Ultrashort Processes of Native Phytochrome: Femtosecond Kinetics of the Far-Red-Absorbing Form Pfr

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The biologically active far-red-absorbing form of the plant photoreceptor phytochrome, Pfr, was studied by femtosecond spectroscopy. The transient absorption changes that occurred upon excitation of Pfr at 730 nm with laser pulses of 150 fs were measured as a function of time at selected probe wavelengths. Immediately after excitation, bleaching of the ground-state absorption bands in the red and blue spectral region was observed. Bleaching was accompanied by an absorption increase at wavelengths between 775 and 825 nm as well as at around 400 nm. The kinetic analysis of the absorption changes $\Delta OD(t)$ at different probe wavelengths yielded two decay components, one with a lifetime of about 700 fs and another with about 4.5 ps. A model that explains the relaxation processes following the excitation of Pfr is suggested. It assumes that the initially excited state decays within about 700 fs toward a secondary excited one which is depopulated with a rate constant of $(4.5\ \text{ps})^{-1}$.

Introduction

Phytochrome is known as one of the most important photoreceptors in green plants that regulates many aspects of plant growth and development. It occurs as a dimeric chromoprotein. Each subunit of ∼125 kDa contains an open chain tetrapyrrole chromophore that is covalently bound to a cystein residue of the protein moiety via a thioether linkage. The apoprotein is encoded by a family of at least five divergent genes. The biological action of phytochrome is based on the photoreversible transformation between a red-light-absorbing form, Pr (λ_{max} = 666 nm), and a far-red-light-absorbing form, Pfr (λ_{max} = 730 nm). Pfr is considered to be the active form and Pr the inactive form of the photoreceptor.[1−3]

$$\text{Pr} \underset{730\ \text{nm}}{\overset{666\ \text{nm}}{\rightleftharpoons}} \text{Pfr} \rightarrow \text{biological response}$$

Because it is very likely that the biological activity of Pfr results from the molecular events that occur in the course of the Pr → Pfr phototransformation, various analytical approaches have been applied to the study of the molecular and kinetic mechanism of this phototransformation. Evidence indicates that the primary photochemical event involves a *Z*,*E* isomerization of the tetrapyrrolic chromophore at the C(15) methine bridge.[4,5] This photoreaction then initiates a complex series of dark reactions leading finally to the formation of the biologically active far-red-absorbing phytochrome.

The results of femtosecond- and picosecond-resolved spectroscopic studies show that the primary photoproduct, lumi-R, is built up with a time constant of ∼30 ps.[6−8] However, a fast relaxational process of ∼3 ps precedes its formation.[7,8] The general assumption is that lumi-R is the isomerized ground-state photoproduct. On the basis of low-temperature studies and nanosecond laser flash experiments, the subsequent dark reactions proceed through another two or three intermediate stages (meta-Ra_1, meta-Ra_2, meta-Rc) which appear and decay in the millisecond time domain (ref 1 and references therein, refs 9−12). The structural changes that accompany the formation of these intermediates are not yet fully understood. Besides the initial *Z*,*E* isomerization, rotations around the single bonds of the methine bridges in the chromophore are postulated to play an important role.[13−15]

The back reaction from Pfr to Pr is not as thoroughly characterized. Thus far, three spectrally and kinetic distinct intermediates (lumi-F, meta-Fa, meta-Fb) have been detected in this phototransformation pathway. They were observed in low-temperature and nanosecond absorption studies.[1,16] The lifetimes associated with these intermediates are in the range from nanoseconds to milliseconds.[16] Nothing is known about the primary photoprocesses occurring immediately after photon absorption in Pfr. We have therefore addressed this problem in our studies. Since efforts to detect the stationary fluorescence of Pfr failed up to now, the fluorescence quantum yield seems to be extraordinarily low. This suggests that the primary photoprocesses of Pfr should be ultrafast and proceed on a very short time scale. To probe these processes, we have therefore applied femtosecond time-resolved spectroscopy. Here, we present the first results of the femtosecond-resolved measurements along with a discussion of a possible kinetic mechanism involved in the first excited-state processes of Pfr.

Materials and Methods

Instrumentation and Measuring Procedure. The femtosecond spectrometer in use was based on a colliding pulse modelocked (CPM) dye laser with a four-stage dye amplifier. The pulses supplied by this amplifier at 618 nm were compressed and spectrally broadened by the generation of a white light continuum in ethylene glycol. The continuum pulses served as seed pulses for a two-stage postamplifier that produced pulses of 150 fs at 730 nm with an energy of 60 μJ after recompression. Pulses of 5 μJ were used for the excitation of Pfr at 730 nm.

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The typical photon fluence at the entrance of the cuvette was 5×10^{15} cm^{-2}.

To measure the kinetics at certain probe wavelengths, the desired wavelengths were selected from the white light continuum by appropriate interference filters with a spectral bandwidth of 10 nm. The cross-correlation width of the pump and probe pulses (fwhm) was ~200 fs. The excitation beam passed the sample under a small angle to the probe beam. The polarization of the excitation beam was parallel to the probe beam. To monitor the time evolution of the absorption changes, the probe pulse was delayed step by step with respect to the excitation pulse by means of a variable optical delay line. By averaging over at least 20 laser shots at each delay time, statistical fluctuations of the excitation and probe pulse were compensated. Absorption changes were measured as ratio of the intensity of the measuring and reference signal by means of a computer-controlled pulse energy detector system (LIEM-II). They are given as difference of the optical densities (ΔOD) with and without excitation. The ΔOD zero level was explicitly measured with the probe pulse preceding the excitation pulse. The obtained kinetic curves were analyzed in terms of a sum of exponentials by a global fit analysis. Time zero of the delay time scale in the kinetic curves was experimentally measured as a function of the probe wavelength using saturable absorbers. Under the experimental conditions applied, the time evolution of the absorption changes did not depend on the laser intensity. Reducing the excitation light intensity by 50% did affect neither the lifetimes nor the relative amplitudes of the kinetic components. Lowering the laser intensity only resulted in a decrease of the absolute amplitudes proportional to the excitation energy. Double excitation can therefore be excluded.

Preparation of Phytochrome. Phytochrome was isolated from 5-day-old etiolated oat seedlings (*Avena sativa L. cv Adamo*) by a method using the partial resolubilization of phytochrome in buffers of different ionic strength. The isolation procedure is a modification of the methods described by Grimm and Rüdiger[17] and Lapko et al.[18] The modifications are as follows: The first ammonium sulfate pellet (230 g/L) was solubilized in 50 mM Tris/HCl buffer at pH 7.8 containing 25% ethylene glycol, 1 mM EDTA, and 0.2 mM Pefabloc SC. It was further fractionated by an additional precipitation with ammonium sulfate (170 g/L) instead of a separation on hydroxylapatite as reported by Grimm and Rüdiger.[17] The second ammonium sulfate precipitate was then washed with defined small volumes of 100 mM phosphate buffer. In contrast to the method of Lapko et al.,[18] the ionic strength of this buffer was increased by the addition of ammonium sulfate in order to reduce the solubility of phytochrome but remaining unaffected that of contaminating proteins. After five successive resuspension steps, phytochrome was dissolved in 10 mM phosphate buffer, pH 7.8, containing 0.2 mM Pefabloc SC. Phytochrome used in the measurements was dissolved in this buffer. In the case of the temperature-dependent measurements, 20% glycerol was added to the buffer solution. The final concentration was about 4 μM, corresponding to an optical density of the Pr form of ~0.6 at 666 nm in a 2 mm path length. Before the experiments, Pr was photoconverted to Pfr by saturating irradiation with red light. The mole fraction of Pfr in the resulting photoequilibrium mixture was 0.85. Red light was obtained by passing the output of a tungsten lamp through an interference filter with peak transmission at 656 nm and half-bandwidth of 10 nm. For the measurements phytochrome was pumped through a flow cuvette. The flow rate was calculated so that under the experimental conditions each laser pulse reached continuously a fresh sample volume. The measurements were carried out at a temperature of 295 K. In the temperature-dependent measurements the temperature was varied between 258 and 295 K.

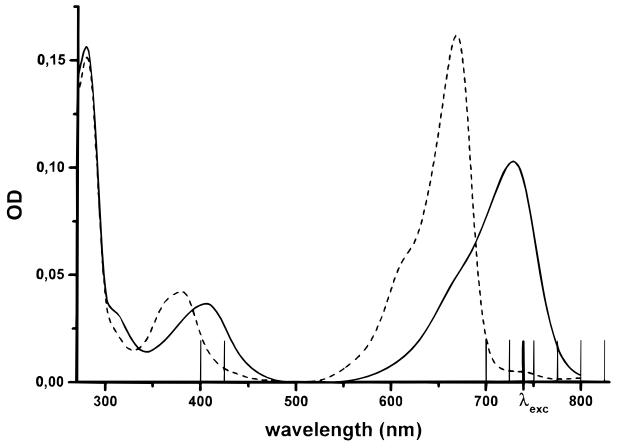

Figure 1. Steady-state absorption spectrum of the red-light-absorbing phytochrome form (Pr) and the far-red-light-absorbing phytochrome form (Pfr) dissolved in 10 mM phosphate buffer, pH 7.8, at 278 K. The spectrum shown for Pfr represents that of 100% Pfr. It is calculated from the spectrum of the photostationary equilibrium between Pfr and Pr under the condition that saturated red light irradiation produces 85% Pfr and 15% Pr. The excitation and probe wavelengths of the femtosecond experiments are indicated. (- - -), spectrum of Pr; (—) spectrum of Pfr.

The photoreversibility and integrity of the phytochrome samples were examined by UV−vis spectroscopy and SDS electrophoresis before and after each experiment. As this analysis revealed, both the spectral and structural properties of Pfr were not affected by the measurements.

Results

Figure 1 depicts the absorption spectrum of the red-light-absorbing phytochrome form (Pr) and the far-red-light-absorbing phytochrome form (Pfr). The spectrum shown for Pfr corresponds to that of 100% Pfr. The excitation and probe wavelengths used in the femtosecond experiments are indicated.

After excitation of Pfr with 150 fs pulses at 730 nm transient absorption changes were observed in the spectral range between 700 and 825 nm as well as 400 and 425 nm. Figure 2 shows the kinetics of the absorption changes at distinct probe wavelengths in the red spectral region. Between 700 and 750 nm, the kinetic curves start with negative ΔOD values at time zero and approach to zero on the ΔOD scale later in time, but there is a clear difference in their time evolution. The most rapid increase in ΔOD up to zero is observed at 750 nm occurring within about 2 ps. Compared with this kinetics, the absorption changes at 700 and 725 nm recover to zero somewhat more slowly. Since the negative absorption changes occur in the spectral region of the Pfr ground-state absorption, they are obviously due to bleaching of the initial absorption of Pfr.

The kinetic curves obtained at the longer wavelengths between 800 and 825 nm show positive transient absorption changes immediately after excitation which then, however, decay over the next few picoseconds. The positive absorption changes can be ascribed to a transient absorption since the Pfr absorption is comparatively small or has even disappeared in this spectral range.

The kinetics as measured at 775 nm is of a more complex nature. This curve starts with small negative absorption changes which rapidly turn to positive ones obviously due to the increase

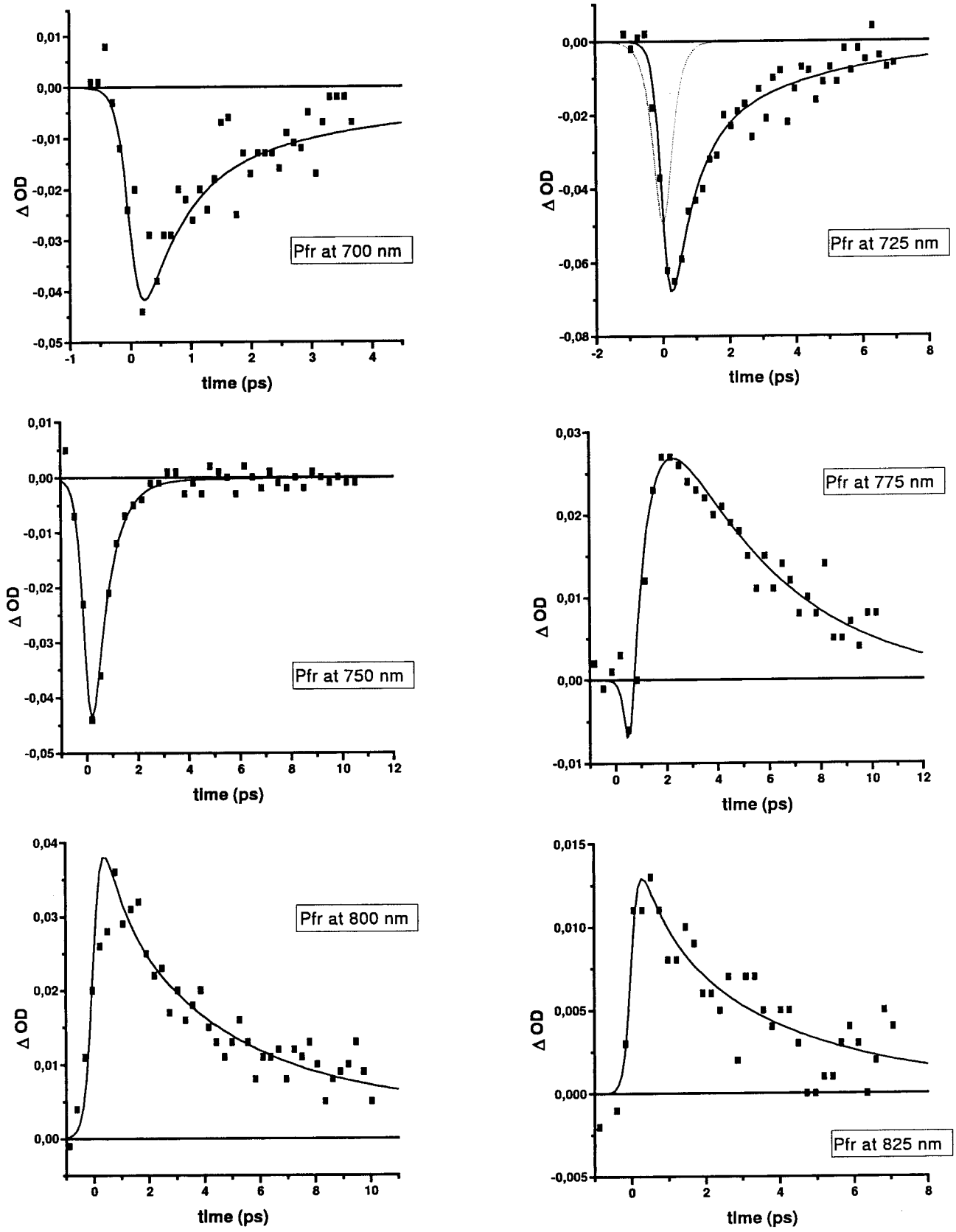

Figure 2. Time-resolved absorption changes $\Delta OD(t)$ of the far-red-light-absorbing form (Pfr) of native oat phytochrome obtained after excitation with 150 fs pulses at 730 nm. The absorption changes are shown for six different probe wavelengths in the red spectral region: 700, 725, 750, 775, 800, and 825 nm as indicated in the plots. The filled squares indicate the experimentally measured data, and the solid curves represent the calculated functions obtained from the best fit parameters of a global data analysis. The estimated lifetimes are as follows: $\tau_1 = 700 \pm 100$ fs and $\tau_2 = 4.5 \pm 0.5$ ps. It should be noted that the ΔOD scale of the individual kinetic curves is different. The instrumental response function is shown as dotted curve in the kinetics at 725 nm.

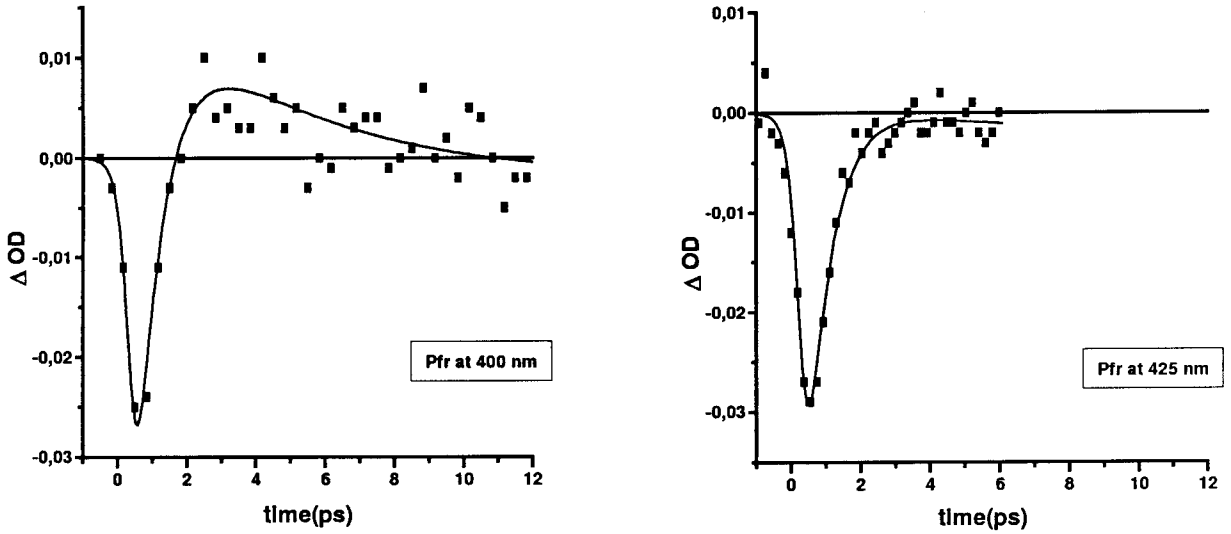

Figure 3. Time-resolved absorption changes $\Delta OD(t)$ of the far-red-light-absorbing form (Pfr) of native oat phytochrome obtained after excitation with 150 fs pulses at 730 nm. The absorption changes are shown for two different probe wavelengths in the blue spectral region: 400 and 425 nm. The filled squares indicate the experimentally measured data, and the solid curves represent the calculated functions obtained from the best fit parameters of a global data analysis. The fitted lifetimes are as follows: $\tau_1 = 700 \pm 100$ fs and $\tau_2 = 4.5 \pm 0.5$ ps.

of a transient absorption which subsequently decays on a slower time. It should be noted that the observed time delay of the absorption increase is true and not only an artifact caused by incorrect determination of the zero point on the time scale. On one hand, the zero point was explicitly measured. On the other hand, the observed increase is clearly less steep than that which would be expected to reflect the rise time of the excitation pulse. Thus, the kinetics at 775 nm apparently reveals first a slight bleaching of the ground-state absorption which is supplanted within some hundreds of femtoseconds by a transient absorption. The transient absorption in turn decays then in about 5 ps.

Figure 3 displays the kinetics of the absorption changes in the blue spectral region at 400−425 nm. At 425 nm the kinetic curve is dominated by a rapid decrease of the negative absorption changes up to zero. The stationary absorption spectrum of Pfr exhibits besides the absorption band in the red wavelength region another one in the blue region (Figure 1). Therefore, the observed negative absorption changes may be attributed to the bleaching of this absorption band. The rapid decay of the bleaching corresponds to that detected in the red spectral region between 700 and 750 nm.

The kinetic changes of the absorption at 400 nm are qualitatively very similar to those observed at 775 nm. At first, bleaching takes place, but after about 1 ps the negative absorption changes turn to positive ones. This indicates the emergence of a transient absorption in this spectral region which, in parallel to the transient absorption in the red wavelength range, decays within the next few picoseconds. It thus seems likely that the kinetics at 400−425 nm reflects the same processes as does the kinetics in the red spectral region.

To determine the lifetimes of the components that can be discerned in the measured kinetics, a global analysis was carried out. The best fit was obtained when a double-exponential function including a constant term was used in the analysis. The constant term was added in order to simulate nonzero asymptotes (ΔOD for $t \rightarrow \infty$) in the kinetics, obviously arising from the formation of a long-living intermediate species. Furthermore, a deconvolution with the cross-correlation function of the pump and probe pulses was included into the fit procedure. Attempts to fit the kinetics to a third exponential term did not result in an improvement of the fit quality. This indicates that the kinetics can be sufficiently described by two exponential components. The lifetimes associated with these components are 700 ± 100 fs and 4.5 ± 0.5 ps, respectively. Table 1 presents the dependence of the amplitudes of the two lifetime components on the different probe wavelengths investigated.

TABLE 1: Dependence of Parameters of the Fit Function on the Probe Wavelengths[a]

λ (nm)	$A_1(\lambda)$	$A_2(\lambda)$	$R(\lambda)$
400	−0.058	0.018	0
425	−0.052	−0.002	0
700	−0.040	−0.018	0
725	−0.070	−0.030	0
750	−0.073	−0.002	0
775	−0.045	0.049	0.0001
800	0.015	0.027	0.0084
825	0.005	0.010	0.0008

[a] $\Delta OD(\lambda,t) = A_1(\lambda) \exp[-t/\tau_1] + A_2(\lambda) \exp[-t/\tau_2] + R(\lambda)$; $\tau_1 = 700 \pm 100$ fs, $\tau_2 = 4.5 \pm 0.5$ ps.

To study the effect of the temperature on the observed kinetic processes, experiments were carried out in which the temperature was varied between 260 and 295 K. Figure 4 shows the dependence of the kinetics of the absorption changes at two representative wavelengths, 725 and 775 nm, on the temperature.

As can be seen, the temperature has no measurable effect within the noise level of our measurements on the decay kinetics of the bleaching at 725 nm. The kinetics measured at the different temperatures yields nearly superimposable curves. This is also the case for the kinetics at 775 nm. Both the rapid rise and the subsequent slower decay remain virtually unaffected upon lowering the temperature. Normally, with this type of kinetics, a small temperature dependence should manifest itself in a significant change of the kinetic behavior. The obtained results thus suggest that the primary processes in excited Pfr are not subject to a measurable temperature effect in the 260−295 K temperature range. Beyond this, they also demonstrate the reproducibility of our femtosecond measurements.

Discussion

The experimental results obtained from our femtosecond-resolved measurements indicate that immediately after excitation bleaching of the Pfr ground-state absorption occurs both in the

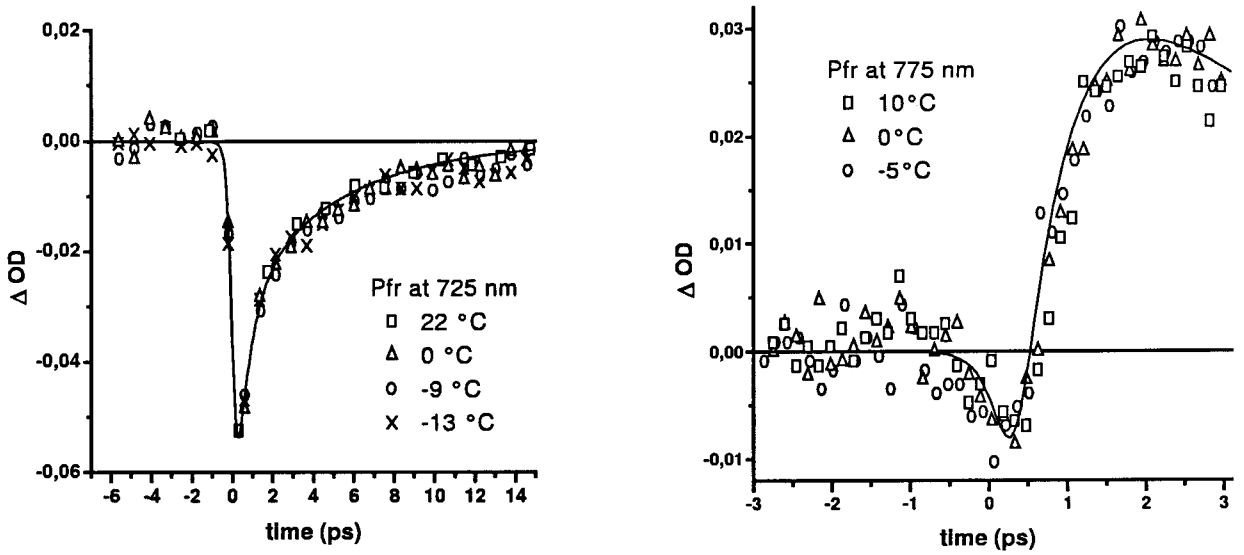

Figure 4. Temperature dependence of the time-resolved absorption changes $\Delta OD(t)$ at 725 and 775 nm obtained after excitation of the Pfr form of phytochrome with 150 fs pulses at 730 nm. The temperature was varied in the 260−295 K temperature range. The temperature of the individual kinetics is indicated in the figure. The solid curve represents the calculated fit function of the respective kinetics at 295 K obtained from the global analysis as described for the data in Figure 2. For the measurements at temperatures below 295 K, 20% glycerol was added to the buffered Pfr solution. At all temperatures investigated, the Pfr solution was pumped through a flow cell at a rate that ensured that each laser pulse excited a fresh sample volume.

red spectral region between 700 and 775 nm (Q-band) and in the blue spectral region between 400 and 425 nm (Soret band). The bleaching observed in the two spectral regions decays very rapidly with time constants of 700 ± 100 fs and 4.5 ± 0.5 ps. In parallel with ground-state bleaching a transient absorption appears at 800−825 nm which decays immediately after its emergence. The decay kinetics of this transient absorption corresponds to that of the bleaching decay. It can be well fitted by the same kinetic components as the bleaching, indicating that these components arise from the same processes that are responsible for the decay of the bleaching. Furthermore, the kinetics observed at both 775 and 400 nm reflects the presence of a component, which rises with ∼700 fs and decays with ∼4.5 ps. Together, these experimental data clearly reveal that two exponential components with lifetimes of ∼700 fs and ∼4.5 ps are involved in the decay of excited Pfr on the femtosecond time scale.

In the following, it is attempted to formulate a kinetic model on the basis of the experimental results obtained. This model is schematically depicted in Figure 5. It describes the simplest kinetic mechanism that can explain the experimental data. However, it should be pointed out that it is of preliminary character. Although the kinetics measured at the distinct probe wavelengths is reproducible and reliable within the signal-to-noise ratio of our measurements, the interpretation of the kinetic data given needs further confirmatory evidence.

Excitation of Pfr with the 730 nm fs pulses brings the molecules to the Franck−Condon state. Because of the impulsive laser excitation, the Franck−Condon state is a vibrationally excited electronic state. It is, therefore, very rapidly depopulated by vibrational relaxation at first. In parallel, polarization dephasing occurs. These first two processes cannot be temporally resolved in our experiments owing to the fwhm of the cross-correlation between the pump and probe pulse and to the signal-to-noise ratio of our measurements which is largely determined by light scattering inherent in the protein solution. What we do observe in the first 100−300 fs of our experiments is an initial rise in the time evolution of the bleaching and of the transient absorption at 800−825 nm. The rise kinetics

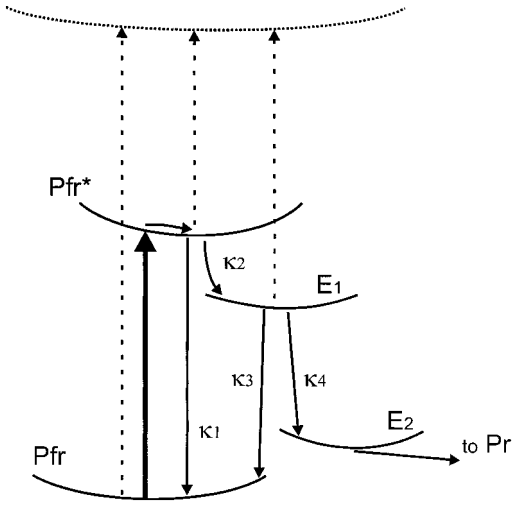

Figure 5. Level scheme proposed for the femtosecond-resolved relaxation processes following the excitation of the far-red-absorbing form (Pfr) of phytochrome with 150 fs pulses at 730 nm. The dark arrow indicates the electronic excitation, dashed arrows indicate transient absorptions from the excited states and ground state, respectively, and full arrows indicate internal conversion to intermediate electronic states and to the ground state, respectively. The parameter k_i represents effective rate constants. The measured lifetimes correspond to k_i as follows: $\tau_1 = (k_1 + k_2)^{-1}$, $\tau_2 = (k_3 + k_4)^{-1}$.

evidently results from the time-dependent increase of the intensity of the pump pulse within the first 200 fs, on one hand. On the other hand, it is somewhat slower than the rise that would be expected from the fwhm of the cross-correlation function. In all probability, this effect reflects the admixture of dephasing and vibrational relaxation of the excited molecules within the first 300 fs. (It should be noted that we have observed a rise term corresponding to the 200 fs cross-correlation in experiments using a cyanine dye in solution as a control.)

Following vibrational relaxation, the excited molecules reach the minimum of the S_1 potential surface. On the basis of the fast decay component observed in the decay kinetics of bleaching and of the transient absorption at 800−825 nm

immediately after excitation, it seems very likely that the lifetime of the S_1 excited state of Pfr (Pfr*) is ~700 fs. From this state (Pfr*) the molecules apparently undergo further relaxation in part toward another electronic state (E_1) and in part toward the Pfr ground state. The latter process is suggested from the ~700 fs decay in the bleaching kinetics. This decay might, however, also result from the rise kinetics of a transient absorption in this spectral region. The existence of the state E_1 can be deduced from the longer-living transient absorption detected at probe wavelengths of 775−825 nm and at around 400 nm which obviously arise from this state. The absorption rise kinetics of ~700 fs as seen at 775 and 400 nm apparently reflects the occupation of the state E_1. On the other hand, the ~4.5 ps decay component detected at 775−825 nm and at 400 nm is very likely related to the depopulation of this state. It can thus be suggested that the lifetime of the state E_1 is ~4.5 ps. We believe that the state E_1 represents an electronically excited state. This proposal is supported by the results obtained from the temperature dependence study. As they reveal, neither the decay rate of the state E_1 nor the amplitude of the corresponding exponential component is significantly affected when the temperature is lowered by 35 K in the 260−295 K temperature range. This implies that the activation energy barrier for the reaction that takes place from the state E_1 is very low, if at all existent. From this finding it seems likely that E_1 is an excited-state rather than a ground-state intermediate. The question could be raised whether E_1 really represents a secondary excited state different from the excited Pfr formed initially or whether it only represents excited-state Pfr after vibrational relaxation. In the latter case, the 700 fs kinetics would reflect vibrational relaxation of the Pfr chromophore. In our opinion, this relaxation time is too long to be ascribed to vibrational relaxation of large molecules such as the Pfr chromophore.[19] Another possibility is that the state E_1 evolves with time due to relaxation of the protein moiety that may occur, to stabilize the structure of the excited Pfr chromophore. But a state resulting from such relaxational interactions between the chromophore and the protein moiety is clearly different from excited-state Pfr formed initially. Finally, it could be argued that solvent relaxation is responsible for the rise time of E_1. Solvent relaxation is, however, unlikely to occur in excited Pfr since the chromophore is located within the immediate vicinity of the protein moiety which adopts the role of the solvent. On the basis of these arguments, it seems justified to assign E_1 to a secondary excited state.

The decay of the state E_1 with a time constant of ~4.5 ps apparently leads to the repopulation of the Pfr ground state. This can be seen from the rapid disappearance of ground-state bleaching which seems to be fully relaxed after the decay of the state E_1. However, the state E_1 cannot exclusively decay back to the ground state since the photoconversion of Pfr yields about 10% Pr.[20,21] Therefore, it is reasonable to assume that a part of the molecule goes over from the state E_1 into another intermediate state, E_2. The state E_2 is poorly characterized at the present stage of our study. It seems likely that its absorption spectrum overlaps with that of Pfr since, as already pointed out, no residual bleaching is left after the decay of E_1. Furthermore, the small positive ΔOD values that remain after the decay of the transient absorption at 775−825 nm might belong to this state. Compared with the time window of our measurements, the state E_2 is relatively long-lived. In our kinetic analysis it is therefore taken into account by the addition of a constant term to the biexponential fit function.

The observed relaxation processes in excited Pfr should result in significant structural changes of the chromophore possibly assisted by local bond rearrangements in the protein structure. At the present stage of our work, the molecular mechanism involved in the early relaxation processes remains unknown. To address this problem, more comprehensive spectroscopic investigations including especially time-resolved resonance Raman and FTIR spectroscopy are required. Nevertheless, the results obtained from the femtosecond-resolved absorption measurements provide an insight into the kinetic mechanism of the primary events in excited Pfr, which is a first necessary step in determining the molecular mechanism.

In conclusion, the results of our study indicate that excitation of the physiologically active Pfr form of phytochrome with 150 fs pulses at 730 nm causes instantaneous bleaching of the two ground-state absorption bands, the Q-band at 730 nm and the Soret band at 400 nm. In parallel with bleaching the appearance of transient absorptions can be observed in both the red and blue spectral region. The decay of these transient absorptions and the decay of the bleaching exhibit two exponential components, one with a lifetime of 700 $\pm$ 100 fs and another with 4.5 $\pm$ 0.5 ps. From the obtained kinetic results it seems reasonable to suggest that the initially excited state of Pfr decays into a secondary excited state, E_1, which in turn goes over into another, as yet poorly characterized, intermediate state, E_2. The molecular changes in the chromophore and/or protein structure that are involved in the early relaxation processes of excited Pfr remain unknown from the present study.

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