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LASER-INDUCED OPTOACOUSTIC SPECTROSCOPY (OAS) OF PHYTOCHROME IN-VITRO

M. Jabben, S.E. Braslavsky and K. Schaffner

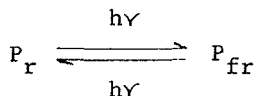
Max-Planck-Institut für Strahlenchemie, D-4330 Mülheim/Ruhr, F.R.G.

Résumé - On étudie le phytochrome photorécepteur des plantes à l'aide d'une méthode de spectroscopie optoacoustique induite par laser. Une solution de phytochrome de (P_r) est irradiée à l'aide de pulses de 15 ns issus d'un laser à colorant accordable de 590 à 720 nm. On effectue une étude comparative à l'aide d'une solution de $CuCl_2$ qui présente la même absorption. Les résultats conduisent à l'interprétation de la desexcitation non radiative du système phytochromique.

Abstract-Laser-induced optoacoustic spectroscopy (OAS) has been applied to the plant photoreceptor phytochrome. A solution of purified oat phytochrome (P_r) was irradiated at room temperature with a 15 ns dye laser pulse tunable from 590 to 720 nm. The first acoustic wave was studied, which is generated by fast heat-emitting processes in less than 0.5 μ s. The P_r solution emitted about 70-90% of the heat produced by a $CuCl_2$ reference of the same absorbance at all wavelengths except for 700 nm, where significantly more heat was produced by P_r . The results are interpreted in terms of a photochromic system consisting of P_r and the first intermediate in the $P_r \longrightarrow P_{fr}$ pathway.

Introduction:

The photoreceptor phytochrome plays a decisive role in plant development throughout the entire life-cycle of a plant. Studies on the photophysical properties of phytochrome are important for the understanding of its biological function. This function relies on the ability of the phytochrome system to exist in two forms, P_r and P_{fr} , which are photochromic. Light absorbed by P_r (absorption maximum at 660 nm) transforms it into P_{fr} (maximum around 730 nm) and vice-versa (Fig. 1).



Phytochrome is a chromoprotein with a monomeric molecular weight of 125.000 D in its native state (1); an linear conjugated tetrapyrrole as the chromophore is linked to the protein (2). Several intermediates have been found to occur during the photoconversion between P_r and P_{fr} . The lifetimes of these intermediates range from about 10 μ s to seconds (3-6). Quantum yields for the photoconversion have been reported to range between 0.1 and 0.2 (7). Since phytochrome shows very low fluorescence yields (ϕ_f ca. 10^{-3} at 293 K) (8,9), it became of interest to study the radiationless processes induced by flash excitation. In this publication we describe experiments on the application of laser-induced optoacoustic spectroscopy (OAS) to phytochrome.

Material and Methods

Phytochrome solutions: Solutions of small (degraded) phytochrome (60.000 D) in 0.1 M Na-phosphate or 0.1 M Tris-H buffer (pH 7.8) were prepared from 4 days old etiolated oat seedlings. Purification was performed by conventional column chromatography to a high degree. The specific absorbance ratio (A_{667}/A_{280}) varied from 1.3 to 0.8 (Fig. 1).

$CuCl_2 \cdot 2 H_2O$ (Merck z.A.), dissolved in 0.1 M Tris-H buffer (pH 7.8), was used as a reference substance (10). Identical results were obtained using either quartz, glass or acrylcuvettes. The temperature of the solution was kept constant at 24.0 ± 0.1 °C. UV-VIS absorption spectra were recorded on a Perkin-Elmer 356 spectrophotometer.

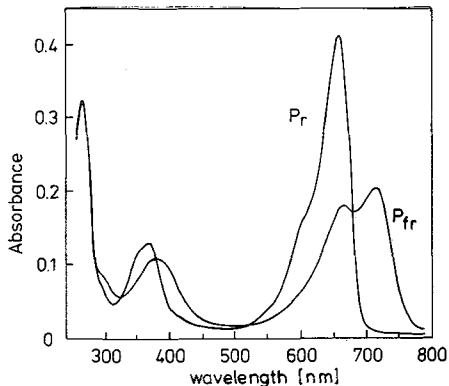


Fig. 1: Absorption spectra of highly purified small phytochrome (60.000 D) from dark-grown oat seedlings.

Instrumentation: The experimental arrangement was similar to that of a previous report (10): A Nd-Yag DLPY₂ laser of 120 mJ output energy in its second harmonic (532 nm) pumped a System 2000 dye laser (J.K. Lasers, Rugby, U.K.). The following laser dyes (all dissolved in methanol) were used: for the 590-620 nm spectral region, Rhodamin B; for 620-680 nm, DCM; for 680-720 nm, Carbazine and Rhodamine 700. A variable neutral density filter served to adjust the laser energy output. The pulse energy was monitored by a pyroelectric detector (EG G, Rj 7100) with a flat spectral response from near UV to near IR. Laser energies varied between 20 and 200 μ J. The 15 ns laser pulse was directed through the middle of the C-clamp held (11) 1 cm acrylic cuvette, the beam diameter being less than 0.5 mm.

Detection system: The piezoelectric transducer (Pb-Zr-Ti, PZT, Siemens H 42, 230 kHz fundamental resonance) was enclosed in a stainless steel casing constructed according to Tam and Patel (12) and attached to the side of the cuvette with a layer of silicone grease in order to enhance acoustic coupling. A Keithley 103A preamplifier was connected as near as possible to the transducer head. The impedance-adapted signal from the preamplifier was fed into a Biomation 8100 transient recorder operated at a sampling time of 10 ns. The first 20 μ s of the signal trace were analysed. Computer (PDP 11/04-VAX 11/780)-aided signal averaging of ten traces (0.5 Hz repetition rate) was performed. The traces are similar to those described by Braslavsky et al. (10); the amplitude of the first maximum (H) was used as the sample response.

The cuvette was continuously irradiated from the opposite side with far-red light in order to keep the phytochrome solution in the P_r form. A 250 W Perkeo R 2500 slide projector with a far-red filter (RG-9, 3 mm, λ_{max} : 740 nm, bandwidth 80 nm; Schott, Mainz/Germany) was used. The continuous irradiation had no detectable effect on the amplitude of the first maximum (H).

Results

A cylindrical acoustic pulse with a pressure amplitude P develops upon pulsed illumination of a thin cylindrical volume in the liquid medium. Tam and Patel (12) calculated that at an observation point perpendicular to the cylinder axis

$$P = \text{const.} \cdot \frac{\beta}{C_p} E_A \quad , \quad //$$

i.e., the pressure amplitude depends on the thermal expansion coefficient (β), the specific heat at constant pressure (C_p), the absorbed pulse energy (E_A), and further factors such as velocity of sound, pulse duration and sound travel distance, which are constant. Eq /1/ assumes that all the absorbed energy is dissipated as heat.

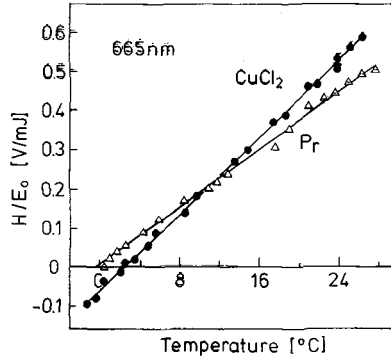


Fig. 2: Optoacoustic signal (H) of P_r and CuCl_2 in 0.1 M Tris buffer as a function of temperature.

The investigation of the optoacoustic effect on phytochrome in an aqueous solution requires constant thermoelastic properties of the medium, i.e., constant β/C_p . The amplitude of the first voltage deflection (H) for CuCl_2 or for phytochrome (P_r) in 0.1 M Tris buffer, when plotted as a function of temperature, demonstrates a pronounced dependence (Fig. 2). It basically reflects the properties of the thermal expansion coefficient β (12). The temperature, at which the observed signal changes sign, is shifted in Tris buffer to values smaller than the one in pure water (4 °C). The strong temperature effect implies that in aqueous solutions the temperature has to be kept at values high enough to record suitable signals. Since 24 °C may cause phytochrome denaturation and/or degradation, the absorbance and photoreversibility of each sample was checked after the measurement. The signal amplitude (H) should depend on the energy specifically absorbed by phytochrome. For constant temperature eq /1/ becomes (g, calibration constant; E_0 , incident laser energy; A, absorbance in the direction of the excitation beam):

$$P = g E_0 (1 - 10^{-A}) \quad /2/$$

for 100% radiationless transfer and homogenous distribution of the sample, and

$$P = g \propto E_0 (1 - 10^{-A}) \quad /3/$$

for radiationless transfer less than 100%, \propto being the yield of radiationless deactivation evolved as heat immediately after the laser pulse ("prompt" heat dissipation). The relationship /3/ has been verified for other systems (10,13).

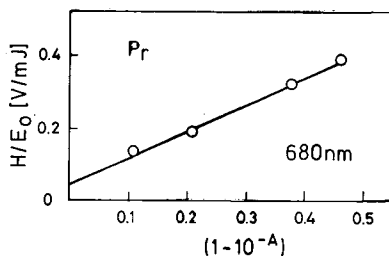


Fig. 3: Optoacoustic signal for constant incident laser energy ($= 20 \mu\text{J}$) as a function of the fraction of light energy absorbed by P_r at 680 nm.

Fig. 3 shows the linear dependence of the amplitude of the first maximum (H) with the fraction of the absorbed energy ($1 - 10^{-A}$). The signal also includes a non-specific response, i.e., the OAS signal produced at $A = 0$. Such non-specific responses were subtracted routinely. For the case of photochemically active substances producing a product which does not revert to ground starting material within the laser pulse duration, the OAS response may reach saturation for certain wavelengths. Irradiation of a phytochrome solution with various energies at three selected wavelengths of different optical absorption at the absorption maximum (665 nm) and at 680 and 590 nm are shown (Fig.4).

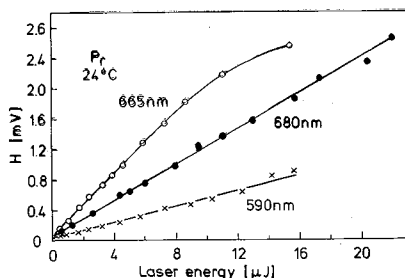


Fig. 4: Dependence of the OAS signal (H) on the incident laser energy (E_0). $A_{590} = 0.11(\times)$; $A_{665} = 0.45(\circ)$; $A_{680} = 0.31(\bullet)$.

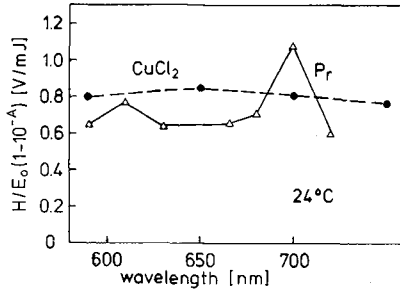


Fig. 5: Spectrum of the first acoustic wave corrected for the light energy $E_0(1-10^{-A})$ absorbed by P_r (Δ) and by CuCl_2 (\bullet).

The energy-response curves of the OAS signal were determined for several wavelengths between 590 and 720 nm. H was measured for different E_0 's within the linear sections of the curves. The spectrum obtained by plotting the ratio of the OAS signal (H) and the absorbed light energy ($E_0(1-10^{-A})$) is depicted in Fig. 5. The system was calibrated using CuCl_2 as a reference substance. When samples of the same absorbance were compared, phytochrome evolved between 70 and 90% ($\alpha = 0.7 - 0.9$) of the heat evolved by the CuCl_2 solution upon laser excitation. At 700 nm significantly more heat was produced by the phytochrome samples than by CuCl_2 . Measurements at wavelengths higher than 720 nm became difficult, since the optical absorption was close to zero.

Discussion

The application of optoacoustic spectroscopy to the plant photo-receptor phytochrome encounters some peculiarities which are related to its proteic and photochromic nature. As a basically hydrophilic protein, phytochrome is kept in an aqueous buffer in order to avoid denaturation. Since the heat expansion coefficient β becomes small around ice temperature (Fig. 2), measurements were made at a higher temperature (24 °C) which may favour protein degradation.

The photochromic nature of phytochrome exerts another restriction on the measurement conditions. A high energy of the exciting pulse will lower the concentration of the substance by product formation. A consequence of the restriction to lower excitation energies is that low concentrations of phytochrome (as,

e.g., encountered in the in-vivo situation in the plant tissue) will yield signals too low (see eq /3/) to be observable with the present detecting system.

CuCl_2 is used as a reference substance for similar reasons as mentioned before (10): CuCl_2 has similar absorption properties as phytochrome, and its excited state decays in less than 10 ns through radiationless processes only. This time is much shorter than the acoustic transit time, i.e., the time required to generate the acoustic wave. In our case the transit time $\mathcal{T} = R/c$ across the beam radius R , c being the acoustic velocity, is less than 300 ns.

We conclude that the behaviour shown in Fig. 5, namely the wavelength dependence of the "prompt" heat dissipation, reflects photophysical properties of the sample. Recently it has been determined that the fluorescence lifetime of P_r is about 60 ps (8). This means that the first radiationless process of excited P_r , leading in part to the first intermediate(s) and in part back to ground state P_r , occurs in a time much shorter than the acoustic transit time (ca. 300 ns) (8,14). It is known from flash photolysis experiments with phytochrome that the initial intermediate has a lifetime of about 10 μs at 300 K (4), which is much longer than the transit time, i.e., the intermediate decays after the first pressure wave has been generated. Therefore, the decay of this intermediate cannot contribute to the "prompt" heat dissipation, as measured by the amplitude of the first acoustic wave.

Since phytochrome (P_r) shows a low fluorescence yield of ca. 10^{-3} (8,9), it is concluded from the results in Fig. 5 that between 590 and 680 nm 70 to 90 % of the absorbed light energy is transferred into heat and the remainder into the photochemical formation of the first intermediate in the P_r to P_{fr} pathway. Maximum absorbance of this intermediate, I-700 (5), has been reported to occur between 685 and 700 nm (4,5). We conclude that the observed strong heat evolution at 700 nm is due to radiationless processes produced by a photochromic system consisting of P_r and its first intermediate I-700, generated within the 15 ns laser flash period. Such a photostationary relation between P_r and I-700 during the first 15 ns confirms and extends earlier observations (15). Such a short lifetime of the photoconversion of I-700 back to P_r would not have been observable by any other method.

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