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PICOSECOND SPECTROSCOPY OF PHOTORECEPTOR MOLECULES

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1. INTRODUCTION

Light is an indispensible prerequisite for life. In the early days of prebiotic evolution, complex organic molecules were formed under the influence of solar UV radiation. Some billions of years ago the invention of photosynthesis by cyanobacteria and green algae laid the basis for the evolution of aerobic organisms. Sunlight is still the only energy source for all kinds of living systems. Eventually, the possibility to form living structures with their high degree of complexity is derived only from the increase in entropy provided by solar radiation while dropping in radiation temperature from the value of the hot surface of the sun to that of the relatively cool one of our planet when reradiated from earth into space. In accordance with the unrivaled importance of light for all kinds of life, sophisticated mechanisms have been developed in the course of evolution to collect and perceive this unique form of high-valued energy.

Light is utilized by living beings mainly for two purposes: to consume energy, or to obtain information. Accordingly, we find two differnt strategies in photoreception. Energy harvesting is most efficiently done by using a large mumber of antenna pigments to funnel excitation into a reaction center, where the conversion

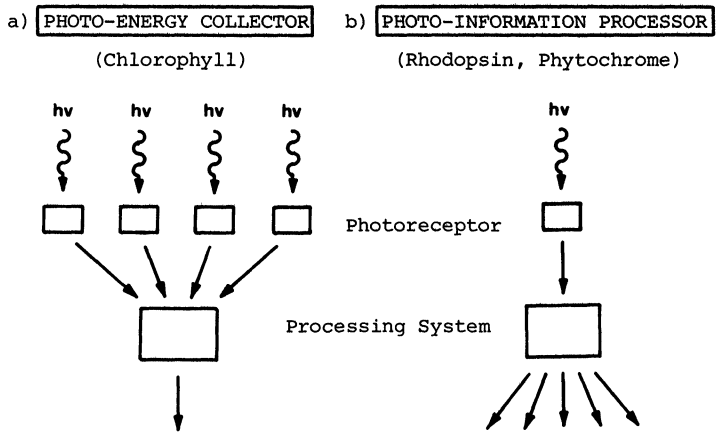

FIGURE 1 Different strategies in light utilization by living organisms a) energy collection, b) information processing

to chemical energy is performed (Figure 1a). This strategy is followed by all photosynthetic organisms. Most surprisingly, apparently only one kind of molecule, chlorophyll, has been invented by evolution to serve as a reaction center. The function of antenna pigments is accomplished by different pigments, e.g. again chlorophyll (in higher plants), or biliproteins (in cyanobacteria and algae). This use of light remained restricted to plants, while using light as a medium for information transport is most conspicious in animals. The sense of vision has evolved on different groups of animals and has reached a high degree of perfection in invertebrates (squids, insects) as well as vertebrates, including man. Nevertheless, also plants are able to perceive light and thereby collect information. Photomorphogenesis, the regulation of morphological development in higher plants by light, is the most important example. Mechanisms for acquiring information work in a way different from that of energy collectors. Usually a single-photon event is sufficient, which triggers a cascade of dark

reactions, forming some kind of signal amplification (FIGURE 1b).

Photoreception was the first biological problem to which picosecond spectroscopy had been applied. Busch et al. showed[1] that the primary photochemical event in vision proceeds in less than 6 ps. In the meantime picosecond measurements have been proven useful for studying various photoreceptive systems, e.g. rhodopsin [2], chlorophyll[2], phytochrome[3], and phycobiliproteins[4].

There exist three different approaches to investigate primary events in photoreception. The first one is to start from the intact photoreceptor pigment, in vitro or even in vivo. This of course gives the most complete picture of the whole process, but usually it is difficult to analyse the highly complex results from measurements of this kind. A method more remote from the intact system, but easier in understanding, is to study only isolated chromophores. In some cases, even the chromophore is too complex or too difficult to handle, so that spectroscopic studies have to be conducted on the level of model compounds and their partial structures. The most complete knowledge can be obtained applying all three methods to the same case.

In the following a limited account will be given of picosecond work done in our laboratory on all three levels of complexity. The photoreceptive systems conserned are the visual system of animals and the photomorphogenetic system of higher plants. It will become apparent that it is most important in picosecond measurements not to rely on a single spectroscopic method but to gain information from different kinds of spectrocopy on different levels of complexity of the system under examination.

2. EXPERIMENTAL

To produce light pulses of picosecond duration, mainly two kinds of lasers are currently being used: synchronously pumped dye lasers, and passively mode-locked solid - state lasers. Because of

the low pulse energies the synchronously pumped systems are only moderately capable of generating new frequencies by nonlinear processes. The spectral region in which these systems are useful is thus more or less limited to the tuning range of common laser dyes like rhodamine. Generation of broadband pulses, as is necessary for single-shot absorption measurements, is hardly possible and can only be achieved by sophisticated amplification techniques. However the high repetition rate of synchronously pumped lasers allows data accumulation and averaging. Thus these systems can be used particularly for picosecond fluorescence studies in photochemically and thermally stable molecules. Passively mode-locked solidstate lasers, on the other hand, due to their high power allow effectice frequency transformations to discrete frequencies (by parametric processes as well as by stimulated scattering) as well as into a broadband continuum (by self-phase modulation and parametric four-photon processes). This opens the possibility in a single-shot mode to measure time-dependent fluorescence, absorption, Raman, and infrared spectra. Thus the versatility of this type of lasers is higher than that of synchronously pumped lasers, even if the pulse-to-pulse reproducibility is inferior. The use of optical multichannel analysers in connection with laboratory computers has opened up the possibility to take spectra over a range of ceveral hundreds of nanometers with a single laser shot. Since the event of reliable streak cameras also the whole time scale from a few picoseconds to many nanoseconds (and longer) has become accesible to single-shot measurements. Careful set-up and calibration of the measuring system now gives the opportunity to obtain with a single laser shot the time-evolution of a complete near UV/visible spectrum (absorption or fluorescence) up to some ten nanoseconds.

The experimental equipment used in our work on photoreceptor

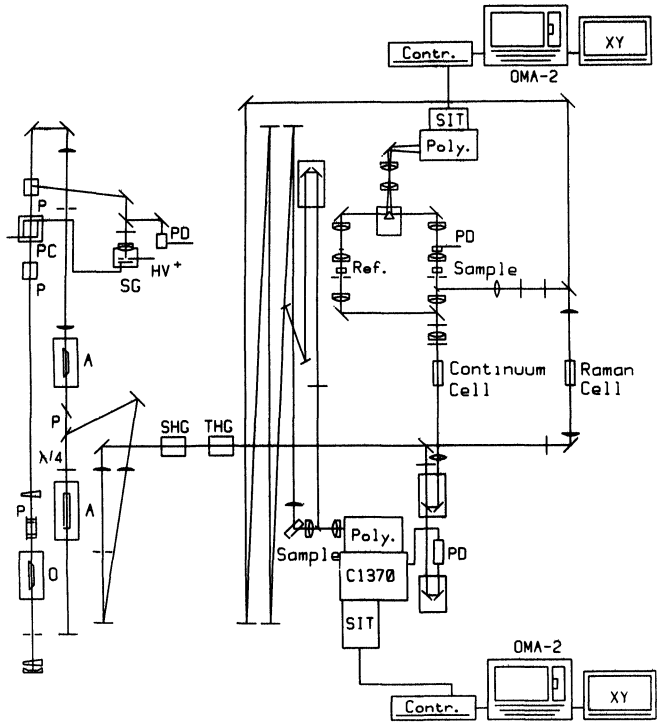

FIGURE 2 Experimental setup for measuring picosecond transient absorption and fluorescence spectra. O laser oscillator, A laser amplifiers, P polarizers, PC Pockels cell, SG spark gap, HV high voltage, λ/4 quarter-wave plate, SHG frequency doubler, THG frequency tripler, PD photodiode. Poly Polychromator, C 1370 streak camera, SIT Silicon intensified target vidicon detector, OMA optical multichannel analyser, XY plotter

molecules is shown schematically in Figure 2. A passively mode-locked Nd-phosphate glass laser (Hoya LHG 8) delivers a train of pulses with duration of ~6 ps each. An electrooptical switch selects a single pulse from the leading edge of the pulse train, having a typical energy of ~100 μJ. Despite the fact that by an intracavity diaphragm the laser is forced to oscillate in the

TEM_{00} mode, spatial filtering is performed after the single-pulse selector to provide optimum beam quality. The pulse is amplified to ~1 mJ in a first amplifier stage. In a second stage the pulse is doubly amplified and coupled out by a combination of a quarter--wave plate and polarizer. After passing another spatial filter, the pulse has a final energy of up to 100 mJ. This energy is high enough to allow all necessary nonlinear frequency conversions with very good efficiency. Frequency doubling and tripling is achieved in nonlinear crystals (KD*P), and further shifting of the frequency can be performed by stimulated Raman scattering in various liquids. The different frequencies are used to excite the sample and thus to initiate the photophysical and photochemical processes under examination. The remaining IR pulse, after appropriate delay, is either used to trigger a 2 ps streak camera (Hamamatsu C1370) when fluorescence is to be measures, or to generate a picosecond continuum (useful range 350 - 800 nm) in the case of absorption measurements.

In fluorescence experiments the exciting pulse is sent along an optical path of ~20 ns to allow for the delay of the steak camera. A small part of the pulse is split off before reaching the sample to act as a time marker (prepulse) later on. The fluorescence emitted from the excited sample is collected to the slit of an ISA HR 320 polychromator, additionally corrected for astigmatism (low dispersion grating, 150 lines/mm). The spectrum is imaged to the cathode of the streak camera. The streak camera output is viewed using a Silicon intensified target vidicon in connection with an optical multichannel analyser (OMA 2, Princeton Applied Research). The digitized timeresolved spectrum then is transmitted to a small laboratory computer (Corvus Concept) or to the central computing facility (Univac 1100/81), respectively, for further processing.

For measurements of transient absorption, the continuum pulse is split into two pulses moving along distinct, but optically similar ways. One pulse transverses the excited sample, while the other one passes an unexcited one, acting as a reference. Both pulses are focussed to the slit of an ISA UFS 200 polychromator. The spectra are registrated and processed by a similar equipment as for the fluorescence measurements. The time resolution in this case is accomplished by delaying the continuum with respect to the exciting pulse step by step, so that in this case a larger number of shots is necessary to get the time evolution of the spectra than in the case of fluorescence measurements. The change in optical density is calculated from the spectra of sample and reference following a procedure given by Greene et al.[5].

The result is a plot of fluorescence intensity or transient absorption vs. wavelength vs. time after excitation. A section parallel to the time axis gives the time evolution for a given wavelength. This can be analysed by a leastsquare routine, fitting an arbitrary function (most frequently a single exponential or a sum of exponentials, but also non-exponentials, if physically reasonable) to the experimental points. A section along the wavelength axis gives the spectrum at a given moment, which also can be fitted using appropriate functions (Gaussian, Lorentzian, and sums hereof), yielding position and halfwidth of transient spectral features. This evaluation process has to be done very carefully, always starting from a reasonable physical model of the kinetics. Since the fitting procedures not always yield unequivocal results, it is not advisable to blindly trust the computer output.

3. THE PLANT PHOTORECEPTOR MOLECULE PHYTOCHROME

Phytochrome is a plant pigment governing photomorphogenesis in higher plants. Among the processes depending on this pigment are germination, phototropism, and photoperiodism, all of practical

interest in crop growing. Phytochrome acts as a photoreceptor due to the fact that it can exist in two modifications (P_r and P_{fr}) interconvertable by light. One modification, P_r, absorbs around 660 nm and is physiologically inactive. Absorption of a red photon transforms it into P_{fr}, which has an absorption band at 730 nm and is the compound triggering the photomorphogenetic actions. It can be converted back to P_r by a dark reaction or upon absorption of a dark-red photon.

Phytochrome is a chromoprotein, containing a chromophore consisting of four pyrrol rings connected by methine bridges. The protein structure is largely unknown. The P_r chromophore structure has recently been elucidated[6,7], while the structure of the P_{fr} chromophore is still a matter of intense investigations. On the primary photoprocess in phytochrome a number of hypotheses have been proposed. A definite decision has been impediated by the lack of time-resolved spectroscopic information. To fill this gap has been the reason of our work on this photoreceptive system.

Since the chromophore structure is not yet fully understood,

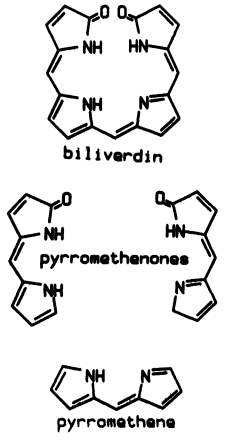

FIGURE 3 Backbone of biliverdin molecule, and partial structures

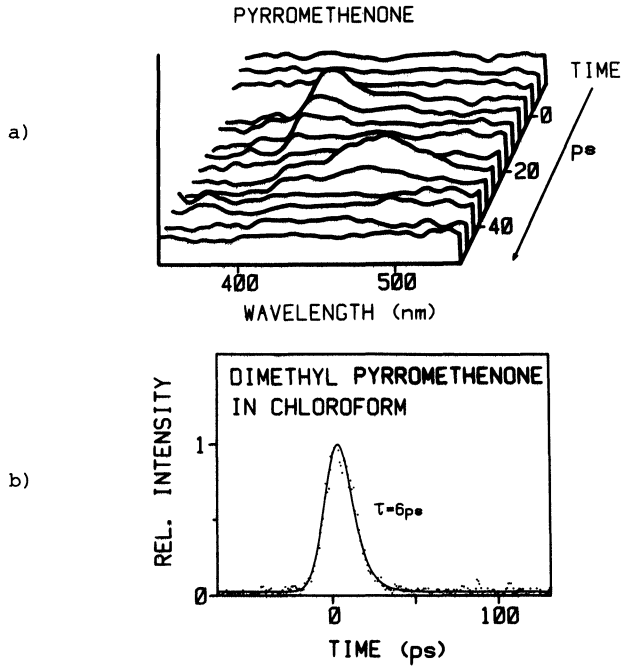

FIGURE 4 Picosecond results for dimethyl pyrromethenone: a) Absorption change vs. wavelength vs. time after excitation. b) fluorescence intensity vs. time after excitation. Fluorescence maximum 420 nm, excitation wavelength 354 nm

the only possibility to really understand time-resolved spectra is by comparison with spectra of well-defined model compounds. Thus, prior to starting with native phytochrome, we decided to conduct a broad picosecond study on pyrrol pigments, four-ringed species as well as their two-ringed partial structures. A short account of our results on this group of compounds shall be given in the following.

The chromophore in the phytochrome molecule is known to have biliverdin structure. This structure can be regarded to be composed of a pyrromethen and two pyrromethenone partial structures,

as schematically shown in Figure 3. So we started our investigations studying primary photoprocesses in these two-ringed molecules. Figure 4a shows a plot of the transient absorption of dimethyl pyrromethenone in chloroform after excitation with a pulse of 354 nm wavelength. Two spectrally overlapping bands can be observed. The first one is centered at 410 nm and shows, after a buildup too fast too resolve ($\sim$2 ps), a decay time of $\sim$ 6 ps. The second band is rather broad and has its center around 460 nm. It appears with a delay of $\sim$ 30 ps and decays within 4 ps. Bleaching of the ground state is observed to last for 40 ps, which is approximately the sum of delay and decay times obseved in the transient absorptions. Figure 4b shows, for comparison, the decay of the fluorscence at 420 nm. The decay time was the same for any part of the fluorescence band, hence no spectrally resolved curve is shown. The observed time constant of 6 ps matches very well the life time of the first transient absorption. Since the fluorescence usually gives the life time of the first excited state S_1, the corresponding absorption is attributed to a $S_1 - S_n$ transition. To understand the delayed absorption, we have to take into account the fact, that pyrromethenone is able to photoisomerise around the exocyclic double bound. A thermal barrier has to be overcome to convert the molecule into a 90° configuration. From Fokker-Planck calculations the measured time of 6 ps seems to be reasonable for this process. Obviously this twisted configuration does not show up within the spectral region covered by our experiment. The broad second transient absorption in our opinion is due to $S_o - S_n$ transitions starting from highly excited vibrational levels, which are populated by internal conversion in the 90° configuration. The delay between the decay of the originally excited A_1 state and the appearance of the new absorption is interpreted as the life time of the twisted excited state. The short time of 4 ps observed for

the decay of the second transient absorption is interpreted as the vibrational relaxation in the ground state, leading to the original Z or the isomerised E configuration. Thus the whole time--course of the isomerisation can be followed by picosecond spectroscopy.

The behaviour of pyrromethene, despite its structural resemblance to pyrromethenone, is quite different. It is known from steady-state experiments that pyrromethene does not isomerise. The fluorescence decay for this partial structure is below 2 ps, and no transient absorption could be observed within the time and wavelength region observed. So we have to conclude that the relaxation from the excited state proceeds very fast, namely in less than 2 ps. We think that this fast relaxation is due to the mobility of the molecule around the methine single bond. This assumption is corroborated by a comparison with a pyrromethene BF_2 complex. By chelation the molecule is now fixed in a flat conformation, for which we would expect a long life time. This expectation is verified experimentally. Ground-state bleaching as well as fluorescence last for 5 ns now (Figure 5).

As an example of an integral pigment suitable as a model chromophore, etiobiliverdin has been investigated. The transient absortion spectrum is given in Figure 6. To allow better interpretation, also spectra of N-methylated and protonated etiobiliverdin have been measured (Figures 7,8). In all three molecules the original ground-state absorption after bleaching recovers within 30 ps. The fluorescence life times are, at least for the protonated and N-methylated molecules, singificantly shorter (27, 11, and 9 ps). Transient and delayed transient absorptions can be observed for all three molecules, none of which have a time behaviour corresponding to the observed fluorescence decay. From this fact it is concluded, in agreement with prior suggestions[8], that the fluorescence observed is due only to a minority of molecules, the majority being essentially non-

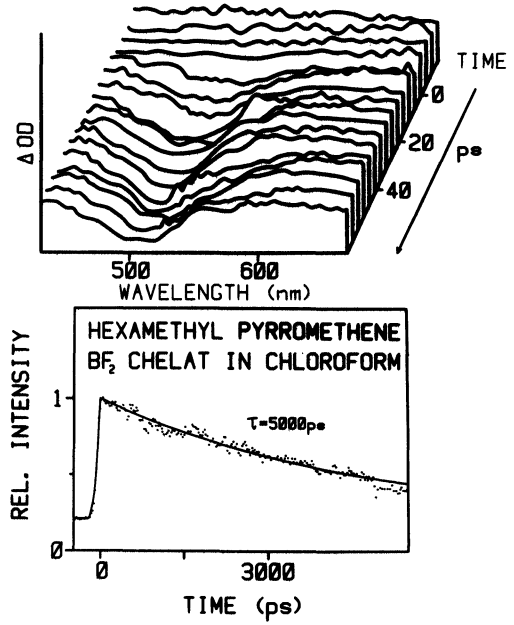

FIGURE 5 Picosecond results for pyrromethene BF_2: a) absorption change vs. wavelength vs. time after excitation, b) fluorescence intensity vs. time after excitation. Fluorescence maximum 540 nm, excitation wavelength 527 nm.

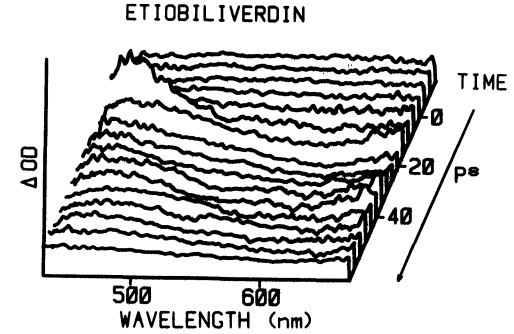

FIGURE 6 Absorption change vs. wavelength vs. time after excitation for etiobiliverdin. Excitation wavelength 621 nm.

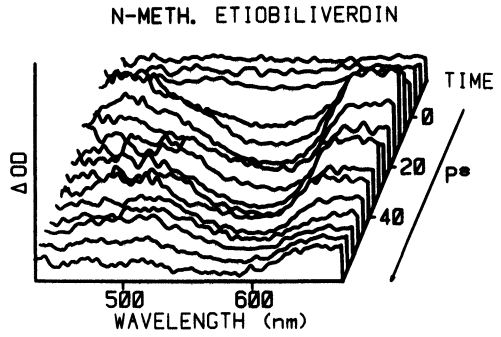

FIGURE 7 Absorption change vs. wavelength vs. time after excitation for N-methyl etiobiliverdin. Excitation wavelength 527 nm.

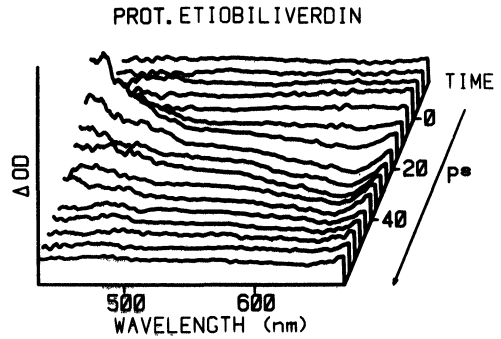

FIGURE 8 Absorption change vs. wavelength vs. time after excitation for protonated etiobiliverdin. Excitation wavelength 621 nm.

-fluorescent. This is a good example proving the application of a single spectroscopic method to be insufficient for analysing the ultrashort time behaviour of complex molecules.

From the results obtained for partial structures, it seems reasonable to assume isomerisation and single-bond rotation to be important also in integral pigments. From steady-state experiments it is known that etiobiliverdin makes no photoisomerisation around

the methine single bond in the pyrromethene fragment of the molecule. Obviously the excited state is non-fluorescent due to vanishing Franck-Condon factors. Transient absorptions with life times of 7, 11, and 15 ps in the three molecules, respectively, are attributed to S_1-S_n absorptions. The S_1 state relaxes, most probably by internal conversion, to a twisted ground-state, to which the delayed transient absorptions are attributed. From force-field calculations[9] it can be assumed that a small barrier ($\sim$2400 cm^{-1}) has to be overcome for return to the original ground-state conformation. The observed life times of the delayed transient absorptions ($\sim$25 ps for all three molecules) are reasonable as concluded from Fokker-Planck calculations. A computer simulation based on PPP and force-field calculations[9,10] and assuming a single-bond rotation gives good agreement between measured and calculated spectra, so that single-bond rotation seems to be verified as the relaxation mechanism. An intramolecular proton transfer, as discussed in the literature[11], can be definitely excluded as a relevant contribution, since it is impossible in N-methyl etiobiliverdin.

Thus, using well-defined phytochrome chromophore model compounds and their partial structures, we were able to show that isomerisation and single-bond rotation are the prevailing relaxation mechanisms in pyrrol pigments.

On the basis of these results on model compounds and their partial structures, it seemed possible to understand picosecond data of native phytochrome itself. The measurements were performed using large phytochrome isolated from rye[12]. Upon excitation with picosecond pulses with a wavelength of 621 nm, a time-resolved fluorescence was observed as shown in Figure 9. The decay was found to be not single-exponential. Following a recent paper[3] we uses a trial function of three exponentials to fit the experimental

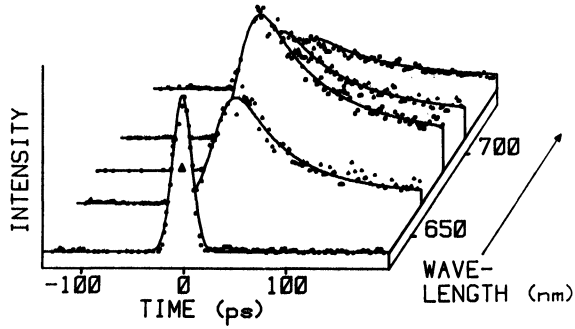

FIGURE 9 Fluorescence intensity vs. wavelength vs. time after excitation for large phytochrome. Excitation wavelength 621 nm. ▲ exciting pulse.

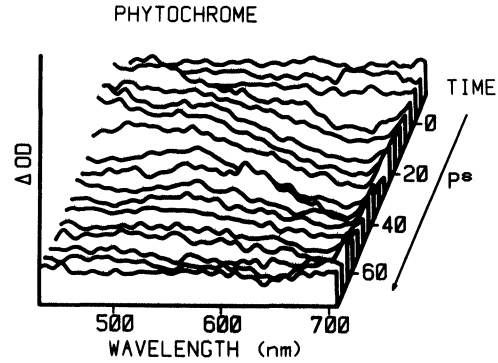

FIGURE 10 Absorption change vs. wavelength vs. time after excitation for large phytochrome. Excitation wavelength 621nm.

values, yielding time constants of ~ 40, 350, and 1250 ps, the shortest decay having the largest amplitude. An interpretation in terms of molecular kinetics based only on these results is impossible, however. Picosecond absorption measurements (Figure 10) showed an immediate bleaching of the 660 nm absorption after excitation, accompanied by a broad transient absorption centered at about 500nm. This transient absorption has a life time of ~35 ps.

The bleaching disappears nearly completely within ~90 ps. It recovers, however, with a time constant exceeding the full time scale of our experiment (1 ns). It should be pointed out, that we cannot follow the relatively slow formation of intermediates described by other authors[13] on our time scale. Anyway, finally the well-known 730 nm absorption of P_{fr} is observed also in our experiment. The disappearance and reappearance of bleaching can only be understood assuming an intermediate with an absorption very nearly similar to that of P_r (absorption maximum ~ 690 nm), forming within ~ 100 ps and decaying with a time constant $\gg$ 1 ns. The absorption centered at 500 nm, from its spectral similarity with equivalent absorptions in the model compounds as well as from its decay time, equalling approximately that of the short-lived fluorescence, can be identified as P_r S_1-S_n absorption. The long-lived fluorescence components might be attributed to impurity emissions, as already argued in ref.[3]. The transient red absorption evolving within ~ 100 ps seems to represent a very early electronically relaxed intermediate. From the resemblance of its absorption to that of the original P_r and the lack of spectral features known to be characteristic for single-bond rotation from our previous experiments, it follows, that in this intermediate conformational changes can be ruled out as relaxation mechanisms. For possible mechanisms leading to this intermediate only proton transfer or Z-E isomerisation remain as reasonable explanation. Proton transfer, as shown above, plays no major role as a relaxation mechanism in pyrrol pigments, however. Furthermore the time of ~ 100 ps is much larger than expected for a proton transfer. So we conclude that the observed intermediate is an isomere, most probably formed by Z-E isomerisation at the C-15 double bond[14]. The process resembles, with a somewhat slower time course, very much the one described above for pyrromethenone, where also a S_1-S_n absorption and a delayed (intemediate) ground--state absorption had been observed. The slowing down has to be

attributed to the stabilizing action of the protein. Thus, the comparison of these phytochrome results with previous results obtained on model compounds gives strong evidence that in phytochrome the S_1 state lives about 40 ps and a Z-E isomerisation at C-15 is accomplished within $\sim$100 ps. So isomerisation seems to be the primary photochemical event, as proposed recently by Rüdiger et al.[14].

4. THE VISUAL CHROMOPHORE RETINAL

Most visual systems investigated up to now use retinal as a chromophore responsible for light reception. Usually it is bound to a protein, forming a chromoprotein called rhodopsin. That the primary photochemical event in rhodopsin goes on in a few picoseconds has been shown a decade ago[1] and repeatedly verified in the meantime[2]. Yet, despite a great number of investigations, also with different picosecond techniques, the process is not fully understood due to the complexity of the problem. It could be assumed that extensive knowledge of the properties of the chromophore itself should be indispensable as a sound basis for an interpretation of the complex results in the chromoprotein. Most surprisingly, however, only very few picosecond investigations have been conducted on retinal[15,16]. Also from those results no clear picture of the primary photoreaction in retinal has emerged. Thus new spectroscopic data with a time-resolution of picoseconds are urgently needed to help elucidating this interesting problem. Especially needed are methods providing new structural information, not obtained by the usual picosecond fluorescence and absorption measurements.

As a new method we have introduced linear dichroism spectroscopy to the picosecond regime. In this method, molecules are incorporated into a polyethylene film and oriented by stretching the film. From previous work[17] the orientation of retinal in the film

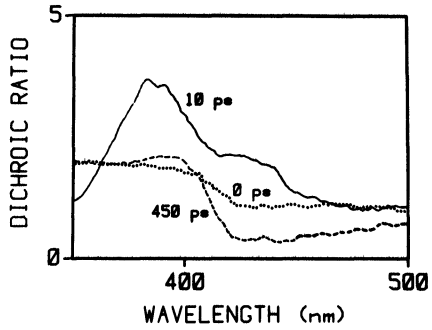

FIGURE 11 Dichroic ratio of all-trans retinal after excitation with 354 nm

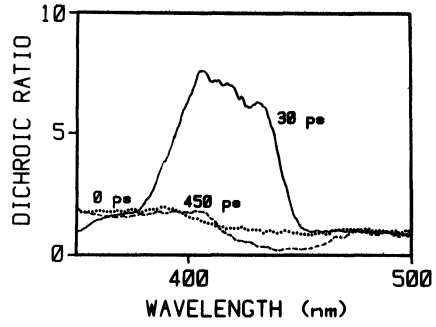

FIGURE 12 Dichroic ratio of 11-cis retinal after excitation with 354 nm

is known. So by measuring transient absorption spectra polarized parallel and perpendicular to the streching direction, the dichroic ratio $D = OD_{\parallel}/OD_{\perp}$ gives information on the direction of the transition-dipole moment and hence on the molecular geometry in the excited and intermediate states. Examples are given in Figures 11 and 12. It is found that a short-lived ($\sim$35 ps) S_1-S_n absorption has a transition-dipole moment oriented well parallel to the stretching direction, indicating that retinal is more planar in

the excited than in the ground state. In the film as well as in n-hexane solution, intersystem crossing is the most dominant relaxation mechanism. The triplet absorption appears within ~ 30 ps. The triplet-triplet absorption observed at 400 nm has a low dichroic ratio (< 1), indicating its transition dipole being directed more or less perpendicular to the stretching direction. The spectral features and relaxation times we observed are in good agreement with values given in ref.[15], but the information on the orientation of the dipole moments could not be obtained previously by other methods. From these results we have to conclude that in discussing the primary event in vision not only geometrical changes at the single and double bonds in the retinal polyene chain should be considered, but also the orientation of the chain with respect to the ring and hence the planarity of the chromophore. This fact has been largely neglected in the theoretical papers on intramolecular motion in retinal, for recent reviews see e.g.[2]. An extension of the picosecond linear dichroism technique to protonated and unprotonated Schiff bases of retinal should give further valuable information.

5 CONCLUSION

Photoreceptor molecules are of high biological interest. Their primary photoreactions proceed on a picosecond time scale. Single-pulse picosecond measurements are especially suited to investigate these processes. In this paper it has been shown, that a reliable understanding of primary photoreactions requires the combined use of different techniques of picosecond spectroscopy, applied at different molecular levels of complexity. The example of the phytochrome molecule gives good evidence, how knowledge gained on the partial-structure and model-compound level is indispensible to interpret spectra obtained from the native molecule. By the example of the visual chromophore, retinal, it has been shown that

the extension of new spectroscopic techniques into the picosecond regime makes obtainable information on the chromophore previously unaccessible, which sheds new light on proposed theories on the primary event in vision.

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