear motions of the retinal skeleton.

The above analogies between the PSB and the protein-embedded chromophore fail to persist on the longer time scales which are associated with the FS decay. In this case, PSB and bR differ in two major respects: (a) The FS (denoted as I_{470}) decay in bR (∼500 fs) is faster, by almost an order of magnitude, relative to *trans*-PSB. (b) Locking of C_{13}═C_{14}, into either *trans* or *13-cis* configuration, does not affect the FS decay in the case of both PSBs. In variance, C_{13}═C_{14} locking in bR markedly extends the FS lifetime (as measured by stimulated[20] or by spontaneous[44] fluorescence), from ∼500 fs to 11−18 ps. It therefore appears that the protein matrix plays a major role in controlling the excited-state dynamics of the chromophore. This catalytic role of the protein should be viewed in terms of the two alternative mechanisms which were presented above:

(i) According to a generally accepted working hypothesis, the FS decay is determined by the rate of reaching a conical intersection between S_1 and S_o. Assuming that in bR this process occurs along the C_{13}═C_{14} torsional coordinate to a 90° configuration, the role of the protein is one of decreasing or eliminating the barrier associated with this motion, as well as to increase the barriers to rotation about other bonds as witnessed by the specificity of the photoreaction. In fact, the FS(I_{470}) decay in bR has been shown to be barrierless[47] as compared to a barrier of ∼600 cm^{-1} observed for PSB.[29]

However, we have argued above that in PSBs the conical intersection (CI_1) may not involve C═C torsional coordinates. This implies that the isomerization process in PSBs may take place in the ground state after channeling sufficient excess vibrational energy into the relevant C═C coordinate. Accordingly, the role of the protein will be to catalyze the transition to a different intersection (CI_2), in which the C_{13}═C_{14} torsional coordinate becomes involved. Upon locking of the double bond in bR5.13, the system reverts to the more slowly populated CI_1, exhibiting the 11−18 ps FS lifetime.

(ii) According to the second approach, the FS decay is not due to the $S_1 \rightarrow S_o$ crossing, but rather reflects a transition to a point on the same $S_1(^1B_u^+)$, or a different ($^1A_g^-$) surface, from which ground-state repopulation takes place at longer times. Accordingly, the role of the protein will be to catalyze this excited-state transition, via a path which involves the C_{13}═C_{14} torsional coordinate.

Conclusions

The ultrafast decay of the fluorescent state of retinal proteins is commonly interpreted in terms of a torsional motion about a specific C═C coordinate, leading to a conical intersection between S_1 and S_o. This study compares the effects of selectively locking the C_{13}═C_{14} bond in a protein embedded chromophore (bR) with those of the related free PSB moiety in solution. The major observation is that in variance with bR, the FS decay in PSB is unaffected by C_{13}═C_{14} locking. We concluded that the primary role of the protein in controlling the early events in the pigment is to hasten molecular action exclusively to isomerization around C_{13}═C_{14}. The exact mechanism of the FS decay in the free PSB is still unclear. It may proceed via a different CI-active coordinate, or via a subsequent (yet undetected) excited state. Future studies will aim at discriminating between such alternatives. A major future goal will be to understand the relation between the molecular structure of the protein binding site, and its catalytic role in controlling the primary photophysical events in the pigments as demonstrated in this paper.

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